About the PaVe-GT AAV9-hPCCA Pre-IND Documents

The following documents are communications between the National Institutes of Health (NIH) National Center for Advancing Translational Sciences (NCATS) and the U.S. Food and Drug Administration (FDA) Center for Biologics, Evaluation and Research (CBER) regarding a pre-IND meeting. The pre-IND meeting focused on the development of AAV9-hPCCA (NCATS-BL0746), a gene therapy for propionic acidemia (PA) resulting from a deficiency of Propionyl-CoA Carboxylase, alpha subunit (PCCA) as part of the Platform Vector Gene Therapy (PaVe-GT) program.

PaVe-GT is a pilot project that will test the hypothesis that the efficiency of gene therapy trial startup can be significantly improved by using similar processes across gene therapies for four different rare diseases. An important goal of PaVe-GT is to share project results and lessons learned with the public in such a way that the information is useful to any party interested in developing a gene therapy efficiently. Specifically, we will make processes, study results, regulatory documentation and knowledge gained from the PaVe-GT program publicly available. To ensure access to the latest information, please visit the PaVe-GT website, subscribe to project updates, and explore the full set of available resources at pave-gt.ncats.nih.gov

Some portions of this document—primarily sections that are highly specific to PCCA-related PA and therefore not relevant to other AAV gene therapy efforts—have been formatted, edited or redacted to improve the clarity of materials, and/or support other project objectives. Modified sections are typically identified with italics, brackets, and highlight, *[as shown here].* The text within the brackets describes the original content. It is important to note that these programs are continually evolving, and some information has changed since the pre-IND meeting.

Disclaimer: NCATS, NHGRI, and NIH provide no warranties, representations or guarantees that PaVe-GT resources will work for any given project or disease condition. The information is specific to each program, and the following documents are meant to serve only as examples. The mention of trade names, commercial products and organizations does not imply endorsement by the U.S. government. Further, NIH disclaims any liability and provides no indemnification. For a full list of terms and conditions for use of PaVe-GT resources, visit pave-gt.ncats.nih.gov/

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Documents

May 10, 2023

U.S. Food and Drug Administration
Center for Biologics Evaluation and Research
Document Control Center
10903 New Hampshire Avenue
WO71, G112
Silver Spring, MD 20993-0002

Re: Request for a Pre-IND Type B Meeting
Sponsor: National Center for Advancing Translational Sciences (NCATS)
Drug name: AAV9-hPCCA
Indication: Treatment of *PCCA*-related propionic acidemia (PA)
Reference Number: *[FDA Pre-IND and INTERACT application numbers]*

With reference to the FDA draft Guidance Document "*Formal Meetings between the FDA and Sponsors or Applicants of PDUFA Products (December 2017)*", NCATS hereby requests a Type B, pre-IND videoconference to seek Agency discussion regarding CMC, non-clinical, and clinical aspects of NCATS' development program for AAV9-hPCCA for treatment of *PCCA*-related PA. This request also follows an INTERACT Meeting held with the Agency on July 14, 2021 *[FDA Pre-IND application number]*.

Enclosed please find a Pre-IND Meeting Request document. A comprehensive Meeting Briefing Package will be provided not later than 30 days before the scheduled meeting.

The *[contractor name]* CRO is performing submission of all Pre-IND materials through the FDA's Electronic Submissions Gateway (ESG). A Letter of Authorization was previously provided to the ESG separately. Please consider the following *[contractor name]* as NCATS' authorized FDA correspondents: *[name and contact information of primary and backup correspondent]*

Should you require any additional information, please contact *[name of primary and backup correspondent]* directly. Alternatively, I can be reached by phone at *[phone number and email of study director]*

Sincerely,

[Signature and name sponsor representative]

[Attachment- Antivirus Statement]

Type B Pre-IND Meeting Request

Center for Biologics Research and Evaluation, Office of Therapeutic Products

Request Date: May 10, 2023

Pre-IND Number: *[FDA Pre-IND and INTERACT application numbers]*

Drug Product: AAV9-hPCCA

Formulation: Sterile, aqueous buffered solution composed of the AAV9-hPCCA drug substance formulated in *[buffer composition]*.

Sponsor: *[Name and contact information of sponsor]*

Confidentiality Statement

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements.*

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1 APPLICATION NUMBER

[FDA pre-IND and INTERACT application numbers]

2 PRODUCT NAME

AAV9-hPCCA; additional name, identifier: NCATS-BL0746.

3 CHEMICAL NAME, ESTABLISHED NAME, AND/OR STRUCTURE

AAV9-hPCCA is an Adeno-Associated Virus 9 vector expressing a functional human codon optimized cDNA encoding the Propionyl-CoA Carboxylase, alpha subunit (*PCCA*), under control of the

The AAV9-hPCCA vector transgene schematic and description are shown in **Figure 1**. The full plasmid map and sequence for the pAAV9-hPCCA plasmid, along with information on the helper plasmid and Rep-Cap plasmid, will be included in the pre-IND briefing package.

Figure 1: [Schematic describing components of the AAV9-hPCCA cassette]

4 PROPOSED REGULATORY PATHWAY

The proposed regulatory pathway is for biologic product under 351(a) of the Public Health Service Act (42 U.S.C. 262), with an application under section 505(b)(1) of the Food, Drug, and Cosmetic Act. An Orphan Drug Designation was obtained on September 27, 2021, and a Rare Pediatric Disease Designation was obtained on September 15, 2022.

5 PROPOSED INDICATION(S)

Treatment of *PCCA*-related propionic acidemia (PA).

6 TYPE OF MEETING BEING REQUESTED

Type B, Pre-IND meeting.

7 PEDIATRIC STUDY PLANS

As PA typically presents in the neonatal period, the clinical development program includes a pediatric population in the first-in-human study. As such, the AAV9-hPCCA development program will comply with the Pediatric Research Equity Act, and an initial pediatric study plan will be provided in accordance with applicable regulation.

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements.*

8 HUMAN FACTORS ENGINEERING

Not applicable.

9 COMBINATION PRODUCT INFORMATION

Not applicable.

10 PURPOSE AND OBJECTIVES OF THE MEETING

The Sponsor seeks to evaluate the safety and preliminary efficacy of the AAV9-hPCCA gene therapy investigational product in a patient population with *PCCA*-related PA, starting with adolescent or pediatric patients and then opening enrolment to eligible patients older than 3 years. The drug product formulation will consist of the AAV9-hPCCA drug substance in a buffered solution, to be administered as a one-time intravenous infusion. Drug substance and drug product will be manufactured in a GMP-compliant manner via triple transfection of a HEK293-S cell line. A stability program will be performed to support all storage conditions and durations for the drug product.

To date, the Sponsor has completed pharmacology studies *in vitro* (CRISPR-induced deletion mutagenesis in parental HepG2 cell line) and *in vivo* (mouse model with a CRISPR-induced $Pcca^{p.Q133LfsX41}$ mutation in Exon 5 of the *Pcca* gene; denoted $Pcca^{-/-}$), including biodistribution endpoints. Subsequent to this meeting, the Sponsor intends to perform a pivotal efficacy/safety study ($Pcca^{-/-}$ mice), as well as a combined GLP biodistribution and dose escalation (by cohort) toxicology study (C57BL/6 mice).

Following the conclusion of the IND-enabling nonclinical development program, the Sponsor intends to perform a first-in-human clinical trial in a patient population with *PCCA*-related PA, starting with adolescent or pediatric patients and then opening enrolment to eligible patients older than 3 years. Given the rarity of the disease population, this dose-escalating study will include both safety and preliminary efficacy endpoints, in line with Agency commentary at the previously held INTERACT meeting (July 14, 2021). The Sponsor ultimately intends to submit a BLA marketing application pursuant to 351 (a) of the Public Health Service Act (42 U.S.C. 262) using the 505(b)(1) marketing pathway.

The purpose of this pre-IND meeting is to obtain FDA feedback on the proposed chemistry, manufacturing, and control (CMC), nonclinical, and clinical development programs for the AAV9-hPCCA investigational product. Specific questions are included herein.

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11 PROPOSED AGENDA

The following agenda is proposed (it is assumed respondents will introduce themselves upon speaking):

Brief Overview	10 minutes
Questions for Agency	40 minutes
Wrap up/Summary of Agreements	10 minutes

12 PROPOSED QUESTIONS

The briefing package will contain all background material relevant for the Agency's review in the context of the Sponsor's proposed questions. A response matrix to the previously held INTERACT meeting will also be included as part of the briefing package.

12.1 Chemistry, Manufacturing, and Controls

Background for Questions 1 and 2:

The AAV9-hPCCA gene therapy investigational product expresses a functional human codon optimized cDNA encoding the *PCCA* gene, under control of the *[specific]* promoter. The AAV9 capsid was selected to further enable hepatic and cardiac transduction. The therapeutic transgene cassette was designed with a *[specific]* promoter to enable wide expression. The inverted terminal repeats (ITRs) of the GMP AAV9-hPCCA have been optimized, strictly conserving all other elements of the research grade and feasibility lots of AAV9-hPCCA used in the proof-of-concept (POC) studies.

Cells from a HEK293-S working cell bank are expanded, grown to achieve the targeted cell density and batched up. Triple transfection with pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids *[ratio]* is then performed, followed by cell expansion and subsequent harvest. Cells are lysed and impurities are removed across multiple steps, including filtration and affinity chromatography. Capsids are enriched via iodixanol gradient ultracentrifugation, and fractions are pooled (depending on yields), filtered, and buffered.

The formulated drug substance is filtered and then aliquoted into 2 mL or 5 mL Crystal Zenith® vials to produce the final drug product. The final drug product vials are labeled and stored at -80°C. The drug product formulation is a sterile, aqueous buffered solution composed of the AAV9-hPCCA drug substance formulated in *[buffer]*.

Stability testing is proposed for frozen drug product (up to five years) and the clinical infusion formulation.

Prior to administration to study participants, drug product will be thawed, diluted for infusion, and administered intravenously via peripheral intravenous (PIV) line or peripherally-inserted

central catheter (PICC) using a syringe pump. Stability will be performed to encompass thawing of the drug product through the duration of administration.

Question 1:

Does the Agency agree with the release specifications for the drug substance and drug product?

Question 2:

Does the Agency agree with the proposed storage, preparation, and stability testing plan for drug product and infusion formulation/procedure?

Background for Question 3:

The Sponsor intends to use a syringe pump (Alaris PC unit, Model 8015 pump, 510(k) Number K091308) for clinical administration of the investigational product via PIV or PICC line. Compatibility of the investigational product with the syringe pump and ancillary materials will be determined by testing appearance, quantity and quality. In addition, we plan to perform 'conditions-in-use' compatibility testing for administration devices used in both pre-clinical and clinical setting, with the goal of understanding if the DP's critical quality attributes are maintained as compared to the DP material used as a control, and of establishing acceptance criteria for acceptable product loss during administration. The compatibility testing conditions will incorporate recommended thaw conditions, such as dose preparation procedures, the duration of storage after preparation, dose or volume range, a flow rate or pressure, duration of delivery, and temperature.

Question 3:

Does the Agency agree that the Sponsor's proposed plan for assessing compatibility of the AAV9-hPCCA drug product with the pre-clinical and clinical administration devices is acceptable?

12.2 Non-Clinical

Background for Questions 4 – 6:

The Sponsor has performed a POC study in *Pcca*$^{-/-}$ mouse pups with research grade investigational product (reference July 14, 2021, INTERACT meeting). A second POC study was performed in *Pcca*$^{-/-}$ mouse pups using a 50L non-GMP batch from the manufacturer, Charles River Laboratories (CRL). A non-GLP, pivotal efficacy/safety study in *Pcca*$^{-/-}$ mouse pups and wild type mouse pups using the investigational product from CRL, manufactured under 50L non-GMP conditions, is planned subsequent to this meeting. This efficacy study will also include select toxicity (clinical pathology and histopathology endpoints; study protocol to be provided in the briefing package).

The Sponsor also plans to conduct a 6-month GLP biodistribution/toxicology study in C57BL/6 mice. This is proposed as a single intravenous dose (slow bolus by tail vein), dose-

escalation (by cohort) study to determine target organ toxicity and biodistribution of the engineering 200L lot AAV9-hPCCA investigational product. On the day of dosing, mice will be approximately 6-8 weeks old, and following dosing will be monitored for up to 6 months. Doses will include a vehicle control, and low (1e13 vg/kg/dose), medium (4e13 vg/kg/dose) and high doses (3e14 vg/kg/dose or maximum feasible dose) of the investigational product. Doses were selected based upon results from the POC studies, with the maximum feasible dose determined based on the AAV concentration of the DP and maximum volume to be injected per animal. A study protocol will be provided in the briefing package.

Additionally, the proposed clinical trial intends to enroll patients with PCCA-related PA who are ≥3 years old and *[NIH Clinical Center specific criteria]*. The first participant in each of the two study cohorts is proposed as an adolescent, ≥12 years to <18 years of age. However, given the rarity of the disease population, this enrollment may not be possible. If an eligible adolescent participant is not able to be identified, enrollment will proceed with an eligible participant ≥3 years to <18 years of age, after which the subsequently enrolled participants would be of any age ≥ 3 years.

Question 4:

Depending on whether the 4e13 vg/kg/dose (mid dose in the GLP toxicity study) or the highest tested dose, 3e14 vg/kg/dose is found to be the No Observed Adverse Effect Level, does the FDA agree that these doses support the proposed starting clinical dose *[starting dose]* and the proposed maximum clinical dose *[higher dose]* respectively?

Question 5:

Does the Agency agree that the combination of the data collected from the six-month GLP toxicology study using slow bolus tail vein injection in adult mice and the toxicity data from the non-GLP efficacy study using facial vein injection in mouse pups, along with the proposed clinical age de-escalation, support the minimum age of three years for clinical study participants?

Question 6:

Does the Agency concur that, pending acceptable results in the planned nonclinical studies, the proposed nonclinical development program is IND-enabling for a clinical study in patients with *PCCA*-related PA, starting with adolescent or pediatric patients and then opening enrolment to eligible patients older than 3 years?

12.3 Clinical

Background for Question 7:

The Sponsor proposes a first-in-human, single-dose, dose escalation clinical trial for administration of AAV9-hPCCA in patients with *PCCA*-related PA, starting with adolescent or pediatric patients and then opening enrolment to eligible patients older than 3 years (and

[NIH Clinical Center specific criteria]). The study will consist of the following two cohorts: Cohort 1 will include ~ 3 - 6 participants who will receive *[starting dose]* AAV9-hPCCA; and Cohort 2, ~ 1-3 participants, who will receive *[higher dose]* AAV9-hPCCA. In addition, there will be a control group (historical control) from the NIH natural history study of PA (NCT02890342). This study intends to assess the safety and preliminary efficacy of the AAV9-hPCCA gene therapy investigational product.

The first participant in each of the two study cohorts is proposed as an adolescent, ≥12 years to <18 years of age. However, given the rarity of the disease population, this enrollment may not be possible. If an eligible adolescent participant is not able to be identified, enrollment will proceed with an eligible participant ≥3 years to <18 years of age, after which the subsequently enrolled participants would be of any age ≥ 3 years. This will be followed by dosing of additional participants which may include those ages 3 years and older at screening. Participants will be enrolled and administered investigational product in a staggered manner. A Data and Safety Monitoring Board (DSMB) review of available safety data will commence following 12 weeks of follow-up after investigational drug product administration for the first participant. If the safety review is sufficient, dosing of the next participants in Cohort 1 may commence up to a total of 6 participants. If clinical assessment and biomarker data do not support the efficacy at this dose in the first 3 participants, the study will proceed to Cohort 2. Prior to this, the DSMB will review all cumulative safety data from Cohort 1 and provide a recommendation for dose escalation. Following dosing of the first participant in Cohort 2, the DSMB will again review 12 weeks of data post-administration of investigational product prior to dosing of the second participant, as well as at the end of Cohort 2.

Investigational product will be administered IV via PICC or PIV over ~30 to 60 min, depending on total infusion volume and rate of infusion. Participants will be followed for five years following administration of the investigational product.

A protocol synopsis will be provided in the briefing package.

Question 7:

Does the Agency agree with the proposed first-in-human study design, including participant inclusion/exclusion criteria, dosing rationale, study population rationale, staggering of investigational product administration, stopping rules, safety oversight, and safety and efficacy endpoints?

Background for Question 8:

The National Human Genome Research Institute (NGHRI), including clinical investigators on the PCCA team, is performing a natural history study of patients with PA ("*Natural History, Physiology, Microbiome and Biochemistry Studies of Propionic Acidemia*"; NCT02890342). The study is prospectively evaluating patients with PA, with special emphasis on the US population. Patients are evaluated by a dedicated team of physicians and healthcare providers who are experts in organic acidemias (OA) and PA. Participants include both pediatric and

adult patients with PA, as well as patients whose frequency and severity of PA symptoms led to treatment with an elective liver transplant procedure. Study visits occur at the NIH Clinical Center or are performed via telehealth platforms supported by NIH. Outcome measures are largely descriptive and encompass correlations between clinical, microbiological, biochemical and molecular parameters. The IRB approved natural history protocol will be included in the briefing package.

A Natural History cohort is proposed as a comparator group in the FIH clinical trial for the AAV9-hPCCA investigational product. This study design is proposed due to the infeasibility of performing a double-blind, randomized, controlled clinical trial. Treatment of PA remains an unmet need, and there exists significant risk of morbidity and mortality that comes with waiting for treatment, and potentially the loss of a spot on a liver transplant list. As such and in aggregate, it would not be ethical to have an untreated control group in pediatric patients with a lethal inborn error of metabolism (IEM), and such a trial would not be supported by patient advocacy groups.

Question 8:

Does the Agency agree with the plan to use historical and concurrent data from the Natural History study (with or without liver transplant, considering the very small number of patients affected by this rare disease) as a comparator arm for the FIH clinical trial?

Background for Question 9:

The primary objectives of the first-in-human study of AAV9-hPCCA are: 1. To assess safety and tolerability of AAV9-hPCCA in subjects with PA; and 2. To assess changes from baseline in response biomarkers, i.e., pharmacodynamic (PD) response to AAV9-hPCCA. The corresponding primary endpoints are: 1. Incidence of treatment-related adverse events, treatment-emergent adverse events, and serious adverse events and their relationship to AAV9-hPCCA administration; and 2. Absolute and percent change from baseline in *[disease specific biomarkers]* at *[XX weeks]* (interim endpoint for DSMB assessment) and at the end of the *[XX weeks]* study period (primary endpoint).

The biomarkers used in this study are based on natural history and clinical experience based on elective liver transplant[1,2]. These biomarkers can assess enzyme activity after AAV9-hPCCA treatment to measure the PD activity of liver targeted PCCA gene replacement. They can be directly compared to historical liver transplant data in this patient population. These biomarkers include changes from baseline in the response biomarkers *[two disease related biomarkers]*.

Question 9:

Does the Agency agree with the proposed safety endpoint, and use of the described surrogate endpoint as the primary efficacy endpoint for the FIH study?

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements.*

Background for Question 10:

In this proposed Phase 1/2 clinical trial, we have selected doses of AAV9-hPCCA that are above 1e13 vg/kg and could carry the risk of toxicities, including thrombotic microangiopathy (TMA) and remote risk of liver failure seen in a small number of patients treated with Zolgensma. The starting dose in our study is *[starting dose]*, which we believe will be effective and, based on data from other trials in unrelated inborn errors of metabolism trials, safe. In this protocol we propose a prophylactic immunosuppression regimen of corticosteroids, given at the accepted dose of 1 mg/kg/day for 30 days followed by taper, which could be extended if clinically indicated. T-cell targeted immunosuppressants may also be considered at the discretion of the site Principal Investigator, if clinically indicated.

Nevertheless, there remains the concern that aggressive immunosuppression may be needed in certain patients to prevent toxicity-related complications, such as TMA. There are a number of immune-modulatory regimens that have been variably implemented. However, it is not well known whether any of these regimens has had an effect on reducing side effects.

Question 10:

Does the Agency agree with the proposed immunosuppressive regimen?

13 LIST OF ATTENDEES

[List of attendees]

14 REQUESTED AGENCY ATTENDEES

Non-specifically, the Sponsor requests Agency attendees familiar with propionic acidemia (and/or associated organic acidemias), gene therapy lifecycle development, and clinical trials in a pediatric population. Where possible, the Sponsor requests attendance of parties present at the INTERACT meeting held on July 14, 2021 *[FDA assigned INTERACT reference number]* due to their predicate understanding of the AAV9-hPCCA development program.

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15 SUGGESTED DATE AND TIME OF THE MEETING

Date	Times Available
July 7, 2023	All day
July 10, 2023	All day
July 11, 2023	All day
July 12, 2023	All day
July 13, 2023	All day

16 MEETING FORMAT

A virtual-only, face-to-face teleconference/video conference is requested.

17 MEETING PACKAGE

The meeting package will be sent at least one month prior to the scheduled meeting date.

18 REFERENCES

Applicable references will be provided in the pre-IND briefing package with associated context described.

1. Shchelochkov, O.A. et al. Severity modeling of propionic acidemia using clinical and laboratory biomarkers. Genet Med 23, 1534-1542 (2021).

2. Manoli, I. et al. 1-(^{13})C-propionate breath testing as a surrogate endpoint to assess efficacy of liver-directed therapies in methylmalonic acidemia (MMA). Genet Med 23, 1522-1533 (2021).

June 9, 2023

U.S. Food and Drug Administration
Center for Biologics Evaluation and Research
Document Control Center
10903 New Hampshire Avenue
WO71, G112
Silver Spring, MD 20993-0002
Copy: *[Name of Regulatory Project Manager]*

Re: ***[FDA-assigned Pre-IND reference number]***
Sponsor: National Center for Advancing Translational Sciences (NCATS)
Drug name: AAV9-hPCCA
Indication: Treatment of *PCCA*-related propionic acidemia (PA)
Pre-IND Meeting Briefing Package

Reference is made to a Meeting Confirmation document received on May 31, 2023, confirming a Pre-IND teleconference to be held on July 10, 2023, from 1:00 – 2:00 PM EST. In accordance with this notification, NCATS hereby provides a Meeting Briefing Package to support questions related to its development program for AAV9-hPCCA for the treatment of *PCCA*-related PA. This Pre-IND meeting follows an INTERACT Meeting held with the Agency on July 14, 2021 *[FDA-assigned INTERACT reference number]*.

All Pre-IND Briefing Package materials are contained in Module 1.6.2 (Meeting background materials), and specifically consist of the following information broken down by subject matter discipline:

- Pre-IND Meeting Briefing Package
- Chemistry, Manufacturing, and Controls Information
    - CMC Information for GMP Production (200L Batch)
    - Certificate of Testing, 50L Batch
    - Device Compatibility Study Protocol *[protocol number]*
- Preclinical Information
    - Study Report NHGRI-PCCA-001
    - Study Report NHGRI-PCCA-002
    - Efficacy Study Protocol *[protocol number]*
    - GLP 6M Toxicity Study Protocol
- Clinical Information
    - Natural History Study Protocol *[protocol number]*
    - Proposed Phase 1/2 Study Protocol Synopsis / Protocol Summary of Changes
    - Test Method – Mitochondrial Metabolites
- INTERACT Meeting Response Matrix
- List of Included Literature References, and Supportive Literature

Should you require any additional information, please contact me at *[Sponsor's Primary Correspondent]*. If I am unavailable, please reach out to *[Sponsor's Secondary Correspondent]*.

Sincerely,

[Signature of Sponsor's Primary Correspondent]

Attachment – Anti Virus Statement

DESCRIPTION OF THE ELECTRONIC SUBMISSION INCLUDING TYPE AND NUMBER OF ELECTRONIC MEDIA USED, APPROXIMATE SIZE OF THE SUBMISSION, STATEMENT THAT THE SUBMISSION IS VIRUS FREE, AND THE SOFTWARE USED TO CHECK THE FILES FOR VIRUSES

Number and Type of Electronic Media:	Electronic transmission via ESG
Size of Submission:	Approximately 25 MB
Virus Protection Statement:	This submission is virus free
Software Information:	Avast Free Antivirus Version 23.4.6062 (build 23.4.8118.762)

Technical Point of Contact for eCTD Publishing Questions

[Name and contact information]

***DISCLAIMER**: This information is specific to AAV9-hPCCA and it does not directly apply to other investigational products. The information may no longer be applicable due to subsequent changes.*

Type B Pre-IND Meeting Package

Center for Biologics Research and Evaluation, Office of Therapeutic Products

Date of Meeting:	July 10, 2023, 1:00 – 2:00 EST
Pre-IND PS Number:	*[FDA-assigned Pre-IND and INTERACT reference numbers]*
Drug Product:	AAV9-hPCCA
Formulation:	Sterile, aqueous buffered solution composed of *[buffer composition]*
Drug Substance:	AAV9-hPCCA DS
Indication:	Treatment of *PCCA*-related propionic acidemia (PA)
Sponsor:	National Center for Advancing Translational Sciences (NCATS) 9800 Medical Center Drive Rockville, MD 20850 301-594-8966

Confidentiality Statement

This document contains information that is confidential within the meaning of the Federal Food, Drug and Cosmetic Act (21 U.S.C. §331 [j]), the Freedom of Information Act (5 U.S.C §552[b][4] & 18 U.S.C. Section 1905) and 21 CFR 314.430 *(Drugs*) and 601.50 (*Biologics*) and may not be revealed or disclosed without the prior written authorization of NCATS, NIH.

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LIST OF TABLES

LIST OF FIGURES

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LIST OF ABBREVIATIONS

Abbreviation	**Full Length Name**
BLA	Biologics License Application
CMC	Chemistry, Manufacturing, and Controls
DP	Drug Product
DS	Drug Substance
FDA	United States Food and Drug Administration
FIH	First-in-human
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
HQ	High Quality
IP	Investigational Product
ITRs	Inverted Terminal Repeats
KO	Knockout
LT	Liver Transplantation
NCATS	National Center for Advancing Translational Sciences
NHGRI	National Human Genome Research Institute
NH	Natural History
NIH	National Institutes of Health
PA	Propionic Acidemia
PAM	Protospacer-adjacent Motif
PBS	Phosphate buffered solution
PCC	Propionyl-CoA Carboxylase
PCCA	Propionyl-CoA Carboxylase, alpha
PCCB	Propionyl-CoA Carboxylase, beta
PD	Pharmacodynamics
PIV	Peripheral Intravenous Line
PICC	Peripherally Inserted Central Catheter
PoC	Proof-of-Concept
RCT	Randomized, Controlled Trial
RoA	Route of Administration
UMMS	University of Massachusetts Medical School
US	United States
WT	Wild type

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1 APPLICATION NUMBER

[FDA-assigned Pre-IND and INTERACT reference numbers]

2 PRODUCT NAME

AAV9-hPCCA; additional name, identifier: NCATS-BL0746

3 CHEMICAL NAME, ESTABLISHED NAME, AND/OR STRUCTURE

AAV9-hPCCA is an Adeno-Associated Virus 9 vector expressing a functional human codon optimized cDNA encoding the Propionyl-CoA Carboxylase, alpha subunit (*PCCA*), under control of *[a specific]* promoter.

The AAV9-hPCCA vector transgene schematic and description are shown in The full plasmid map and sequence for the pAAV9-hPCCA plasmid, along with information on the helper plasmid and Rep-Cap plasmid, is included herein.

Figure 1. ***[Schematic describing components of the AAV9-hPCCA cassette]***

4 PROPOSED REGULATORY PATHWAY

The proposed regulatory pathway is for biologic product under 351(a) of the Public Health Service Act (42 U.S.C. 262), with an application under section 505(b)(1) of the Food, Drug, and Cosmetic Act. An Orphan Drug Designation was obtained on September 27, 2021, and a Rare Pediatric Disease Designation was obtained on September 15, 2022.

5 PROPOSED INDICATION(S)

Treatment of *PCCA*-related propionic acidemia (PA).

6 DOSAGE FORM, ROUTE OF ADMINISTRATION, AND DOSING REGIMEN

AAV9-hPCCA drug product will be administered as an intravenous infusion in over approximately 30-60 minutes using a syringe pump. Pending review of initial data, dosing will proceed in two cohorts. Cohort 1 will receive a dose of *[starting dose]* of AAV9-hPCCA, and Cohort 2 will receive a dose of *[higher dose]*.

7 PEDIATRIC STUDY PLANS

As PA typically presents in the neonatal period, the clinical development program includes a pediatric population in the first-in-human study. As such, the AAV9-hPCCA development program will comply with the Pediatric Research Equity Act, and an initial pediatric study plan will be provided in accordance with applicable regulation.

***DISCLAIMER**: This information is specific to AAV9-hPCCA and it does not directly apply to other investigational products. The information may no longer be applicable due to subsequent changes.*

8 HUMAN FACTORS ENGINEERING

Not applicable

9 COMBINATION PRODUCT INFORMATION

Not applicable

10 LIST OF ATTENDEES

[Names of attendees and their designations]

11 DEVELOPMENT HISTORY AND PRODUCT DEVELOPMENT STATUS

11.1 Brief History of Development

11.1.1 AAV9-hPCCA Gene Therapy Program

PA is a rare autosomal recessive disorder of organic acid metabolism in humans. It is caused by a deficiency of propionyl-CoA carboxylase (PCC), a ubiquitously expressed, heteropolymeric mitochondrial enzyme involved primarily in the catabolism of propiogenic amino acids, particularly isoleucine, valine, methionine, and threonine, as well as odd-chain fatty acids [1]. The enzyme is composed of α- and β-subunits encoded by their respective genes, *PCCA* and *PCCB*.

Most frequently, PA presents in the neonatal period with hyperammonemia, vomiting, poor feeding and hypotonia, and progresses into a life-threatening metabolic crisis. Patients who survive suffer from recurrent metabolic instability and can develop multisystem complications, including cardiomyopathy. The long-term prognosis for survival in severely affected patients is poor, where PA patients with an early and severe clinical course experience increased mortality and disease associated morbidity [1, 2]. The recalcitrant nature of the disorder to conventional medical management, including the dietary restriction of amino acid precursors, L-carnitine supplementation, and administration of metronidazole to reduce the generation of propionic acid by intestinal bacteria, has led to the implementation of elective liver transplantation (LT) as an experimental surgical treatment for PA. While not curative of all aspects of the disorder, successful LT in the setting of PA provides restoration of metabolic stability and protection from early death, and therefore represents a clinical benchmark for gene replacement therapy that may increase hepatic PCC expression and activity. There is currently no US Food and Drug Administration (FDA)-approved drug or biologic for the treatment of PA, though it is noted the FDA has approved Carbaglu® (carglumic acid) as **adjunctive** therapy to standard of care for the treatment of acute hyperammonemia due to PA, among other indications.

The Sponsor is developing an AAV9 gene therapy candidate, AAV9-hPCCA, for the treatment of *PCCA*-related PA (proposed patient population does not include those with *PCCB*-related PA). Previously, an INTERACT meeting was held with the Agency on July 14, 2021, in support of this development program. Additionally, an Orphan Drug Designation was obtained

on September 27, 2021, and a Rare Pediatric Disease Designation was obtained on September 15, 2022.

11.1.2 PaVe-GT Platform Program

Platform Vector Gene Therapy (PaVe-GT) is an NIH program whose main goal is to test whether the efficiency of gene therapy development and clinical testing can be increased by standardizing, where applicable, preclinical and manufacturing processes for four different rare disease gene targets [3]. PaVe-GT will attempt to use AAV as a platform vector to develop gene therapy products for four very low prevalence orphan diseases. The rare monogenic diseases selected include two organic acidemias and two congenital myasthenic syndromes. The organic acidemias under study are PA (caused by PCCA deficiency) and isolated methylmalonic acidemia (MMAB deficiency/cobalamin type B methylmalonic acidemia). The congenital myasthenic syndromes under study are characterized by deficiency of the protein DOK7 (downstream of tyrosine kinase 7) or deficiency of Collagen Q (ColQ), a specific nonfibrillar collagen.

The AAV9 vector used in clinical trials for organic acidemias will be produced in the same manufacturing facility, using the same production and purification methods, with the only difference being the transgene. While proof-of-concept (PoC) studies will be conducted individually for the selected disorders, it is hypothesized that additional efficiencies will be gained in the biodistribution studies and potentially, antidrug antibody evaluations and toxicology, as the preclinical testing advances. The PA program described herein details the first proposed clinical utilization of the PaVe-GT platform.

11.2 Substantive Changes in Development Plans

Since the INTERACT meeting, revisions have been made to the proposed first-in-human (FIH) clinical trial. A revised protocol synopsis is included as an addendum to Section **15.3**. Additionally, a Summary of Changes is provided, highlighting the revisions made to the FIH synopsis discussed at the INTERACT meeting. Lastly, a Response Matrix to the Agency's commentary provided prior to the INTERACT meeting is included in the briefing package.

11.3 Current Status of Product Development

To date, the Sponsor has completed pharmacology studies *in vitro* (CRISPR-induced deletion mutagenesis in parental HepG2 cell line) and *in vivo* (mouse model with a CRISPR-induced Pcca$^{p.Q133LfsX41}$ mutation in Exon 5 of the *Pcca* gene; denoted *Pcca*$^{-/-}$), including biodistribution endpoints. Subsequent to this meeting, the Sponsor intends to perform a pivotal efficacy study inclusive of a safety arm (*Pcca*$^{-/-}$ and wild type (WT) counterpart mouse pups) and biodistribution measures, as well as a combined Good Laboratory Practice (GLP) biodistribution and dose escalation (by cohort) toxicology study in C57BL/6 mice.

Following the conclusion of the IND-enabling preclinical development program, the Sponsor intends to perform a FIH clinical trial starting with adolescent or pediatric patients with PA, then opening enrollment to eligible patients older than three years of age. Given the rarity of the disease population, this dose-escalating study will include both safety and preliminary efficacy endpoints, in line with Agency commentary at the previously held INTERACT meeting. The Sponsor ultimately intends to submit a Biologics License Application (BLA)

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marketing application pursuant to 351 (a) of the Public Health Service Act (42 U.S.C. 262) using the 505(b)(1) marketing pathway.

A summary of the investigational product (IP) lifecycle development activities to date, and through the proposed FIH clinical trial, is shown in **Table 1**. This table also notes the similarities and differences for key manufacturing considerations and clinical tenets throughout the lifecycle development activities and provides context for the subsequent data and rationale contained herein.

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Table 1. Lifecycle development for the AAV9-hPCCA investigational product

Parameter	NHGRI-PCCA-001 (PoC / Biodistribution)	NHGRI-PCCA-002 (AAV Infection Study)	NHGRI-PCCA-002 (PoC / Biodistribution)	Proposed Efficacy Study (*[protocol number]*)	Proposed Toxicology Study	Proposed FIH Clinical Study	Brief Description of Lifecycle Changes / Additional Notes
IP Nomenclature	AAV9-hPCCA (Research Grade)	AAV9-hPCCA (10L)	AAV9-hPCCA (small batch 10L & 50L)	AAV9-hPCCA (50L batch)	AAV9-hPCCA (200L engineering batch)	AAV9-hPCCA (200L batch)	N/A
Manufacturing Location (DS / DP)	• UMass Medical School Gene Therapy Center • Vigene Biosciences	Charles River Laboratories (CRL; formerly Vigene)	CRL (formerly Vigene)	CRL (formerly Vigene)	CRL (formerly Vigene)	CRL (formerly Vigene)	Following introductory manufacturing activities at UMMS and Vigene Biosciences, manufacture has moved to CRL for batches inclusive of the efficacy, tox study and FIH study
Manufacturing (e.g., GMP, GMP-like, GLP)	Non-GMP	Non-GMP	Non-GMP	Non-GMP	Non-GMP (engineering run for 200L clinical batch)	GMP	Manufacturing has moved from non-GMP in early phases to full GMP for the 200L clinical batch.
Plasmid grade; Vendor	• Research grade; UMass • Research Grade; Vigene Biosciences	Research grade; CRL	Research grade; CRL	Research grade; CRL	HQ grade; CRL	HQ grade; CRL	Same vector plasmid was used between 50L and 200L batches, though the former was research grade as compared to HQ grade for the latter. Same helper plasmids were used between the 50L and 200L batches, though for the former they were also research grade as compared to HQ grade for the latter.
Additional plasmid information	*[components of plasmid vector]*	*[components of plasmid vector]*	*[components of plasmid vector]*	*[components of plasmid vector]*	*[components of plasmid vector]*	*[components of plasmid vector]*	*[Changes in components of plasmid vector]*

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Parameter	NHGRI-PCCA-001 (PoC / Biodistribution)	NHGRI-PCCA-002 (AAV Infection Study)	NHGRI-PCCA-002 (PoC / Biodistribution)	Proposed Efficacy Study (*[protocol number]*)	Proposed Toxicology Study	Proposed FIH Clinical Study	Brief Description of Lifecycle Changes / Additional Notes
Cell type used in gene therapy manufacture	HEK-293	HEK293-S	HEK293-S	HEK293-S (research lot)	HEK293-S cells (working cell bank lot)	HEK293-S cells (working cell bank lot)	50L and 200L batches manufactured in cell line derived from the same Master Cell Bank lot.
Release Testing (Drug product)	Quality testing only (titer, vector purity by silver staining)	Quality testing only (titer, vector purity by silver staining)	Quality testing only (titer, vector purity by silver staining)	Residual host cell DNA/protein, vector purity, residual benzonase, endotoxin, genomic titer (ddPCR/qPCR), capsid titer, percent full capsid, potency	Appearance, Genomic titer (qPCR), sterility, bacteristasis/fungistasis, subvisible particle analysis, endotoxin, osmolality, pH, purity, aggregation, and potency	Appearance, Genomic titer (qPCR), sterility, bacteristasis/fungistasis, subvisible particle analysis, endotoxin, osmolality, pH, purity, aggregation, and potency	Similarity in release testing between 50L and 200L lots, though all release specifications were "report results" for the 50L batch (**Note**: DS testing in clinical batch to also include additional bioburden, endotoxin, replication competent AAV, purity, sequencing, percent full capsid, and residuals)
Formulation	Aqueous, buffered solution	Aqueous, buffered solution	Aqueous, buffered solution	Aqueous, buffered solution	Aqueous, buffered solution	Aqueous, buffered solution	N/A
Doses (vector genomes/kg/dose)	7E12 – 2.8E14	N/A	7E11 – 2.8E14	1. 7E12 (Low) 2. 3.5E13 (Mid) 3. 7E13 (Mid-High) 4. 3E14 (High) (approximate doses)	1. 1E13 (Low) 2. 4E13 (Mid) 3. 3E14 (High)	1. *[starting dose]* 2. *[higher dose]*	Doses in PoC studies informed doses for the pivotal efficacy study, tox study, and proposed FIH dose; tox study will inform safety margins for FIH study
Excipient	Phosphate buffered solution (PBS)	PBS	PBS plus Sorbitol and Tween-80	PBS plus Sorbitol and Tween-80	PBS plus Sorbitol and Tween-80	PBS plus sorbitol and Tween-80	PBS is noted as the buffer, though the proposed 200L batch also has sorbitol and Tween-80
Control Arm	Untreated / PBS	Untreated	Untreated	Vehicle	Vehicle	Natural History comparator group	Controlling mechanism for the FIH study added due to FDA INTERACT commentary

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Parameter	NHGRI-PCCA-001 (PoC / Biodistribution)	NHGRI-PCCA-002 (AAV Infection Study)	NHGRI-PCCA-002 (PoC / Biodistribution)	Proposed Efficacy Study (*[protocol number]*)	Proposed Toxicology Study	Proposed FIH Clinical Study	Brief Description of Lifecycle Changes / Additional Notes
RoA / Location	Retro-orbital sinus	N/A	Retro-orbital sinus	Intravenous in facial vein	Intravenous (slow bolus by tail vein, over 30-60 seconds)	Intravenous via PIV or PICC line	FDA concurred that a tail vein injection in the tox study could inform proposed RoA in the FIH study
Injection Device(s)	Syringe/needle	N/A	Syringe/ needle	Syringe/needle	Syringe/needle	Syringe pump via PIV or PICC line	N/A

Abbreviations: CRL: Charles River Laboratories, DP: Drug product, DS: Drug substance, FIH: First-in-human, GLP: Good Laboratory Practices, GMP: Good Manufacturing Practices, HQ: High quality, ITRs: inverted terminal repeats, N/A: Not Applicable, PBS: Phosphate buffered solution, PIV: Peripheral intravenous line, PICC: Peripherally inserted central catheter, RoA: Route of Administration

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12 PURPOSE AND OBJECTIVES OF MEETING

The purpose of this pre-IND meeting is to obtain FDA feedback on the proposed chemistry, manufacturing, and control (CMC), preclinical, and clinical development programs for the AAV9-hPCCA IP. Specific questions are included herein.

13 AGENDA

The following agenda is proposed (it is assumed respondents will introduce themselves upon speaking):

Brief Overview	10 minutes
Questions for Agency	40 minutes
Wrap up/Summary of Agreements	10 minutes

14 LIST OF QUESTIONS FOR DISCUSSION

14.1 Chemistry, Manufacturing, and Controls

Background for Questions 1 and 2:

The AAV9-hPCCA gene therapy IP expresses a functional human codon optimized cDNA encoding the *PCCA* gene, under control of *[a specific promoter]*. The AAV9 capsid was selected to further enable hepatic and cardiac transduction. The therapeutic transgene cassette was designed with a *[a specific promoter]*to enable wide expression. The inverted terminal repeats (ITRs) of the GMP AAV9-hPCCA have been optimized, strictly conserving all other elements of the research grade and feasibility lots of AAV9-hPCCA used in the PoC studies.

Cells from a HEK293-S working cell bank are expanded, grown to achieve the targeted cell density and batched up. Triple transfection with pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids *[ratio]* is then performed, followed by cell expansion and subsequent harvest. Cells are lysed and impurities are removed across multiple steps, including filtration and affinity chromatography. Capsids are enriched via iodixanol gradient ultracentrifugation, and fractions are pooled (depending on yields), filtered, and buffered.

The formulated drug substance is filtered and then aliquoted into 2 mL or 5 mL Crystal Zenith® vials to produce the final drug product. The final drug product vials are labeled and stored at -80°C. The drug product formulation is a sterile, aqueous buffered solution composed of the AAV9-hPCCA drug substance formulated in *[buffer composition]*.

Stability testing is proposed for frozen drug product (up to five years) and the clinical infusion formulation.

Prior to administration to study participants, drug product will be thawed, diluted for infusion, and administered intravenously via peripheral intravenous (PIV) line or peripherally inserted

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central catheter (PICC) using a syringe pump. Stability will be performed to encompass thawing of the drug product through the duration of administration.

Question 1:

Does the Agency agree with the release specifications for the drug substance and drug product?

Question 2:

Does the Agency agree with the proposed storage, preparation, and stability testing plan for drug product and infusion formulation/procedure?

Background for Question 3:

The Sponsor intends to use a syringe pump (Alaris PC unit, Model 8015 pump, 510(k) Number K091308) for clinical administration of the IP via PIV or PICC line. Compatibility of the IP with the syringe pump and ancillary materials will be determined by testing appearance, quantity and quality. In addition, we plan to perform 'conditions-in-use' compatibility testing for administration devices used in both preclinical (pivotal efficacy study) and clinical settings, with the goal of understanding if the DP's critical quality attributes are maintained as compared to the DP material used as a control, and of establishing acceptance criteria for acceptable product loss during administration. Studies will be performed using the 50L batch of AAV9-hPCCA DP, which was manufactured in the same process as the 200L batch, but not under GMP conditions. The compatibility testing conditions will inform the recommendations for thaw conditions, such as dose preparation procedures, the duration of storage after preparation, dose or volume range, duration of delivery, and temperature.

Question 3:

Does the Agency agree that the Sponsor's proposed plan for assessing compatibility of the AAV9-hPCCA drug product with the preclinical and clinical administration devices is acceptable?

14.2 Preclinical

Background for Questions 4 – 6:

The Sponsor has performed a PoC study in *Pcca*$^{-/-}$ mouse pups with research grade IP (as described for the July 14, 2021, INTERACT meeting). A second PoC study was performed in *Pcca*$^{-/-}$ mouse pups using a 50L non-GMP batch from the manufacturer, Charles River Laboratories (CRL). A non-GLP, pivotal efficacy study (with safety and biodistribution endpoints) in *Pcca*$^{-/-}$ mouse pups and wild-type mouse pups using the IP from CRL, manufactured under 50L non-GMP conditions, is planned subsequent to this meeting. This efficacy study will also include select toxicity (clinical pathology and histopathology) endpoints.

The Sponsor also plans to conduct a 6-month GLP biodistribution/toxicology study in C57BL/6 mice. This is proposed as a single intravenous dose (slow bolus by tail vein), dose escalation (by cohort) study to determine target organ toxicity and biodistribution of the

engineering 200L lot AAV9-hPCCA IP. On the day of dosing, mice will be approximately 6-8 weeks old, and following dosing will be monitored for up to six months. Doses will include a vehicle control, and low (1E13 vg/kg/dose), medium (4E13 vg/kg/dose) and high doses (3E14 vg/kg/dose or maximum feasible dose) of the IP. Doses were selected based upon results from the PoC studies, with the maximum feasible dose determined based on the AAV concentration of the DP and maximum volume to be injected per animal.

Additionally, the proposed clinical trial intends to enroll patients with PCCA-related PA who are ≥3 years old and *[meet NIH Clinical Center specific criteria]*. The first participant in each of the two study cohorts is proposed as an adolescent, ≥12 years to <18 years of age. However, given the rarity of the disease population, this enrollment may not be possible. If an eligible adolescent participant is not able to be identified, enrollment will proceed with an eligible participant ≥3 years to <18 years of age, after which the subsequently enrolled participants would be of any age ≥ 3 years.

Question 4:

Depending on whether the 4E13 vg/kg/dose (mid-dose in the GLP toxicity study) or the highest tested dose, 3E14 vg/kg/dose, is found to be the No Observed Adverse Effect Level, does the FDA agree that these doses support the proposed starting clinical dose *[starting dose]* and the proposed maximum clinical dose *[higher dose]*, respectively?

Question 5:

Does the Agency agree that the combination of the data collected from the 6-month GLP toxicology study using slow bolus tail vein injection in adult mice and the toxicity data from the non-GLP efficacy study using facial vein injection in mouse pups, along with the proposed clinical age de-escalation, support the minimum age of three years for clinical study participants?

Question 6:

Does the Agency concur that, pending acceptable results in the planned preclinical studies, the proposed preclinical development program is IND-enabling for a clinical study in patients with *PCCA*-related PA, starting with adolescent or pediatric patients and then opening enrolment to eligible patients older than 3 years?

14.3 Clinical

Background for Question 7:

The Sponsor proposes a first-in-human, single-dose, dose escalation clinical trial for administration of AAV9-hPCCA in patients with *PCCA*-related PA, starting with adolescent or pediatric patients and then opening enrolment to eligible patients older than 3 years (*[who meet NIH Clinical Center specific criteria]*). The study will consist of the following two cohorts: Cohort 1 will include ~ 3 - 6 participants who will receive *[starting dose]* AAV9-hPCCA; and Cohort 2, ~1-3 participants, who will receive *[higher dose]* AAV9-hPCCA. In addition, there will be a control group (historical/concurrent control) from the NIH natural history study of PA

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(NCT02890342). This study intends to assess the safety and preliminary efficacy of the AAV9-hPCCA gene therapy IP.

The first participant in each of the two study cohorts is proposed as an adolescent, ≥12 years to <18 years of age. However, given the rarity of the disease population, this enrollment may not be possible. If an eligible adolescent participant cannot be identified, enrollment will proceed with an eligible participant ≥3 years to <18 years of age, after which the subsequently enrolled participants would be of any age ≥3 years. This will be followed by dosing of additional participants who may include those aged 3 years and older at screening. Participants will be enrolled and administered IP in a staggered manner. A Data and Safety Monitoring Board (DSMB) review of available safety data will commence following 12 weeks of follow-up after investigational drug product administration for the first participant. If the safety review is sufficient, dosing of the next participants in Cohort 1 may commence up to a total of six participants. If clinical assessment and biomarker data do not support the efficacy at this dose in the first three participants, the study will proceed to Cohort 2. Prior to this, the DSMB will review all cumulative safety data from Cohort 1 and provide a recommendation for dose escalation. Following dosing of the first participant in Cohort 2, the DSMB will again review 12 weeks of data post-administration of IP prior to dosing of the second participant, as well as at the end of Cohort 2.

IP will be administered IV via PICC or PIV over ~30 to 60 min, depending on total infusion volume and rate of infusion. Participants will be followed for five years following administration of the IP.

Question 7:

Does the Agency agree with the proposed first-in-human study design, including participant inclusion/exclusion criteria, dosing rationale, study population rationale, staggering of IP administration, stopping rules, safety oversight, and safety and efficacy endpoints?

Background for Question 8:

The National Human Genome Research Institute (NGHRI), including clinical investigators on the PCCA team, is performing a natural history study of patients with PA ("*Natural History, Physiology, Microbiome and Biochemistry Studies of Propionic Acidemia*"; NCT02890342). The study is prospectively evaluating patients with PA, with special emphasis on the US population. Patients are evaluated by a dedicated team of physicians and healthcare providers who are experts in organic acidemias (OA) and PA. Participants include both pediatric and adult patients with PA, as well as patients whose frequency and severity of PA symptoms led to treatment with an elective liver transplant procedure. Study visits occur at the NIH Clinical Center or are performed via telehealth platforms supported by NIH. Outcome measures are largely descriptive and encompass correlations between clinical, microbiological, biochemical and molecular parameters (refer to Section 15.3.2.2 for additional detail). The IRB-approved natural history protocol is included in the briefing package.

A Natural History cohort is proposed as a comparator group in the FIH clinical trial for the AAV9-hPCCA IP. This study design is proposed due to the infeasibility of performing a

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double-blind, randomized, controlled clinical trial. Treatment of PA remains an unmet need, and there exists significant risk of morbidity and mortality that comes with waiting for treatment and potentially the loss of a spot on a liver transplant list. As such and in aggregate, it would not be ethical to have an untreated control group in pediatric patients with a lethal inborn error of metabolism (IEM), and such a trial would not be supported by patient advocacy groups.

Question 8:

Does the Agency agree with the plan to use historical and concurrent data from the Natural History study (with or without liver transplant, considering the very small number of patients affected by this rare disease) as a comparator arm for the FIH clinical trial?

Background for Question 9:

The primary objectives of the FIH study of AAV9-hPCCA are: 1. To assess safety and tolerability of AAV9-hPCCA in subjects with PA; and 2. To assess changes from baseline in response biomarkers, i.e., pharmacodynamic (PD) response to AAV9-hPCCA. The corresponding primary endpoints are: 1. Incidence of treatment-related adverse events, treatment-emergent adverse events, and serious adverse events and their relationship to AAV9-hPCCA administration; and 2. Absolute and percent change from baseline in *[two disease related biomarkers]* at *[XX]* weeks (interim endpoint for DSMB assessment) and at the end of the *[XX weeks]* study period (primary endpoint).

The biomarkers used in this study are based on natural history and clinical experience accumulated from elective liver transplant. These biomarkers reflect enzyme activity after AAV9-hPCCA treatment to measure the PD activity of liver targeted PCCA gene replacement. They can be directly compared to historical liver transplant data in this patient population. These biomarkers include *[two disease-related biomarkers]* as measured by the percent change from baseline.

Question 9:

Does the Agency agree with the proposed safety endpoint, and use of the described surrogate endpoints *[two disease related biomarkers]* as the primary efficacy endpoint for the FIH study?

Background for Question 10:

In this proposed Phase 1/2 clinical trial, we have selected doses of AAV9-hPCCA that are above 1E13 vg/kg and could carry the risk of toxicities, including thrombotic microangiopathy (TMA) and remote risk of liver failure seen in a small number of patients treated with Zolgensma®. The starting dose in our study is *[starting dose]*, which we believe will be effective and based on data from other trials in unrelated inborn errors of metabolism trials, safe. In this protocol, we propose a prophylactic immunosuppression regimen of corticosteroids, given at the accepted dose of 1 mg/kg/day for 30 days followed by taper, which could be extended if clinically indicated. T-cell targeted immunosuppressants may also be considered at the discretion of the site Principal Investigator, if clinically indicated.

Nevertheless, there remains the concern that aggressive immunosuppression may be needed in

certain patients to prevent toxicity-related complications, such as TMA. There are a number of immune-modulatory regimens that have been variably implemented. However, it is not well known whether any of these regimens has had an effect on reducing side effects.

Question 10:

Does the Agency agree with the proposed immunosuppressive regimen?

15 DATA SUMMARIES

15.1 Chemistry, Manufacturing, and Controls

15.1.1 CMC Introduction and Lifecycle Development History

Manufacture of the AAV9-hPCCA drug product began with research batches produced at]Vigene Biosciences, and at the UMass Medical School Gene Therapy Center under non-Good Manufacturing Practices (GMP) conditions. Subsequently, all drug substance (DS) and drug product (DP) manufacturing activities (excluding testing) have been performed by Charles River Laboratories, with some potency testing performed by Leidos Biomedical Research Inc., Biodevelopment Pharmaceutical Program.

As described in **Table 1**, since this transfer, key manufacturing materials (e.g., plasmids and cell line used) and parameters (e.g., additional release testing on the 50L and 200L batches), have been either unchanged, modified slightly, or strengthened. However, the table demonstrates how the early-stage batches used in the PoC studies are informative for batches to be used in the preclinical pivotal efficacy, toxicology and proposed FIH studies.

The most recent completed manufacturing run is for the 50L batch, which was used in Study NHGRI-PCCA-002, and will be used for the proposed pivotal efficacy study *[protocol number]*. It is anticipated that the 200L batch to be administered in the pivotal toxicity and FIH clinical studies will use an equivalent process. The 200L clinical batch will also be manufactured under GMP conditions, using the highest quality reagents available.

Table 2 explicitly links each batch of DP to each study performed and proposed.

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Table 2. AAV9-hPCCA lots used for each study performed and proposed

Study Name	Batch Nomenclature	Batch / Lot Numbers	Manufacturer
NHGRI-PCCA-001	Research grade	1. VCAV-06223 2. VCAV-06109 3. No lot number (Date: 05/28/2020)	1 & 2: UMass Gene Therapy Center 3: Vigene Biosciences
AAV Infection Study	10L	Feasibility batch (LE14DEC20)	CRL
NHGRI-PCCA-002	10L	Feasibility batch (LE14DEC20)	CRL
NHGRI-PCCA-002	50L	Lot # TL21-001-41	CRL
Pivotal Efficacy Study *[protocol number]*	50L	Lot # TL21-001-41	CRL
6M GLP Tox / Biodistribution Study	200L Engineering	TBD	CRL
Phase I/II FIH Trial	200L	TBD	CRL

CRL: Charles River Laboratories; *FIH: First-in-human*

Continuing, **Table 3** shows a comparison of some of the key process attributes between the early stage (non-GMP) batches. A comparison of key process parameters between the 50L and 200L batches is provided in the document entitled "CMC Information for GMP Production (200L Batch)" (Table 2, "High-Level Differences Between 50L and 200L Process").

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Table 3. Key process attributes for AAV9-hPCCA development batches

Attribute	10L	10L (CRL)	10L (CRL)	10L (CRL)	50L
Lot Number	Feasibility batch (LE14DEC20)	Lot # TL-21-001-17*	Lot # TL-21-001-20*	Lot # TL-21-001-27*	Lot # TL-21-001-41
DS titer by qPCR	6.05E14 in 5 mL	4.10E14 in 3.6 mL	3.09E14 in 5 mL	3.37E14 in 4.5 mL	2.98E15 in 17.1 mL
DS final titer by ddPCR	Not performed	3.96E14 in 3.6 mL	3.29E14 in 5 mL	4.40E14 in 4.5 mL	1.83E15 in 3.6 mL
Potency – AAV9-hPCCA transduction and expression of Pcca in KO cells by Western blot	Dose-dependent effects of PCCA expression in HepG2 KO cells	Not performed	Not performed	Dose-dependent effects of PCCA expression in HepG2 KO cells	Dose-dependent effects of PCCA expression in HepG2 KO cells
Empty to full (E:F) ratio by cryo-TEM	74.4% full	Not performed	42.6% full**	92.8% full	91.6% full
VP1:VP2:VP3 by silver staining	See gels in subsequent figures	See gels below	See gels below	See gels in subsequent figures	See gels in subsequent figures
Host DNA content (pg/mL)	Not performed	6.69E05	1.9E06	2.1E06	2.51E06
HCP content (ng/mL)	Not performed	< DL (2 ng/mL)	6.3	< DL (2 ng/mL)	< DL (2 ng/mL)
HEK host cell line passage # at transfection	P3	P5	P5	P5	P6

**Lots were used for process optimization/development runs and to ensure product is functional (feasibility material)*

***Pooling of fractions after ultracentrifugation may have been the cause for the low E:F fraction*

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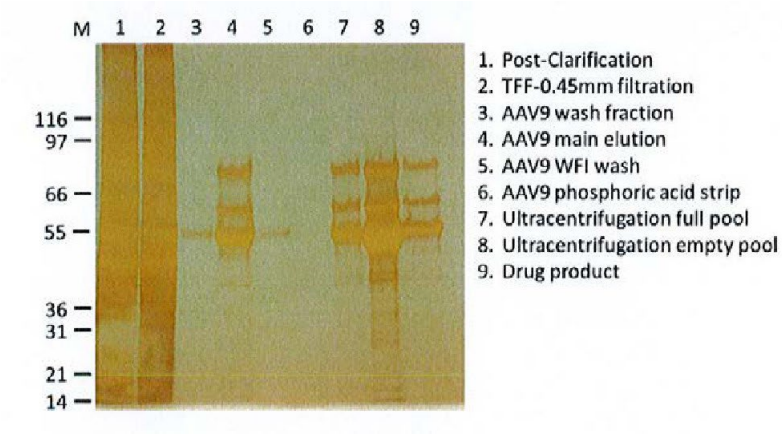
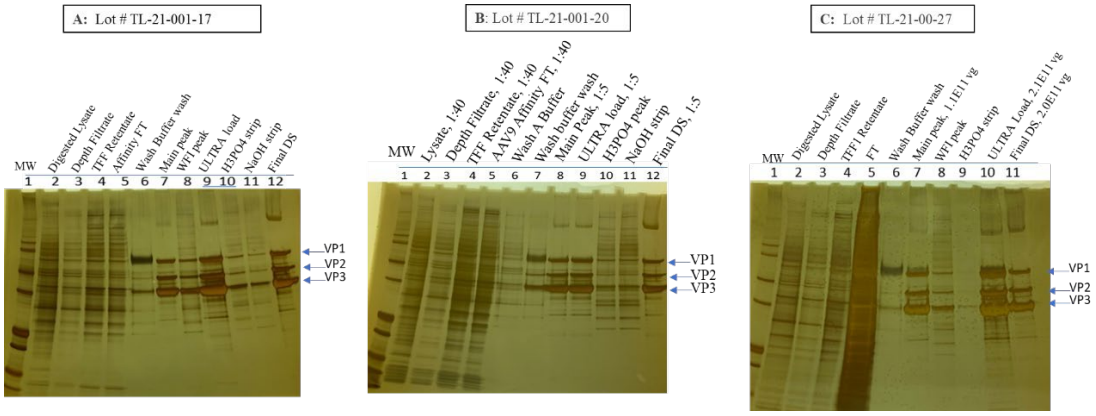
VP1:VP2:VP3 was performed by silver staining. Gels are shown below for each lot described in **Table 3**.

Figure 2. SDS-PAGE/silver stain analysis of DS samples from the purification of AAV9-hPCCA 10L feasibility lot



Aliquots from each step were analyzed by SDS-PAGE/silver staining. Three bands corresponding to capsid proteins VP1 (84 kDa), VP2 (65 kDa), and VP3 (57 kDa) are clearly shown for the AAV9 DS (lane 9). The result shows the removal of impurities. Relative band density ratios of viral capsid proteins (VP1, VP2 and VP3) in the final DS correspond with AAV9 vector protein theoretical ratio of 1:1:10.

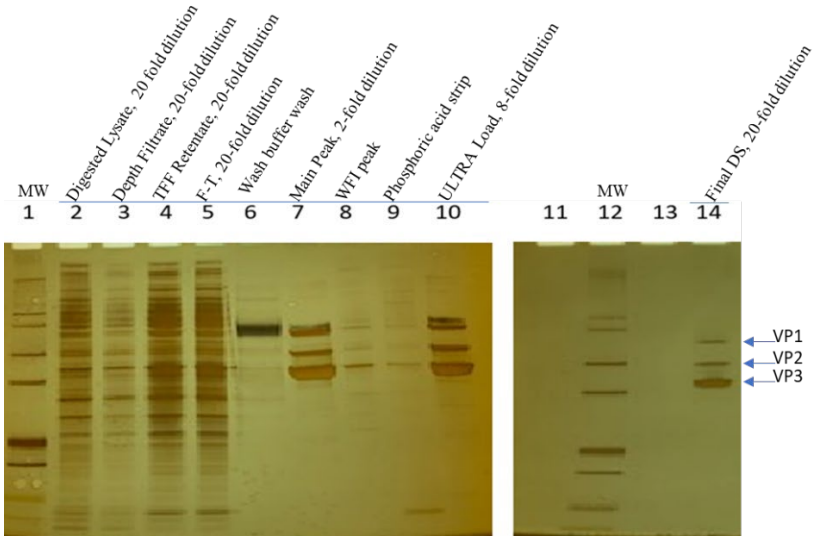
Figure 3. SDS-PAGE/silver stain analysis of in-process and final DS samples from down-stream purification of AAV9-hPCCA 10L lots.



Aliquots from each step were analyzed by SDS-PAGE/silver staining. The band pattern was similar in all three (3) runs. Three bands corresponding to capsid proteins VP1 (84 kDa), VP2 (65 kDa), and VP3 (57 kDa) are clearly shown for the AAV9 DS (lane 7 in panels A and C and Lane 8 in panel B). The result shows the removal of impurities, and the molecular weight and relative band density ratios of viral capsid proteins (VP1, VP2 and VP3) in the final DS of all 10L runs correspond with AAV9 vector protein theoretical ratio of 1:1:10 (Lane 12 in panels A and B, and lane 11 in panel C).

***DISCLAIMER**: This information is specific to AAV9-hPCCA and it does not directly apply to other investigational. products. The information may no longer be applicable due to subsequent changes.*

Figure 4. SDS-PAGE/silver stain analysis of in-process and final DS samples from down-stream purification of AAV9-hPCCA 50L lot# TL-21-001-41.



Aliquots taken at various steps were analyzed via SDS-PAGE with silver stain for purity. Panels show good similarity with the 10L runs. In the final DS, only the three VP proteins (VP1, 84 kDa; VP2, 65 kDa; and VP3, 57 kDa) are detected and the density of these bands corresponds well with the theoretical 1:1:10 ratio (Ln 14) for these VP proteins in AAV9 vector.

15.1.2 AAV9-hPCCA Manufacturing Process

Please refer to the document entitled "CMC Information for GMP Production (200L Batch)" for information related to DS and DP manufacture, testing, stability, and container closure.

As noted in **Table 4**, the analytical methods (tests, vendors and operating procedures) proposed for the 200L DS batch are equivalent to or in excess of the release testing performed on the 50L DS batch ("in excess" refers to testing being performed on the 200L DS that was not performed on the 50L DS). Two potential differences in testing are for the determination of Emply/Full particles (potential difference in vendor and/or method), and genomic titer for information only by ddPCR (potential difference in vendor and/or method). These rows are bolded in the table. A Certificate of Testing for the 50L batch is included with this package.

Release testing performed on the 200L GMP engineering lot will be equivalent to those performed on the 200L GMP lot.

***DISCLAIMER**: This information is specific to AAV9-hPCCA and it does not directly apply to other investigational. products. The information may no longer be applicable due to subsequent changes.*

Table 4. AAV9-hPCCA DS testing comparison for 50L and 200L batches

Test	50L Contractor Test/Identification	200L Contractor/Test Identification
Genomic Titer (Gene-Specific qPCR)[1]	*[Method number]*	*[Method number]*
Total AAV9 Capsid Titer (ELISA)	*[Method number]*	*[Method number]*
Bioburden (Spread Plate Count)	Not Done (N/D)	*[Method number]*
Bacterial Endotoxin per USP<85> (LAL/Chromogenic Method)	N/D	*[Method number]*
Replication Competent AAV (Culture/qPCR)	N/D	To be determined
Vector Purity (SDS-PAGE and Silver Stain)	*[Method number]*	*[Method number]*
Genome Sequencing of Viral Product (Sanger or NGS)	N/D	To be determined
Empty/Full Particles (Cryo-TEM)	*[Contractor name]*	To be determined
Residual HEK293 Host Cell Protein (ELISA)	*[Method number]*	*[Method number]*
Residual Host Cell DNA (qPCR)	*[Method number]*	*[Method number]*
Residual Benzonase (ELISA)	*[Method number]*	*[Method number]*
Residual Adenovirus 5 E1A DNA (qPCR)	N/D	*[Method number]*
Residual Plasmid DNA (qPCR)	N/D	*[Method number]*
Residual Tween-20 (UV/Vis Spectrophotometry)	N/D	*[Method number]*
Residual Polyethyleneimine (HPLC)	N/D	*[Method number]*
Residual Iodixanol (HPLC)	N/D	HPLC
Capillary Gel Electrophoresis	N/D	To be determined
Genomic Titer by ddPCR	*[Method number]*	To be determined

[1]*Preclinical and clinical dose calculation based on qPCR titer. ddPCR performed for information only.*

15.1.3 DS and DP Manufacturing Facility

Appropriate manufacturing facility information will be provided in the IND application.

***DISCLAIMER**: This information is specific to AAV9-hPCCA and it does not directly apply to other investigational. products. The information may no longer be applicable due to subsequent changes.*

15.1.4 AAV9-hPCCA Administration Devices and Compatibility

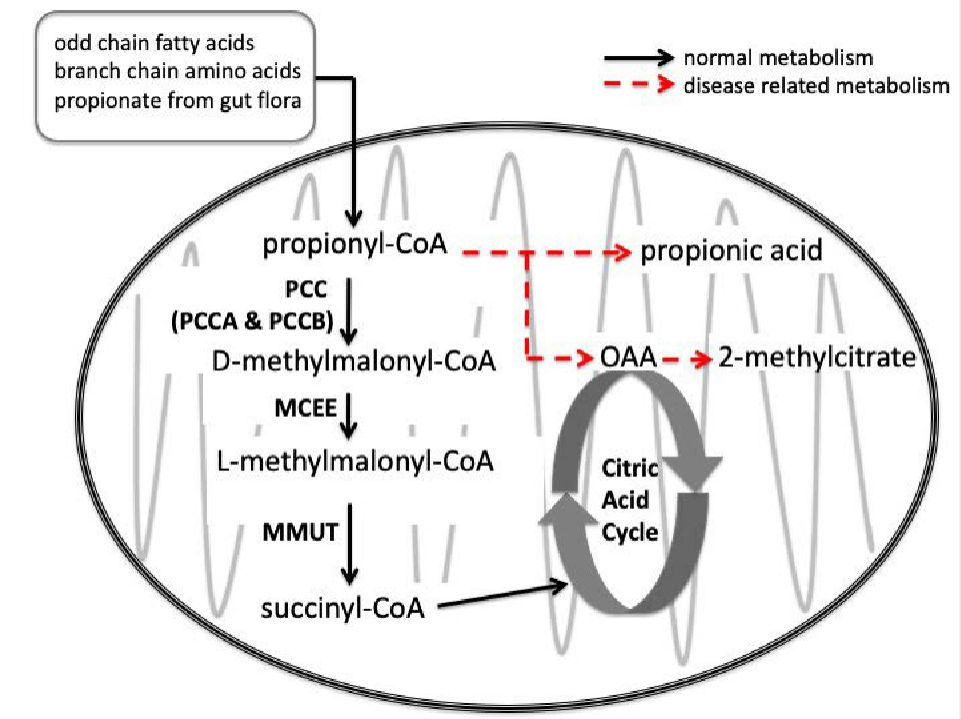
The Sponsor will perform "conditions-in-use" compatibility testing for devices used in both the pivotal efficacy study and the FIH clinical trial. Plans for each suite of testing are described in the document entitled "*[Protocol number]* Testing of compatibility, in-use, and stability of the gene therapy product AAV9-hPCCA."

15.2 Preclinical

15.2.1 Introduction

PA is a well-recognized autosomal recessive disorder of organic acid metabolism in humans caused by a deficiency of PCC, a ubiquitously expressed, heteropolymeric mitochondrial enzyme involved primarily in the catabolism of propiogenic amino acids, particularly isoleucine, valine, methionine, and threonine, as well as odd-chain fatty acids [1]. The enzyme is composed of α- and β-subunits encoded by their respective genes, *PCCA* and *PCCB*. PCC catalyzes the first step in the conversion of propionyl-CoA to D-methylmalonyl-CoA in the pathway of propionyl-CoA oxidation, depicted in **Figure 5**. PA is caused by mutations in either the *PCCA* or *PCCB* gene. The formation of 2-methylcitrate (2-MC), an important biomarker generated through the condensation of oxaloacetic acid and propionyl-CoA is also noted, as are downstream enzymatic steps in the pathway, including D-methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase, in the metabolism of propionyl-CoA into the citric acid (Krebs) cycle.

Figure 5. Catabolism of Propionyl-CoA



***DISCLAIMER**: This information is specific to AAV9-hPCCA and it does not directly apply to other investigational. products. The information may no longer be applicable due to subsequent changes.*

The Sponsor is developing an AAV9 gene therapy candidate, AAV9-hPCCA, for the treatment of *PCCA*-related PA. To date, the Sponsor has completed pharmacology studies *in vitro* (CRISPR-induced deletion mutagenesis in parental HepG2 cell line) and *in vivo* (mouse model with a CRISPR-induced $Pcca^{p.Q133LfsX41}$ mutation in Exon 5 of the *Pcca* gene; denoted $Pcca^{-/-}$), including biodistribution endpoints. Subsequent to this meeting, the Sponsor intends to perform a pivotal efficacy study ($Pcca^{-/-}$ and WT counterpart mouse pups), as well as a combined GLP biodistribution and dose escalation (by cohort) toxicology study (C57BL/6 mice, 6-8 weeks old at time of DP administration).

Table 5 describes the preclinical studies both performed and proposed by the Sponsor.

***DISCLAIMER**: This information is specific to AAV9-hPCCA and it does not directly apply to other investigational. products. The information may no longer be applicable due to subsequent changes.*

Table 5. Preclinical lifecycle development program for AAV9-hPCCA

5A. Pharmacology: *Primary Pharmacodynamics*

Study Number (Reference)	Species	GLP	Route of Administration	Dose (vg/kg/dose)*	Objective	Noteworthy Findings
AAV Infection Study	Cellular model of PCCA knockout (HEPG2 cell line, 10e6 cells)	N	*In vitro* treatment with AAV9-hPPCA, or untreated	1. Untreated 2. 5E4 vg AAV9-hPCCA 3. 1E5 vg AAV9-hPCCA 4. 5E5 vg AAV9-hPCCA 5. 1E6 vg AAV9-hPCCA (**Note**: dosing strengths are in terms of total vg)	To test the *in vitro* potency of AAV9-hPCCA	Results suggest a dose-dependent response for generation of the PCCA protein
NHGRI-PCCA-001	*Pcca*$^{+/-}$, *Pcca* $^{-/-}$, and WT mice	N	Retro-orbital sinus injection	1. Untreated (*Pcca* $^{-/-}$ N=24; *Pcca* $^{+/-}$ N=3; WT N=3) 2. PBS buffer (N=11) 3. 7E12 (*Pcca* $^{-/-}$; N=4) 4. 7E13 (*Pcca* $^{-/-}$; N=28) 5. 2.8E14 (*Pcca* $^{-/-}$; N=12) 6. 7E12 – 2.8E14 (*Pcca* $^{+/-}$; N=28)	To assess survival and biomarker expression *in vivo* in a mouse disease model following treatment with AAV9-hPCCA produced at UMMS or Vigene (now Charles River Laboratories)	• *Pcca* $^{-/-}$ mouse survival appears to be dose-dependent after treatment with a single dose of AAV9-hPCCA in homozygous mouse models of disease • Plasma 2-methylcitrate levels are significantly and dose-dependently decreased up to Day 101 post-injection in homozygous mouse models of disease (*Pcca*$^{-/-}$) treated with a single dose of AAV9-hPCCA

*Unless explicitly noted otherwise

Study Number (Reference)	Species	GLP	Route of Administration	Dose (vg/kg/dose)*	Objective	Noteworthy Findings
NHGRI-PCCA-002	*Pcca* $^{+/-}$, *Pcca* $^{-/-}$, and WT mice	N	Retro-orbital sinus injection	1. Untreated (*Pcca* $^{-/-}$ N=48; *Pcca* $^{+/-}$ N=6; WT N=7) 2. PBS buffer (*Pcca* $^{-/-}$; N=11, 50L batch only) 2. 7E11 (*Pcca* $^{-/-}$; N=12, 10L batch only) 3. 7E12 (*Pcca* $^{-/-}$; N=19) 4. 7E13 (*Pcca* $^{-/-}$; N=49) 5. 2.8E14 (*Pcca* $^{-/-}$; N=39) 6. 7E12 – 2.8E14 (*Pcca* $^{+/-}$; N=143)	To assess survival, biomarker expression, and function *in vivo* in a mouse disease model following treatment with AAV9-hPCCA from two batches (10L and 50L)	• *Pcca* $^{-/-}$ mouse survival appears to be dose-dependent after treatment with a single dose of AAV9-hPCCA in homozygous mouse models of disease • Plasma 2-methylcitrate levels are significantly decreased at Day 30 post-injection in homozygous mouse disease models; in heterozygous littermates, plasma 2-methylcitrate levels without any treatment are similar to those observed in wild-type mice and AAV9-hPCCA treatment has no effect on plasma 2-methylcitrate levels • PCC enzyme activity is observed at Day 30 post-injection in both heart and liver tissue of homozygous mouse disease models, and increased in a dose-dependent manner compared to untreated controls; in heterozygous littermates, AAV9-hPCCA treatment increases PCC enzyme activity in heart tissue in a dose-dependent manner but does not increase it in liver tissue • PCC is observed to be functional (81% of wild-type function), as assessed by propionate oxidation, in a treated homozygous mouse disease model

*Unless explicitly noted otherwise

Study Number (Reference)	Species	GLP	Route of Administration	Dose (vg/kg/dose)*	Objective	Noteworthy Findings
Proposed Efficacy Study *[protocol number]*	*Pcca* $^{-/-}$ and WT counterpart mouse pups	N	Intravenous in facial vein	1. Vehicle 2. 7E12 (Low) 3. 3.5E13 (Mid) 4. 7E13 (Mid-High) 5. 3E14 (High) (approximate doses)	To assess survival and key metabolites, biodistribution, and histopathology (at select timepoints) of mice receiving treatment with AAV9-hPCCA (50L batch). There will be an additional safety arm	TBD

*Unless explicitly noted otherwise

5B. Pharmacokinetics: *Biodistribution*

Study Number (Reference)	Species	GLP	Route of Administration	Dose (vg/kg/dose)*	Objective	Noteworthy Findings
NHGRI-PCCA-001	*Pcca* $^{+/-}$ and *Pcca* $^{-/-}$ mice	N	Retro-orbital sinus injection	1. 7E13 (*Pcca* $^{-/-}$, N=6; *Pcca* $^{+/-}$, N=3) 2. 2.8E14 (*Pcca* $^{-/-}$, N=5; *Pcca* $^{+/-}$; N=2)	To assess copy numbers of the *Pcca* transgene in liver and heart tissue at Day 30 and Day 101 post-injection in heterozygous and homozygous mouse models of disease	• AAV9-hPCCA genome is readily detected in liver and heart tissue through Day 101 post-injection in homozygous mouse models of disease and heterozygous littermates; levels detected appear to be dose-dependent, with better signal observed in the heart • AAV-driven human *PCCA* mRNA expression is detected in the liver tissue of homozygous mouse disease models through Day 101 post-injection of a single dose of AAV9-hPCCA • AAV-driven PCCA protein expression is detected in the heart and liver tissue of homozygous mouse disease models through Day 101 post-injection of a single dose of AAV9-hPCCA • AAV-driven *PCCA*/PCCA mRNA and protein expression do not appear to be dose-dependent

*Unless explicitly noted otherwise

Study Number (Reference)	Species	GLP	Route of Administration	Dose (vg/kg/dose)*	Objective	Noteworthy Findings
NHGRI-PCCA-002	*Pcca* $^{+/-}$ and *Pcca* $^{-/-}$ mice	N	Retro-orbital sinus injection	1. Untreated (*Pcca*$^{-/-}$, N=3; Pcca+/-, N=3; WT, N=5) 2. 7E13 (*Pcca* $^{-/-}$, N=6; *Pcca* $^{+/-}$, N=7)	To assess expression of *Pcca* transgene in liver and heart tissue at Day 30 post-injection in homozygous mouse models of disease, as compared to wild-type	• AAV-driven PCCA protein and human *PCCA* mRNA are detected in the heart and liver tissue of homozygous mouse disease models at Day 30 post-injection of a single dose of AAV9-hPCCA • AAV-driven *PCCA* mRNA expression is observed in liver tissue and heart tissue of homozygous mouse disease models at Day 30 post-injection of a single dose of AAV9-hPCCA, with *PCCA* mRNA levels in liver tissue reaching ~5% of WT endogenous murine *Pcca* levels and *PCCA* mRNA levels in heart tissue reaching ~275% of WT *Pcca* mRNA expression
Proposed Efficacy Study *[protocol number]*	*Pcca* $^{-/-}$ and WT counterpart mouse pups	N	Intravenous in facial vein	1. Vehicle (3M/3F per time point for WT) 2. 7E12 (Low) (3M/3F per time point per genotype) 3. 3E14 (High) (3M/3F per time point per genotype) (approximate doses)	To assess vector DNA biodistribution and transgene mRNA expression analysis via qPCR	TBD

*Unless explicitly noted otherwise

Study Number (Reference)	Species	GLP	Route of Administration	Dose (vg/kg/dose)*	Objective	Noteworthy Findings
Proposed 6-month toxicity study (GLP 6M mice protocol)	C57BL6 mice (1:1 M/F)	Y	Intravenous in tail vein	1. Vehicle (Day 7, and Months 1, 3, 6; N=40) 2. Low dose, 1E13 (Day 7, and Months 1, 3, 6; N=40) 3. Mid-dose, 4E13 (Day 7, and Months 1, 3, 6; N=40) 4. High dose, 3E14 (Day 7, and Months 1, 3, 6; N=40) 5M/5F from each dose group and euthanasia time point will be designated for biodistribution evaluation	To assess *Pcca* transgene expression in blood, injection site, heart, brain, liver, lung, spleen, kidneys, and ovary/testis in dosed animals as compared to untreated animals	TBD

5C. Toxicology: *Single Dose Toxicity*

Study Number (Reference)	Species	GLP	Route of Administration	Dose (vg/kg/dose)*	Objective	Noteworthy Findings
Proposed 6-month toxicity study (GLP 6M mice protocol)	C57BL6 mice (1:1 M/F)	Y	Intravenous in tail vein	1. Vehicle (N=80) 2. Low dose, 1E13 (N=80) 3. Mid-dose, 4E13 (N=80) 4. High dose, 3E14 (N=80) 20 animals apiece in each dosing group to be sacrificed at four time points: Day 7, Month 1, Month 3, and Month 6	To determine target organ toxicity of AAV9-PCCA gene vector administered once via intravenous injection in mice with a 6-month observation period	TBD

**Unless explicitly noted otherwise*

15.2.2 Pharmacology

All pharmacology studies performed and proposed are primary pharmacodynamics (PD) studies.

15.2.2.1 Primary Pharmacodynamics

Primary pharmacodynamics studies were performed with both *in vitro* and *in vivo* models. For the *in vitro* study, a parental human hepatocyte derived (HepG2) cell line with a *PCCA* knockout (KO) mutation was used. Notably, there does not exist a validated animal model of PA, and as such a de novo KO mouse model of pediatric PA (*Pcca*$^{-/-}$ mouse pups) was utilized for *in vivo* studies. The mice have no detectable PCCA protein, display immediate neonatal lethality, and effectively recapitulate the phenotype of the human disorder.

15.2.2.1.1 *IN VITRO* PRIMARY PHARMACODYNAMICS

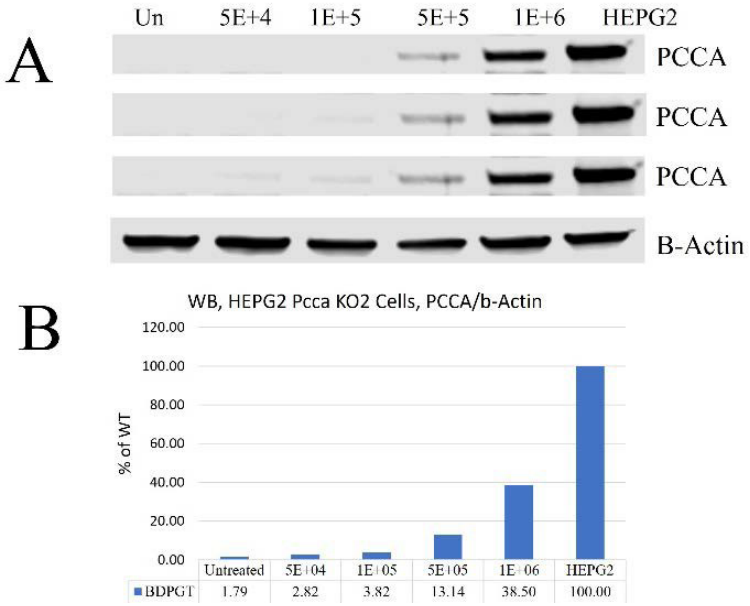
A deletion mutation (CRISPR/Cas9-based deletion mutagenesis) in *PCCA* was engineered in a HepG2 cell line to create the *PCCA* KO cell model. These cells were used to test the *in vitro* infection potency of AAV9-hPCCA (10L batch manufactured by CRL) applied at infection doses ranging from 5E4 to 1E6 vector genomes.

PCCA KO cells were seeded in 6-well dishes at 1E7 cells/well and grown to confluency (~48 hours). At harvest, monolayers of infected *PCCA* KO cells were rinsed, collected, and spun to pellet the unbroken cells. The supernatant was pipetted off, and the cell pellet was lysed. PCCA protein concentration was determined by a micro bicinchoninic acid (µBCA) assay. Cellular protein was subjected to Western Blot analysis using a PCCA polyclonal antibody (with a *B*-Actin loading control). Untreated KO cells and WT HepG2 cells were used as negative and positive controls, respectively.

Figure 6 shows the results of the infection study. Briefly, there was a dose-dependent generation of PCCA protein as shown in the Western Blot, with the highest infection dose (1E6 vg) resulting in nearly 40% of WT PCCA protein expression, thereby noting a mechanistic PoC for potency.

***DISCLAIMER**: This information is specific to AAV9-hPCCA and it does not directly apply to other investigational. products. The information may no longer be applicable due to subsequent changes.*

Figure 6. *In vitro* infection study results



In vitro infection study results shown as A) Western Blot comparing four dosing strengths of AAV9-hPCCA from 5E4 – 1E6 vg with untreated ("un"; negative) and WT ("HEPG2"; positive) controls, and B) quantitation of % WT protein expression for each dosing group and control).

15.2.2.1.2 *IN VIVO* PRIMARY PHARMACODYNAMICS

As it is hypothesized that the ability of PA patients to oxidize propionic acid *in vivo* will exhibit significant associations with residual enzymatic function of propionyl-CoA carboxylase, efficacy and PoC endpoints for the *Pcca*$^{-/-}$ mouse studies included metabolic measures, such as plasma 2-MC levels, PCC enzyme activity and 1-^{13}C-propionate oxidation. Additional measures included mouse survival by dosing cohort, as well as expression of PCCA protein and *PCCA* transgene-derived mRNA in dosed mouse liver and heart tissue compared to positive and negative controls.

15.2.2.1.2.1 Justification/Generation of Species

As described previously in the INTERACT meeting briefing package, early mouse models of PA were found to be too fragile for utilization in PD studies [4, 5]. As such, the National Human Genome Research Institute (NHGRI) utilized CRISPR/Cas9 gene editing to directly engineer the Pcca$^{p.Q133LfsX41}$ mutation in Exon 5 of a mouse *Pcca* gene, thus mimicking the human *PCCA*-related PA phenotype. These mice have been studied previously and were shown to mimic symptoms of pediatric PA [6].

PA is well-recognized to be inherited in an autosomal recessive fashion, hence the use of *Pcca*$^{-/-}$ mice as a disease model. Additionally, as part of the NHGRI Natural History (NH) clinical study (NCT02890342), clinical data through history on many parents of affected patients has been obtained and, as with mice, this disorder is inherited in an autosomal recessive fashion.

Pcca$^{-/-}$ mice were created in a research setting at NHGRI using FvB/N background mice obtained from The Jackson Laboratory. All reagents used to generate these mice were research grade, obtained from IDT. Off-target effects of the CRISPR/Cas9-based mutation was assessed via computation and by subsequent breeding to characterize the resulting homozygous mutant (*Pcca*$^{-/-}$) compared to a preexisting deletion allele. The mice have

a pure metabolic phenotype and as such no off-target analysis was performed due to the uniform phenotype and lack of additional phenotypic features, such as malformations or developmental disorders in the homozygous mice.

Briefly, DESKGEN was used to identify protospacer-adjacent motif sequences from the *Streptococcus pyogenes* (SpyCas9) targeting Exon 5 of the *Pcca* gene for mutagenesis. A synthetic single-guide RNA (sgRNA) was custom synthesized by Horizon Discovery (Edit-R predesigned synthetic sgRNA, Horizon Discovery). Zygotes were co-injected with the Cas9 mRNA and sgRNA and cultured at 37°C under 5% CO_2 until the blastocyst stage then transferred into the uterus of pseudo-pregnant FvBN females. These manipulations were performed by the NHGRI mouse and embryonic stem cell core facility.

After embryo injection, FvBN F0 animals were screened and those that carried the mutation *Pcca*$^{c.398\_401delAAGC}$ (PCCA$^{p.Gln133Leufs*41}$) were crossed with WT FvBN mice to generate F1 carriers, which were intercrossed to generate homozygous *Pcca*$^{c.398_401delAAGC/c.398_401delAAGC}$ mice, designated *Pcca*$^{-/-}$. *Pcca*$^{-/-}$ mice lack immunoreactive PCCA and perish in the immediate neonatal period. Male *Pcca*$^{-/-}$ mice rescued by AAV9 gene delivery were bred with *Pcca*$^{+/-}$ females to generate the animals used to study AAV9-hPCCA (10L) and AAV9-hPCCA (50L). Breeder male *Pcca*$^{-/-}$ mice were not included in the experimental treatment cohorts.

Since all mice are treated in the neonatal period and are generated by AAV-rescued homozygous male crossed to *Pcca*$^{+/-}$ females, 50% of the treated animals are *Pcca*$^{+/-}$ heterozygous mice. Due to their inherent fragility, all mice (homozygous recessive and heterozygous) are treated prior to genotyping and sex determination, and as such results are reported for both genotypes. However, it is the *Pcca*$^{-/-}$ mice which are utilized to best recapitulate the clinical manifestation of *PCCA*-related PA.

Pre-planned cohort sizes were not utilized in the initial PoC *in vivo* studies, because the experimental animals were generated by mating treated mutant males to carrier females and treating the affected animals on day one of life. Availability of breeders was the main limiting factor in determining the number of mice available for each group. Mothers produce different size litters, and it is unpredictable how many mice will be born, and further what the sex distribution will be.

Both *Pcca*$^{-/-}$ mouse pups and age-matched WT mouse pups will be bred as described above and used in the proposed pivotal efficacy study. This study will also have select toxicity endpoints, and in addition to PoC objectives seeks to support the dosing of a clinical pediatric population.

15.2.2.1.2.2 Dose Level Justification

The relevance of the doses is related to studies that have previously defined effective neonatal rescue of lethal metabolic phenotypes at or around 1E11 and 4E11 vg/pup. This is based on another animal model of a related disorder, methylmalonic acidemia [7, 8]. Therefore, studies in the PCCA mouse model were initiated with a dose of 1E11 vg/pup based on this precedent and proceeded to deescalate by logs in the *Pcca*$^{-/-}$ mice to assess differences between the 1E11 vg/pup dose (approximately 7E13 vg/kg) and the 1E10 vg/pup dose (7E12 vg/kg). The 4E11 vg/pup cohort was added to determine whether an increase in efficacy could be noted. Benefit was shown relative to lower doses, but this dose projection for humans (4E14 vg/kg) exceeds doses where toxic effects have been seen in patients treated with other disorders, such as spinal muscular atrophy [9]. Finally, the sub-therapeutic dose of 1E9 vg/pup was explored (7E11 vg/kg) to determine whether any signal of efficacy could be detected.

15.2.2.1.2.3 Justification of Measures

Clinical studies have demonstrated the utility of the measurement of *[disease related biomarker]* as a biomarker in patients with PA, and gene therapy studies where mice were treated with variable AAV doses showed this biochemical response [10, 11]. To date, the preclinical development program and available real-world data have not identified a "percent wild-type" expression that may lead to rescue, but based on the RNAScope images provided (please see included PoC study reports), it is suggested that even 5-10% transduction produces an appreciable and potentially clinically relevant effect. It is also noted that the vector used in studies described herein exhibits robust expression in cardiomyocytes. Further study development may lead to additional prospective quantitation of desired efficacy marker values.

Further clinical relevance of sampling liver and heart tissue is related to PA patients being routinely treated using elective LT to provide metabolic stability and mitigate biomarker changes, including propensity towards hyperammonemia [12-14]. The heart is also affected in patients (cardiomyopathy) and is therefore an important target in gene therapy studies of this disorder. Enzyme activity measurements are significant in the liver and the heart because these tissues are where substantial and potentially lethal pathology develops in patients. With restoration of hepatic PCC enzyme activity, it is believed the mice (and patients) will have improved metabolic function and the ability to oxidize propionyl-CoA. Further, the restoration of PCC activity in the cardiomyocytes could protect mice (and patients) from developing cardiomyopathy. For these reasons, the liver and heart were studied extensively in the preclinical development program [15-17].

15.2.2.1.2.4 NHGRI-PCCA-001 Study

This PoC efficacy study served to gauge survival and biomarker response in *Pcca*$^{-/-}$ mouse pups rescued by varying dosing strengths of AAV9-hPCCA manufactured by the University of Massachusetts Medical School and Vigene. Please refer to the Study Report NHGRI-PCCA-001 for full details.

15.2.2.1.2.5 NHGRI-PCCA-002 Study

This PoC efficacy study served to gauge survival and biomarker response in *Pcca*$^{-/-}$ mouse pups rescued by varying dosing strengths of AAV9-hPCCA manufactured by CRL across multiple batches (denoted as "10L" and "50L" batches). Please refer to the Study Report NHGRI-PCCA-002 for full details.

15.2.2.1.2.6 Proposed Pivotal Efficacy Study (*[protocol number]*)

A non-GLP, pivotal efficacy/safety study in *Pcca*$^{-/-}$ mouse pups and WT mouse pups using the IP from CRL, manufactured under 50L non-GMP conditions, is planned subsequent to this meeting. This efficacy study will also include select toxicity (clinical pathology and histopathology endpoints). Please see Protocol *[protocol number]* for the proposed study design.

15.2.2.2 Analytical Methods / Method Development

Please refer to Section 6.2 of both Study Report NHGRI-PCCA-001 and Study Report NHGRI-PCCA-002 for a description of measurements and assessments.

Table 6 shows assays that will be replicated in the pivotal efficacy study. Most of these were used in the NHGRI-PCCA-001 and NHGRI-PCCA-002 studies.

Table 6. Analytical method consistency across preclinical efficacy studies

Assay Title	Vendor for the Efficacy study	Validation
Determination of 2-Methylcitrate and Propionyl-L-Carnitine in Mouse Plasma using LC-MS/MS	*[name of contractor]*	Will be qualified
PCC Enzymatic Activity Assay	*[name of contractor]* (Assay method has been developed by NCATS, and description is included in Study Report NHGRI-PCCA-002)	Will be qualified
Quantitative Determination of AAV9 PCCA in Mouse Tissues by qPCR	*[name of contractor]*	Will be qualified
Quantitative Determination of AAV9 PCCA mRNA in Mouse Tissues by RT-qPCR	*[name of contractor]*	Will be qualified

15.2.2.3 Pharmacology Conclusions

A single retro-orbital sinus injection of AAV9-hPCCA, across multiple batches of DP, was found to increase survival duration (significantly and dose-dependently) of *Pcca*$^{-/-}$ mouse pups, thereby informing study doses for subsequent preclinical and clinical development. 2-MC, a plasma biomarker of PA, was also found to be reduced in treated pups (as compared to untreated controls), and *in vivo* 1-13C-propionate oxidation showed similar levels between untreated WT mice and treated *Pcca*$^{-/-}$mice.

15.2.3 Biodistribution

Efficacy studies NHGRI-PCCA-001, NHGRI-PCCA-002 and Protocol *[protocol number]* are designed to examine endpoints of biodistribution. The proposed single-dose, GLP 6M Toxicology Study is also prospectively designed to assess biodistribution across multiple tissues at four distinct doses and time points.

15.2.3.1 NHGRI-PCCA-001 Study

This study examined biodistribution of the AAV9-hPCCA DP in heart and liver tissue of treated *Pcca*$^{-/-}$ mouse pups (and heterozygous pups) via *PCCA* transgene expression, *PCCA* mRNA expression, and PCCA protein levels. Please refer to the Study Report NHGRI-PCCA-001 for full details.

15.2.3.2 NHGRI-PCCA-002 Study

This study examined biodistribution of the AAV9-hPCCA DP in heart and liver tissue of treated *Pcca*$^{-/-}$ mouse pups (and heterozygous pups) using multiple batches of DP via *PCCA* mRNA expression and PCCA protein levels. Please refer to the Study Report NHGRI-PCCA-002 for full details.

15.2.3.3 Protocol *[protocol number]*

Six *Pcca*$^{-/-}$ mouse pups (3M/3F) and six wild-type mouse pups (3M/3F) in the low dose and high dose cohorts sacrificed at Days 7, 30 and 90 will be assessed for biodistribution measures. An additional six wild-type mouse pups (3M/3F) in the vehicle dosing group sacrificed at each time point will be used as controls. Tissues assessed will include brain, gonad, heart, eye with optic nerve, kidneys, liver, lungs, spleen, stomach, and skeletal muscle (quadriceps femoris). Please refer to Protocol *[protocol number]* for additional detail.

15.2.3.4 GLP 6-Month Toxicity Study with Biodistribution

Ten (10) C57BL6 mice apiece (5M/5F) across four dosing groups (one vehicle control, and three doses of AAV9-hPCCA) and four sacrifice time points (Day 7, and Months 1, 3 and 6) will be designated for evaluation of biodistribution and hematology. qPCR and RT-PCR methods for biodistribution will be used and the following tissues will be collected from all animals at necropsy: blood, injection site, heart, brain, liver, lung, spleen, kidneys, and ovary/testis. Please see the proposed GLP 6M Toxicity Study Protocol for additional detail.

15.2.3.5 Analytical Methods

Please refer to Section 6.2 of both Study Report NHGRI-PCCA-001 and Study Report NHGRI-PCCA-002 for a description of measurements and assessments.

15.2.3.6 Biodistribution Conclusions

Sentinel efforts suggest AAV9-hPCCA DP uptake in the hearts and livers of disease model mice. The pivotal efficacy and/or toxicity studies will further examine the biodistribution profile of AAV9-hPCCA.

15.2.4 Toxicology

As AAV9-hPCCA is proposed as a single clinical administration, the planned GLP single-dose toxicity study will function as the pivotal preclinical study. The proposed pivotal efficacy study in *Pcca*$^{-/-}$ mouse pups and WT mouse pups will also examine select safety endpoints.

15.2.4.1 GLP 6-Month Toxicity Study with Biodistribution

Twenty (20) C57BL6 mice apiece (10M/10F) across four dosing groups (one vehicle control, and three doses of AAV9-hPCCA) and four sacrifice time points (Day 7, and Months 1, 3 and 6) will be assessed for clinical signs, body weight, food consumption, functional observation, and clinical pathology (day of sacrifice, prior to sacrifice). Following sacrifice, animals will be necropsied and assessed for immunogenicity. Anti-AAV9 and anti-PCCA antibodies will be determined the day of necropsies, and spleen samples will be processed for T-cell responses to AAV9 and PCCA by interferon-gamma (IFN-γ) ELISPOT assay. Please see the proposed GLP 6M Toxicity Study Protocol for additional detail.

15.2.4.1.1 JUSTIFICATION OF SPECIES

WT mice are being utilized instead of disease model mice because it is not feasible to perform all proposed measures on a model with a 1–2-day lifespan.

15.2.4.1.2 DOSE LEVEL JUSTIFICATION

Doses were selected based on results from the PoC studies (where potential efficacy was demonstrated), with the maximum feasible dose determined based on the AAV concentration of the DP and maximum volume to be injected per animal. The proposed high dose, 3E14 vg/kg/dose, represents a *[XX]* safety margin over the proposed clinical high dose (*[higher dose]*), and a *[XX]* safety margin over the proposed clinical starting dose (*[starting dose]*). Similar preclinical doses have been observed in the development of a marketed AAV9 gene therapy, Zolgensma, and the Sponsor's proposed FIH high dose is lower than what was observed in Zolgensma's clinical development program [9].

15.2.4.2 Proposed Pivotal Efficacy Study (*[protocol number]*)

This efficacy study will include select toxicity (clinical pathology and histopathology endpoints). Please see Protocol *[protocol number]* for the proposed study design.

15.2.5 Justification for AAV-hPCCA Routes of Administration

Clinical delivery of AAV-hPCCA will be systemic, proposed as intravenous route of administration. For *Pcca*$^{-/-}$ neonatal mouse pups, tail vein injections are not possible on the first day following birth, and facial vein injections could only be accomplished on day two following birth (at the NHGRI lab facility), at which point untreated pups would be dead. As such, injection via retro-orbital sinus plexus to the systemic circulation was used to best recapitulate the anticipated route of administration in humans. In the pivotal efficacy study, facial vein injection will be the route of administration and will be dosed within few hours of birth, as feasible.

C57BL/6 mice, to be used in the GLP toxicity study, will receive AAV-hPCCA via slow bolus tail vein injection (over approximately 30 – 60 seconds) to best mimic proposed clinical administration.

15.2.6 Device, Administration and Endpoint Consistency

15.2.6.1 Pivotal Efficacy Study

Mouse pups will receive a temporal vein (facial) injection via an insulin syringe attached to a 30- or 31-gauage needle. Given their fragility, the proposed clinical duration of administration is not able to be recapitulated in the disease model mouse pup. As noted in previous sections, this injection will provide for systemic release of the AAV9-hPCCA DP. Please refer to the document entitled "*[Protocol number]:* Testing of compatibility, in-use, and stability of the gene therapy product AAV9-hPCCA" for additional information on devices to be used in this study, as well as the FIH clinical trial.

Metabolite *[disease related biomarkers]* (where possible), serum chemistry and hematology measures also mirror those to be performed in the proposed clinical trial.

15.2.6.2 6M GLP Toxicity/Biodistribution Study

Please refer to **Table 7** for a comparison of key parameters for the proposed toxicity study and FIH clinical trial. Please refer to the document entitled "*[Protocol number]*: Testing of compatibility, in-use, and stability of the gene therapy product AAV9-hPCCA" for additional device and container closure description for the FIH clinical trial.

Table 7. Parameter comparison between the GLP toxicity study and the FIH clinical trial

Parameter	GLP Toxicity Study	FIH Clinical Trial
Route of Administration	Tail vein injection	Peripheral IV or Peripherally-implanted central catheter
Vector Concentration (in DP)	5E13 vg/mL	5E13 vg/mL
Infusion Volume	Volume will vary depending on the mass of the mouse and the dosing cohort	This will be a range of up to 100 mL over participants, dependent upon subject mass and dosing cohort
Flow Rate	Slow bolus over 30-60 seconds, based on vendor's SOP	Administration over 30-60 minutes
Administration Device	BD Insulin Syringes with 3/10 mL capacity and 30G needle	PICC line and PIV, with syringe pump (Carefusion, Alaris PC unit, Model 8015 pump with the Alaris modules)
Biodistribution Profile	Systemic (tissues directly assessed)	Systemic (surrogate biomarkers assessed)

Mice in the toxicity study will also be followed for clinical pathology (hematology and clinical chemistry), T-cell response (to both AAV9 and PCCA) and anti-drug antibody and anti-PCCA antibody determinations.

15.3 Clinical

15.3.1 Introduction

PA is an IEM resulting from deleterious variants in the *PCCA* or *PCCB* genes leading to impaired activity of PCC. PCC is a ubiquitously expressed mitochondrial enzyme whose function is closely linked to energy metabolism. The clinical course of PA is characterized by chronic multiorgan dysfunction punctuated by episodes of metabolic instability due to metabolic acidosis, ketonuria, hyperammonemia, and hypoglycemia leading to emergency room visits and hospitalizations. Most patients with PA present in the newborn period with poor feeding, vomiting, irritability and lethargy. Without treatment, these infants may develop neonatal encephalopathy, seizures, coma, and respiratory failure, which if left untreated can result in death. Universal newborn screening implemented in the United States (US) can identify most affected infants.

PA is a rare disorder with birth prevalence varying widely by region. Published literature suggests PA is an ultra-rare disease affecting ~1 in 243,000 individuals in the US with approximately half due to variants in the *PCCA* gene [18, 19]. Based on birth rate and incidence data, it can be estimated there are ~50 *PCCA*-type PA patients living in the US

as detailed in prior applications for Orphan Drug and Rare Pediatric Disease designations.

There is no cure for PA. Current standards of care include strict adherence to low-protein diet with and without poorly palatable medical foods, supplementation with levocarnitine and carglumic acid for acute hyperammonemia. Other treatments may apply if patients develop severe complications of PA, for example the use of cardiac medications in patients with cardiomyopathy or antiepileptic drugs in PA patients with epilepsy [1]. Some PA patients, who experience frequent hospitalizations due to metabolic instability and/or who develop dilated cardiomyopathy may undergo a liver or a combined liver-kidney or heart transplantation and require life-long immunosuppression.

15.3.2 PA Natural History Study

The NHGRI is conducting a natural history (NH) study, "The Natural History, Physiology, Microbiome and Biochemistry Studies of Propionic Acidemia" (*[Protocol number];* NCT02890342), with *[name of principal investigator]* as the Principal Investigator (this is in addition to a nearly 20 year-long NH protocol for methylmalonic acidemia, NCT00078078). The propionic acidemia protocol is included as a separate document within the briefing package and is described herein. Briefly, this study seeks to better understand PA patient outcomes and their associations with clinical, pharmacological, laboratory and dietary factors. Additionally, the study assesses the patients' *in vivo* oxidation of propionic acid, and its association with residual enzymatic function of PCC, propiogenic gut microflora, and transplantation status.

Individuals with PA are evaluated at the NIH Clinical Center over a week and return every 1-3 years to undergo standardized, deep phenotyping studies, including clinical (genetics, cardiology, neurology, neuropsychology, ophthalmology, audiology, rehabilitation medicine, nephrology, endocrine, ob/gyn), biochemical, nutritional, imaging, and laboratory evaluations. Resting energy expenditure (REE) is measured using indirect calorimetry. Body composition is evaluated using whole-body dual energy X-ray absorptiometry (DXA), and data are adjusted for age and sex. Study participants undergo a thorough cardiac phenotyping, including echocardiography, EKG and 24-hour Holter evaluation.

Neurological outcomes are documented with the help of clinical evaluation, brain MRI and MRS, and neurocognitive testing (full-scale IQ and adaptive scoring). Standardized ophthalmology evaluation is supplemented by optical coherence tomography to evaluate participant's optic nerve thickness, if clinically indicated. The severity of PCC deficiency is evaluated using the non-invasive *in vivo* whole-body *[disease related biomarker assay]* calculated as a percent of *[disease related biomarker]* in 1 to 2 hours after a single enteral dose. Past medical records are obtained and recorded in detail to allow retrospective analysis of laboratory trends, number of hospitalizations for metabolic decompensations, hyperammonemic or pancreatitis episodes, dietary information, growth charts, and other relevant medical history.

15.3.2.1 Data Collection / Uniformity for NH Study Participants with *PCCA*-Related PA

A dedicated group of associate investigators at the NIH Clinical Center conduct all protocol evaluations and interpret results of laboratory, imaging, cardiac, neurocognitive and other testing, allowing for standardized and consistent data collection and analysis. The PA and methyl malonic acidemia studies are the largest single-center cohorts of OA. In addition, the same group of experienced investigators will evaluate patients enrolled in the proposed gene therapy clinical trial. This, combined with a 1-3 month screening period for each participant for repeat assessments of study biomarkers prior to dosing in the clinical trial will allow for reliable comparisons of treatment effects.

Notably, efforts derived from the NH history study summarize both *PCCA*- and *PCCB*-related PA, and included patients of variable ages and severities of PA who may not meet the inclusion/exclusion criteria for the proposed FIH clinical trial. As such efforts were undertaken to provide additional data specificity. To date, the study has enrolled *[XX]* individuals with PA, including a subset who have undergone organ transplantation.

A summary table (**Table 8**) and representative graphs are provided to illustrate the range of data available in the NH study, supporting its use as a comparator dataset in the proposed AAV9 PCCA gene therapy clinical trial. Data has been sorted to segregate participants with *PCCA*- and *PCCB*-related PA, as well as to distinguish between cohorts who have and have not undergone organ transplant. Data is inclusive of proposed primary efficacy endpoints for the proposed AAV9-hPCCA gene therapy program, along with certain exploratory endpoints.

Table 8. ***[Clinical and biochemical summary data from the NIH propionic acidemia protocol]***

Continuing, *in vivo* POBT has been previously defined as one of the most impactful variables associated with PA severity. **Figure 7** shows the correlations of enzymatic activity as assessed by POBT with clinical parameters and biomarkers in the non-transplanted *PCCA*-only cohort, as well as the age ranges of *PCCA* and *PCCB* patients with or without an organ transplant. Representative statistically significant correlations are presented from data collected via a series of standardized outcome measures in the NH study cohort, with additional data continuing to be collected and assessed, thus forming the foundation for the NH study to serve as the direct comparator for the proposed clinical trial.

Figure 7. ***[Correlations between proposed assay and disease related clinical parameters]***

15.3.2.2 PA Biomarker Development

Previously, results generated in the NH study were aggregated and analyzed in an effort to discern key biomarkers related to the clinical progression of PA and potential measures of disease improvement [10]. By combining knowledge of the NHGRI subject matter experts with supervised machine learning using support vector machine with a polynomial kernel function, it was determined that severity biomarkers of PA could be effectively evaluated as potential tools in a clinical trial development

program.

Briefly and as described previously in support of the INTERACT meeting, it was determined that *[disease specific biomarker]* closely correlated with established diagnostic biomarkers *[biomarker names],* as shown in **Figure 8**, as well as clinically relevant outcomes *[clinical endpoints]*. Additionally, this *[biomarker assay*] does not depend on the renal function (see Cystatin C-based eGFR graph).

Figure 8. ***[Correlation between proposed biomarker assay and diagnostic biomarkers]***

Additionally, and as shown in **Figure 9,** select established and novel biomarkers correlate to PA disease severity, across the NH cohort (all PA types). Measurements in the liver transplant subgroup showed values in the range of the milder PA patients for *[several disease related biomarkers]*. Importantly, *[a specific biomarker]* results showed no overlap in values between severe PA participants and PA after LT

Figure 9. ***[Levels of proposed disease related biomarkers in patients with mild or severe symptoms and patients who have undergone liver transplant]***

As noted in prior sections of this document, there is no approved treatment for PA disease (recently approved Carbaglu is indicated for hyperammonemia in PA), and as such elective LT has been viewed as the terminal option for restoration of metabolic stability and protection from early death. However, it is noted that LT does not guarantee restoration of clinical function. NH data from UK and French cohorts noted that seven of 12 pediatric PA patients (58%) died within the first year after LT. Further, out of 17 LT procedures followed, 13 had early and severe complications [13], and as such restoring function while obviating the need for LT represents an overarching clinical goal of the proposed AAV9-hPCCA program. Coupled with the results of the PoC measures in the preclinical development program, change in *[two disease-specific biomarkers]* are proposed as primary endpoint measures of potential efficacy. Secondary and exploratory endpoints will also include additional biomarker measures, along with subjective measures *[endpoints]*.

15.3.2.2.1 *[Disease related biomarker]* MEASUREMENT

The *[disease related biomarker assay]* in the NH study, and proposed for the FIH clinical trial utilizes the Exalenz BreathID device, a 510(k)-cleared device (K130524), a non-invasive breath test system for the detection of *Helicobacter pylori* (*H. pylori*). Though the Sponsor intends to leverage device outputs as a surrogate for biomarker unrelated to *H. pylori*, it is believed the device's utilization falls within its intended use to "continually and non-invasively measure changes in *[disease related biomarker]* ratio of exhaled breath" [20]. Previous clinical use of this device was performed in a NH study for a related methylmalonic acidemia (MMA) patient population (NCT00078078). In this study, 83 breath test measures were performed across *[XX]* MMA patient participants, along with measures for a control arm comprised of healthy adult volunteers and MMA heterozygote parents. Results described the method for and

clinical feasibility of the measure with the Exalenz device, along with the correlation of *[biomarker assay]* with MMA disease severity and clinical outcomes [21]. This Exalenz device was also used in the PA NH study [10].

Measurement of this biomarker was performed preclinically in the NHGRI-PCCA-002 study and is described in the report provided as part of this briefing package. It is also proposed for the pivotal preclinical efficacy study, though it is noted that due to the difficulty level of the measurement in disease model mouse pups it may not be feasible. The pivotal efficacy study protocol *[protocol number]* describes the method for CO_2 collection, which includes collection of expired breath into airtight containers with CO_2 probes, followed by ^{13}C measurement in an aliquot of the expired air.

Additional information on the utilization of this device and its proposed clinical utility will be provided in the IND. Development of the Exalenz device as an *in vitro* diagnostic will be considered as the Sponsor gains additional information from the clinical trial.

15.3.2.2.2 *[Disease-related biomarker assay]*

The *[disease-related biomarker]* measurement is performed in the NH study. This measurement was also performed in both studies NHGRI-PCCA-001 and NHGRI-PCCA-002 and is proposed for the pivotal efficacy study. To date, collected serum samples in completed and ongoing studies were sent to the University of Colorado for analysis, in accordance with the previously described method [22]. Briefly, labeled *[disease-related biomarker]* is added to samples in a known amount, followed by extraction, evaporation and silylation. Prepared samples are analyzed by gas chromatography/mass spectrometry with a mass selective detector.

For the proposed FIH clinical trial, samples will be sent to a CLIA-certified laboratory within the Mayo Clinic Laboratories. Analogously, measures from the parallel NH study will also be analyzed at the Mayo Clinic Laboratories, utilizing the same method. A description of the method is included as part of this package. The method description includes multiple mitochondrial metabolites but will be used primarily for *[disease-specific biomarker]* in support of the FIH trial.

Development of the *[disease-specific biomarker]* test as an *in vitro* diagnostic will be considered as the Sponsor gains additional information from the clinical trial.

15.3.2.3 Observation of Patients with PCCA-Related PA

In their preliminary INTERACT commentary, the FDA noted that "…*given the rarity of this disease, we recommend that you also include an appropriate primary efficacy endpoint that reflects how a patient with PA will feel, function, or survive if they benefit from therapy with your product*." Importantly, the NHGRI investigatory staff on the NH study will be the same as those proposed for the FIH clinical trial. As such they will have intimate knowledge of the patients' disease lifecycles, and are best-positioned to subjectively assess potential clinical benefit following AAV9-hPCCA treatment. This includes practical measures of clinical meaningfulness, such as the *[primary*

efficacy endpoint] including but not limited to:

The following will be recorded at scheduled protocol visits in the study eCRFs:

- Annualized frequency of hospitalizations, reasons for admission, severity of metabolic acidosis and/or hyperammonemia, and number of days the subject spends in the hospital.
- Evaluation of diet and growth.
- Need for modification to a sick-day (low protein and high carbohydrate/fat diet), oral vs tube feeding ratio, complete protein vs propiogenic precursor-free formula.
- Disease progression: Decline in cardiac and renal function and other major clinical events, including pancreatitis, metabolic stroke, optic nerve injury, hearing loss will be monitored and recorded.
- Vineland Adaptive Behavior Scales-3nd Edition will be administered under the supervision of a psychologist. This is a semi-structured caregiver interview designed to evaluate adaptive functioning in four domains: Communication, Daily Living Skills, Socialization, and Motor Skills.
- Peds neuroQOL and Quality of Life Inventory-completed by the patient or proxy will be recorded annually.
- Caregiver questionnaires will be employed to capture changes in functioning.

As noted previously, secondary and exploratory endpoints will also include subjective measures (e.g., patient and caretaker reported outcomes) of quality of life.

15.3.3 Proposed Phase I/II FIH Clinical Trial

In their preliminary INTERACT commentary, Agency respondents noted:

"*Due to rarity of PA and the substantial unmet medical need, we recommend that this early phase clinical trial should be adequately designed to demonstrate preliminary effectiveness to support marketing approval. To achieve this goal, we recommend that you modify your study design to employ a randomized control design, if feasible. If not feasible, please consider a prospective concurrent control with patients on standard of care who are not interested in gene therapy. If you ultimately determine that only a natural history control is feasible, please note that control patients should be as similar as possible to the population that will receive the experimental therapy with respect to disease sub-type, genotype, demographics, baseline functional status, and concomitant therapy with the exception of receiving the IP. Additionally, study observations should be performed using the same methodology and timing in the controls and the experimental subjects*."

In seeking responsiveness to Agency commentary, the Sponsor's proposed FIH trial has been redesigned to include a comparator arm, and to incorporate both primary safety and efficacy endpoints.

15.3.3.1 Mechanism of Control

Clinical trials for patients with PA and associated acidemias (e.g., MMA) present many challenges, including but not limited to the rarity of *PCCA*-related PA, heterogeneity of clinical manifestation, prior therapies utilized (or elective LT), and selection of both comparator arms and suitable biomarkers [23]. Continuing, given the severity of disease progression, and the massive unmet clinical need, a randomized, controlled trial (RCT) design would be infeasible and unethical. Further, per discussion members of PA patient advocacy groups such as the Organic Acidemia Association and the Propionic Acidemia Foundation may not be receptive RCT design.

Correspondingly the Sponsor proposes a comparator group from the NIH's NH study in PA, described in the sections above. The Sponsor intends to leverage both historic and ongoing data from patients with *PCCA*-related PA, agnostic to whether they enroll into the FIH trial. Specifically, the Sponsor proposes the following potential mechanisms of control for the Agency's consideration:

- **Nonconcurrent/concurrent aggregate external data**: The NH study both predates and will run parallel to the proposed clinical trial. This allows for both historical and concurrent data to be utilized in aggregate, as relevant clinical parameters (primary efficacy and exploratory endpoints) have been and will continue to be accounted for, allowing for direct contrast of disease biomarkers. Comparator data will include that from participants who enroll into and are dosed in the clinical trial (nonconcurrent), as well as those who do not enroll into the clinical trial (nonconcurrent and concurrent).
    - Additional stratification factors for this cohort will include whether NH patients have received LT, and also the severity of *PCCA*-related PA (mild or severe) as defined biochemically.
- **Intra-patient data**: For patients in the NH history study who meet the relevant inclusion/exclusion criteria for the AAV9-hPCCA FIH trial and wish to enroll, there will already be patient-level efficacy biomarker data in hand that will mirror those collected to support the trial's primary and exploratory outcomes (in addition to the data collected during the trial's screening period). As noted previously in this section, the Sponsor also has obtained patients' medical records and recorded relevant medical history, which may serve as a comparator for the trial's proposed secondary endpoint related to healthcare utilization. This intra-patient data may serve to strengthen the aggregate data control and more narrowly focus individual treatment effect.

    In line with the Agency's Draft Guidance '*Rare Diseases: Natural History Studies for Drug Development*' (2019), the Sponsor believes the NH study participants represent a cohort "*very similar to the treated group in all respects, including disease severity, duration of illness, prior treatments, and any other aspects of the disease that could affect outcomes and the timing of outcomes*,"

while accounting for the inherent heterogenous nature of PA. Continuing, bias is viewed as mitigated due to the predictability of the disease course and the potentially dramatic treatment effect of the proposed gene therapy, which is again in line with the Draft Guidance wherein it is noted data is most interpretable when a treatment effect:

- Is large in comparison to potential biases and the known variability in progression.
- Is not affected by patient or investigator motivation or choice of subjects for treatment.
- Can be objectively measured.
- Is measured in a manner that reasonably manages and minimizes bias.
- Has a strong temporal association with administration of the investigational drug.
- Is consistent with expected pharmacological activity based on the target and perhaps shown in animal models.

Given the available NH data and the plans for the NH study to continue data collection, and taking into account the infeasibility of an RCT, the Sponsor believes the proposed represents a sensible solution for an ethical trial that will inform efficacy signals for a future pivotal trial and potentially begin the onset of data collection for a future integrated summary of efficacy supporting a BLA submission.

15.3.3.2 Justification of Population

With respect for the Agency's commentary during the INTERACT meeting, the Sponsor perceives practical and ethical limitations of a study that enrolls a homogeneous patient population and first doses adult participants. PA is an inherently heterogeneous disease given the large number >150 of *PCCA* variants and manifests differently across individual patients. Given the ultra-rarity of the disease, enrollment of a homogeneous population would be infeasible. **Figure 10** depicts a flowchart derived in good faith, starting with the totality of the NH study patient population and applying various study inclusion/exclusion criteria, which sees a reduced patient pool as few as five individuals. Notably, the Sponsor anticipates additional enrollment into the NH study, as well as participation in the clinical trial from patients not enrolled in the NH study.

Figure 10. ***[Flowchart showing the derivation of a feasible FIH patient population]***

Continuing and as described in preceding sections, the overarching clinical goal of the proposed AAV9-hPCCA lifecycle development program is to effectively obviate the need for LT in patients with *PCCA*-related PA by restoring clinically meaningful levels of function (in addition to potential benefit across other organ functions). Many PA patients receive elective liver transplant (LT) early in life, and median ages have been reported for multiple patient cohorts:

- 2.7 years, with a minimum of 0.6 years and maximum of 23.0 years, as reported in a European cohort using questionnaire-based data collection [12]
- 3.2 years, with a minimum of 1.1 years and maximum of 9.0 years, as reported in UK and French cohorts [13]
- 1.9 years, with a minimum of 0.4 years and a maximum 9.4 years, as reported in a retrospective study [14]

This serves to highlight the importance of treating a pediatric population directly in the FIH study, as they would receive the most potential clinical benefit. Because patients with PA who have received a LT do not qualify for the FIH study, the AAV gene therapy represents an alternative to LT.

Compatibly, given the severity of their disease adult patients with PA who have not received elective LT would be less likely to derive clinical benefit, including but not limited to reasons such as already having accumulated potentially irreversible organ damage. Given this, utilization of adults as sentinels could be considered unethical, given the questionable risk/benefit profile.

Given these considerations, the Sponsor proposes to enroll a sentinel adolescent participant into the first study cohort, followed by subsequent age de-escalation to the youngest pediatric age tolerated by the NIH Clinical Center (3 years). If an adolescent participant is not available, the Sponsor will seek to proceed with an eligible participant ≥3 years to <18 years of age, after which the subsequently enrolled participants would be of any age ≥ 3 years. Given the PoC data with disease model mouse pups, and the proposed efficacy and safety data collection in the remainder of the IND-enabling preclinical development program, the Sponsor believes the risk/benefit profile of this proposed enrollment is justified.

Continuing and as previously described, the proposed site investigatory staff is intimately familiar with the patients with PA given they are investigators in the NH study. As such they are uniquely positioned in their knowledge of available study participants and potential clinical trial candidates. Still, the Sponsor does not seek to be exclusionary of participants who may achieve clinical benefit solely on the basis of age, and if an adult participant meeting all other inclusion/exclusion criteria is discovered (e.g., internationally) and interested in enrolling into the FIH clinical trial, this can be accommodated. However, the Sponsor does not intend to delay pediatric enrollment in order to seek out eligible adult participants, as this could lead to an increase in disease severity for eligible pediatric patients.

15.3.3.3 Proposed Clinical Trial Protocol Synopsis

The Sponsor proposes a FIH clinical trial protocol entitled "*A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (PCCA)-Related Propionic Acidemia*." Please refer to the document entitled "Proposed Phase

1/2 Study Protocol Synopsis" contained within this package. Please also refer to the accompanying Proposed Phase 1/2 Study Protocol Summary of Changes, which describes high-level changes to the synopsis made since the previously held INTERACT meeting.

16 REFERENCES

1. Shchelochkov, O.A., N. Carrillo, and C. Venditti, *Propionic Acidemia*, in *GeneReviews(®)*, M.P. Adam, et al., Editors. 1993, University of Washington, Seattle
Copyright © 1993-2023, University of Washington, Seattle. GeneReviews is a registered trademark of the University of Washington, Seattle. All rights reserved.: Seattle (WA).
2. Surtees, R.A., E.E. Matthews, and J.V. Leonard, *Neurologic outcome of propionic acidemia.* Pediatr Neurol, 1992. **8**(5): p. 333-7.
3. Brooks, P.J., et al., *The Platform Vector Gene Therapies Project: Increasing the Efficiency of Adeno-Associated Virus Gene Therapy Clinical Trial Startup.* Hum Gene Ther, 2020. **31**(19-20): p. 1034-1042.
4. Miyazaki, T., et al., *Fatal propionic acidemia in mice lacking propionyl-CoA carboxylase and its rescue by postnatal, liver-specific supplementation via a transgene.* J Biol Chem, 2001. **276**(38): p. 35995-9.
5. Guenzel, A.J., et al., *Generation of a hypomorphic model of propionic acidemia amenable to gene therapy testing.* Mol Ther, 2013. **21**(7): p. 1316-23.
6. Chandler, R.J., et al., *Adeno-associated virus serotype 8 gene transfer rescues a neonatal lethal murine model of propionic acidemia.* Hum Gene Ther, 2011. **22**(4): p. 477-81.
7. Chandler, R.J., et al., *Promoterless, Nuclease-Free Genome Editing Confers a Growth Advantage for Corrected Hepatocytes in Mice With Methylmalonic Acidemia.* Hepatology, 2021. **73**(6): p. 2223-2237.
8. Senac, J.S., et al., *Gene therapy in a murine model of methylmalonic acidemia using rAAV9-mediated gene delivery.* Gene Ther, 2012. **19**(4): p. 385-91.
9. FDA. *ZOLGENSMA®(onasemnogene abeparvovec-xioi) Package Insert*. Available from: https://www.fda.gov/media/126109/download
10. Shchelochkov, O.A., et al., *Severity modeling of propionic acidemia using clinical and laboratory biomarkers.* Genet Med, 2021. **23**(8): p. 1534-1542.
11. Longo, N., et al., *Biomarkers for drug development in propionic and methylmalonic acidemias.* J Inherit Metab Dis, 2022. **45**(2): p. 132-143.
12. Molema, F., et al., *Liver and/or kidney transplantation in amino and organic acid-related inborn errors of metabolism: An overview on*

European data. J Inherit Metab Dis, 2021. **44**(3): p. 593-605.
13. Charbit-Henrion, F., et al., *Early and late complications after liver transplantation for propionic acidemia in children: a two centers study.* Am J Transplant, 2015. **15**(3): p. 786-91.
14. Pillai, N.R., et al., *Liver transplantation in propionic and methylmalonic acidemia: A single center study with literature review.* Mol Genet Metab, 2019. **128**(4): p. 431-443.
15. Chakrapani, A., et al., *Safety, efficacy, and timing of transplantation(s) in propionic and methylmalonic aciduria.* J Inherit Metab Dis, 2023. **46**(3): p. 466-481.
16. Sen, K., et al., *Solid organ transplantation in methylmalonic acidemia and propionic acidemia: A points to consider statement of the American College of Medical Genetics and Genomics (ACMG).* Genet Med, 2023. **25**(2): p. 100337.
17. Vara, R., et al., *Liver transplantation for propionic acidemia in children.* Liver Transpl, 2011. **17**(6): p. 661-7.
18. Almási, T., et al., *Systematic literature review and meta-analysis on the epidemiology of propionic acidemia.* Orphanet J Rare Dis, 2019. **14**(1): p. 40.
19. Chapman, K.A., et al., *Incidence of maple syrup urine disease, propionic acidemia, and methylmalonic aciduria from newborn screening data.* Mol Genet Metab Rep, 2018. **15**: p. 106-109.
20. FDA. *Substantial Equivalence Determination Decision Summary* Available from: https://www.accessdata.fda.gov/cdrh_docs/reviews/K130524.pdf
21. Manoli, I., et al., *1-(13)C-propionate breath testing as a surrogate endpoint to assess efficacy of liver-directed therapies in methylmalonic acidemia (MMA).* Genet Med, 2021. **23**(8): p. 1522-1533.
22. Allen, R.H., et al., *Elevation of 2-methylcitric acid I and II levels in serum, urine, and cerebrospinal fluid of patients with cobalamin deficiency.* Metabolism, 1993. **42**(8): p. 978-88.
23. Vockley, J., et al., *Challenges and strategies for clinical trials in propionic and methylmalonic acidemias.* Mol Genet Metab, 2023. **139**(3): p. 107612.

Study Report

Survival and Biomarker Response in Pcca$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study

Name of Investigational Drug:	AAV9-hPCCA
Study Number:	NHGRI-PCCA-001
Sponsor:	*[sponsor's contact information]*
Study Director:	*[name and designation of study director]*
Testing Facility:	*[name and location of testing facility]*
Study Initiation Date:	March 2020
Study End Date:	February 2023
Date of Report:	26 May 2023

SIGNATURE PAGE

Study Number: NHGRI-PCCA-001

Study Title: Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study

This report accurately describes the data obtained in the study. I have reviewed the study and agree that the data supports the conclusions stated herein:

[signature and date]

[name, designation and contact information of study director]

SYNOPSIS

Name of Sponsor Company: NCATS

Name of Investigational Drug: AAV9-hPCCA

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study
Study Objective:	The objectives of the present study were to: • Assess survival in a mouse model of *PCCA*-related PA (*Pcca*$^{-/-}$ mice) following treatment of escalating dosing cohorts with AAV9-hPCCA (single retro-orbital injection) compared to untreated and PBS-treated *Pcca*$^{-/-}$ mice and AAV9-hPCCA-treated *Pcca*$^{+/-}$ mice (primary). • Assess efficacy of treatment with escalating doses of AAV9-hPCCA in *Pcca*$^{-/-}$ mice and control animals (secondary): o Evaluate propionyl-CoA metabolism by measuring plasma 2-MC levels in AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice when compared to untreated *Pcca*$^{-/-}$ mice at fixed time points post-treatment. o Evaluate *PCCA* messenger RNA (mRNA) and PCCA protein expression in AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice when compared to untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice at fixed time points post-treatment. • Assess AAV9-hPCCA biodistribution following treatment with escalating doses in *Pcca*-/- mice and control animals (biodistribution): o Evaluate AAV9-hPCCA vector copy numbers in the heart and liver of AAV9-hPCCA-treated *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice at fixed time points post-treatment. o Evaluate PCCA protein expression in the heart and liver of AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice when compared to untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice at fixed time points post-treatment.

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study
Investigator and Study Centers:	*[name, designation and affiliation of study director]* Additional Study Centres: **AAV9-hPCCA research grade manufacturing**: • University of Massachusetts, Chan Medical School, Viral Vector Core, 368 Plantation Street, AS6-2024, Worcester, MA 01605; https://www.umassmed.edu/research/cores/viralvectorcore/ • Vigene Biosciences (now Charles River Laboratories; 5 Research Court, Rockville, MD 20850) **Metabolite 2-methylcitrate testing:** University of Colorado, School of Medicine, Division of Hematology, Anemia Metabolite Laboratory https://medschool.cuanschutz.edu/hematology/research/anemia-metabolite-lab **ddPCR**: NIH/NCI Center for Cancer Research (CCR) Genomics Core: https://genomics.ccr.cancer.gov/

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study
Study Design:	On postnatal day (P)0-1, pups that were the result of crossing mice that were heterozygous null for propionyl-coenzyme A (CoA) carboxylase, alpha subunit (*Pcca*$^{+/-}$) received a single retroorbital injection of adeno-associated virus serotype 9 human propionyl- CoA carboxylase, alpha subunit (AAV9-hPCCA) or its vehicle phosphate-buffered saline (PBS), or received no treatment. The genotype of the pups was unknown at the time of injection. Once genotyping was performed, the dosing groups were quantified as follows: *Pcca*$^{-/-}$ mice received 1E10 (N=4), 1E11 (N=28) or 4E11 (N=12) viral genomes (vg) (i.e., 7E12, 7E13 or 2.8E14 vg/kg) of AAV9-hPCCA per pup or PBS (N=11), and *Pcca*$^{+/-}$ mice received either 1E10 (N=3), 1E11 (N=20) or 4E11 (N=5) vg of AAV9-hPCCA per pup (i.e., 7E12, 7E13 or 2.8E14 vg/kg). An additional group of *Pcca*$^{-/-}$ mice were untreated (N=24). Survival was monitored prospectively, and mice were either found dead or euthanized if meeting specific criteria. At Day 30, Day 90 and Day 101 post-injection, blood samples were taken from a subset of AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice and at P1, blood samples were taken from a subset of untreated *Pcca*$^{-/-}$ mice to measure a biomarker response (plasma levels of 2-methylcitrate [2-MC]). A subset of the *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA were sacrificed at Day 30 and Day 101 post-injection, and a subset of the *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA were sacrificed at Day 101 post-injection, and their hearts and livers were collected to measure endogenous murine *Pcca* messenger ribonucleic acid (mRNA), AAV-encoded human *PCCA* (mRNA) and PCCA protein expression and vector biodistribution. In addition, at Day 30 and Day 101 post-injection, a subset of AAV9-hPCCA-treated *Pcca*$^{+/-}$ mice were sacrificed, and their hearts and livers were collected to measure endogenous *Pcca* mRNA, AAV-encoded *PCCA* mRNA and PCCA protein expression and vector biodistribution. Untreated wild-type (WT) mice (N=3: N=2 for mRNA extraction and N=3 for protein extraction), untreated *Pcca*$^{+/-}$ mice (N=3: N=1 for mRNA extraction and N=3 for protein extraction) and untreated *Pcca*$^{-/-}$ mice (N=3, N=1 for mRNA extraction and N=3 for protein extraction) were sacrificed, and their livers were collected to measure PCCA protein levels and *Pcca* and *PCCA* mRNA levels. Hearts were also collected from some of the untreated WT (N=2), *Pcca*$^{+/-}$ (N=2) and *Pcca*$^{-/-}$ mice (N=2) for protein extraction and measurement of PCCA protein levels.
Study Period (months/years):	March 2020 to February 2023

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study
Methods:	After retro-orbital injection of AAV9-hPCCA or PBS on P0-P1 or no treatment administration, survival was monitored and survival curves were generated. Differences in percent survival rates between the groups were compared using a log-rank (Mantel-Cox) test. Data from mice that were sacrificed at planned time points were censored from the statistical analysis. Some animals exhibited signs of morbidity and thus were sacrificed and analyzed at interim timepoints. Plasma levels of 2-MC were measured by gas chromatography–mass spectrometry with stable isotopic internal calibration. Plasma 2-MC levels were compared between untreated *Pcca*$^{-/-}$ mice at the time of birth and AAV9-hPCCA-treated mice at Day 30, Day 90 and Day 101 post-injection using a Student's t test. Endogenous murine *Pcca* and AAV-encoded human *PCCA* mRNA levels were measured in the liver of untreated *Pcca*$^{-/-}$ mice on P1 and *Pcca*$^{-/-}$ mice treated with 1E11 and 4E11 vg/pup (7E13 and 2.8E14 vg/kg) of AAV9-hPCCA at Day 30 and Day 101 post-injection by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and normalized to endogenous beta-actin mRNA levels. *Pcca* and *PCCA* mRNA levels were measured in the liver of untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice by RT-qPCR and normalized to endogenous beta-actin mRNA levels. PCCA protein levels were measured in the liver of untreated *Pcca*$^{-/-}$ mice at P1, untreated WT and *Pcca*$^{+/-}$ mice, and *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice treated with 1E11 and 4E11 vg/pup (7E13 and 2.8E14 vg/kg) of AAV9-hPCCA at Day 30 and Day 101 post-injection by western blotting and normalized to endogenous beta-actin protein levels. PCCA protein levels were measured in the heart of untreated *Pcca*$^{-/-}$ mice at P1, untreated WT and *Pcca*$^{+/-}$ mice, and *Pcca*$^{-/-}$ mice treated with 1E11 and 4E11 vg/pup (7E13 and 2.8E14 vg/kg) of AAV9-hPCCA at Day 101 post-injection by western blotting and normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels. Vector copy numbers of the *PCCA* transgene in the heart and liver of *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice treated with 1E11 and 4E11 vg/pup (7E13 and 2.8E14 vg/kg) of AAV9-hPCCA were measured at Day 30 ($1x10^{11}$ vg dose) and Day 101 (1E11 vg/pup [7E13 vg/kg] and 4E11 vg/pup [2.8E14 vg/kg] doses) post-injection by digital droplet polymerase chain reaction (ddPCR) and normalized to the number of endogenous *Gapdh* alleles.
Animals:	79 *Pcca*$^{-/-}$ mice, 28 *Pcca*$^{+/-}$ mice, and 3 WT mice
Test Articles:	AAV9-hPCCA, 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg), single retro-orbital injection, and PBS buffer control.

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study
Results:	Retro-orbital injection of AAV9-hPCCA significantly and dose-dependently prolonged the survival of *Pcca*$^{-/-}$ mice at all 3 tested doses (Figure 2). *Pcca*$^{-/-}$ mice treated with 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA had mean survival times of ~41, ~55.9 and > 112.5 days, respectively, compared to untreated and PBS-treated *Pcca*$^{-/-}$ mice, which died shortly after birth (AAV9-hPCCA-treated vs. untreated *Pcca*$^{-/-}$ mice: $p<0.005$ for the 1E10 vg/pup [7E12 vg/kg] dose and $p<0.0001$ for the 1E11 and 4E11 vg/pup [7E13 and 2.8E14 vg/kg] doses). In the highest AAV9-hPCCA dose group (4E11 vg/pup [2.8E14 vg/kg]), the last mouse survived up to ~370 days post-injection. Treatment of *Pcca*$^{-/-}$ mice with 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA significantly and dose-dependently decreased mean±standard deviation (SD) plasma levels of 2-MC at Day 30 (20.36±7.54, 8.52±3.85 and 8.17±2.47 µM, respectively), Day 90 (19.27±4.74 µM for the 1E11 vg/pup [7E13 vg/kg] dose and 13.13±2.89 µM for the 4E11 vg/pup [2.8E14 vg/kg] dose) and Day 101 (17.83±5.98 µM for the 1E11 vg/pup [7E13 vg/kg] dose and 15.72±2.58 µM for the 4E11 vg/pup [2.8E14 vg/kg] dose) post-injection when compared to untreated *Pcca*$^{-/-}$ mice (51.20±3.03 µM, $p<0.01$) (Figure 3). No *Pcca*$^{-/-}$ mice in the 1E10 vg/pup (7E12 vg/kg) dose group survived at Day 90, and the effects observed in the 1E11 vg/pup (7E13 vg/kg) and 4E11 vg/pup (2.8E14 vg/kg) groups were lesser at Day 90 and Day 101 post-injection than at Day 30. Mean±SD *PCCA* mRNA levels measured at Day 30 and Day 101 post-injection in the livers of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA were 27.4±8.3% and 18.8±3.0%, respectively, of *Pcca* mRNA levels observed in WT mice and mean±SD *PCCA* mRNA levels measured at Day 101 post-injection in the livers of *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA were 14.2±3.4% of the *Pcca* mRNA levels observed in WT mice (Figure 4). Mean±SD PCCA protein levels measured at Day 30 and Day 101 post-injection in the livers of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA were 44.70±4.67% and 40.50±4.53%, respectively, of the PCCA protein levels observed in WT mice and mean±SD PCCA protein levels in the livers of *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA were 45.20±2.44% of the PCCA protein levels observed in WT mice (Figure 4).

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study
Results (continued):	PCCA protein levels in the livers and hearts of *Pcca*$^{+/-}$ mice were lower than the PCCA protein levels observed in WT mice, and PCCA was not detected in untreated *Pcca*$^{-/-}$ mice. PCCA protein expression was detected in the livers of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA at Day 30 post-injection and in the livers and hearts of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) and $4x10^{11}$ vg/pup (2.8E14 vg/kg) of AAV9-hPCCA at Day 101 post-injection (Figure 5). The *PCCA* transgene was detected at Day 30 in the livers and hearts of *Pcca*$^{-/-}$ mice and a *Pcca*$^{+/-}$ mouse treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA and at Day 101 in the livers and hearts of *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) and 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA (Figure 6). *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA had 1.83±0.14 transgene copies at Day 30 and 1.08±0.03 transgene copies at Day 101 in heart tissue, and 0.60±0.35 transgene copies at Day 30 and 0.17±0.06 transgene copies at Day 101 in liver tissue (values are in units of number of copies of *PCCA* transgene adjusted to the number of endogenous *Gapdh* alleles). *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA had 4.05±1.05 transgene copies in heart tissue and 0.35±0.12 transgene copies in liver tissue at Day 101. *Pcca*$^{+/-}$ mice treated with 1E11 vg/pup (7E13vg/kg) of AAV9-hPCCA had 3.00 transgene copies (N=1, no SD) at Day 30 and 0.87±0.38 transgene copies at Day 101 in heart tissue, and 0.50 transgene copies (N=1, no SD) at Day 30 and 0.08±0.08 transgene copies at Day 101 in liver tissue. *Pcca*$^{+/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA had 3.27±0.27 transgene copies in heart tissue and 0.25±0.10 transgene copies in liver tissue at Day 101. The PCCA transgene copy number was thus higher in heart tissue than in liver tissue and was similar between *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice that received the same treatment. At Day 101, the transgene copy number increased with the dose of AAV9-hPCCA. In addition, in the mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA, the transgene copy number decreased over time.

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Pilot Non-GLP Study
Conclusions:	In conclusion: • A single retro-orbital injection of AAV9-hPCCA at 1E10, 1E11 or 4E11 vg per pup (i.e., 7E12, 7E13 or 2.8E14 vg/kg), administered on P1, prolonged survival in *Pcca*$^{-/-}$ mice, a model of PCCA-related PA that has no PCCA immunoreactivity and does not survive the immediate neonatal period. AAV9-hPCCA treatment significantly and dose-dependently increased the length of survival time of the Pcca$^{-/-}$ mice, with mice in the highest dose group surviving up to approximately 370 days post-injection. - Survival data indicate that clinical efficacy may be anticipated starting with the human equivalent of the middle dose, 1E11 vg per pup (7E13 vg/kg). • Administration of AAV9-hPCCA at 1E10, 1E11 or 4E11 vg/pup (i.e., 7E12, 7E13 or 2.8E14 vg/kg) significantly and dose-dependently decreased plasma levels of 2-MC in *Pcca*$^{-/-}$ mice when compared to untreated *Pcca*$^{-/-}$ mice, although the decrease became less marked over time between Day 30, Day 90 and Day 101 post-injection. • Administration of AAV9-hPCCA at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) to *Pcca*$^{-/-}$ mice resulted in lasting expression of the *PCCA* transgene (up to Day 101), with *PCCA* mRNA levels reaching between 15% and 25% of WT *Pcca* mRNA levels, and PCCA protein levels reaching approximately 40% of WT PCCA protein levels in the livers of the treated mice. PCCA protein expression was also observed in the hearts of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) at Day 101 post-injection. • The number of copies of the *PCCA* transgene were higher in the hearts than in the livers of *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice that received AAV9-hPCCA at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg), appeared to slightly decrease between Day 30 and Day 101, and were similar between the *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice.
Funding:	This work is funded by NCATS Cures Acceleration Network and Intramural Research Program of the NHGRI, NIH *[funding/grant award number]*.
Date of Report:	26 May 2023

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ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviation or specialist term	Explanation
AAV	Adeno-associated virus
AAV9	Adeno-associated virus serotype 9
AAV9-hPCCA	Adeno-associated virus serotype 9 human propionyl-CoA carboxylase, alpha subunit
ACUC	Animal Care and Use Committee
Cas9	CRISPR associated protein 9
cDNA	Complementary deoxyribonucleic acid
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
CRM	Cross-reactive material
ddPCR	Digital droplet polymerase chain reaction
DNA	Deoxyribonucleic acid
F	Female
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase gene (mouse)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase protein
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
ITR	Inverted terminal repeat
LT	Liver transplantation
M	Male
MMA	Methylmalonic acidemia
mRNA	Messenger ribonucleic acid
NA	Not applicable
NCATS	National Center for Advancing Translational Sciences
ND	Not determined
ns	Not statistically significant
ori	Origin of replication
qPCR	Quantitative polymerase chain reaction
P	Postnatal day
PA	Propionic acidemia
PBS	Phosphate-buffered saline

Abbreviation or specialist term	Explanation
PCC	Propionyl-CoA carboxylase
Pcca	Propionyl-CoA carboxylase, alpha subunit gene (mouse)
PCCA	Propionyl-CoA carboxylase, alpha subunit protein
PCCA	Propionyl-CoA carboxylase, alpha subunit gene (human)
PCCB	Propionyl-CoA carboxylase, beta subunit gene (human)
RNA	Ribonucleic acid
retro-orbital	Retro-orbital
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
UMMS	University of Massachusetts Medical School
vg	Vector genome
WT	Wild type

1 INTRODUCTION

Propionic acidemia (PA) is a well-recognized autosomal recessive disorder of organic acid metabolism in humans with an estimated incidence of 1:250,000-1:750,000 births. It is caused by a deficiency of propionyl-coenzyme A (CoA) carboxylase (PCC), a ubiquitously expressed, heteropolymeric mitochondrial enzyme involved primarily in the catabolism of propiogenic amino acids, particularly isoleucine, valine, methionine, and threonine, as well as odd-chain fatty acids. PCC is composed of alpha and beta subunits encoded by their respective genes, PCC subunit A (*PCCA*) and PCC subunit B (*PCCB*). PCC catalyzes the first step in the conversion of propionyl-CoA to D-methylmalonyl-CoA in the pathway of propionyl-CoA oxidation. PA is caused by mutations in either the *PCCA* or *PCCB* gene.

Most frequently, PA presents in the neonatal period with hyperammonemia, vomiting, poor feeding, and hypotonia and progresses into a life-threatening metabolic crisis. Patients who survive suffer from recurrent metabolic instability and can develop multisystemic complications, including cardiomyopathy. The long-term prognosis for survival in severely affected patients is poor as illustrated by an early and relatively large (for the disease prevalence) single center study of 20 patients with PA treated at a tertiary care center: those who presented in the first week of life (11 patients) largely perished by the age of 6 years [1]. Over the decades, it has been recurrently noted that PA patients with an early and severe clinical course experience increased mortality and disease associated morbidity [2]. There is no curative therapy for PA; the disorder is managed via dietary restriction of amino acid precursors, L-carnitine supplementation, and administration of metronidazole to reduce the generation of propionic acid by intestinal bacteria. Elective liver transplantation (LT) is also used as an experimental surgical treatment for PA; while not curative of all aspects of the disorder, successful LT provides restoration of metabolic stability and protection from early death to PA patients, and therefore represents a clinical benchmark for gene replacement or additional approaches that might increase hepatic PCC expression and activity.

The *Pcca*$^{-/-}$ mouse is a model of *PCCA*-related PA that recapitulates several features of the human disorder, including high plasma levels of 2-methylcitrate (2-MC), an important biomarker generated through the condensation of oxaloacetic acid and propionyl-CoA, and early lethality. Untreated *Pcca*$^{-/-}$ mice experience 100% mortality by P2. More details about this mouse model are provided in Section 5.1.

The test article used in the present study, adeno-associated virus serotype 9 (AAV9) human PCCA (hPCCA), manufactured at the University of Massachusetts Medical School (UMMS) and at Vigene Biosciences (company acquired by Charles River Laboratories in May 2021; referred to from here on as Vigene), designated as AAV9-hPCCA in this study, is an AAV9 vector that expresses a functional human codon-optimized complementary DNA (cDNA) encoding the *PCCA* gene under control of *[a specific promoter]*. It was administered on postnatal day (P) 1 as a single retro-orbital injection of 1E10, 1E11 or 4E11 viral genomes (vg) per pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) to homozygous *Pcca*$^{-/-}$ mice and to heterozygous *Pcca*$^{+/-}$ littermates. Survival was monitored, and transgene expression (messenger RNA [mRNA] and protein) levels and vector genome copy number and biodistribution were evaluated at 30, 90, and 101 days post-injection of AAV9-hPCCA. Wild type (WT) mice were used as controls for PCCA protein, endogenous murine *Pcca* mRNA and AAV-encoded human *PCCA* mRNA expression, with untreated and phosphate-buffered

saline (PBS)-treated *Pcca*$^{-/-}$ mice, and untreated *Pcca*$^{+/-}$ mice used as controls for survival measurements, untreated *Pcca*$^{-/-}$ mice used as controls for plasma 2-MC levels and *Pcca*/*PCCA* mRNA and PCCA protein expression, and untreated *Pcca*$^{+/-}$ mice used as controls for *Pcca*/*PCCA* mRNA and protein expression.

2 STUDY OBJECTIVES

The objectives of the present study were to:

- Assess survival in a mouse model of *PCCA*-related PA (*Pcca*$^{-/-}$ mice) following treatment of escalating dosing cohorts with AAV9-hPCCA (single retro-orbital injection) compared to untreated and PBS-treated *Pcca*$^{-/-}$ mice and AAV9-hPCCA-treated *Pcca*$^{+/-}$ mice (primary).

- Assess efficacy of treatment with escalating doses of AAV9-hPCCA in *Pcca*$^{-/-}$ mice and control animals (secondary):

    - Evaluate propionyl-CoA metabolism by measuring plasma 2-MC levels in AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice when compared to untreated *Pcca*$^{-/-}$ mice at fixed time points post-treatment.

    - Evaluate *PCCA* messenger RNA (mRNA) and PCCA protein expression in AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice when compared to untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice at fixed time points post-treatment.

- Assess transgene biodistribution following treatment with escalating doses of AAV9-hPCCA in *Pcca*-/- mice and control animals (biodistribution):

    - Evaluate AAV9-hPCCA vector copy numbers in the heart and liver of AAV9-hPCCA-treated *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice at fixed time points post-treatment.

    - Evaluate PCCA protein expression in the heart and liver of AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice when compared to untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice at fixed time points post-treatment.

3 STUDY DESIGN

3.1 Overall Study Design and Plan

As untreated *Pcca*$^{-/-}$ mice die within 24 hours of birth, all mice born from crossing pairs of *Pcca*$^{+/-}$ mice, with the exception of untreated controls, were injected with AAV9-hPCCA within a few hours of birth and thus, genotype and mouse body weight were not immediately determined as this would be stressful to the pups. Additionally, animal sacrifice was not prospectively designed for the present study but rather determined on an individual basis predicated upon mouse morbidity/mortality, with relevant biological specimen collection immediately following. The genotypes, sex, and treatment groups of the WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice used in the present study are provided in Table 1. Sex was not determined for mice that did not survive the immediate postnatal period.

The mice in group 1 (*Pcca*$^{-/-}$) received no treatment, and the mice in group 2 (*Pcca*$^{-/-}$) received a single retro-orbital injection of PBS on P1 (Day 0). The mice in groups 3-5 (*Pcca*$^{-/-}$) and groups 6-8 (*Pcca*$^{+/-}$) received a single retro-orbital injection of AAV9-hPCCA ($1x10^{10}$, $1x10^{11}$ or $4x10^{11}$ vg/pup [i.e., 7E12, 7E13 or 2.8E14 vg/kg]) on P1. The WT and *Pcca*$^{+/-}$ mice in groups 9 and 10, respectively, received no treatment.

Survival of the mice in groups 1-8 was monitored twice daily, and mice were either found dead or euthanized if meeting specific morbidity criteria.

Blood samples were collected from a subset of the animals in groups 1 and 3-5 for measurement of plasma 2-MC levels on Day 0 (untreated *Pcca*$^{-/-}$ mice only), Day 30 (*Pcca*$^{-/-}$ mice treated with $1E10^{10}$, $1E10^{11}$ and 4E11 vg/pup [i.e., 7E12, 7E13 and 2.8E14 vg/kg] of AAV9-hPCCA), Day 90 (*Pcca*$^{-/-}$ mice treated with 1E11 and 4E11 vg/pup [i.e., 7E13 and 2.8E14 vg/kg] of AAV9-hPCCA) and Day 101 post-injection (*Pcca*$^{-/-}$ mice treated with 1E11 and 4E11 vg/pup [i.e., 7E13 and 2.8E14 vg/kg] of AAV9-hPCCA).

Tissue samples (liver and heart) were collected from a subset of the *Pcca*$^{-/-}$ animals in groups 1, 4 and 5 for measurement of human *PCCA* transgene copy number, PCCA protein expression, and/or human *PCCA* mRNA expression: on P1 in group 1, on Day 30 post-injection in group 4 (1E11 vg/pup [i.e., 7E13 vg/kg] of AAV9-hPCCA), and on Day 101 in groups 4 and 5 (1E11 and 4E11 vg/pup [i.e., 7E13 and 2.8E14 vg/kg] of AAV9-hPCCA, respectively).

Heart and liver samples were also collected from the animals in groups 1, 9 and 10 to serve as controls for measurement of PCCA protein expression and/or endogenous murine *Pcca* and AAV-encoded *PCCA* mRNA expression.

Table 1. Animals and AAV9-hPCCA Doses Tested

Group Number	Genotype	Species	Route	Dose of AAV9-hPCCA	M[a]	F[a]	ND*
1	*Pcca*$^{-/-}$**	Mouse	retro-orbital	NA (untreated)	-	-	24
2	*Pcca*$^{-/-}$	Mouse	retro-orbital	0 (PBS)	-	-	11
3	*Pcca*$^{-/-}$	Mouse	retro-orbital	1E10 vg/pup (7E12 vg/kg)	1	2	1
4	*Pcca*$^{-/-}$	Mouse	retro-orbital	1E11 vg/pup (7E13 vg/kg)	9	11	8
5	*Pcca*$^{-/-}$	Mouse	retro-orbital	4E11 vg/pup (2.8E14 vg/kg)	4	8	-
6	*Pcca*$^{+/-}$	Mouse	retro-orbital	1E10 vg/pup (7E12 vg/kg)	-	3	-
7	*Pcca*$^{+/-}$	Mouse	retro-orbital	1E11 vg/pup (7E13 vg/kg)	8	12	-
8	*Pcca*$^{+/-}$	Mouse	retro-orbital	4E11 vg/pup (2.8E14 vg/kg)	2	3	-
9	WT (FvBN)	Mouse	retro-orbital	NA (untreated)	-	3	-
10	*Pcca*$^{+/-}$	Mouse	retro-orbital	NA (untreated)	-	2	1

NA: not applicable; ND: not determined; M, male; F, female.

[a] Number of animals.

*The mice that were not found after injection, presumably died and were cannibalized, and the mice that were genotyped but died before the sex could be established are reported as "not determined" (ND). These mice were counted in the survival curve.

**The *Pcca*$^{-}$ allele is a missense/frameshift stop mutation, *Pcca*$^{p.Q133LfsX41}$, which results in neonatal lethality and is null at the PCCA protein expression level. More details about this mouse model are provided in Section 5.1.

3.2 Additional Test Sites

The following study activities were performed outside of the NIH Campus:

- AAV9-hPCCA research grade manufacturing:
  - University of Massachusetts, Chan Medical School, Viral Vector Core, 368 Plantation Street, AS6-2024, Worcester, MA 01605; https://www.umassmed.edu/research/cores/viralvectorcore/
  - Vigene Biosciences (now Charles River Laboratories; 5 Research Court, Rockville, MD 20850)
- Metabolite (2-methylcitrate) measurements:

  - University of Colorado, School of Medicine, Division of Hematology, Anemia Metabolite Laboratory

- Digital droplet polymerase chain reaction (ddPCR):
  - NIH/NCI Center for Cancer Research (CCR) Genomics Core: https://genomics.ccr.cancer.gov/

3.3 Storage of Data

Study data were stored in notebooks (written) and on NIH-provided computers (electronically).

4 STUDY MATERIALS

4.1 Test Article

The test article consists of an AAV9 vector expressing a functional human codon optimized PCCA cDNA under control of the *[specific promoter]*. In humans, endogenous PCCA protein is ubiquitously expressed, therefore a therapeutic transgene cassette with *[a specific promoter]* was designed to enable wide expression, and the AAV9 capsid was selected to further enable hepatic and cardiac transduction. A schematic of the vector transgene and description of cassette features summarizing the salient features of the AAV9-hPCCA vector is presented in Figure 1 and details concerning the lots of AAV9-hPCCA vector used as test material in this study are provided in Table 2. In addition, the vector contains a kanamycin resistance gene and an F1 origin of replication (ori). Research-grade AAV9-hPCCA was manufactured at the University of Massachusetts Medical School (UMMS) Gene Therapy Center and at Vigene using a standard triple transfection production method in 293T cells to produce the AAV9 vector [3].

Figure 1. AAV9-hPCCA Vector Schematic *[Schematic of the vector including length of different cassette elements]*

Table 2. Test Material

Test Article	Manufacturer	Excipient	Vector length (base pairs)	Vector Grade	Vector Lots
AAV9-hPCCA	UMMS Gene Therapy Center	PBS	[XX]	Research grade	VCAV-06223
AAV9-hPCCA	UMMS Gene Therapy Center	PBS	[XX]	Research grade	VCAV–06109
AAV9-hPCCA	Vigene	PBS	[XX]	Research grade	Date: 05/28/2020

4.2 Control Article

The control article used in this study was PBS (BioWhittaker, Cat No. 17-516F).

4.3 Dose Formulations

AAV9-hPCCA drug product was resuspended in PBS at final doses of 1E10, 1E11 or 4E11 vg per pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) in a volume of 40 μL. Master vector mixes were made according to the total number of pups per dose, and 40 μL of drug product were injected retro-orbitally per pup.

5 ANIMAL INFORMATION

The *Pcca*$^{-/-}$ mouse model was generated using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing to engineer mutations that are compatible with those seen in PA patients, such as frameshift-stop and missense changes. One mutation, *Pcca*$^{p.Q133LfsX41}$, in Exon 5 of the *Pcca* gene, is severe and null at the level of protein expression (cross-reactive material [CRM]-negative).

5.1 Source

WT FvBN mice were obtained from the NHGRI mouse core and derived from FvBN mice obtained from Jackson Labs.

DESKGEN was used to identify protospacer-adjacent motif (PAM) sequences (NGG) from the *Streptococcus pyogenes* (SpyCas9) targeting exon 5 of the *Pcca* gene for mutagenesis. A synthetic single-guide RNA (sgRNA) was custom synthesized by Horizon Discovery (Edit-R predesigned synthetic sgRNA, Horizon Discovery). Zygotes were co-injected with the Cas9 mRNA and sgRNA and cultured at 37°C under 5% CO_2 until the blastocyst stage then transferred into the uterus of pseudo-pregnant females. These manipulations were performed by the NHGRI mouse and embryonic stem cell core facility on the NIH campus in Bethesda, MD.

After embryo injection, FvBN F0 animals were screened and those that carried the mutation *Pcca*$^{c.398\_401delAAGC}$ (PCCA$^{p.Gln133Leufs*41}$) were crossed with WT FvBN mice to generate F1 carriers, which were intercrossed to generate homozygous *Pcca*$^{c.398_401delAAGC/c.398_401delAAGC}$ mice, designated *Pcca*$^{-/-}$. *Pcca*$^{-/-}$ mice lack immunoreactive PCCA and perish in the immediate neonatal period. Male *Pcca*$^{-/-}$ mice rescued by AAV9 gene delivery were bred with *Pcca*$^{+/-}$ females to generate the animals used to study AAV9-hPCCA. Breeder male *Pcca*$^{-/-}$ mice were not included in the experimental treatment cohorts.

5.2 Animal Care Committee

Animal work was approved by and performed in accordance with the guidelines for animal care at the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Animal experiments were performed at National Institutes of Health (NIH) with the approval of the Animal Care and Use Committee (ACUC) of NHGRI protocol.

5.3 Housing

All mice were housed in micro-isolators in the animal facility and the animal experiments were performed in accordance with the guidelines for animal care at NIH.

5.4 Animal Identification

Animals were identified using ear punches performed at weaning. Tail snips were collected and analyzed for genotyping.

5.5 Husbandry

All mice were maintained under a 12h light/12h dark cycle.

5.6 Feed and Water

Mice were maintained on a standard mouse chow (PicoLab Mouse Diet 20, LabDiet, St. Louis, MO) and water, which were available *ad libitum*.

5.7 Environmental Enrichment

Mice were housed in the animal facility and centrally monitored. Animals received *ad libitum* access to food and water, nestlets for bedding, and were routinely monitored by the facility staff.

5.8 Genotyping

Tail clips were sent to Transnetyx, Inc. for genotyping. Genotyping was performed by quantitative PCR (qPCR), using tail lysate and the following primers:*[primer sequences]*

5.9 Allocation to Study Groups

Immediately after birth, all pups (with the exception of untreated controls) were injected with the vehicle (PBS) or the test article and returned to the nest for routine care and monitoring. Mice were allocated prospectively to dosing cohorts, but not by sex or genotype.

5.9.1 Untreated Controls

Mice that were not treated were observed and dead pups removed when they were discovered in the cages. By 48 hours, there was 100% lethality noted in the *Pcca*$^{-/-}$ mice. Genotyping proceeded as described in Section 5.8 and at weaning, only *Pcca*$^{+/-}$ mice were alive. These mice were followed longitudinally.

5.9.2 PBS Treated Controls

Mice that were treated with PBS were observed and dead pups removed when they were discovered in the cages. By 48 hours, there was 100% lethality noted in the *Pcca*$^{-/-}$ mice. Genotyping proceeded as described in Section 5.8 and at weaning, only *Pcca*$^{+/-}$ mice were alive. These mice were followed longitudinally.

5.9.3 AAV9-hPCCA Treated Animals

Mice that were treated with AAV9-hPCCA and that survived to weaning were genotyped and followed longitudinally. Blood was removed by orbital puncture to measure metabolites, and at selected times, mice were harvested for tissue studies and removed from the survival cohort.

6 EXPERIMENTAL PROCEDURES

6.1 Test Article Administration

On P1, the mouse pups received a single retro-orbital injection of AAV9-hPCCA (1E10, 1E11, or 4E11 vg/pup [i.e., 7E12, 7E13 or 2.8E14 vg/kg]) or PBS only or were not treated, as shown in Table 3.

Tail vein injections are not possible in neonatal mice on P1, and facial vein injections are most easily accomplished on P2. Because the untreated *Pcca*$^{-/-}$ mice experience 100% lethality by P2, AAV9-hPCCA was injected via retro-orbital plexus to the systemic circulation, to recapitulate the anticipated route of administration in humans, and allow for historical comparisons to neonatal lethal mice treated using a similar delivery route [4-9].

The doses of AAV9-hPCCA were selected based on a long experience with AAV gene therapy to treat neonatal lethal mouse models of methylmalonic acidemia (MMA) and PA [4-9]. These studies used AAV vectors with a variety of transgenes, encapsidated with AAV8, 9, or 44.9 capsids. Doses ranging from 1E10 to 4E11 vg/pup (i.e., 7E12 to 2.8 vg/kg) were administered with variable effects on survival and metabolic correction. Doses of 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) of an AAV8 methylmalonyl-CoA mutase vector produced pronounced effects on the long-term survival of mice with MMA [4, 6], while an AAV8 PCCA vector used to treat a distinct lethal mouse model of PA at a dose of 1E10 vg/pup (i.e., 7E12 vg/kg) increased survival but less than what was observed in the MMA mice [9]. Because our older studies used transgenes that contain viral enhancers, a direct comparison to the AAV9-hPCCA vector studied here is not feasible [4-10]. The dose ranges previously used provide guidance and were therefore used to select the low 1E10, medium 1E11, and high 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) doses for the pilot studies.

Table 3. AAV9-hPCCA Retro-Orbital Dosing Information

Group Number	Genotype	Dosage	Dosing Volume
1	*Pcca*$^{-/-}$	NA (untreated)	NA
2	*Pcca*$^{-/-}$	0 (PBS)	40 µL
3	*Pcca*$^{-/-}$	1E10 vg/pup (7E12 vg/kg)	40 µL
4	*Pcca*$^{-/-}$	1E11 vg/pup (7E13 vg/kg)	40 µL
5	*Pcca*$^{-/-}$	4E11 vg/pup (2.8E14 vg/kg)	40 µL
6	*Pcca*$^{+/-}$	1E10 vg/pup (7E12 vg/kg)	40 µL
7	*Pcca*$^{+/-}$	1E11 vg/pup (7E13 vg/kg)	40 µL
8	*Pcca*$^{+/-}$	4E11 vg/pup (2.8E14 vg/kg)	40 µL
9	WT	NA (untreated)	NA
10	*Pcca*$^{+/-}$	NA (untreated)	NA

NA: not applicable.

6.2 Measurements and Assessments

6.2.1 Survival Assessment

Homozygous *Pcca*$^{-/-}$ males rescued by AAV9 gene delivery were mated with heterozygous females. All the pups obtained in the litter were treated with AAV9-hPCCA within a few hours after birth (at P0-1). To minimize stress on the newborns and mothers, the pups were not weighed before AAV9-hPCCA administration. The vector was dosed as vg/pup and approximated for vg/kg using the average body weight of 1.4 g (N=20). The genotypes of all pups were not determined until weaning. AAV particles were suspended in PBS and delivered via retro-orbital injection to *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice in the immediate newborn period at doses ranging from 1E10 to 4E11 vg/pup (i.e., 7E12 to 2.8E14 vg/kg) as previously described [4-9]. The treated mice were assessed for survival, weight gain, and metabolic improvement. PBS and no treatment (untreated) were used as controls.

The animals were observed 2 times a day for any clinical signs or mortality. The day of death was recorded for any animal that was found dead or was euthanized for humane reasons.

If any animal exhibited lethargy and/or weight loss, it was euthanized (per the Animal Study Proposal guidelines [11]) as described in Section 6.3.1 for humane reasons.

A graph depicting the percent survival of different cohorts of animals compared to the survival of untreated *Pcca*$^{-/-}$ mice was generated (Figure 2). P values for the comparison of the survival of the different cohorts to the untreated *Pcca*$^{-/-}$ mice were calculated using a log-rank (Mantel-Cox) test. Data from animals that were sacrificed at planned time points for the assays described in Sections 6.2.2, 6.2.3, and 6.2.4 were censored from the statistical analysis at the time of euthanasia.

6.2.2 Plasma 2-Methylcitrate Measurements

Blood samples were collected from a subset of the mice described in Table 1 by orbital bleeding at the following time points:

- Day 0 (N=7 untreated *Pcca*$^{-/-}$ mice).

- Day 30 post-injection (N=3 *Pcca*$^{-/-}$ mice treated with 1E10 vg/pup [i.e., 7E12 vg/kg] of AAV9-hPCCA, N=15 *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup [i.e., 7E13 vg/kg] of AAV9-hPCCA, and N=15 *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup [i.e., 2.8E14 vg/kg] of AAV9-hPCCA).
- Day 90 post-injection (N=3 *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup [i.e., 7E13 vg/kg] of AAV9-hPCCA and N=3 *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup [i.e., 2.8E14 vg/kg] of AAV9-hPCCA).
- Day 101 post-injection (N=3 *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup [i.e., 7E13 vg/pup] of AAV9-hPCCA and N=5 *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup [i.e., 2.8E14 vg/kg] of AAV9-hPCCA).

Plasma was isolated from the blood samples. The samples were immediately centrifuged, and the plasma was removed, diluted in water, and stored at −80 °C in a screw-top tube for later analysis. 2-MC was analyzed by gas chromatography–mass spectrometry with stable isotopic internal calibration as previously described [12, 13].

A bar graph depicting the mean ± standard deviation (SD) plasma 2-MC levels (µM) in each group of mice was generated (Figure 3).

P values for the comparison of plasma 2-MC levels between AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice and *Pcca*$^{-/-}$ mice that were untreated at birth were calculated using a Student's t test.

6.2.3 Transgene Expression Measurements

After blood sampling as described in Section 6.2.2, at the following time points, some of the animals had tissue samples (heart and liver) harvested as described in Section 6.3.2:

- Day 0 (N=3 untreated *Pcca*$^{-/-}$ mice).
- Day 30 post-injection (N=3 *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup [i.e., 7E13 vg/kg] of AAV9-hPCCA).
- Day 101 post-injection (N=3 *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup [i.e., 7E13 vg/kg] of AAV9-hPCCA and N=5 *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup [i.e., 2.8E14 vg/kg] of AAV9-hPCCA).

Additional, untreated animals were sacrificed as controls and tissue samples were collected as follows:

- Livers were harvested from:
  - N=3 untreated wild-type (WT) mice:
    - N=2 (92 days old) for mRNA extraction.
    - N=3 (75, 92 and 92 days old, respectively) for protein extraction.
  - N=3 untreated *Pcca*$^{+/-}$ mice:
    - N=1 (116 days old) for mRNA extraction.
    - N=3 (1, 115 and 116 days old, respectively) for protein extraction.
  - N=3 untreated *Pcca*$^{-/-}$ mice:

- N=1 (1 day old) for mRNA extraction.
- N=3 (1 day old) for protein extraction.

- Hearts were harvested for protein extraction from:
  - N=2 untreated WT mice (92 days old).
  - N=2 untreated *Pcca*$^{+/-}$ mice (115 and 116 days old, respectively).
  - N=2 untreated *Pcca*$^{-/-}$ mice (1 day old).

PCCA and *Pcca* mRNA levels were measured by quantitative reverse transcription polymerase chain reaction (RT-PCR). PCCA protein levels were measured by western blotting.

The tissue samples collected from the untreated age-matched WT and *Pcca*$^{+/-}$ mice at Day 30 and Day 101 were only used to measure protein expression.

A bar graph was generated to show the mean ±SD *PCCA* mRNA levels and the mean±SD PCCA protein levels (normalized as described in Section 6.2.3.1 and Section 6.2.3.2, respectively) in the livers of untreated WT mice (N=3), untreated *Pcca*$^{-/-}$ mice (N=3), *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA at Day 30 (N=3) and Day 101 (N=3) post-injection, and *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA at Day 101 (N=5) (Figure 4). *PCCA* mRNA levels were shown as percentage of endogenous *Pcca* mRNA levels detected in the WT mice, and PCCA protein levels were shown as percentage of PCCA protein levels detected in the WT mice. No statistical analysis was performed to compare the groups.

6.2.3.1 Measurement of *Pcca* and *PCCA* mRNA levels

Total ribonucleic acid (RNA) from frozen tissue was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). DNase digestion was performed using RNase-Free DNase Set (Qiagen, Valencia, CA) and 2 µg of RNA was reverse transcribed using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA). Taqman® gene expression assays were performed in triplicate according to the manufacturer's instructions using the Fast Advanced Master Mix (Applied Biosystems, Foster City, CA) and the Bio-Rad CFX96 Real-Time System. PCCA mRNA expression levels were normalized to either β-actin (Mm00607939_s1; Applied Biosystems) or GAPDH (Mm99999915_g1; Applied Biosystems).

TaqMan® Assay (20X):

- Human codon optimized PCCA: APKA7P4 SYNPCCA 1 FAM.
- Murine PCCA: Mm00454899 m1 Pcca FAM.
- Murine β-actin: Mm00607939_s1 Actb VIC PL.
- Murine GAPDH: Mm99999915_g1 Gapdh VIC PL.

Quantification of relative gene expression, presented as percentage of the relevant baseline, was calculated using the 2^-[delta][delta]Cq (quantification cycle) method.

6.2.3.2 Measurement of PCCA protein levels

Previously snap frozen livers and hearts were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Waltham, MA) with complete tablets, Mini EDTA-free (Roche, Indianapolis, IN). Protein concentrations were determined using a Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. A total of 50 µg of total protein was separated by gel electrophoresis using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell with Mini-PROTEAN TGX Gels (Bio-Rad,Hercules, CA, respectively), Protein transfer was done using Trans-Blot Turbo Transfer Pack (Bio-Rad, Hercules, CA). EveryBlot Blocking Buffer (Bio-Rad, Hercules, CA) was used for blocking and antibody hybridization.

The following primary antibodies were used:

- PCCA (abcam, ab187686) at a dilution 1:1,000.
- GAPDH (Proteintech Cat. # 60004-1-Ig) at a dilution of 1:10,000.
- β-actin (Proteintech, Cat. # 66009-1-Ig) at a dilution of 1:10,000.

The following secondary antibodies were used:

- LI-COR, 926-32213, at a dilution of 1:20,000.
- LI-COR, 926-68072, at a dilution of 1:20,000.

Blots were imaged using LI-COR's Odyssey DLx imaging system and LICOR Acquisition Software.

PCCA protein levels were normalized to β-actin protein levels (liver) or to GAPDH protein levels (heart).

6.2.4 Transgene Copy Number Measurements

After blood sampling as described in Section 6.2.2, at the following time points, some AAV9-hPCCA-treated *Pcca*$^{+/-}$ mice had tissue samples (heart and liver) harvested as described in Section 6.3.2:

- Day 30 (N=1 *Pcca*$^{+/-}$ mouse and N=3 *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup [i.e., 7E13 vg/kg] of AAV9-hPCCA).
- Day 101 (N=2 *Pcca*$^{+/-}$ mice and N=3 *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup [i.e., 7E13 vg/kg] of AAV9-hPCCA, and N=2 *Pcca*$^{+/-}$ mice and N=5 *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup [i.e., 2.8E14 vg/kg] of AAV9-hPCCA).

Genomic deoxyribonucleic acid (DNA) from tissue samples was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). ddPCR was performed using a Bio-Rad QX200 AutoDG ddPCR system with following probes: BIO- RAD (Hercules, CA) ddPCR CNV assay Gapdh (assay-ID dMumCNS300520369) and codon optimized PCCA (assay-ID dHsaCPE5056812) according to the manufacturer's recommendations.

The ddPCR probes used are shown in Table 4.

Table 4. ddPCR probes

ddPCR probes	Fluorescence	Bio-Rad Catalog #	Bio-Rad Catalog # (Cont.)
PCCA (codon-optimized)	FAM	Cat # 1042958 FAM	dCNS481508363
Gapdh	HEX	Cat # 1042961 HEX	dMmuCNS300520369

Gapdh: glyceraldehyde 3-phosphate dehydrogenase gene (mouse); PCCA: propionyl-CoA carboxylase, alpha subunit gene (human).

A bar graph was generated to show the mean±SD copy numbers of the PCCA transgene (normalized to the number of copies of *Gapdh*) in *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA at Day 30 and Day 101, and in *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA at Day 101 (Figure 6). No statistical analysis was performed to compare the groups.

6.3 Termination and Postmortem Procedures

6.3.1 Euthanasia

Mice were euthanized per NHGRI ACUC Guideline 01.1 Guidelines for the Euthanasia of Mice and the ARAC Guideline for Euthanasia of Rodents Using Carbon Dioxide. The CO_2 asphyxiation was followed by cervical dislocation.

6.3.2 Tissue Harvesting

After mice were euthanized, the antral side of animal was treated with 70% ethanol to minimize interference from hair. The skin was then cut using scissors, and the abdominal cavity was opened, followed by the removal of the liver and heart. Half of the tissues were placed in 10% formalin for pathology and the rest was placed in Eppendorf tubes on dry ice for western blot, RT-qPCR and DNA copy number analysis.

7 RESULTS

7.1 Survival of *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ Mice After Single Retro-orbital Injection of AAV9-hPCCA or PBS, or No Treatment

The mice from groups 1-8 (see Table 1) were either left untreated (*Pcca*$^{-/-}$ only) or received a single retro-orbital injection of PBS (*Pcca*$^{-/-}$ only) or 1E10, 1E11 or 4E11 vg/pup (i.e., 7E12, 7E13 or 2.8E14 vg/kg) of AAV9-hPCCA (*Pcca*$^{-/-}$ and *Pcca*$^{+/-}$) on P1, and were followed until survival post-injection.

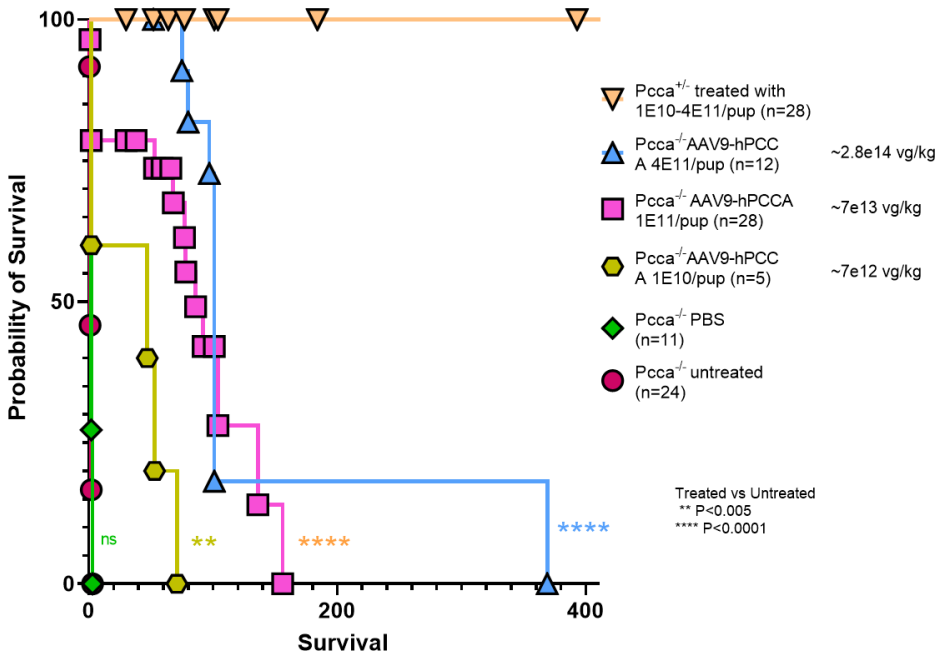
Survival curves are shown in Figure 2.

As shown in Figure 2, *Pcca*$^{-/-}$ mice that were untreated and *Pcca*$^{-/-}$ mice that were treated with PBS all died shortly after birth, within 24 hours. Conversely, 100% of the *Pcca*$^{+/-}$ mice treated with 1E10-4E11 vg/pup (i.e., 7E12-2.8E14 vg/kg) of AAV9-hPCCA were still alive at the end of the experiment (Day 400).

Administration of AAV9-hPCCA prolonged the survival of *Pcca*$^{-/-}$ mice in a dose-dependent manner (see Figure 2).

- *Pcca*$^{-/-}$ mice treated with 1E10 vg/pup (i.e., 7E12 vg/kg) of AAV9-hPCCA had a mean survival of 47 days, with the last mouse in this treatment group surviving ~70 days post-injection ($p > 0.005$ vs. untreated *Pcca*$^{-/-}$ mice).
- *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA had a mean survival of 86 days, with the last mouse in this treatment group surviving ~155 days ($p<0.0001$ vs. untreated *Pcca*$^{-/-}$ mice).
- *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA had a mean survival of 101 days, with the last mouse in this treatment group surviving ~370 days ($p<0.0001$ vs. untreated *Pcca*$^{-/-}$ mice).

Figure 2. Survival Curves for Untreated *Pcca*$^{-/-}$ Mice, *Pcca*$^{-/-}$ Mice Treated with PBS or AAV9-hPCCA* at 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg), and *Pcca*$^{+/-}$ Mice Treated with AAV9-hPCCA* at 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg)



*AAV9-hPCCA = UMMS batch VCAV-06223, UMMS batch VCAV–06109 and Vigene batch 05/28/20; ns: not statistically significant.
Estimated doses by body weight (vg/kg) are provided using the assumption that a pup's average weight was 1.4 g. The p values for the comparisons between PBS- or AAV9-hPCCA-treated and untreated *Pcca*$^{-/-}$ mice were calculated using a Mantel-Cox test.
All treated *Pcca*$^{+/-}$ mice survived to the last observed time point, regardless of the dose of AAV9-hPCCA administered.

7.2 Plasma 2-MC Levels Over Time in *Pcca*$^{-/-}$ Mice After Single Retro-orbital Injection of AAV9-hPCCA

Plasma 2-MC levels were measured in a subset of untreated *Pcca*$^{-/-}$ mice at birth and in subsets of *Pcca*$^{-/-}$ mice treated with 1E10, 1E11 or 4E11 vg/pup (i.e., 7E12, 7E13 or 2.8E14 vg/kg) of AAV9-hPCCA at Day 30, Day 90, and Day 101 post-injection.

Mean±SD 2-MC plasma levels are shown in Figure 3.

As shown in Figure 3, untreated *Pcca*$^{-/-}$ mice at birth had a mean±SD 2-MC plasma concentration of 51.20±3.03 µM.

Overall, the plasma 2-MC levels in the *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA were significantly decreased when compared to those observed in untreated *Pcca*$^{-/-}$ mice, with $p<0.01$ (see Figure 3).

Compared to untreated *Pcca*$^{-/-}$ mice, at Day 30 post-injection of 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA, plasma 2-MC levels in *Pcca*$^{-/-}$

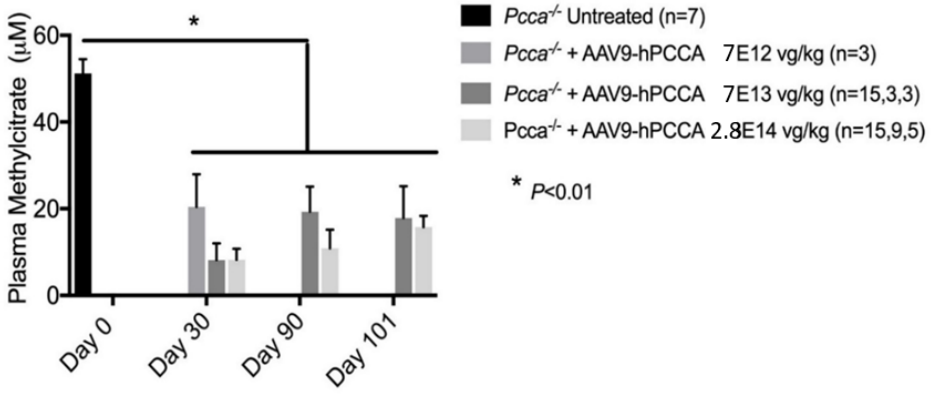
mice were decreased in a dose-dependent manner, with mean±SD concentrations of 20.36±7.54, 8.52±3.85 and 8.17±2.47 µM, respectively (see Figure 3).

At Day 90 post-injection, the plasma 2-MC levels in *Pcca*$^{-/-}$ mice treated with 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA were higher than at Day 30, but remained dose-dependently decreased compared to untreated *Pcca*$^{-/-}$ mice, with mean±SD concentrations of 19.27±4.74 and 13.13±2.89 µM, respectively (see Figure 3).

At Day 101 post-injection, plasma 2-MC levels in the *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA were similar to those observed on Day 90, and plasma 2-MC concentrations in the *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA were slightly higher than those observed on Day 90. However, the plasma levels of 2-MC in the *Pcca*$^{-/-}$ mice treated with 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA remained dose-dependently decreased when compared to untreated *Pcca*$^{-/-}$ mice, with mean±SD concentrations of 17.83±5.98 and 15.72±2.58 µM, respectively (see Figure 3).

There are no plasma 2-MC data for the *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA at 1E10 vg/pup (i.e., 7E12 vg/kg) at Day 90 or Day 101 as no mice in that treatment group survived to Day 90.

Figure 3. Plasma 2-MC* Concentrations (µM) in Plasma of Untreated *Pcca*$^{-/-}$ Mice and *Pcca*$^{-/-}$ Mice treated with AAV9-hPCCA at 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg)**



*Methylcitrate = 2-MC.
**AAV9-hPCCA = UMMS batch VCAV-06223, UMMS batch VCAV-06109 and Vigene batch 05/28/20). Estimated doses by body weight (vg/kg) are provided using the assumption that a pup's average weight was 1.4 g; 7E12 vg/kg = 1E10 vg/pup, 7E13 vg/kg = 1E11 vg/pup, and 2.8E14 vg/kg = 4E11 vg/pup. Results are shown as mean±SD. The p value for the comparison between AAV9-hPCCA-treated and untreated *Pcca*$^{-/-}$ mice was calculated using a Student's t test.

7.3 Transgene Expression (mRNA and Protein) in *Pcca*$^{-/-}$ Mice After Single Retro-orbital Injection of AAV9-hPCCA

PCCA mRNA expression was measured by quantitative RT-PCR in the livers of untreated WT and *Pcca*$^{-/-}$ mice, *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA at Day 30 and Day 101 post-injection, and *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA at Day 101 post-injection. PCCA protein expression was measured in the hearts and livers of untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice, *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA at Day 30 and Day 101 post-injection, and *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA at Day 101.

Relative *PCCA* mRNA levels as a percentage of WT *Pcca* mRNA levels and relative PCCA protein levels as a percentage of WT PCCA expression in the livers of untreated WT and *Pcca*$^{-/-}$ mice and of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA are shown in Figure 4 and the results are summarized in Table 5. Representative western blots showing PCCA protein levels in the hearts and livers of untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice and of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA are presented in Figure 5.

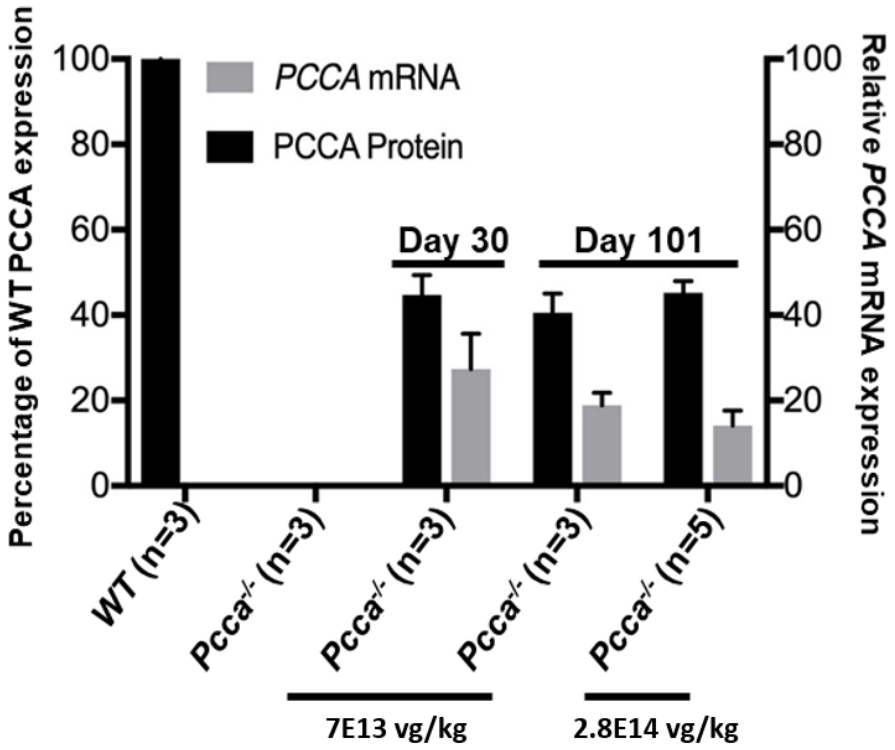
As shown in Figure 4 and Table 5, untreated WT mice expressed *PCCA* protein but no transgene-derived PCCA mRNA, and untreated *Pcca*$^{-/-}$ mice expressed neither PCCA protein nor *PCCA* mRNA.

In the livers of *Pcca*$^{-/-}$ mice treated with 1E11^{11} vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA, the AAV-encoded *PCCA* mRNA was expressed at 27.4±8.3% of WT *Pcca* mRNA levels at Day 30 and 28.8±3.0% of WT *Pcca* mRNA levels at Day 101. Treatment of *Pcca*$^{-/-}$ mice with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA rescued PCCA protein levels to 44.70±4.67% of WT PCCA protein expression levels at Day 30 and to 40.50±4.53% of WT PCCA protein expression levels at Day 101. In *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA, at Day 101, AAV-encoded *PCCA* mRNA was expressed at 14.2±3.4% % of WT *Pcca* mRNA levels and PCCA protein expression levels were rescued to 45.20±2.44% of WT PCCA protein expression levels (Figure 4 and Table 5).

Thus, in the mice that received AAV9-hPCCA at 1E11 vg/pup (i.e., 7E13 vg/kg), *PCCA* mRNA and PCCA protein expression levels slightly decreased between Day 30 and Day 101, with a more pronounced decrease in mRNA levels than in protein levels (Figure 4 and Table 5). At Day 101, *PCCA* mRNA expression levels were slightly lower, but PCCA protein expression levels appeared slightly higher in the mice that received AAV9-hPCCA at 4xE11 vg/pup (i.e., 2.8E14 vg/kg) than in the mice that received the 1E11 vg/pup (i.e., 7E13 vg/kg) dose (Figure 4 and Table 5).

As shown in Figure 5, as expected, PCCA protein expression was not detected in the heart or the liver of untreated *Pcca*$^{-/-}$ mice and was lower in the heart and the liver of untreated *Pcca*$^{+/-}$ mice than in the heart and the liver of untreated WT mice. Treatment of *Pcca*$^{-/-}$ mice with 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA resulted in clear expression of PCCA protein at Day 30 (1E11 vg/pup [7E13 vg/kg] dose only) and Day 101 post-injection.

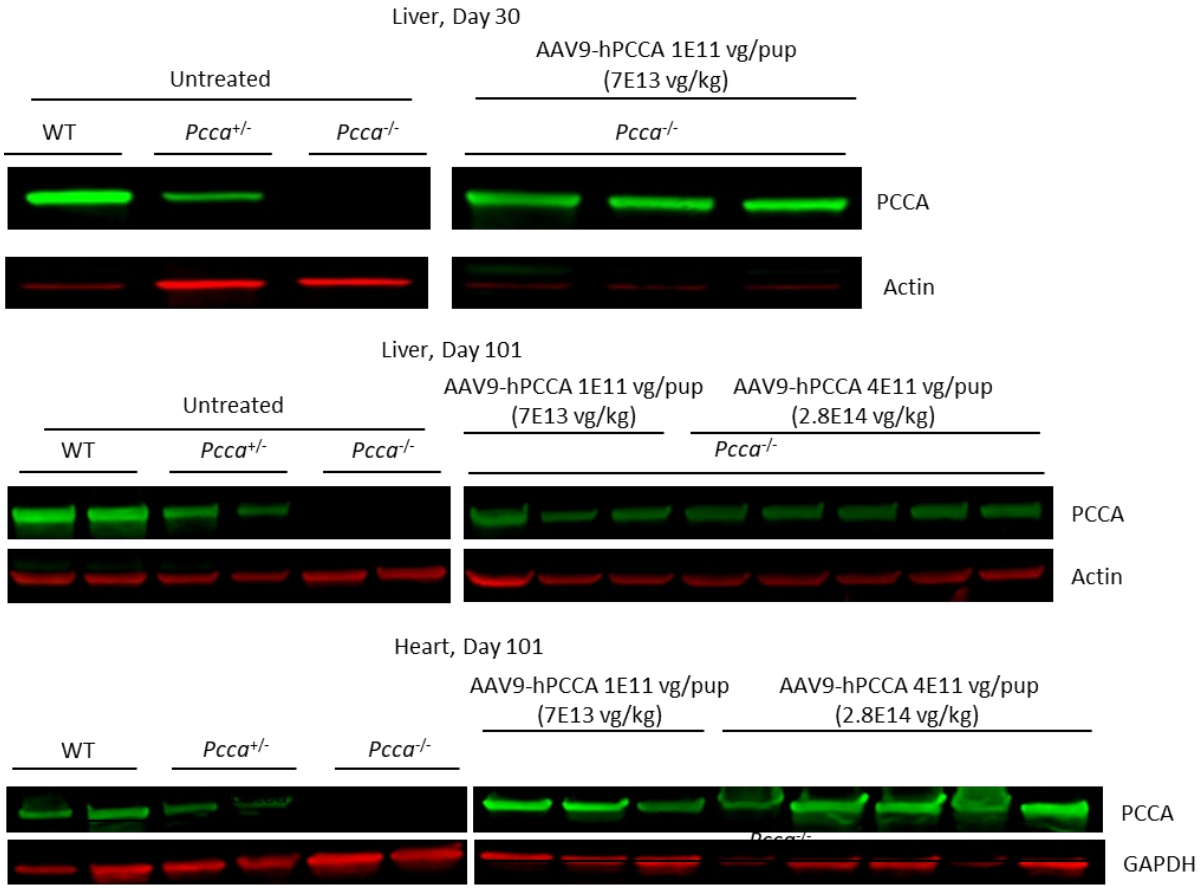
Figure 4. Relative *PCCA* mRNA levels and Percentage of WT PCCA Protein Expression in the Livers of Untreated WT and *Pcca*$^{-/-}$ Mice and *Pcca*$^{-/-}$ Mice treated with AAV9-hPCCA at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg)



AAV9-hPCCA = Day 30, 1E11 vg/pup (7E13 vg/kg) dose and Day 101, 4E11 vg/pup (2.8E14 vg/kg) dose: UMMS batch VCAV-06109; Day 101, 1E11 vg/pup (7E13 vg/kg) dose: Vigene batch 05/28/20.

Estimated doses by body weight (vg/kg) are provided using the assumption that a pup's average weight was 1.4 g; 7E13 vg/kg = 1E11 vg/pup, and 2.8E14 vg/kg = 4E11 vg/pup. Results are shown as mean±SD.

Figure 5. PCCA Protein Expression in the Livers and Hearts of Untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ Mice and *Pcca*$^{-/-}$ Mice treated with AAV9-hPCCA* at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg)



*AAV9-hPCCA = Day 30, 1E11 vg/pup (7E13 vg/kg) dose and Day 101, 4E11 vg/pup (2.8E14 vg/kg) dose: UMMS batch VCAV-06109; Day 101, 1E11 vg/pup (7E13 vg/kg) dose: Vigene batch 05/28/20.

Table 5. AAV-Encoded Transgene mRNA and Protein Expression in Liver Results Tabulated

Group Number	Genotype	Treatment	N	Day Post-Injection	Mean±SD Relative *PCCA* mRNA Expression (%)	Mean±SD Percentage of WT PCCA Protein Expression
1	*Pcca*$^{-/-}$	NA (untreated)	3	NA	0	0
4	*Pcca*$^{-/-}$	AAV9-hPCCA 1E11 vg/pup (7E13 vg/kg)	3	30	26.53±7.21	47.91±11.56
4	*Pcca*$^{-/-}$	AAV9-hPCCA 1E11 vg/pup (7E13 vg/kg)	3	101	18.85±3.01	40.49±4.53
5	*Pcca*$^{-/-}$	AAV9-hPCCA 4E11 vg/pup (2.8E14 vg/kg)	5	101	14.17±3.45	45.21±2.72
9	WT	NA (untreated	3	NA	0	100

7.4 Transgene Copy Number in *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ Mice after Single Retro-orbital Injection of AAV9-hPCCA

Copy numbers of the PCCA transgene were measured by ddPCR in the livers and hearts of *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA at Day 30 and Day 101 post-injection and in *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA at Day 101 post-injection.

Mean±SD numbers of copies of the *PCCA* transgene, normalized to the number of *Gapdh* alleles, are presented in Figure 6.

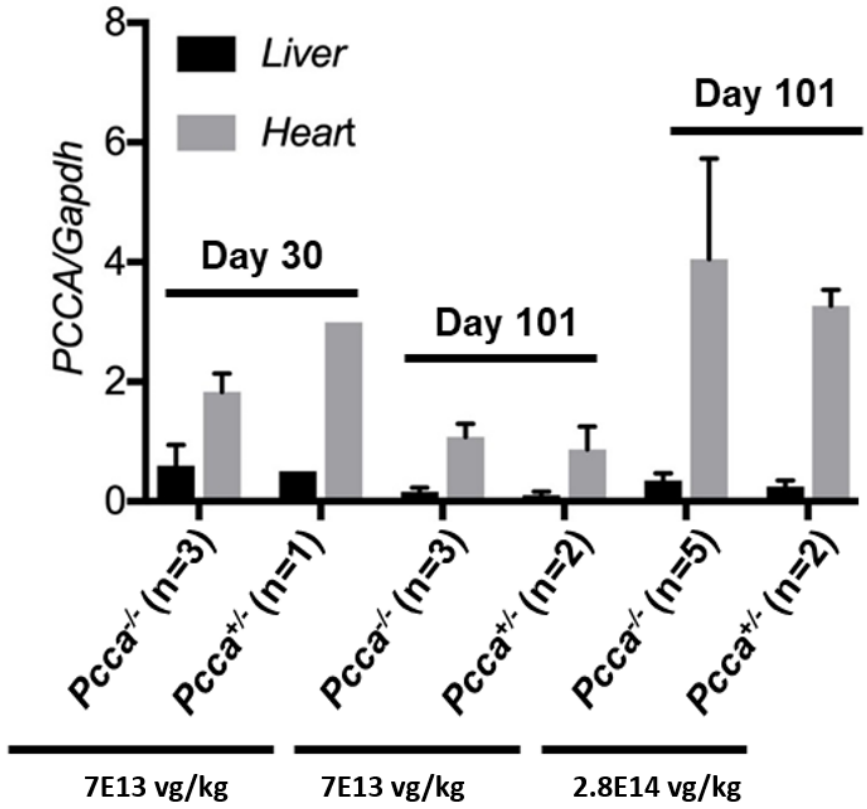
As shown in Figure 6, *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA had 1.83±0.14 transgene copies at Day 30 and 1.08±0.03 transgene copies at Day 101 in heart tissue, and 0.60±0.35 transgene copies at Day 30 and 0.17±0.06 transgene copies at Day 101 in liver tissue (values are in units of number of copies of *PCCA* transgene adjusted to the number of endogenous *Gapdh* alleles). *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA had 4.05±1.05 transgene copies in heart tissue and 0.35±0.12 transgene copies in liver tissue at Day 101.

Pcca$^{+/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA had 3.00 (N=1, no SD) transgene copies at Day 30 and 0.87±0.38 transgene copies at Day 101 in heart tissue, and 0.50 (N=1, no SD) transgene copies at Day 30 and 0.08±0.08 transgene copies at Day 101 in liver tissue. *Pcca*$^{+/-}$ mice treated with 4E11 vg/pup (i.e., 2.8E14 vg/kg) of AAV9-hPCCA had 3.27±0.27 transgene copies in heart tissue and 0.25±0.10 transgene copies in liver tissue at Day 101 (Figure 6).

The number of copies of the AAV-encoded *PCCA* transgene was thus higher in the heart than in the liver regardless of the treatment group, the time point, or the genotype of the

mice. The reduction in the number of genomes in the liver compared to the heart over time is most certainly the result of rapid hepatic growth after the neonatal period in mice which results in the loss of AAV genomes in the daughter cells, as has been noted and studied by others previously [14, 15]. *PCCA* transgene copy numbers were similar between the *Pcca*$^{-/-}$ mice and the *Pcca*$^{+/-}$ mice, regardless of the treatment group, the tissue or the time point (Figure 6). Finally, in the mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA, the number of copies of the PCCA transgene was lower in mice of both genotypes and in both tissues examined on Day 101 than it was on Day 30

Figure 6. *PCCA* Transgene Copy Number in the Livers and Hearts of P*cca*$^{-/-}$ Mice treated with AAV9-hPCCA at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg)



AAV9-hPCCA = Day 30, 1E11 vg/pup (7E13 vg/kg) dose and Day 101, 4E11 vg/pup (2.8E14 vg/kg) dose: UMMS batch VCAV-06109; Day 101, 1E11 vg/pup (7E13 vg/kg) dose: Vigene batch 05/28/20.

Estimated doses by body weight (vg/kg) are provided, using the assumption that a pup's average weight was 1.4 g; 7E13 vg/kg = 1E11 vg/pup, and 2.8E14 vg/kg = 4E11 vg/pup. Results are shown as mean±SD.

8 STUDY DEVIATIONS

No major deviations to the study protocol were reported. Though study parameters and measures were prospectively designed, sacrifice time points were dependent upon morbidity and mortality of the animals.

9 GOOD LABORATORY PRACTICE (GLP) COMPLIANCE STATEMENT

This study was not conducted under the standards for Good Laboratory Practice (GLP) for Non-clinical Laboratory Studies as promulgated by the United States Food and Drug Administration (21 Code of Federal Regulations part 58) as it was a research, proof-of-concept study.

10 CONCLUSIONS

In conclusion:

- A single retro-orbital injection of AAV9-hPCCA at 1E10, 1E11 or 4E11 vg per pup (i.e., 7E12, 7E13 or 2.8E14 vg/kg), administered on P1, prolonged survival in *Pcca*$^{-/-}$ mice, a model of PCCA-related PA that has no PCCA immunoreactivity and does not survive the immediate neonatal period. AAV9-hPCCA treatment significantly and dose-dependently increased the length of survival time of the *Pcca*$^{-/-}$ mice, with mice in the highest dose group surviving up to approximately 370 days post-injection.
    - Proof-of-concept results showing effective rescue of the *Pcca-/-* mouse model from neonatal lethality with AAV9-hPCCA inform dose translation. Based on the survival data in the neonatal mouse studies, ~ 7E12 to ~7E13 vg/kg is the therapeutic window where we would expect to see therapeutic effects in research participants.
- Administration of AAV9-hPCCA at 1E10, 1E11 or 4E11 vg/pup (i.e., 7E12, 7E13 or 2.8E14 vg/kg) significantly and dose-dependently decreased plasma levels of 2-MC in *Pcca*$^{-/-}$ mice when compared to untreated *Pcca*$^{-/-}$ mice, although the decrease became less marked over time between Day 30, Day 90 and Day 101 post-injection.
- Administration of AAV9-hPCCA at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) to *Pcca*$^{-/-}$ mice resulted in lasting expression of the *PCCA* transgene (up to Day 101), with *PCCA* mRNA levels reaching between 15% and 25% of WT *Pcca* mRNA levels, and PCCA protein levels reaching approximately 40% of WT PCCA protein levels in the livers of the treated mice. PCCA protein expression was also observed in the hearts of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) at Day 101 post-injection.
- The number of copies of the *PCCA* transgene were higher in the hearts than in the livers of *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice that received AAV9-hPCCA at 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg), appeared to slightly decrease between Day 30 and Day 101, and were similar between the *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice.

11 REFERENCES

1. Surtees, R.A., E.E. Matthews, and J.V. Leonard, *Neurologic outcome of propionic acidemia.* Pediatr Neurol, 1992. **8**(5): p. 333-7.
2. Shchelochkov, O.A., N. Carrillo, and C. Venditti, *Propionic Acidemia*, in *GeneReviews((R))*, M.P. Adam, et al., Editors. 1993: Seattle (WA).
3. Rashnonejad, A., et al., *Large-Scale Production of Adeno-Associated Viral Vector Serotype-9 Carrying the Human Survival Motor Neuron Gene.* Mol Biotechnol, 2016. **58**(1): p. 30-6.
4. Chandler, R.J., et al., *Systemic gene therapy for methylmalonic acidemia using the novel adeno-associated viral vector 44.9.* Mol Ther Methods Clin Dev, 2022. **27**: p. 61-72.
5. Chandler, R.J. and C.P. Venditti, *Pre-clinical efficacy and dosing of an AAV8 vector expressing human methylmalonyl-CoA mutase in a murine model of methylmalonic acidemia (MMA).* Mol Genet Metab, 2012. **107**(3): p. 617-9.
6. Chandler, R.J. and C.P. Venditti, *Long-term rescue of a lethal murine model of methylmalonic acidemia using adeno-associated viral gene therapy.* Mol Ther, 2010. **18**(1): p. 11-6.
7. Carrillo-Carrasco, N., et al., *Liver-directed recombinant adeno-associated viral gene delivery rescues a lethal mouse model of methylmalonic acidemia and provides long-term phenotypic correction.* Hum Gene Ther, 2010. **21**(9): p. 1147-54.
8. Senac, J.S., et al., *Gene therapy in a murine model of methylmalonic acidemia using rAAV9-mediated gene delivery.* Gene Ther, 2012. **19**(4): p. 385-91.
9. Chandler, R.J., et al., *Adeno-associated virus serotype 8 gene transfer rescues a neonatal lethal murine model of propionic acidemia.* Hum Gene Ther, 2011. **22**(4): p. 477-81.
10. Chandler, R.J., et al., *Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy.* J Clin Invest, 2015. **125**(2): p. 870-80.
11. *NHGRI Animal Care and Use Committee (ACUC) Guidelines for Euthanasia of Rodents*. Available from: https://theodora.com/rodent_laboratory/guideline_01_1.html.
12. Marcell, P.D., et al., *Quantitation of methylmalonic acid and other dicarboxylic acids in normal serum and urine using capillary gas chromatography-mass spectrometry.* Anal Biochem, 1985. **150**(1): p. 58-66.
13. Allen, R.H., et al., *Elevation of 2-methylcitric acid I and II levels in serum, urine, and cerebrospinal fluid of patients with cobalamin deficiency.* Metabolism, 1993. **42**(8): p. 978-88.
14. Cunningham, S.C., et al., *Gene Delivery to the Juvenile Mouse Liver Using AAV2/8 Vectors.* Mol Ther, 2008. **16**(6): p. 1081-1088.
15. Hu, C., R.W. Busuttil, and G.S. Lipshutz, *RH10 provides superior transgene expression in mice when compared with natural AAV serotypes for neonatal gene therapy.* J Gene Med, 2010. **12**(9): p. 766-78.

Study Report

Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Non-GLP Proof-of -Concept Study

Name of Investigational Drug:	AAV9-hPCCA (10L and 50 L)
Study Number:	NHGRI-PCCA-002
Sponsor:	*[sponsor's contact information]*
Study Director:	*[name and designation of study director]*
Testing Facility:	*[name and location of testing facility]*
Study Initiation Date:	January 2021
Study End Date:	February 2023
Date of Report:	31 May 2023

SIGNATURE PAGE

Study Number: NHGRI PCCA 002

Study Title: Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Non-GLP Proof-of -Concept Study

This report accurately describes the data obtained in the study. I have reviewed the study and agree that the data supports the conclusions stated herein:

[Signature and date]

[name, designation and contact information of study director]

SYNOPSIS

Study Synopsis

Name of Sponsor Company: National Center for Advancing Translational Sciences

Name of Investigational Drug: AAV9-hPCCA (10L) and AAV9-hPCCA (50L)

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Non-GLP Proof-of -Concept Study
Study Objective:	The objectives of the present study were to: • Assess the survival following treatment in a dose-escalating, controlled study and determine percent survival of cohorts following dose administration • Assess the weight change following Investigational Product (IP) administration compared to baseline (average) • Assess efficacy (propionyl-CoA metabolism) by measuring: o Plasma 2-MC of dosed groups as compared to positive and negative controls o PCC enzyme activity in liver and heart tissue as compared to positive and negative controls o Propionate oxidation measurement compared to positive controls • Assess the biodistribution of IP in liver and heart tissues by evaluating: o Expression of PCCA protein and messenger ribonucleic acid (mRNA) in dosed mouse liver and heart tissue compared to positive and negative controls o RNAScope hybridization to detect PCCA mRNA in liver and heart
Investigator(s) and Study Center(s):	*[name, designation and affiliation of study director]* **AAV9-hPCCA research and development (R&D) grade manufacturing**: Charles River Laboratories, 5 Research Court, Rockville, MD 20850 **Metabolite 2-methylcitrate testing:** University of Colorado, School of Medicine, Division of Hematology, Anemia Metabolite Laboratory https://medschool.cuanschutz.edu/hematology/research/anemia-metabolite-lab

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Non-GLP Proof-of -Concept Study
Study Design:	On postnatal Day (P)0-1, mice that were homozygous null for propionyl-coenzyme A (CoA) carboxylase, alpha subunit (*Pcca*$^{-/-}$) received a single retro-orbital injection of 1E9, 1E10, 1E11 or 4E11 viral genomes (vg) per pup (i.e., 7E11, 7E12, 7E13 or 2.8E14 vg/kg) of adeno-associated virus serotype 9 human propionyl-CoA carboxylase, alpha subunit, AAV9-hPCCA (10L) or AAV9-hPCCA (50L), manufactured by Charles River Laboratories (Section 4.1) or vehicle (phosphate-buffered saline [PBS]). In addition, heterozygous (*Pcca*$^{+/-}$) mice received 1E9, 1E10, 1E11 or 4E11 vg/pup (i.e., 7E11, 7E12, 7E13 or 2.8E14 vg/kg) of AAV9-hPCCA (10L and 50L). An additional group of *Pcca*$^{-/-}$ mice were untreated. Survival was monitored prospectively, and mice were either found dead or euthanized if meeting specific criteria. At Day 30 post-injection, blood samples were taken from a subset of AAV9-hPCCA (10L and 50L)-treated *Pcca*$^{-/-}$ mice and at P1, blood samples were taken from a subset of untreated *Pcca*$^{-/-}$ mice to measure a biomarker response (plasma levels of 2-methylcitrate [2-MC]). The mice were sacrificed, and their hearts and livers were collected to measure endogenous murine *Pcca* and AAV-encoded human *PCCA* messenger ribonucleic acid (mRNA) and PCCA protein expression and biodistribution. In addition, at Day 30 post-injection, a subset of AAV9-hPCCA (10L and 50L)-treated *Pcca*$^{+/-}$ mice were sacrificed, and their hearts and livers were collected to measure AAV-encoded PCCA protein expression and biodistribution, and, for mice treated with AAV9-hPCCA (10L) at 1E11 vg/pup (i.e., 7E13 vg/kg) only, *Pcca* and *PCCA* mRNA expression. Untreated wild type (WT) mice and untreated *Pcca*$^{+/-}$ mice were sacrificed, and their hearts and livers were collected to measure PCCA protein levels.
Study Period (months/years):	January 2021 to February 2023

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Non-GLP Proof-of -Concept Study
Methods:	After retro-orbital injection of AAV9-hPCCA (10L), AAV9-hPCCA (50L) or PBS on P1 or no treatment, survival was monitored, and survival curves were generated. Differences in percent survival rates between the groups were compared using a log-rank (Mantel-Cox) test. Data from mice that were sacrificed at planned time points were censored from the statistical analysis. Some animals exhibited signs of morbidity and thus were sacrificed and analyzed at interim timepoints. Plasma levels of 2-MC were measured by gas chromatography–mass spectrometry with stable isotopic internal calibration. Plasma 2-MC levels were compared between untreated *Pcca*$^{-/-}$ mice at the time of birth and AAV9-hPCCA-treated mice at Day 30 post-injection using a Student's t test. Tissue samples (livers and hearts) were collected from pups at each dose tested (1E9, 1E10, 1E11 and 4E11 vg/pup [i.e., 7E11, 7E12, 7E13 and 2.8E14 vg/kg]) at Day 30 post-dosing with AAV9-hPCCA (10L). RNAscope® probes were designed against the human codon optimized PCCA RNA by ACDbio using their proprietary technology; the probes do not cross-react with other transcripts in the murine transcriptome, including *Pcca*. Livers were fixed in 4% paraformaldehyde and processed into paraffin blocks. Five-micron sections were cut and stained with the RNAScope® 2.5 HD Assay-Brown (ACDBio 322300) following the manufacturer's instructions. Untreated wild-type (WT) mice (N=5), *Pcca*$^{+/-}$ mice (N=3) and *Pcca*$^{-/-}$ mice (N=3 for liver and N=2 for heart) and *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L) at 1E11 vg/pup (i.e., 7E13 vg/kg, N=3) were sacrificed, and their livers and hearts were collected to measure endogenous murine *Pcca* and AAV-encoded human *PCCA* mRNA levels using reverse transcription quantitative polymerase chain reaction (RT-qPCR); *Pcca* and *PCCA* mRNA levels were normalized to endogenous beta-actin (for liver) and *Gapdh* (for heart) mRNA levels. PCCA protein levels were measured in the livers and hearts of untreated *Pcca*$^{-/-}$ mice at P1, untreated WT and *Pcca*$^{+/-}$ mice, and *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice treated with 1E11 and 4E11 vg/pup (i.e., 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA (10L) at Day 30 post-injection by western blotting.
Animals:	*Pcca*$^{-/-}$ mice, *Pcca*$^{+/-}$ mice, and WT mice
Test Articles:	AAV9-hPCCA (10L), 1E9, 1E10, 1E11, or 4E11 vg/pup (i.e., 7E11, 7E12, 7E13 or 2.8E14 vg/kg), single retro-orbital injection (Section 4.1). AAV9-hPCCA (50L), 1E10, 1E11, or 4E11 vg/pup (i.e., 7E12, 7E13 or 2.8E14 vg/kg), single retro-orbital injection (Section 4.1).

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Non-GLP Proof-of -Concept Study
Results:	In vitro studies showed that HepG2 cells infected with 5E4, 1E5, 5E5 and 1E6 vg of AAV9-hPCCA (10L) expressed the PCCA protein in a dose-dependent manner (Figure 2). *Pcca*$^{-/-}$ mice that were untreated all died shortly after birth. Administration of AAV9-hPCCA (10L and 50L) prolonged the survival of *Pcca*$^{-/-}$ mice in a dose-dependent manner (Figure 3 and Figure 11). The plasma 2-MC levels in the *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L and 50L) were significantly decreased when compared to untreated *Pcca* $^{-/-}$ mice, with $p<0.0001$ (Figure 5 and Figure 12). Liver and heart both expressed PCCA protein on Day 30 post-treatment of *Pcca*$^{-/-}$ mice with AAV9-hPCCA (10L) (Figure 5). Qualitative analysis showed that the number of cells expressing *PCCA* stained in liver and heart of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L) increased dose-dependently (Figure 7). The endogenous *Pcca* mRNA expression was slightly lower in the livers and hearts of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L) than in the livers and hearts of untreated *Pcca*$^{-/-}$ mice. AAV-encoded mRNA expression in the hearts of AAV9-hPCCA (10L)-treated mice was higher compared to endogenous *Pcca*$^{-/-}$ mRNA expression (275.96 ± 36.21% vs 7.73 ± 0.95% of WT *Pcca* mRNA levels) (Figure 10). The PCC enzyme activity in the livers and hearts of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L) increased dose-dependently compared to untreated *Pcca*$^{-/-}$ mice (Figure 8). The treated *Pcca*$^{-/-}$ mice had propionate oxidation levels similar to those seen in WT controls (81% of WT) (Figure 9).

Title of Study:	Survival and Biomarker Response in *Pcca*$^{-/-}$ Mice after Single Retro-Orbital Injection of AAV9-hPCCA – Non-GLP Proof-of -Concept Study
Conclusions:	• A single retro-orbital injection of AAV9-hPCCA (10L and 50L) at 1E9 (10L only), 1E10, 1E11 or 4E11 vg per pup (i.e., 7E11, 7E12, 7E13 and 2.8E14 vg/kg), administered on P1, increased survival duration in *Pcca*$^{-/-}$ mice, a model of PCCA-related PA that has no PCCA immunoreactivity and does not survive the immediate neonatal period. AAV9-hPCCA (10L and 50L) treatment significantly and dose-dependently increased the length of survival of the mice, with mice in the highest dose group surviving up to approximately 200 days after AAV9-hPCCA (10L) injection and 250 days after AAV9-hPCCA (50L) injection. • At Day 30 and 51, Pcca-/- mice treated with the 1E11 vg/pup dose of AAV9-hPCCA (10L) showed significant increase in body weight compared to Pcca-/- mice treated with 1E10 vg/pup of AAV9-hPCCA (10L). At Day 51, the body weight of Pcca-/- mice treated with 1E11 vg/pup of AAV9 hPCCA (10L) was significantly higher ($p=0.0093$) than that of Pcca-/- mice treated with 4E11 vg/pup of AAV9-hPCCA (10L). The increase in body weight may reflect enhanced overall well-being of the treated animals. • Administration of AAV9-hPCCA (10L) at 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) significantly and dose-dependently decreased plasma levels of 2-MC in Pcca-/- mice when compared to untreated Pcca-/- mice. • Administration of AAV9-hPCCA (10L) at 1E10 to 4E11 vg/pup (i.e., 7E12 to 2.8E14 vg/kg) to Pcca-/- mice resulted in lasting expression of the PCCA transgene (on Day 30), supported by western blot and in situ hybridization methods. • Administration of AAV9-hPCCA (50L) at 1E11 vg/pup (i.e., 7E13 vg/kg) to Pcca-/- mice resulted in lasting expression of the PCCA transgene (at Day 30) in the liver and heart tissue. • In vivo 1-13C-propionate oxidation showed similar levels between untreated WT mice and treated Pcca-/-mice on Day 30 after injection of AAV9-hPCCA (10L) at 1E11 vg/pup (7E13 vg/kg).
Funding:	This work is funded by NCATS Cures Acceleration Network and Intramural Research Program of the NHGRI, NIH *[funding/grant award number].*
Date of Report:	May 2023

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ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviation / Term	Definition
2-MC	2-methylcitrate
AAV	Adeno-associated virus
AAV9	Adeno-associated virus serotype 9
AAV9-hPCCA	Adeno-associated virus serotype 9 human propionyl-CoA carboxylase, alpha subunit
ACUC	Animal Care and Use Committee
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid
BDP	Biopharmaceutical Development Program
BSA	Bovine serum albumin
Cas9	CRISPR associated protein 9
cDNA	Complementary deoxyribonucleic acid
CE	Collision energy
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
F	Female
fPCR	Fluorescent polymerase chain reaction
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase protein
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
IP	Investigational Product
ITR	Inverted terminal repeat
LT	Liver transplantation
M	Male
MMA	Methylmalonic acidemia
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

Abbreviation / Term	Definition
NA	Not applicable
NCATS	National Center for Advancing Translational Sciences
ND	Not determined
ori	Origin of replication
P	Postnatal day
PA	Propionic acidemia
PAM	Protospacer-adjacent motif
PBS	Phosphate-buffered saline
PCC	Propionyl-CoA carboxylase
Pcca	Propionyl-CoA carboxylase, alpha subunit gene (mouse)
PCCA	Propionyl-CoA carboxylase, alpha subunit protein
PCCA	Propionyl-CoA carboxylase, alpha subunit gene (human)
PCCB	Propionyl-CoA carboxylase, beta subunit gene (human)
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
SD	Standard deviation
sgRNA	Single-guide RNA
SRM	Selected reaction monitoring
TCA	Trichloroacetic acid
UPLC	Ultra-performance liquid chromatography
vg	Vector genome
WB	Western blot
WT	Wild type

1. INTRODUCTION

Propionic acidemia (PA) is a well-recognized autosomal recessive disorder of organic acid metabolism in humans with an estimated incidence of 1:250,000-1:750,000 births. It is caused by a deficiency of propionyl-coenzyme A (CoA) carboxylase (PCC), a ubiquitously expressed, heteropolymeric mitochondrial enzyme involved primarily in the catabolism of propiogenic amino acids, particularly isoleucine, valine, methionine, and threonine, as well as odd-chain fatty acids. PCC is composed of alpha and beta subunits encoded by their respective genes, PCC subunit A (*PCCA*) and PCC subunit B (*PCCB*). PCC catalyzes the first step in the conversion of propionyl-CoA to D-methylmalonyl-CoA in the pathway of propionyl-CoA oxidation. PA is caused by mutations in either the *PCCA* or *PCCB* gene.

Most frequently, PA presents in the neonatal period with hyperammonemia, vomiting, poor feeding, and hypotonia and progresses into a life-threatening metabolic crisis. Patients who survive suffer from recurrent metabolic instability and can develop multisystemic complications, including cardiomyopathy. The long-term prognosis for survival in severely affected patients is poor as illustrated by an early and relatively large (for the disease prevalence) single center study of 20 patients with PA treated at a tertiary care center: those who presented in the first week of life (11 patients) largely perished by the age of 6 years [1]. Over the decades, it has been recurrently noted that PA patients with an early and severe clinical course experience increased mortality and disease associated morbidity [2]. There is no curative therapy for PA; the disorder is managed via dietary restriction of amino acid precursors, L-carnitine supplementation, and administration of metronidazole to reduce the generation of propionic acid by intestinal bacteria. Elective liver transplantation (LT) is also used as an experimental surgical treatment for PA; while not curative of all aspects of the disorder, successful LT provides restoration of metabolic stability and protection from early death to PA patients, and therefore represents a clinical benchmark for gene replacement or additional approaches that might increase hepatic PCC expression and activity.

The *Pcca*$^{-/-}$ mouse is a model of *PCCA*-related PA that recapitulates several features of the human disorder, including high plasma levels of 2-methylcitrate (2-MC), an important biomarker generated through the condensation of oxaloacetic acid and propionyl-CoA, and early lethality. Untreated *Pcca*$^{-/-}$ mice experience 100% mortality by P2. More details about this mouse model are provided in Section 5.1.

The test articles used in the present study, adeno-associated virus serotype 9 (AAV9) human PCCA (hPCCA), were manufactured at Charles River Laboratories (CRL), designated as AAV9-hPCCA (10L) and AAV9-hPCCA (50L) based on the batch size. The test articles are AAV9 vectors that express a functional human codon optimized complementary DNA (cDNA) encoding the *PCCA* gene under control of the *[specific promoter]*. It was administered on postnatal Day (P) 0-1 as a single retro-orbital injection of 1E9, 1E10, 1E11 or 4E11 viral genomes (vg) per pup (i.e., 7E11, 7E12, 7E13 or 2.8E14 vg/kg) to homozygous *Pcca*$^{-/-}$ mice and to heterozygous *Pcca*$^{+/-}$ littermates. *In vitro* potency, body weight, survival, plasma 2-MC levels, PCC enzyme activity and 1-^{13}C-propionate oxidation were monitored, and transgene expression (messenger RNA [mRNA] and protein) levels and biodistribution were evaluated at 30 days post-injection of AAV9-hPCCA (10L) and AAV9-hPCCA (50L). Untreated wild type (WT) mice were used as positive controls for endogenous PCCA protein

and endogenous murine *Pcca* mRNA expression and 1-^{13}C-propionate oxidation, and negative controls for AAV-encoded human *PCCA* mRNA expression and plasma 2-MC levels, and untreated *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice were used as negative controls.

2. STUDY OBJECTIVES

Objective	Endpoint
To assess the survival following IP administration in a dose-escalating, controlled study	Percent survival by cohort following date of dose
To assess the weight change following IP administration	Change in body weight from baseline (average by cohort)
To assess efficacy (propionyl-CoA metabolism)	• Plasma 2-MC of dosed groups as compared to positive and negative controls • PCC enzyme activity in liver and heart tissue as compared to positive and negative controls • Propionate oxidation measurement compared to positive controls
To assess the biodistribution of IP in liver and heart tissues	• Expression of PCCA protein and mRNA in dosed mouse liver and heart tissue compared to positive and negative controls • RNAScope hybridization to detect *PCCA* mRNA in liver, heart, and skeletal muscle tissue

3. STUDY DESIGN

3.1 Overall Study Design and Plan

As *Pcca*$^{-/-}$ mice die within 24 hours of birth, all mice born from crossing pairs of *Pcca*$^{+/-}$ mice (see Section 5.1), with the exception of untreated controls, were injected with AAV9-hPCCA (10L or 50L) within a few hours of birth and thus, genotype and mouse weight were not immediately determined as this would be stressful to pups. Additionally, prospective design could not be used for the present study and animal sacrifice was determined on an individual basis, with relevant biological specimen collection immediately following.

On postnatal Day P0-1, mice that were homozygous null for PCCA (*Pcca*$^{-/-}$) received a single retro-orbital injection of 1E9, 1E10, 1E11 or 4E11 vg/pup (i.e., 7E11. 7E12, 7E13 or 2.8E14 vg/kg) of AAV9-hPCCA (10L) or AAV9-hPCCA (50L), manufactured by Charles River Laboratories. Heterozygous (*Pcca*$^{+/-}$) mice received 1E9, 1E10, 1E11 or 4E11 vg/pup (i.e., 7E11, 7E12, 7E13 or 2.8E14 vg/kg) of AAV9-hPCCA (10L or 50L). Additional groups of *Pcca*$^{-/-}$ mice were untreated, along with untreated heterozygotes and WT mice. Survival was monitored prospectively, and mice were either found dead or euthanized if meeting specific morbidity criteria.

At P1, blood samples were taken from a subset of untreated *Pcca*$^{-/-}$ mice to measure a biomarker response (plasma levels of 2-MC). At Day 30 post-injection, blood samples were taken from a subset of AAV9-hPCCA (10L and 50L)-treated *Pcca*$^{-/-}$ mice to measure biomarker response (2-MC). The mice were sacrificed, and their hearts and livers were collected to measure endogenous *Pcca* mRNA and AAV-encoded *PCCA* mRNA and PCCA protein expression and biodistribution. In addition, at Day 30 post-injection, a subset of AAV9-hPCCA (10L and 50L)-treated *Pcca*$^{+/-}$ mice were sacrificed, and their hearts and livers were collected to measure endogenous *Pcca* mRNA, AAV-encoded PCCA mRNA, and PCCA protein expression and biodistribution. Untreated wild type (WT) mice and untreated *Pcca*$^{+/-}$ mice were sacrificed, and their hearts and livers were collected to measure *Pcca* mRNA, *PCCA* mRNA, and PCCA protein levels.

Animals and doses are noted in Table 1 and Table 2. Sex was not determined for each animal; due to their fragile phenotype and the stress it places on the disease model pups upon birth. Pups that died or were sacrificed before weaning did not have their sex determined.

Table 1: Animals and AAV9-hPCCA (10L) Doses Tested

Group Number	Genotype*	Species	Route	Dose of AAV9-hPCCA (10L)	M[a]	F[a]	ND*
1.	*Pcca*$^{-/-**}$	Mouse	NA	NA (untreated)	-	-	24
2.	*Pcca*$^{-/-}$	Mouse	retro-orbital	1E9 vg/pup (7E11 vg/kg)	-	-	12
3.	*Pcca*$^{-/-}$	Mouse	retro-orbital	1E10 vg/pup (7E12 vg/kg)	6	3	4
4.	*Pcca*$^{-/-}$	Mouse	retro-orbital	1E11 vg/pup (7E13 vg/kg)	15	14	7
5.	*Pcca*$^{-/-}$	Mouse	retro-orbital	4E11 vg/pup (2.8E14 vg/kg)	8	6	1
6.	*Pcca*$^{+/-}$	Mouse	retro-orbital	1E10 vg/pup (7E12 vg/kg)	11	21	0
7.	*Pcca*$^{+/-}$	Mouse	retro-orbital	1E11 vg/pup (7E13 vg/kg)	34	26	0
8.	*Pcca*$^{+/-}$	Mouse	retro-orbital	4E11 vg/pup (2.8E14 vg/kg)	4	9	0
9.	*Pcca*$^{+/-}$	Mouse	retro-orbital	Untreated	1	2	0
10.	*Pcca*$^{+/+}$	Mouse	retro-orbital	WT untreated	1	3	0

NA: not applicable; ND: not determined; M: male; F: female.

[a] number of animals

*The mice that were not found after injection, presumably died and were cannibalized, and the mice that were genotyped but died before the sex could be established are reported as "not determined" (ND). These mice were accounted for as part of the survival data for each cohort.

**The *Pcca*$^{-}$ allele is a missense/frameshift stop mutation, *Pcca*$^{p.Q133LfsX41}$, which results in neonatal lethality and is null at the PCCA protein expression level. More details about this mouse model are provided in Section 5.1.

Table 2: Animals and AAV9-hPCCA (50L) Doses Tested

Group Number	Genotype*	Species	Route	Dose of AAV9-hPCCA (50L) (vg/pup)	M[a]	F[a]	ND*
1.	*Pcca*$^{-/-}$	Mouse	NA	NA (untreated)	-	-	24
2.	*Pcca*$^{-/-}$	Mouse	Retro-orbital	NA (Phosphate-buffered saline)	-	-	11
3.	*Pcca*$^{-/-}$	Mouse	retro-orbital	1E10	1	0	5
4.	*Pcca*$^{-/-}$	Mouse	retro-orbital	1E11	7	3	3

Group Number	Genotype*	Species	Route	Dose of AAV9-hPCCA (50L) (vg/pup)	M[a]	F[a]	ND*
5.	*Pcca*$^{-/-}$	Mouse	retro-orbital	4E11	6	8	10
6.	*Pcca*$^{+/-}$	Mouse	retro-orbital	1E10	6	5	0
7.	*Pcca*$^{+/-}$	Mouse	retro-orbital	1E11	3	0	0
8.	*Pcca*$^{+/-}$	Mouse	retro-orbital	4E11	10	14	0
9.	*Pcca*$^{+/-}$	Mouse	retro-orbital	Untreated	2	1	0
10.	*Pcca*$^{+/+}$(WT)	Mouse	retro-orbital	Untreated	0	3	0

NA: not applicable; ND: not determined; M, male; F: female.

[a] Number of animals

*The mice that were not found after injection, presumably died and were cannibalized, and the mice that were genotyped but died before the sex could be established are reported as "not determined" (ND).

3.2 Additional Test Site(s)

The following study activities were performed outside of the NIH Campus:

- AAV9-hPCCA research and development (R&D) grade manufacturing:
  - Charles River Laboratories, 5 Research Court, Rockville, MD 20850
- Metabolite (2-methylcitrate) measurements:
  - University of Colorado, School of Medicine, Division of Hematology, Anemia Metabolite Laboratory
    https://medschool.cuanschutz.edu/hematology/research/anemia-metabolite-lab

3.3 Storage of Data

Study data were stored in notebooks (written) and on NIH-provided computers (electronically).

4. STUDY MATERIALS

4.1 Test Articles

4.1.1 AAV9-hPCCA (10L)

The test article consists of an AAV9 vector expressing a functional human codon optimized PCCA cDNA under control of *[specific promoter]*. In humans, endogenous PCCA protein is ubiquitously expressed, therefore a therapeutic transgene cassette with a *[specific promoter]* to enable wide expression and selected the AAV9 capsid to further enable hepatic and cardiac transduction was designed. A schematic of the vector transgene and description of cassette features summarizing the salient features of the AAV9-hPCCA (10L) vector is presented in Figure 1 and details concerning the lots of AAV9-hPCCA (10L) vector used as test material in this study are provided in Table 3. In addition, the vector contains a kanamycin resistance gene and an F1 origin of replication (ori).

Research grade AAV9-hPCCA (10L) was manufactured at Charles River Laboratories (CRL) using standard production methods for AAV9 vectors [3].

Figure 1: AAV9-hPCCA (10L) Vector Schematic

[Schematic describing components of the AAV9-hPCCA cassette]

Table 3: AAV9-hPCCA (10L) Batch Details

Test Article Name	Manufacturer	Excipient	Vector length	Vector Grade	Vector Lot
AAV9-hPCCA (10L)	Charles River Laboratories (CRL)	PBS	[XX] base pairs	R&D grade	Feasibility Batch (10L)

4.1.2 AAV9-hPCCA (50L)

The vector used to prepare the AAV9-hPCCA (50L) batch is of the same sequence as the one used to prepare the AAV9-hPCCA (10L) batch (see Figure 1). Details concerning the batch of AAV9-hPCCA (50L) vector used as test material in the present study are provided in Table 4.

AAV9-hPCCA (50L) was manufactured at Charles River Laboratories (CRL) using standard production methods for AAV9 vectors.

Table 4: AAV9-hPCCA (50L) Batch Details

Test Article Name	Manufacturer	Excipient	Vector length	Vector Grade	Vector Lot
AAV9-hPCCA (50L)	Charles River Laboratories (CRL)	PBS	[XX] base pairs	R&D grade	TL-21-001-41

4.2 Control Article

PBS (BioWhittaker, Cat No. 17-516F) was used as the control article for mice treated with the 50L drug product from Charles River Laboratories (CRL).

4.3 Dose Formulations

AAV9-hPCCA drug product was resuspended in PBS at final doses of 1E9, 1E10, 1E11 or 4E11 vg per pup (i.e., 7E11, 7E12, 7E13 or 2.8E14 vg/kg) in a volume of 40 µL. Master vector mixes (a dosing solution of enough volume to treat all pups in a cohort, overage as appropriate) were made according to the total number of pups per dose, and 40 µL of the diluted drug product were injected retro-orbitally per pup.

5. ANIMAL INFORMATION

The *Pcca*$^{-/-}$ mouse model was generated using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing to engineer mutations that are compatible with those seen in PA patients, such as frameshift-stop and missense changes. One mutation, *Pcca*$^{p.Q133LfsX41}$, in exon 5 of the *Pcca* gene, is severe and null at the level of protein expression (cross-reactive material-negative).

5.1 Source

WT FvBN mice were obtained from the NHGRI mouse core and derived from FvBN mice obtained from Jackson Labs.

DESKGEN was used to identify protospacer-adjacent motif (PAM) sequences (NGG) from the *Streptococcus pyogenes* (SpyCas9) targeting exon 5 of the *Pcca* gene for mutagenesis. A synthetic single-guide RNA (sgRNA) was custom synthesized by Horizon Discovery (Edit-R predesigned synthetic sgRNA, Horizon Discovery). Zygotes were co-injected with the Cas9 mRNA and sgRNA and cultured at 37°C under 5% CO_2 until the blastocyst stage then transferred into the uterus of pseudo-pregnant females. These manipulations were performed by the NHGRI mouse and embryonic stem cell core facility on the NIH campus in Bethesda, MD.

After embryo injection, FvBN F0 animals were screened and those that carried the mutation *Pcca*$^{c.398\_401delAAGC}$ (PCCA$^{p.Gln133Leufs*41}$) were crossed with WT FvBN mice to generate F1 carriers, which were intercrossed to generate homozygous *Pcca*$^{c.398_401delAAGC/c.398_401delAAGC}$ mice, designated *Pcca*$^{-/-}$. *Pcca*$^{-/-}$ mice lack immunoreactive PCCA and perish in the immediate neonatal period. Male *Pcca*$^{-/-}$ mice rescued by AAV9 gene delivery were bred with *Pcca*$^{+/-}$ females to generate the animals used to study AAV9-hPCCA (10L) and AAV9-hPCCA (50L). Breeder male *Pcca*$^{-/-}$ mice were not included in the experimental treatment cohorts.

5.2 Animal Care Committee

Animal work was approved by and performed in accordance with the guidelines for animal care at the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Animal experiments were performed at National Institutes of Health (NIH) with the approval of the Animal Care and Use Committee (ACUC) of NHGRI protocol.

5.3 Housing

All mice were housed in micro-isolators in the animal facility and the animal experiments were performed in accordance with the guidelines for animal care at NIH.

5.4 Animal Identification

Animals were identified using ear punches performed at weaning. Tail snips were collected and analyzed for genotyping.

5.6 Husbandry

All mice were maintained under a 12h light/12h dark cycle.

5.7 Feed and Water

Mice were maintained on a standard mouse chow (PicoLab Mouse Diet 20, LabDiet, St. Louis, MO) and water, which were available *ad libitum*.

5.8 Environmental Enrichment

Mice were housed in the animal facility and centrally monitored. Animals received *ad libitum* access to food and water, nestlets for bedding, and were routinely monitored by the facility staff.

5.9 Genotyping

Tail clips were sent to Transnetyx, Inc. for genotyping. Genotyping was performed by quantitative PCR (qPCR), using tail lysate and the following primers: *[primer sequences]*

5.10 Allocation to Study Groups

Immediately after birth, all pups (with the exception of untreated controls) were injected with the vehicle (PBS) or the test article and returned to the nest for routine care and monitoring. Mice were allocated prospectively to dosing cohorts, but not by sex or genotype.

5.10.1 Untreated Controls

Mice that were not treated were observed and dead pups removed when they were discovered in the cages. By 48 hours, there was 100% lethality noted in the *Pcca*$^{-/-}$ mice. Genotyping proceeded as described in Section 5.8 and at weaning, only *Pcca*$^{+/-}$ mice were alive. These mice were followed longitudinally.

5.10.2 PBS Treated Controls

Mice that were treated with PBS (control for mice treated with the 50L IP, only) immediately after birth were observed and dead pups removed when they were discovered in the cages. By 48 hours, there was 100% lethality noted in the *Pcca*$^{-/-}$ mice. Genotyping proceeded as described in Section 5.8 and at weaning, only *Pcca*$^{+/-}$ mice were alive. These mice were followed longitudinally.

5.10.3 Treated Animals

Mice that were treated with AAV9-hPCCA and that survived to weaning were genotyped and followed longitudinally. Blood was removed by orbital puncture to measure metabolites, and at selected times, mice were harvested for tissue studies and removed from the survival cohort.

6. EXPERIMENTAL PROCEDURES

6.1 Test Article Administration

6.1.1 AAV9-hPCCA (10L) Administration

On P1, the mouse pups received a single retro-orbital injection of AAV9-hPCCA (10L) (1E9, 1E10, 1E11, or 4E11 vg/pup [i.e., 7E11, 7E12, 7E13 or 2.8E14 vg/kg]) or were not treated, as shown in Table 5.

Tail vein injections are not possible in neonatal mice on P1, and facial vein injections are most easily accomplished on P2. Because the untreated *Pcca*$^{-/-}$ mice experience 100% lethality by P2 (see Section 7.1), AAV9-hPCCA was injected via retro-orbital plexus to the systemic circulation, to recapitulate the anticipated route of administration in humans, and allow for historical comparisons to neonatal lethal metabolic mice treated using a similar delivery route [4-9].

The doses of AAV9-hPCCA were selected based on a long experience with AAV gene therapy to treat neonatal lethal mouse models of methylmalonic acidemia (MMA) and PA [4-9]. These studies used AAV vectors with a variety of transgenes, encapsidated with AAV8, 9, or 44.9 capsids. Doses ranging from 1E10 to 4E11 vg/pup (i.e, 7E12 to 2.8E14 vg/kg) were administered with variable effects on survival and metabolic correction. Doses of 1E11 and 4E11 vg/pup (i.e, 7E13 and 2.8E14 vg/kg) of an AAV8 methylmalonyl-CoA mutase vector produced pronounced effects on the long-term survival of mice with MMA [4, 6], while an AAV8 PCCA vector used to treat a distinct lethal mouse model of propionic acidemia at a dose of 1E10 vg/pup (i.e., 7E12 vg/kg) increased survival but less than what was observed in the MMA mice [9]. Because our older studies [4-10] used transgenes that contain viral enhancers, a direct comparison to the AAV9-hPCCA vector studied here is not feasible. The dose ranges previously used do provide guidance and were therefore used to select the low 1E10, medium 1E11, and high 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) doses for the pilot studies. A dose of 1E9 vg/kg was selected to examine a predicted subtherapeutic response.

Table 5: AAV9-hPCCA (10L) Retro-Orbital Dosing Information

Group Number	Genotype	Dosage	Dosing Volume
1	*Pcca*$^{-/-}$	NA (untreated)	NA
2	*Pcca*$^{-/-}$	1E9 vg/pup (7E11 vg/kg)	40 µL
3	*Pcca*$^{-/-}$	1E10 vg/pup (7E12 vg/kg)	40 µL
4	*Pcca*$^{-/-}$	1E11 vg/pup (7E13 vg/kg)	40 µL
5	*Pcca*$^{-/-}$	4E11 vg/pup (2.8E14 vg/kg)	40 µL
6	*Pcca*$^{+/-}$	1E10 vg/pup (7E12 vg/kg)	40 µL
7	*Pcca*$^{+/-}$	1E11 vg/pup (7E13 vg/kg)	40 µL
8	*Pcca*$^{+/-}$	4E11 vg/pup (2.8E14 vg/kg)	40 µL
9	WT	NA (untreated)	NA
10	*Pcca*$^{+/-}$	NA (untreated)	NA

NA: not applicable; WT: wild type.

6.1.2 AAV9-hPCCA (50L) Administration

On P1, the mouse pups received a single retro-orbital injection of AAV9-hPCCA (50L) (1E10, 1E11, or 4E11 vg/pup [i.e., 7E12, 7E13 or 2.8E14 vg/kg]) or PBS or were not treated, as shown in Table 6.

AAV9-hPCCA (50L) injections were performed as described in Section 6.1.1.

The doses of AAV9-hPCCA (50L) were selected for the reasons listed in Section 6.1.1 with the lowest dose of 1E9 omitted due to lack of efficacy.

Table 6: AAV9-hPCCA (50L) Dosing Information

Group Number	Genotype	Dosage	Dosing Volume
1	*Pcca*$^{-/-}$	NA (untreated)	NA
2	*Pcca*$^{-/-}$	0 (PBS)	40 µL
3	*Pcca*$^{-/-}$	1E10 vg/pup (7E12 vg/kg)	40 µL
4	*Pcca*$^{-/-}$	1E11 vg/pup (7E13 vg/kg)	40 µL
5	*Pcca*$^{-/-}$	4E11 vg/pup (2.8E14 vg/kg)	40 µL

Group Number	Genotype	Dosage	Dosing Volume
7	*Pcca*$^{+/-}$	1E10vg/pup (7E12 vg/kg)	40 µL
8	*Pcca*$^{+/-}$	1E11 vg/pup (7E13 vg/kg)	40 µL
9	*Pcca*$^{+/-}$	4E11 vg/pup (2.8E14 vg/kg)	40 µL
10	*Pcca*$^{+/-}$	NA (untreated)	NA
11	WT	NA (untreated)	NA

NA: not applicable; WT: wild type.

6.2 MEASUREMENTS AND ASSESSMENTS

6.2.1 In *Vitro* Potency of AAV9-hPCCA (10L)

PCCA KO cells were seeded in 6-well dishes at 1E7 cells/well and grown to confluency (~48h). At harvest, monolayers of infected PCCA KO cells were rinsed with 2 mL ice cold PBS pH 7.4 2x, which was removed. A volume of 1 mL ice cold PBS pH7.4 was added, and cells were scraped off the surface and collected in a 1.5 mL microtube. The tube was spun at 300 g for 10 minutes to pellet the unbroken cells. The supernatant was pipetted off, and the cell pellet was lysed in 500 µL 1X RIPA. Protein concentration was determined by µBCA assay. A total of 25 µg of total cellular protein was subjected to western blot analysis using a PCCA polyclonal antibody. See Section 6.2.5 for more details on gel electrophoresis, transfer, blocking and primary and secondary antibodies used for the detection of PCCA. Beta-actin was used as a loading control.

6.2.2 Survival Assessment

Homozygous *Pcca*$^{-/-}$ males rescued by AAV9 gene delivery were mated with heterozygous females. All the pups obtained in the litter were treated with AAV9-hPCCA (10L or 50L) within a few hours after birth (at P0-1). To minimize stress on the newborns and mothers, the pups were not weighed before AAV9-hPCCA (10L) administration. The vector was dosed as vg/pup and approximated for vg/kg using the average body weight of 1.4 g (N=20). The genotypes of all pups were not determined until weaning. AAV particles were suspended in PBS and delivered via retro-orbital injection to *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice in the immediate newborn period at doses ranges. AAV9-hPCCA (10L) treatment doses ranged from 1E9 to 4E11 vg/pup (i.e., 7E11 to 2.8E14 vg/kg). AAV9-hPCCA (50L) treatment doses ranged from 1E10 to 4E11 vg/pup, i.e., 7E12 to 2.8E14 vg/kg (*Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice) as previously described [4-9]. The treated mice were assessed for survival, weight gain (AAV9-hPCCA (10L) only), and metabolic improvement. *Pcca*$^{-/-}$ mice that received no treatment (untreated) and mice that received PBS were used as a control groups (PBS control was used as a control for animals dosed with AAV9-hPCCA 50L only).

The animals were observed 2 times a day for any clinical signs or mortality. The day of death was recorded for any animal that was found dead or was euthanized for humane reasons.

If any animal exhibited lethargy and/or weight loss, it was euthanized (per the Animal Study Proposal guidelines [11]) as described in Section 6.4.1 for humane reasons.

A graph depicting the percent survival of different cohorts of animals compared to the survival of untreated *Pcca*$^{-/-}$ mice was generated. P values for the comparison of the survival of the different cohorts to the untreated *Pcca*$^{-/-}$ mice were calculated using a log-rank (Mantel-Cox) test. Data from mice that were sacrificed at pre-planned time points for the assays described in Section 6.2.4 to Section 6.2.9 were censored from the statistical analysis at the time of euthanasia.

6.2.3 Body Weight

After the mice treated with AAV9-hPCCA (10L) at 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) were genotyped, they were individually removed from their cages over time and weighed on a digital scale with accuracy to within 0.01 g. Numbers of mice varied over time due to mortality/morbidity or sacrifice. Bar graphs were generated showing the mean ± SD body weight of each group at Day 30, Day 51, Day 72, and Day 100.

6.2.4 Plasma 2-Methylcitrate Measurements

6.2.4.1 AAV9-hPCCA (10L) Experiment

Blood samples were collected from a subset of the mice described in Table 1 by orbital bleeding at the following time points:

- Day 0 (N=10 untreated *Pcca*$^{+/-}$ mice).
- Day 0 (N=7 untreated *Pcca*$^{-/-}$ mice).
- Day 30 *Pcca*$^{-/-}$ + AAV9-hPCCA (10L) 1E10 vg/pup, i.e., 7E12 vg/kg (N=5).
- Day 30 *Pcca*$^{+/-}$ + AAV9-hPCCA (10L) 1E10 vg/pup, i.e., 7E12 vg/kg (N=2).
- Day 30 *Pcca*$^{-/-}$ + AAV9-hPCCA (10L) 1E11 vg/pup, i.e., 7E13 vg/kg (N=11).
- Day 30 *Pcca*$^{+/-}$ + AAV9-hPCCA (10L) 1E11 vg/pup, i.e., 7E13 vg/kg (N=4).
- Day 30 *Pcca*$^{-/-}$ + AAV9-hPCCA (10L) 4E11 vg/pup, i.e., 2.8E14 vg/kg (N=14).

6.4.2.2 AAV9-hPCCA (50L) Experiment

In addition, blood samples were collected from a subset of the mice described in Table 2 by orbital bleeding at the following time points:

- Day 0 (N=10 untreated *Pcca*$^{+/-}$ mice) (same mice as in AAV9-hPCCA [10L] experiments).
- Day 0 (N=7 untreated *Pcca*$^{-/-}$ mice) (same mice as in AAV9-hPCCA [10L] experiments).
- Day 30 *Pcca*$^{-/-}$ + AAV9-hPCCA (50L) 1E10 vg/pup, i.e., 7E12 vg/kg (N=1).
- Day 30 *Pcca*$^{-/-}$ + AAV9-hPCCA (50L) 1E11 vg/pup, i.e., 7E13 vg/kg (N=10).
- Day 30 *Pcca*$^{-/-}$ + AAV9-hPCCA (50L) 4E11 vg/pup, i.e., 2.8E14 vg/kg (N=7).

6.4.2.3 Both AAV9-hPCCA Batches

Plasma was isolated from the blood samples. The samples were immediately centrifuged, and the plasma was removed, diluted in water (5 µL of plasma in 195 µL of water), and stored at −80 °C in a screw-top tube for later analysis. 2-MC was analyzed by gas chromatography–mass spectrometry with stable isotopic internal calibration as previously described [12, 13].

A bar graph depicting the mean ± standard deviation (SD) plasma 2-MC levels (µM) in each group of mice was generated.

P values for the comparison of plasma 2-MC levels between AAV9-hPCCA (10L)-treated *Pcca*$^{-/-}$ mice and *Pcca*$^{-/-}$ mice that were untreated at birth were calculated using a Student's t test.

6.2.5 PCCA Protein Expression [AAV9-hPCCA (10L) only]

Previously snap frozen livers and hearts from untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice and from *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ mice 30 days after administration of 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA (10L) were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Waltham, MA) with complete tablets, Mini ethylenediaminetetraacetic acid (EDTA)-free (Roche, Indianapolis, IN). Protein concentrations were determined using a Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. A total of 50 µg of total protein was separated by gel electrophoresis using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell with Mini-PROTEAN TGX Gels (Bio-Rad, Hercules, CA, respectively). Protein transfer was done using Trans-Blot Turbo Transfer Pack (Bio-Rad, Hercules, CA). EveryBlot Blocking Buffer (Bio-Rad, Hercules, CA) was used for blocking and antibody hybridization.

The following primary antibodies were used:

- PCCA (abcam, ab187686) at a dilution 1:1,000,
- Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Proteintech Cat. # 60004-1-Ig) at a dilution of 1:10,000.
- β-actin (Proteintech, Cat. # 66009-1-Ig) at a dilution of 1:10,000

The following secondary antibodies were used:

- LICOR, 926-32213, at a dilution of 1:20,000
- LICOR, 926-68072, at a dilution of 1:20,000.

Blots were imaged using LICOR's Odyssey DLx imaging system and LICOR Acquisition Software.

PCCA protein levels were normalized to β-actin protein levels (liver) or to GAPDH protein levels (heart).

6.2.6 PCCA RNA *In Situ* Hybridization [AAV9-hPCCA (10L) only]

Tissue samples (liver and heart) were collected from treated *Pcca*$^{-/-}$ mice (N=1 to 3) at each dose tested (1E10, 1E11 and 4E11 vg/pup [i.e., 7E12, 7E13 and 2.8E14 vg/kg]) at Day 30 post-dosing with AAV9-hPCCA (10L). RNAscope® probes were designed against the human codon optimized *PCCA* RNA by ACDbio using their proprietary technology and do not cross-react with other transcripts in the murine transcriptome, including *Pcca*. Livers were fixed in 4% PFA and processed into paraffin blocks. Five-micron sections were cut and stained with the RNAScope® 2.5 HD Assay-Brown (ACDBio 322300) following the manufacturer's instructions. Slide images were captured using the Zeiss AxioScan Z1 slide scanner and analyzed using Image Pro premier 3D version 9.3 by Media Cybernetics.

6.2.7 PCC Enzyme Activity Assay [AAV9-hPCCA (10L) only]

Mouse organs were harvested as quickly as possible following euthanasia in a CO_2 chamber. For each mouse, tissue was removed and placed into a tissue culture dish. Each tissue was divided into several parts: one part was used for the enzyme activity assay, and the remaining part was snap frozen and reserved for other assays. All samples were stored in a -80°C freezer until they were assayed.

For the PCC enzyme assay, tissue was rinsed 2X with ice cold PBS pH7.4, to which 10 volumes/weight (100 mg tissue = 1 mL) 1X radioimmunoprecipitation assay (RIPA) buffer was added, and then homogenized with a bead mill. This tissue lysate was then assayed by micro bicinchoninic Acid (BCA) assay (Thermo) to determine mg/mL total protein. Protein was then adjusted to 1mg/mL by addition of 1X RIPA, and this tissue lysate was added to the assay as the source of PCC in a total of 20 µg protein (20 µL) per reaction. Tissue collection tubes were placed on dry ice prior to the sample collection.

A modification of the activity assay method of Y-N Liu et al [14] was used. To evaluate the PCC enzyme activity, the cellular extracts were incubated with 56.3 µL of 50 mM TrisCl pH 8.0, 125 mM KCl, 10 mM MgCl2, 10 mM NaHCO3, 0.5% bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 2 mM adenosine triphosphate (ATP), 5 mM propionyl-CoA (Sigma) for 0, 15, and 30 min at 37°C. 25 µL incubation samples were quenched with 25 µL of 8% trichloroacetic acid (TCA) to stop enzymatic reactions and precipitate proteins. The samples were vortexed, then centrifuged at 3000 rpm and 4°C for 30 min. The supernatants were transferred into a 96-well plate for ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) analysis. The calibration curve and quality controls were prepared under the same conditions.

Quantification of Methylmalonyl-CoA and Propionyl-CoA

The UPLC separation was performed on a Waters Acquity system with an Atlantis PREMIER BEH C18 AX column (1.7 µm, 2.1 x 50 mm; Waters). The mobile phases consisted of 100 mM ammonium formate/water (A) and 10% water/90% acetonitrile (B). Mass spectrometry (MS) analyses were conducted on a Waters Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization in positive mode and selected reaction monitoring (SRM). The SRM for propionyl-CoA were m/z 824→317 and 824 →428, with

collision energy (CE) of 30 and 24 eV, respectively. The SRM was 868→317 (CE= 38) for methylmalonyl-CoA. The ion source temperature was set at 150°C, and desolvation temperature was set to 600°C. Supernatant (1 µL) from the TCA acid quench/protein precipitation (see above underlined for PCCA enzyme activity assay) was injected onto the high-performance-liquid chromatography column.

6.2.8 In *Vivo* 1-^{13}C-Propionate Oxidation [AAV9-hPCCA (10L) only]

Pcca$^{-/-}$ mice (N=3) treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA (10L) were compared to untreated *Pcca*$^{+/+}$ controls (N=3) to determine the ability of PCC in the AAV9-hPCCA (10L)-treated *Pcca*$^{-/-}$ mice to mediate the metabolism of 1-^{13}C-propionate into CO_2.

In vivo 1-^{13}C-propionate oxidation was determined by collecting expired gas from control and treated mice after the animals were injected by the intraperitoneal route with 200 µg of 1-^{13}C-sodium propionate as previously described [6], using an adaptation of a method developed to study propionate oxidation in patients with methylmalonic and propionic acidemia. 1-C^{13}-sodium propionate was purchased from Cambridge Isotope Laboratories. The mice were placed into a respiratory chamber that contained a CO_2 probe to allow the direct measurement of the CO_2 generated by each animal. An aliquot of expired air was removed from the chamber at each time point for analysis of ^{13}C enrichment in CO_2. The isotope ratio (^{13}C/^{12}C) of the expired gas was determined with a gas isotope ratio mass spectrometer (Metabolic Solutions, Nashua, NH). The percent dose metabolized at each time point was calculated as % dose metabolized = total ^{13}C excreted (mmol/dose (mmol) × 100%). The percent dose metabolized was compared between the untreated WT mice and the *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA (10L) at each time point using an unpaired t test and the difference was considered statistically significant at $p<0.05$.

6.2.9 Measurement of *Pcca* and *PCCA* mRNA levels

Total ribonucleic acid (RNA) from frozen tissue was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). DNase digestion was performed using RNase-Free DNase Set (Qiagen, Valencia, CA) and 2 µg of RNA was reverse transcribed using the High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA). Taqman® gene expression assays were performed in triplicate according to the manufacturer's instructions using the Fast Advanced Master Mix (Applied Biosystems, Foster City, CA) and the Bio-Rad CFX96 Real-Time System. PCCA mRNA expression levels were normalized to either β-actin (Mm00607939_s1; Applied Biosystems) or GAPDH (Mm99999915_g1; Applied Biosystems).

TaqMan® Assay (20X):

- Human codon optimized PCCA: APKA7P4 SYNPCCA 1 FAM.
- Murine PCCA: Mm00454899 m1 Pcca FAM.
- Murine β-actin: Mm00607939_s1 Actb VIC PL.
- Murine GAPDH: Mm99999915_g1 Gapdh VIC PL.

Quantification of relative gene expression, presented as percentage of the relevant baseline, was calculated using the 2^-[delta][delta]Cq (quantification cycle) method.

6.3 TERMINATION AND POSTMORTEM PROCEDURES

6.3.1 Euthanasia

Mice were euthanized per NHGRI ACUC Guideline 01.1 Guidelines for the Euthanasia of Mice and the ARAC Guideline for Euthanasia of Rodents Using Carbon Dioxide. The CO_2 asphyxiation was followed by cervical dislocation.

6.3.2 Tissue Harvesting

After mice were euthanized, the antral side of animal was treated with 70% ethanol to minimize interference from hair. The skin was then cut using scissors, and the abdominal cavity was opened, followed by the removal of the liver and heart. The upper leg was removed, and the quadriceps dissected from the femur with scissors and snap-frozen. Half of the tissues were placed in 10% formalin for pathology and the rest was placed in Eppendorf tubes on dry ice for western blot, RNAScope and PCC enzyme activity analysis.

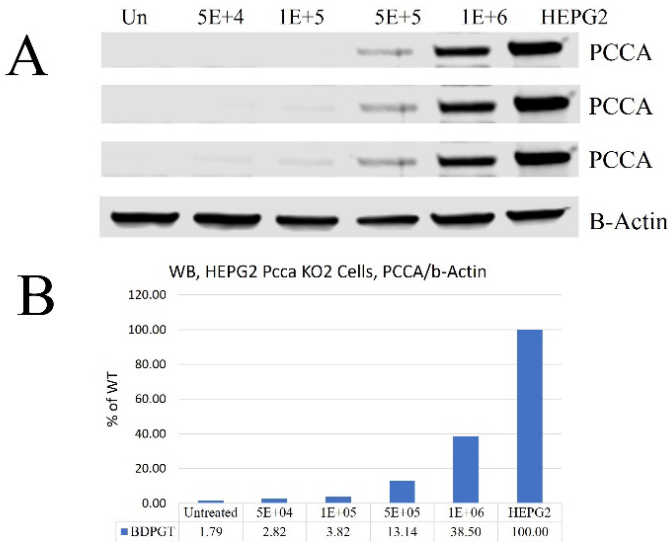
7. RESULTS

7.1 AAV9-hPCCA (10L) Results

7.1.1 In *Vitro* Potency of AAV9-hPCCA (10L)

Figure 2 shows that HepG2 cells infected with 5E4, 1E5, 5E5 and 1E6 vg of AAV9-hPCCA (10L) expressed the PCCA protein in a dose-dependent manner.

Figure 2: *In vitro* AAV9-hPCCA (10L) Infection of Human Liver PCCA Knockout Cell Line



B-actin: beta-actin; Un: untreated; WB: western blot.

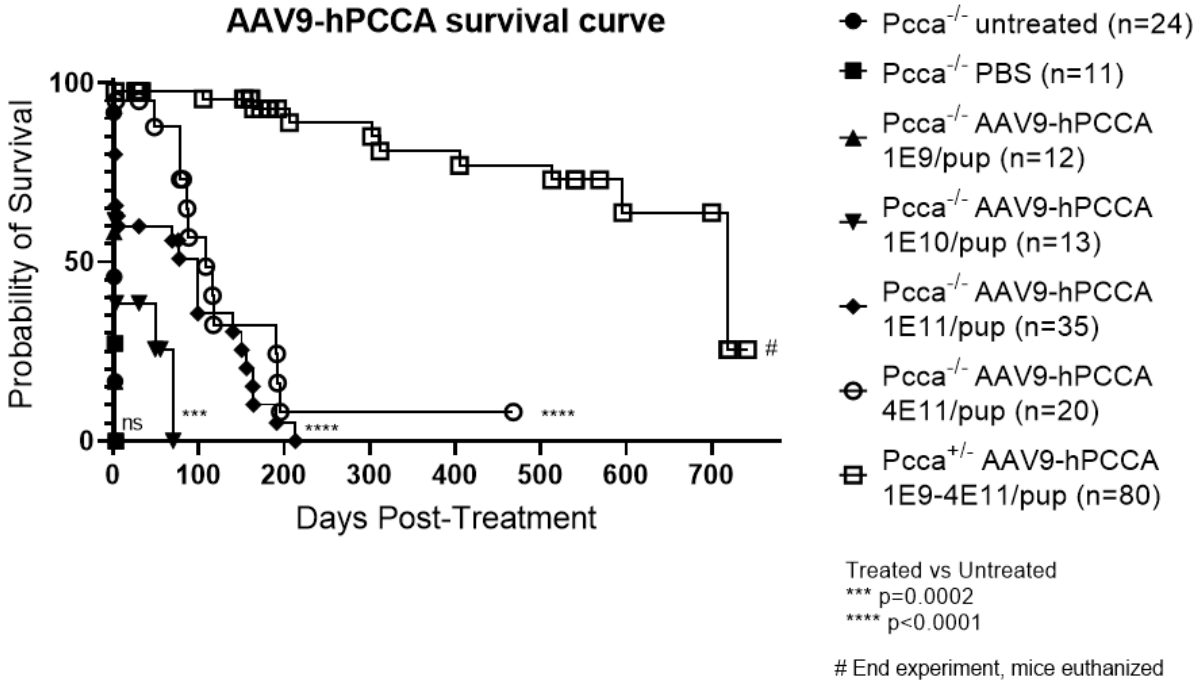
7.1.2 Survival of *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ Mice After Single Retro-Orbital Injection of AAV9-hPCCA (10L) or No Treatment

The graph depicts the percent survival of different cohorts of animals compared to survival of untreated vs treated *Pcca*$^{-/-}$ mice. *P* values were calculated using a Log_rank (Mantel-Cox) test. The mice from groups 1-8 (see Table 1) were either left untreated (*Pcca*$^{-/-}$ only) or received a single retro-orbital injection of AAV9-hPCCA (10L) (*Pcca*$^{-/-}$ and *Pcca*$^{+/-}$) on P1 and were followed until date of survival following injection/control.

As shown in Figure 3, *Pcca*$^{-/-}$ mice that were untreated all died shortly after birth, within 24 hours. Administration of AAV9-hPCCA (10L) prolonged the survival of *Pcca*$^{-/-}$ mice in a dose-dependent manner (Figure 3).

- *Pcca*$^{-/-}$ mice treated with a low dose of AAV9-hPCCA (10L), 1E9 vg/pup (i.e., 7E11 vg/kg), had a mean survival similar to untreated mice. All mice died by Day 2.
- *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L), 1E10 vg/pup (i.e., 7E12 vg/kg), had a mean survival of 3 days ($p<0.001$ vs. untreated *Pcca*$^{-/-}$ mice).
- *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L) 1E11 vg/pup (i.e., 7E13 vg/kg), had a mean survival of 99 days, ($p<0.0001$ vs. untreated *Pcca*$^{-/-}$ mice).
- *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L), 4E11 vg/pup (i.e., 2.8E14 vg/kg), had a mean survival of 108 days, ($p<0.0001$ vs. untreated *Pcca*$^{-/-}$ mice).

Figure 3: Survival Curves for Mice Treated with AAV9-hPCCA (10L)



BDPGT = AAV9-hPCCA (10L); ns: not statistically significant.

Estimated doses by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, were as follows: 1E9 vg/pup = 7E11 vg/kg; 1E10 vg/pup = 7E12 vg/kg; 1E11 vg/pup = 7E13 vg/kg; and 4E11 vg/pup = 2.8E14 vg/kg. The p values for the comparisons between AAV9-hPCCA (10L)-treated and untreated *Pcca*$^{-/-}$ mice were calculated using a Mantel-Cox test.

7.1.3 Body Weight of Mice Treated with AAV9-hPCCA (10L)

The body weight of mice treated with AAV9-hPCCA (10L) from Day 30 to Day 100 post-treatment is presented in Figure 4.

At Day 30 post-treatment, *Pcca*$^{-/-}$ mice treated with the 1E11 vg/pup (7E13 vg/kg) dose of AAV9-hPCCA (10L) showed a significant ($p=0.0048$) increase in body weight compared to *Pcca*$^{-/-}$ mice treated with the low dose of 1E10 vg/pup (7E12 vg/kg). The body weight change between *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) and 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA (10L) was not significant.

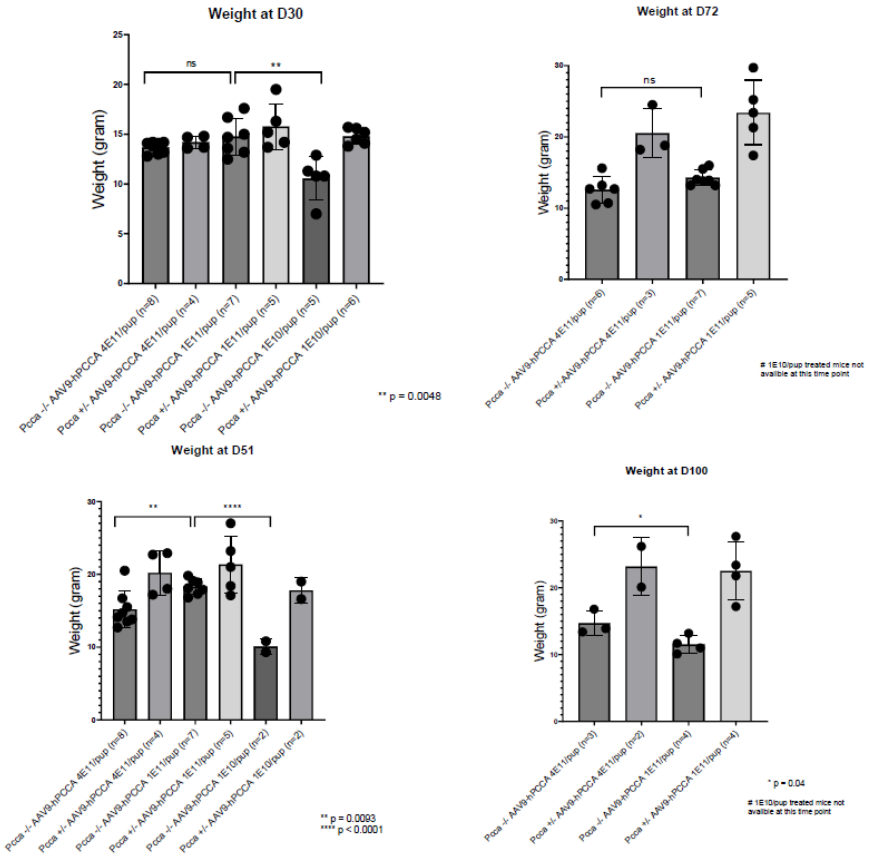
At Day 51 post-treatment, *Pcca*$^{-/-}$ mice treated with the 1E11 vg/pup (7E13 vg/kg) dose of AAV9-hPCCA (10L) showed a significant ($p<0.0001$) increase in body weight compared to *Pcca*$^{-/-}$ mice treated with the low dose of 1E10 vg/pup (7E12 vg/kg). The body weight of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA (10L) was significantly higher ($p=0.0093$) than that of *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg).

At Day 72 post-treatment, there was no significant difference in the body weight of *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA (10L) compared to that of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA (10L).

At Day 100 post-treatment, the body weight of *Pcca*$^{-/-}$ mice treated with the 4E11 vg/pup (2.8E14 vg/kg) dose of AAV9-hPCCA (10L) was significantly higher ($p=0.04$) than that of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA (10L).

In aggregate, the weight trend in the treated mutant mice demonstrates a dose response to AAV9-hPCCA: *Pcca*$^{-/-}$ mice treated with 1E10 vg/pup (7E12 vg/kg) were smaller at D30 and onward compared to those treated with either 1E11 vg/pup (7E13 vg/kg) or 4E11 vg/pup (2.8E14 vg/kg). As discussed in the following sections, the different doses yielded variable levels of PCCA transgene expression in the liver and heart, with the lowest dose having the fewest corrected cells in either organ. Of note, the very low levels of hepatic PCCA expression at D30 and beyond in the 1E10 vg/pup (7E12 vg/kg) treated *Pcca*$^{-/-}$ mice may explain the poor growth given the critical role PCCA plays in hepatic metabolism.

Figure 4: Body Weight of Mice Treated with AAV9-hPCCA (10L)



Estimated doses by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, were as follows: 1E10 vg/pup = 7E12 vg/kg; 1E11 vg/pup = 7E13 vg/kg; and 4E11 vg/pup = 2.8E14 vg/kg.

7.1.4 Clinical and Metabolic Response to AAV9-hPCCA (10L)

The metabolic responses to AAV9-hPCCA (10L)-treated mice were assessed by plasma 2-MC levels.

Plasma 2-MC levels were measured in a subset of untreated mice at birth and in mice treated with a dose range of 1E10 to 4E11 vg/pup (i.e., 7E12 to 2.8E14 vg/kg) of AAV9-hPCCA (10L) at Day 30 post-injection. Mean ± SD 2-MC plasma levels are shown in Figure 5.

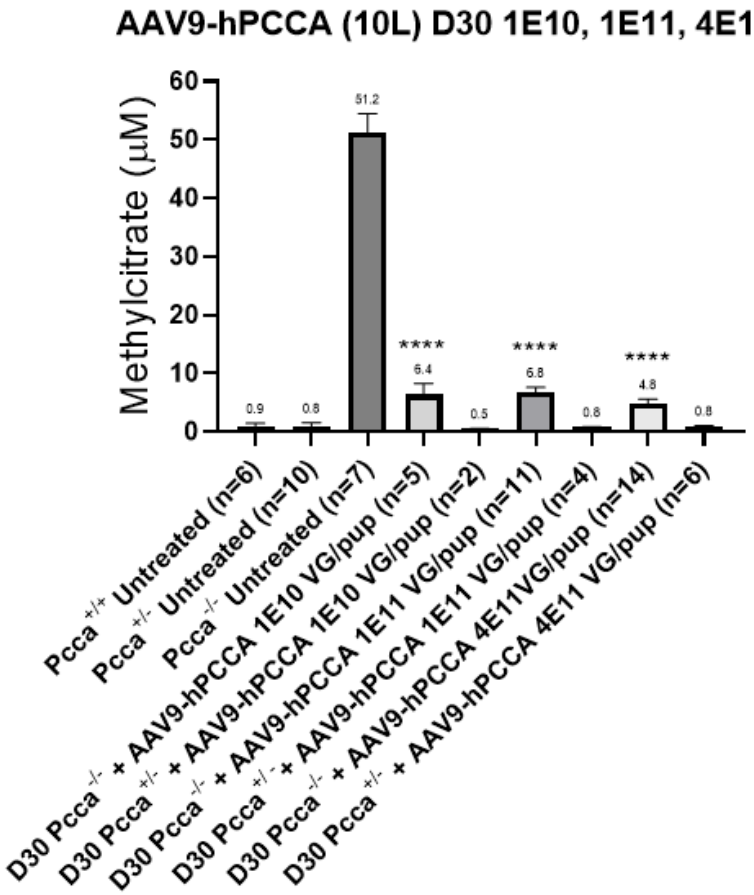
As shown in Figure 5, untreated *Pcca* $K^{+/+}$, *Pcca* $K^{+/-}$ and *Pcca* $K^{-/-}$ mice at birth had mean 2-MC plasma concentrations of 0.9, 0.8 and 51.2 µM, respectively.

At Day 30 post-injection, the plasma 2-MC levels in the *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L) were significantly decreased when compared to untreated *Pcca* $^{-/-}$mice at birth, with 6.4, 6.8 and 4.8 µM 2-MC in plasma after treatment with AAV9-hPCCA at 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg), respectively, versus 51.2 µM 2-MC in the plasma of untreated *Pcca*$^{-/-}$ mice, p<0.0001 (Figure 5).

At Day 30 post-injection with different doses of AAV9-hPCCA (10L), the plasma 2-MC levels in treated *Pcca*$^{+/-}$ mice remained at levels similar to those observed in untreated *Pcca*$^{+/-}$

mice, with 0.5, 0.8 and 0.8 µM 2-MC in plasma after treatment with AAV9-hPCCA at 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg), respectively, versus 0.8µM 2-MC in the plasma of untreated *Pcca*$^{+/-}$ mice (Figure 5).

Figure 5: Plasma Methylcitrate Levels Following AAV9-hPCCA (10L) Treatment

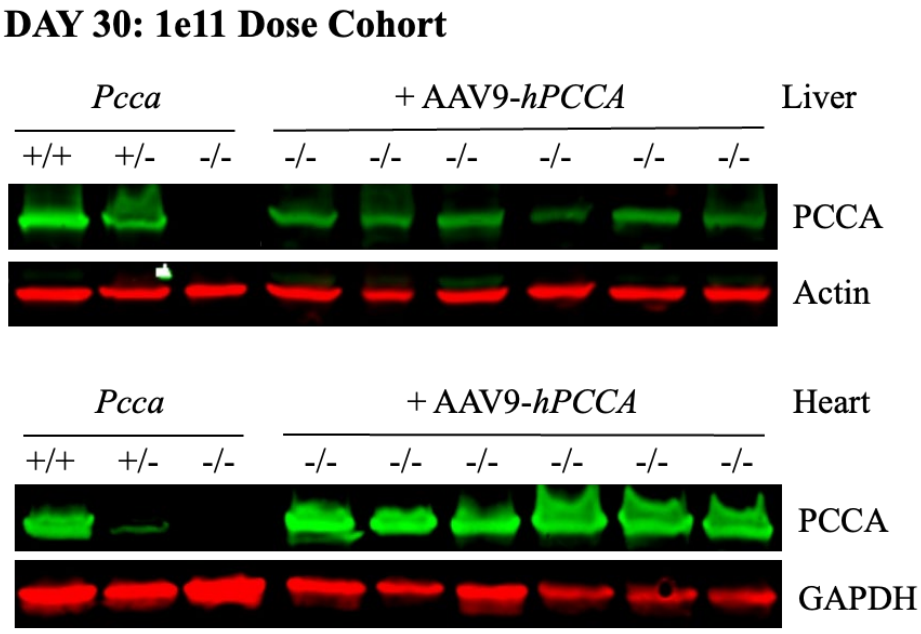


Estimated doses by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, were as follows: 1E10 vg/pup = 7E12 vg/kg; 1E11 vg/pup = 7E13 vg/kg; and 4E11 vg/pup = 2.8E14 vg/kg. The p values for the comparison of plasma 2-MC levels between AAV9-hPCCA (10L)-treated mice untreated mice were calculated using a Student's t test.

7.1.5 PCCA Protein Expression on Day 30 after Injection of AAV9-hPCCA (10L)

Representative findings of PCCA protein expression in mice treated with AAV9-hPCCA (10L) at a dose of 1E11 vg/pup (i.e., 7E13 vg/kg) on Day 30 post-treatment in the liver and heart are shown in Figure 6. The livers and hearts of AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice both expressed PCCA protein on Day 30 post-treatment with AAV9-hPCCA (10L).

Figure 6: Western Blot Analysis of Liver and Heart in Untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ Mice and in *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ Mice Treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA (10L)

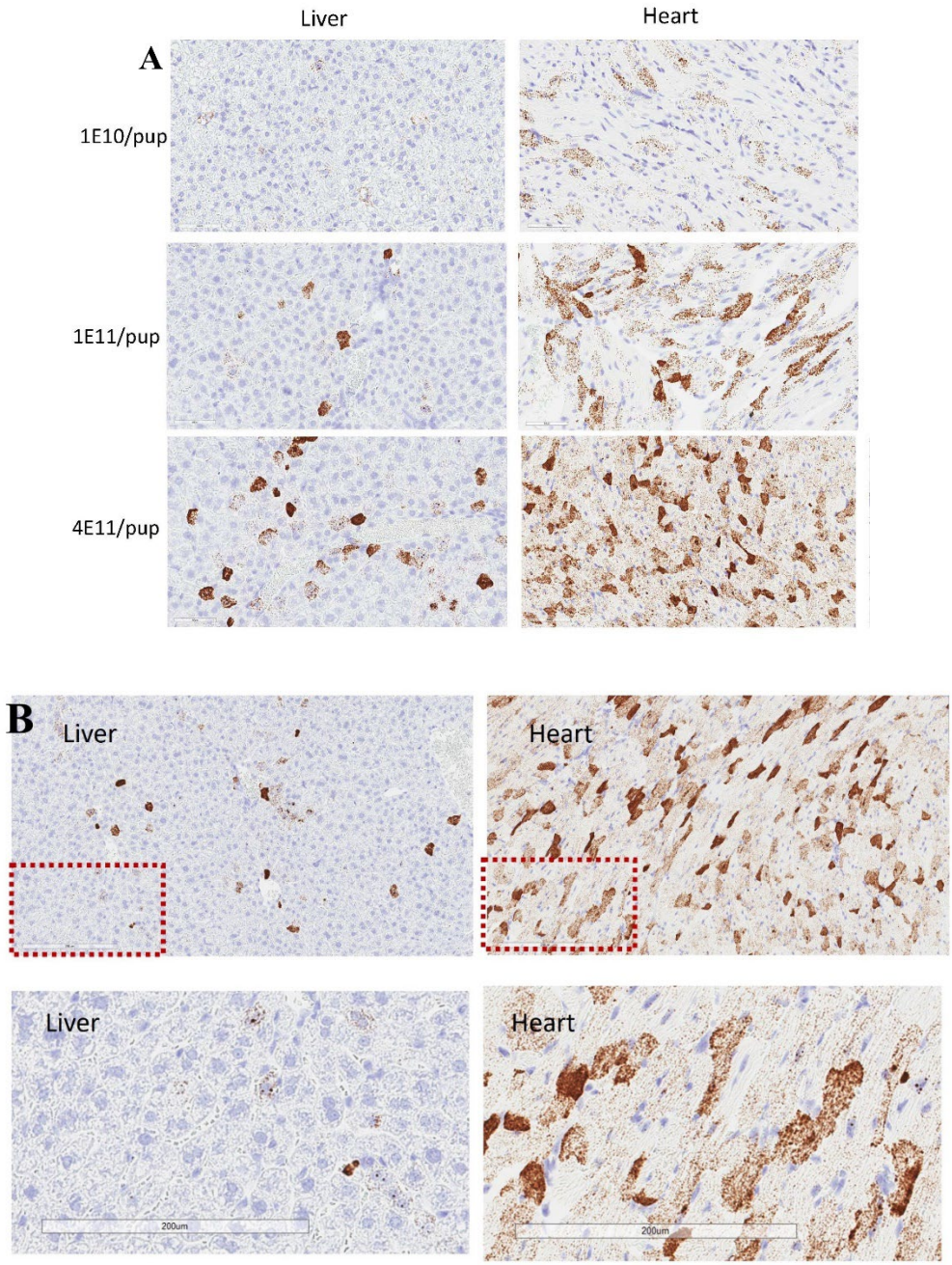


The estimated dose by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, was as follows: 1E11 vg/pup = 7E13 vg/kg.

7.1.6 PCCA RNA Expression (*by In Situ* Hybridization) on Day 30 after Injection of AAV9-hPCCA (10L)

Representative RNAscope® images from the liver and heart of *Pcca*$^{-/-}$ mice treated with each dose of AAV9-hPCCA (10L) tested (1E10, 1E11, and 4E11 vg/pup [7E12, 7E13 and 2.8E14 vg/kg]) are presented in Figure 7.

Figure 7: *PCCA* RNA *in situ* hybridization in the liver and heart of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L)



Estimated doses by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, were as follows: 1E10 vg/pup = 7E12 vg/kg; 1E11 vg/pup = 7E13 vg/kg; and 4E11 vg/pup = 2.8E14 vg/kg.

A: *In Situ* hybridization images of liver, and heart from mice treated with increasing doses of AAV9-hPCCA (10L)

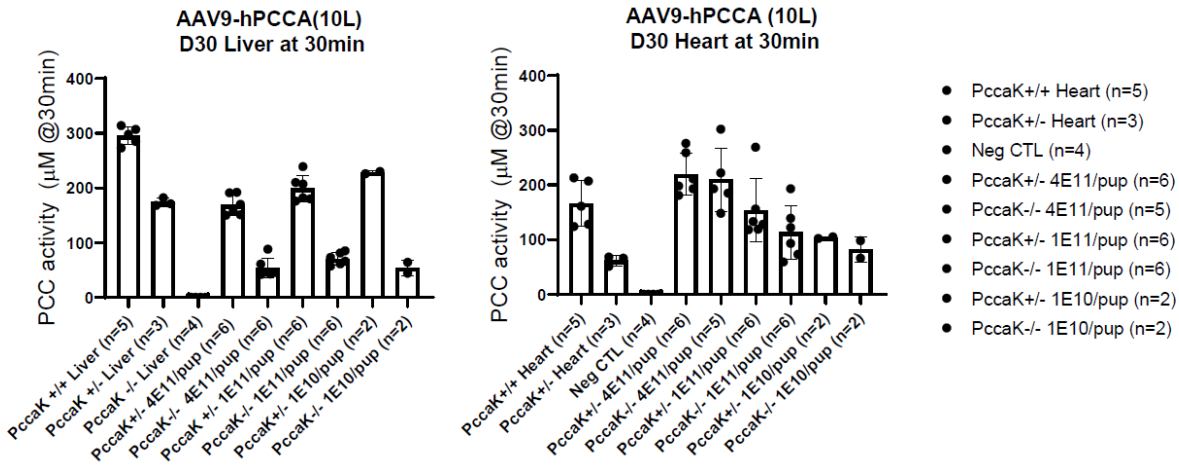
B: *In Situ* hybridization images of liver and heart from mice treated at the high dose (4E11 vg/pup [i.e., 2.8E14 vg/kg]) of AAV9-hPCCA (10L). Bottom images are magnified images of the red boxes. Scale bar = 200 µM.

7.1.7 PCC Enzyme Activity on Day 30 after Injection of AAV9-hPCCA (10L)

The PCCA enzyme activity over 30 min in the livers and hearts of mice treated with AAV9-hPCCA (10L) at escalating doses on Day 30 post-treatment is shown in Figure 8. The PCC enzyme activity in the livers and hearts of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L) increased dose-dependently compared to untreated *Pcca*$^{-/-}$ mice.

The PCC enzyme activity in the livers of *Pcca*$^{+/-}$ mice treated with AAV9-hPCCA (10L) remained similar to that observed in the livers of untreated *Pcca*$^{+/-}$ mice. The PCC enzyme activity in the hearts of *Pcca*$^{+/-}$ mice treated with AAV9-hPCCA (10L) increased dose-dependently compared to that observed in the hearts of untreated *Pcca*$^{+/-}$ mice.

Figure 8: PCC Enzyme Activity in the Livers and Hearts of Mice treated with AAV9-hPCCA (10L)



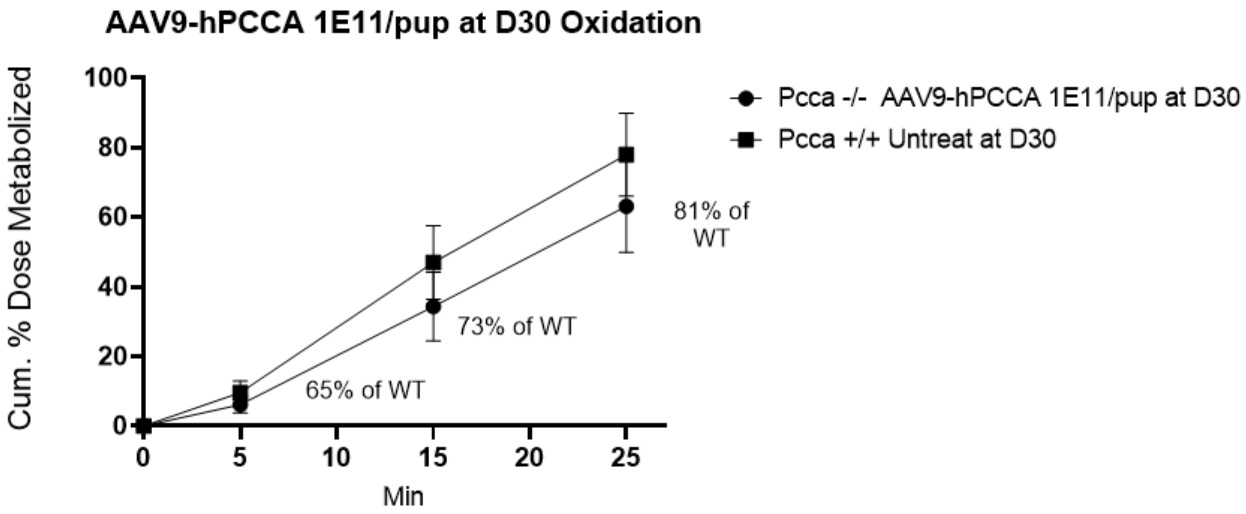
*PccaK = *Pcca;* Neg CTL: negative control (i.e., untreated *Pcca*$^{-/-}$ mice).

Estimated doses by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, were as follows: 1E10 vg/pup = 7E12 vg/kg; 1E11 vg/pup = 7E13 vg/kg; and 4E11 vg/pup = 2.8E14 vg/kg.

7.1.8 In *vivo* 1-^{13}C-Propionate Oxidation on Day 30 after Injection of AAV9-hPCCA (10L)

In vivo 1-^{13}C-propionate oxidation in untreated WT mice and *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup of AAV9-hPCCA (10L) on Day 30 post-treatment (N=3 for each) is shown in Figure 9. The treated *Pcca*$^{-/-}$ mice had propionate oxidation levels similar to those seen in untreated WT controls (81% of WT at 30 minutes).

Figure 9: In vivo 1-^{13}C-propionate oxidation after Injection of AAV9-hPCCA



Cum.: cumulative; D: Day; Untreat = untreated
The estimated dose by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, was as follows: 1E11 vg/pup = 7E13 vg/kg.

7.1.9 mRNA Expression in Pcca-/- Mice After Single Retro-orbital Injection of AAV9-hPCCA (10L)

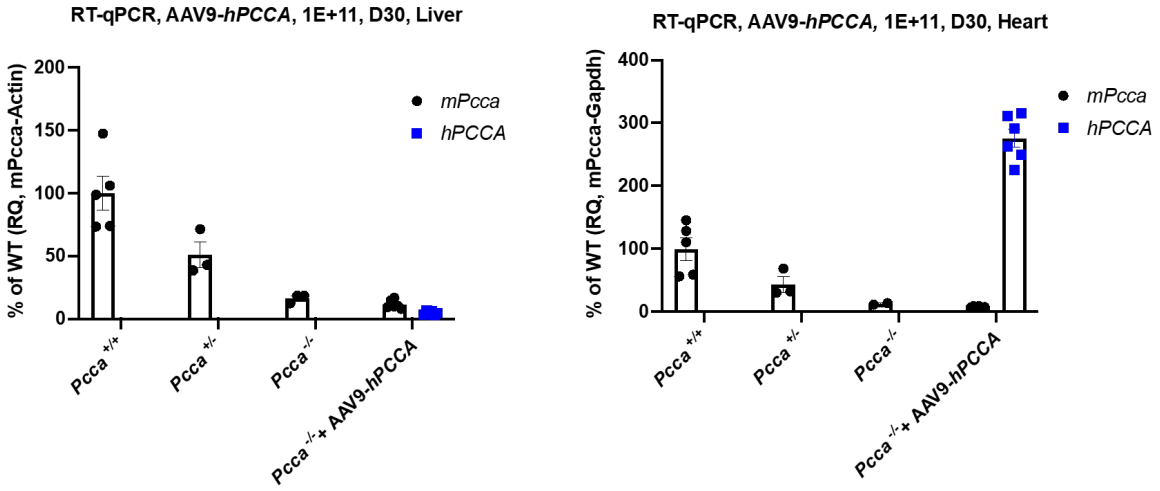
PCCA mRNA expression was measured by quantitative RT-PCR in the livers and hearts of untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice and of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA (10L) at Day 30 post-injection. Relative endogenous *Pcca* mRNA and transgene-derived *PCCA* mRNA levels as a percentage of WT *Pcca* mRNA levels in the livers and hearts of untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice and of *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA (10L) are shown in Figure 10 and the results are summarized in Table 7.

As shown in Figure 10 and Table 7, untreated WT mice expressed endogenous *Pcca* mRNA in liver at 100.00 ± 30.26% and heart at 100.00 ± 40.58%. In *Pcca*$^{+/-}$ mice, the endogenous *Pcca* mRNA was expressed in liver at 51.05 ± 17.79% and in heart at 43.80 ± 21.64% of WT *Pcca* mRNA levels. In untreated *Pcca*$^{-/-}$ mice, the endogenous *Pcca* mRNA was expressed in liver at 16.54 ± 3.49% and in heart at 12.66 ± 1.63% of WT *Pcca* mRNA levels. In *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA (10L), at Day 30, endogenous *Pcca* mRNA was expressed in liver at 11.59 ± 3.50% and in heart at 7.73 ± 0.95% of WT *Pcca* mRNA levels. In *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (i.e., 7E13 vg/kg) of AAV9-hPCCA (10L), at Day 30, AAV-encoded *PCCA* mRNA was expressed in liver at 4.75 ± 1.48% and in heart at 275.96 ± 36.21% of WT *Pcca* mRNA levels.

In the livers and hearts of *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (10L), the endogenous *Pcca* mRNA expression was slightly lower than in the untreated *Pcca*$^{-/-}$ mice. AAV-encoded mRNA expression in the hearts of AAV9-hPCCA (10L) treated mice was higher compared to endogenous *Pcca*$^{-/-}$ mRNA expression (275.96 ± 36.21% vs 7.73 ± 0.95% of WT *Pcca*

mRNA levels), consistent with the measurement of increased PCC activity in the hearts of these mice (Figure 8). The pattern of decreased expression in the liver vs heart is likely caused by dilution of the vector genomes in the liver caused by hepatocyte growth compared to the heart which is effectively transduced by AAV9 and is not subject to vector loss as a consequence of an increase in cell numbers over time.

Figure 10: Relative PCCA mRNA levels in the Liver and Heart of Untreated WT, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice and *Pcca*$^{-/-}$ Mice treated with AAV9-hPCCA on Day 30



HT = *Pcca*$^{+/-}$; MT = *Pcca*$^{-/-}$; mPcca = endogenous (mouse) *Pcca* mRNA; RQ = RT-qPCR; hPcca = AAV-encoded (human) *PCCA* mRNA.
The estimated dose by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, was as follows: 1E11 vg/pup = 7E13 vg/kg.

Table 7: AAV-Encoded Transgene mRNA in Liver and Heart on Day 30

Genotype and Treatment	Liver (N)	Heart (N)	Liver (D)	Heart (D)	*Pcca* mRNA[a] (liver)	*PCCA* mRNA[a] (liver)	*Pcca* mRNA[a] (heart)	*PCCA* mRNA[a] (heart)
WT untreated	5	5	NA	NA	100.00 ± 30.26	-	100.00 ± 40.58	-
Pcca+/- untreated	3	3	NA	NA	51.05 ± 17.79	-	43.80 ± 21.64	-
Pcca-/- untreated	3	3	NA	NA	16.54 ± 3.49	-	12.66 ± 1.63	-
Pcca-/- 1E11 vg/pup (7E13 vg/kg) AAV9-hPCCA (10L)	6	6	30	30	11.59 ± 3.50	4.75 ± 1.48	7.73 ± 0.95	275.96 ± 36.21

[a]Mean ± SD Relative to WT *Pcca* mRNA Expression (%)
NA: not applicable; Data was represented as mean ± SD; N, number of animals; D, days post injection.
The estimated dose by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, was as follows: 1E11 vg/pup = 7E13 vg/kg.

7.2 AAV9-hPCCA (50L) Results

7.2.1 Survival of *Pcca*$^{-/-}$ and *Pcca*$^{+/-}$ Mice After Single Retro-Orbital Injection of AAV9-hPCCA (50L) or No Treatment

The graph depicts the percent survival of different cohorts of animals compared to survival of untreated versus AAV9-hPCCA-treated *Pcca*$^{-/-}$ mice.

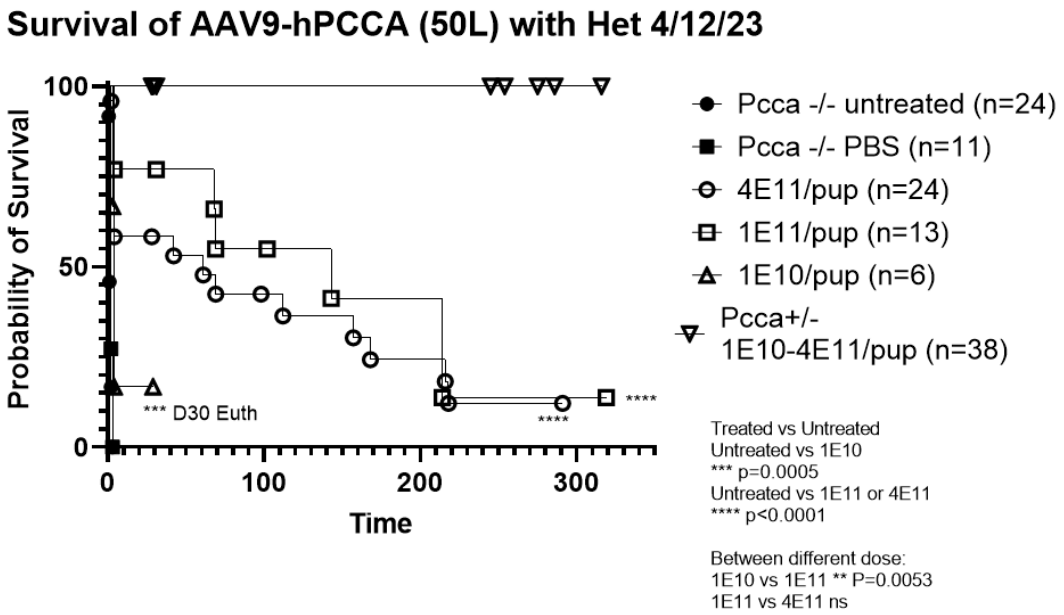
The mice received a single retro-orbital injection of 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA (50L) on P1 and were followed until date of survival following injection/control.

As shown in Figure 11, *Pcca*$^{-/-}$ mice that were untreated and *Pcca*$^{-/-}$ mice that were treated with PBS all died shortly after birth, within 24 hours. Conversely, *Pcca*$^{+/-}$ mice treated with mid and high doses of AAV9-hPCCA (50L) were alive up to ~250 and ~220 days respectively.

Administration of AAV9-hPCCA (50L) rescued the mice from neonatal lethality and prolonged the survival of *Pcca*$^{-/-}$ mice in a dose-dependent manner (Figure 11). *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (50L) showed significant [$p=0.0001$ (1E10 group) and $p<0.0001$ (1E11 and 4E11 groups)] increase in the survival compared with the untreated group.

- *Pcca*$^{-/-}$ mice treated with 1E10 vg/pup (7E12 vg/kg) of AAV9-hPCCA (50L) had a mean survival of 4 days (maximum survival of ~30 days post-injection).
- *Pcca*$^{-/-}$ mice treated with 1E11 vg/pup (7E13 vg/kg) of AAV9-hPCCA (50L) had a mean survival of 143 days (maximum survival of ~250 days post-injection).
- *Pcca*$^{-/-}$ mice treated with 4E11 vg/pup (2.8E14 vg/kg) of AAV9-hPCCA (50L) had a mean survival of 61 days (maximum survival of ~230 days post-injection).

Figure 11: Survival Curves for Mice Treated with AAV9-hPCCA (50L)



AAV9-hPCCA (50L) Median survival	Untreated	PBS	1E10/pup	1E11/pup	4E11/pup	AAV9-hPCCA (50L) Treated Het
Days	1	2	4	143	61	Undefined

Estimated doses by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, were as follows: 1E10 vg/pup = 7E12 vg/kg; 1E11 vg/pup = 7E13 vg/kg; and 4E11 vg/pup = 2.8E14 vg/kg. The p values for the comparisons between AAV9-hPCCA (10L)-treated and untreated *Pcca*$^{-/-}$ mice and between the different dose groups were calculated using a Mantel-Cox test.

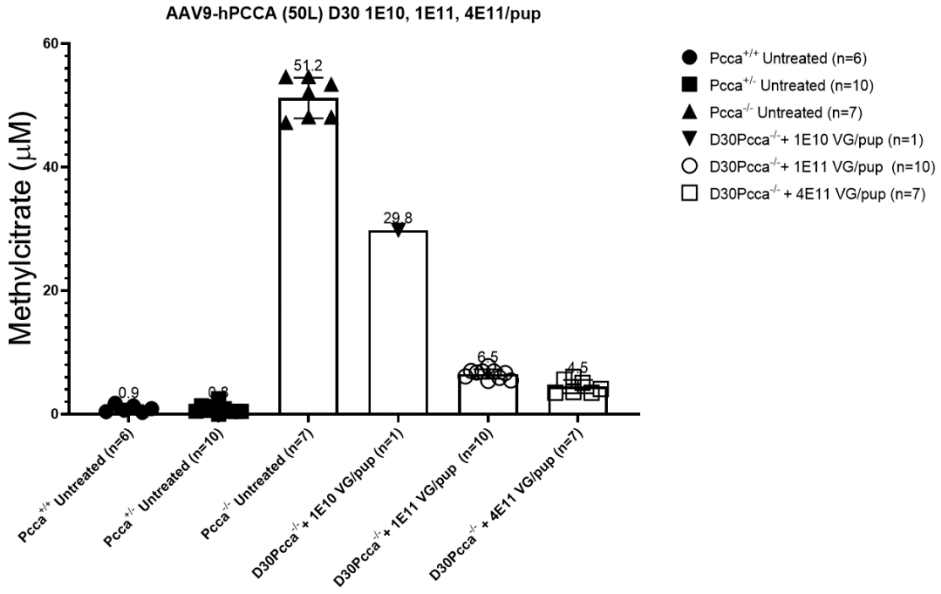
7.2.2 Plasma 2-Methylcitrate Levels in Plasma at Day 30 after Injection of AAV9-hPCCA (50L)

Plasma 2-MC levels were measured in a subset of untreated mice (measured at birth) and treated mice with doses of 1E10, 1E11 and 4E11 vg/pup (7E12, 7E13 and 2.8E14 vg/kg) of AAV9-hPCCA (50L) (measured at Day 30 post-injection). Mean ± SD 2-MC plasma levels are shown in Figure 12.

As shown in Figure 12, untreated *Pcca*$^{+/+}$, *Pcca*$^{+/-}$ and *Pcca*$^{-/-}$ mice at birth had mean 2-MC plasma concentrations of 0.9, 0.8 and 51.2 µM respectively.

The plasma 2-MC levels in the *Pcca*$^{-/-}$ mice treated with AAV9-hPCCA (50L) at 1E11 and 4E11 vg/pup (7E13 and 2.8E14 vg/kg) were significantly decreased when compared to those observed in untreated *Pcca*$^{-/-}$ mice, with $p<0.001$.

Figure 12: Plasma Methylcitrate Levels on Day 30 Following Administration of AAV9-hPCCA (50L) at 1E10, 1E11 and 4E11 vg/pup (7E12, 7E13 and 2.8E14 vg/kg)



Estimated doses by body weight (vg/kg), using the assumption that a pup's average weight was 1.4 g, were as follows: 1E10 vg/pup = 7E12 vg/kg; 1E11 vg/pup = 7E13 vg/kg; and 4E11 vg/pup = 2.8E14 vg/kg.

8. STUDY DEVIATIONS

No major deviations to the study protocol were reported. Though study parameters and measures were prospectively designed, sacrifice time points were dependent upon morbidity and mortality of the animals.

9. GLP COMPLIANCE STATEMENT

This study was not conducted under the standards for Good Laboratory Practice (GLP) for Non-clinical Laboratory Studies as promulgated by the United States Food and Drug Administration (21 Code of Federal Regulations part 58) as it was a proof-of-concept study.

10. CONCLUSIONS

- A single retro-orbital injection of AAV9-hPCCA (10L and 50L) at 1E9 (10L only), 1E10, 1E11 or 4E11 vg per pup (i.e., 7E11, 7E12, 7E13 and 2.8E14 vg/kg), administered on P1, increased survival duration in *Pcca*$^{-/-}$ mice, a model of PCCA-related PA that has no PCCA immunoreactivity and does not survive the immediate neonatal period. AAV9-hPCCA (10L and 50L) treatment significantly and dose-dependently increased the length of survival of the mice, with mice in the highest dose group surviving up to approximately 200 days after AAV9-hPCCA (10L) injection and 250 days after AAV9-hPCCA (50L) injection.
- At Day 30 and 51, *Pcca*$^{-/-}$ mice treated with the 1E11 vg/pup dose of AAV9-hPCCA (10L) showed significant increase in body weight compared to Pcca-/- mice treated with 1E10 vg/pup of AAV9-hPCCA (10L). At Day 51, the body weight of *Pcca*$^{-/-}$ mice treated

with 1E11 vg/pup of AAV9-hPCCA (10L) was significantly higher (p=0.0093) than that of Pcca-/- mice treated with 4E11 vg/pup of AAV9-hPCCA (10L). The increase in body weight may reflect enhanced overall well-being of the treated animals.

- Administration of AAV9-hPCCA (10L) at 1E10, 1E11 and 4E11 vg/pup (i.e., 7E12, 7E13 and 2.8E14 vg/kg) significantly and dose-dependently decreased plasma levels of 2-MC in *Pcca*$^{-/-}$ mice when compared to untreated *Pcca*$^{-/-}$ mice.
- Administration of AAV9-hPCCA (10L) at 1E10 to 4E11 vg/pup (i.e., 7E12 to 2.8E14 vg/kg) to *Pcca*$^{-/-}$ mice resulted in lasting expression of the *PCCA* transgene (on Day 30), supported by western blot and *in situ* hybridization methods.
- Administration of AAV9-hPCCA (50L) at 1E11 vg/pup (i.e., 7E13 vg/kg) to *Pcca*$^{-/-}$ mice resulted in lasting expression of the *PCCA* transgene (at Day 30) in the liver and heart tissue.
- *In vivo* 1-^{13}C-propionate oxidation showed similar levels between untreated WT mice and treated *Pcca*$^{-/-}$mice on Day 30 after injection of AAV9-hPCCA (10L) at 1E11 vg/pup (7E13 vg/kg).

11. REFERENCES

1. Surtees, R.A., E.E. Matthews, and J.V. Leonard, *Neurologic outcome of propionic acidemia.* Pediatr Neurol, 1992. **8**(5): p. 333-7.
2. Shchelochkov, O.A., N. Carrillo, and C. Venditti, *Propionic Acidemia*, in *GeneReviews((R))*, M.P. Adam, et al., Editors. 1993: Seattle (WA).
3. Rashnonejad, A., et al., *Large-Scale Production of Adeno-Associated Viral Vector Serotype-9 Carrying the Human Survival Motor Neuron Gene.* Mol Biotechnol, 2016. **58**(1): p. 30-6.
4. Chandler, R.J., et al., *Systemic gene therapy for methylmalonic acidemia using the novel adeno-associated viral vector 44.9.* Mol Ther Methods Clin Dev, 2022. **27**: p. 61-72.
5. Chandler, R.J. and C.P. Venditti, *Pre-clinical efficacy and dosing of an AAV8 vector expressing human methylmalonyl-CoA mutase in a murine model of methylmalonic acidemia (MMA).* Mol Genet Metab, 2012. **107**(3): p. 617-9.
6. Chandler, R.J. and C.P. Venditti, *Long-term rescue of a lethal murine model of methylmalonic acidemia using adeno-associated viral gene therapy.* Mol Ther, 2010. **18**(1): p. 11-6.
7. Carrillo-Carrasco, N., et al., *Liver-directed recombinant adeno-associated viral gene delivery rescues a lethal mouse model of methylmalonic acidemia and provides long-term phenotypic correction.* Hum Gene Ther, 2010. **21**(9): p. 1147-54.
8. Sénac, J.S., et al., *Gene therapy in a murine model of methylmalonic acidemia using rAAV9-mediated gene delivery.* Gene Ther, 2012. **19**(4): p. 385-91.
9. Chandler, R.J., et al., *Adeno-associated virus serotype 8 gene transfer rescues a neonatal lethal murine model of propionic acidemia.* Hum Gene Ther, 2011. **22**(4): p. 477-81.
10. Chandler, R.J., et al., *Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy.* J Clin Invest, 2015. **125**(2): p. 870-80.
11. *NHGRI Animal Care and Use Committee (ACUC) Guidelines for Euthanasia of Rodents*. Available from: https://theodora.com/rodent_laboratory/guideline_01_1.html.
12. Marcell, P.D., et al., *Quantitation of methylmalonic acid and other dicarboxylic acids in normal serum and urine using capillary gas chromatography-mass spectrometry.* Anal Biochem, 1985. **150**(1): p. 58-66.
13. Allen, R.H., et al., *Elevation of 2-methylcitric acid I and II levels in serum, urine, and cerebrospinal fluid of patients with cobalamin deficiency.* Metabolism, 1993. **42**(8): p. 978-88.
14. Liu, Y.N., et al., *Measuring propionyl-CoA carboxylase activity in phytohemagglutinin stimulated lymphocytes using high performance liquid chromatography.* Clin Chim Acta, 2016. **453**: p. 13-20.

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

PROTOCOL

Study Title:	Evaluation of the efficacy, biodistribution, and safety of an Adeno-associated virus 9 (AAV9) Propionyl-CoA Carboxylase Subunit A (PCCA) gene therapy candidate in a propionic acidemia (PA) disease mouse model
Protocol Number:	*[protocol number]*
Sponsor:	*[sponsor name and contact information]*
Test Facility:	*[testing facility name and contact information]*
Study Director:	*[name of study director]*
Sponsor Representative:	*[name of sponsor representative]*
Version:	01

SPONSOR SIGNATURE

Sponsor Representative Date

SIGNATURES

Study Director Date Management Date

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

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***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

1 OBJECTIVE

The purpose of this study is to evaluate the pharmacology and toxicology of the sponsor's AAV9-hPCCA gene therapy candidate in homozygous knockout (KO) *Pcca* mice and their wildtype (WT) counterparts.

2 JUSTIFICATION

Propionic acidemia (PA) is an inherited autosomal recessive metabolic disorder that is characterized by deficiency of propionyl-CoA carboxylase (PCC), which is an enzyme necessary to break down proteins and fats. This disorder presents early in infants and without treatment, coma and death may occur. The sponsor has been developing a new AAV9 gene therapy that replaces the deficit in PCCA, one of two genes that encodes the PCC enzyme. Treatment with this AAV9 therapy has shown a reversal in the loss of PCC protein and an increase in survival of *PCCA* knock out (i.e., $Pcca^{Q133Lfs*41/Q133Lfs*41}$ homozygous) mice.

3 REGULATORY COMPLIANCE

This study will not be conducted in accordance with U.S. FDA 21 CFR Part 58 (Good Laboratory Practices for Nonclinical Laboratory Studies, GLP). Standard operating procedures (SOPs) will be followed.

All animal work will be conducted in designated animal research facilities, which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulation (9 CFR Parts 1, 2, and 3, as applicable), as well as the *Guide for the Care and Use of Laboratory Animals* (2011).

4 KEY STUDY PERSONNEL

Changes in the Study Director, if any, will be added by revision. Any additional key study personnel changes or additions will be identified in the final report.

Study Director:	*[name and contact information of study director]*
Sponsor Representative:	*[name and contact information of sponsor representative]*
Attending Veterinarian:	*[name and contact information of attending veterinarian]*
Contributing Scientist Vector Biodistribution and Transgene Expression:	*[name and contact information of contributing scientist]*
Contributing Scientist – Bioanalysis/LC-MS/MS:	*[name and contact information of contributing scientist]*
Contributing Scientist – Pathology:	Will be included in the final report

5 TEST ARTICLE

The AAV9 test article and diluent (if necessary) will be provided and characterized by the Sponsor or designee. Documentation on, at a minimum, the identity (supplier/manufacturer), viral titer, injection volume, injection titer, batch number and/or lot number, mass/concentration, sterility, and stability for the test and carrier article will be provided, as certificates of analysis or equivalent documents, for inclusion in the final report.

5.1 Test Article

Identity:	AAV9-hPCCA (50L)
Description:	*[test article, including promoter, gene of interest, and other key elements]*.
Supplier/Manufacturer:	*[name of contractor]*
Stock Viral Titer:	5.64 e+13 gc/ml viral genomic titer by qPCR
Batch/Lot Number:	TL-21-001-41
Storage Conditions:	-90°C to -70°C

5.2 AAV9 Diluent

Identity:	Phosphate-buffered saline (PBS)
Description:	Balanced salt solution
Supplier/Manufacturer:	Will be included in the final report
Batch #/Lot#:	Will be included in the final report
Storage Conditions:	Ambient

6 TEST SYSTEM

Species/Strain:	PccaQ133Lfs*41/Q133Lfs*41 (KO) Pcca+/+ (WT) Mice will be bred in-house
Target Age at Arrival/ Study Start:	Postnatal Day 0-1 (P0-1)
Body Weight range at Arrival/ Study Start:	< 2 grams. Actual body weight range will be included in the final report
Identification:	Cage cards/tail marks/tattoos/microchips/ear tags or marks/shaving per *[SOP number and name]*

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

Number on Study/Sex: KO: 72 males (M) and 72 females (F) on study; WT: 90 males (M) and 90 females (F) on study. Additional mice will be bred to meet study numbers and replace found dead or moribund animals

Source: Bred in-house

7 ANIMAL HUSBANDRY

Housing: Pups will remain with the dam until weaning age (P21) or until euthanasia. After weaning, mice will be housed up to 5 animals per cage in polycarbonate shoebox cages on racks with SaniChip, with bedding changed per *[SOP number and name]*. Fighting among animals may result in single housing to avoid injury and preserve group size.

Quarantine: Not applicable (N/A)

Feed: Mice will be fed Harlan Global Certified Rodent Chow (2016C) *ad libitum*. Each lot of feed is analyzed for contaminants by the manufacturer and will be used within designated shelf-life, per *[SOP number and name]*. The Attending Veterinarian or designee and Study Director will review the feed analyses documentation and copies of the analyses will be included in the study file.

Water: Municipal water will be provided *ad libitum*. Water is analyzed according to the schedule outlined in *[SOP number and name]*. The Attending Veterinarian or designee and the Study Director will review the water analyses documentation and copies of the analyses will be included in the study file.

Environmental Conditions: The targeted indoor conditions for temperature and photoperiod will be as follows:

Temperature: 20 – 26ºC
Relative Humidity: 30-70%
Light Cycle: 12 hours

The light cycle may be briefly interrupted for experimental timepoints. Excursions that may have an impact on the study will be reviewed by the Study Director and noted in the study file. Light, humidity, and temperature excursions are defined as a sustained reading that falls out of range for more than 3 hours. Other excursions will be dealt with on a case-by-case basis. The Study Director will review deviations and report any excursions that may have an impact on the study.

Health Status (including Serology): Only otherwise healthy animals will be used on study (accounting for disease model).

Palliative and/or Prophylactic Treatments: The Study Director in consultation with the Veterinary staff may approve palliative and/or prophylactic treatments under emergency situations. If needed, the animal may be removed from the study upon consultation with the Sponsor Representative. In addition, the Sponsor Representative will be notified of the treatment and treatment will be documented in the Study file.

8 EXPERIMENTAL DESIGN

Table 1 summarizes the experimental design. Animals (both WT and KO) will receive Vehicle, Low Dose, Mid-Dose, Mid-High Dose, or High Dose TA formulation via temporal vein injection on postnatal day 0-1 (P0-1). Animals will be monitored for clinical signs and body weight throughout the study. Samples will be collected for bioanalysis, biodistribution, and histology.

Table 1. Experimental Design

Group	Treatment	Dose[a] (vg/pup)	Genotype	7 Day M[b]	7 Day F[b]	30 Day M[b]	30 Day F[b]	90 Day M[b]	90 Day F[b]
1	Vehicle	N/A	WT	6	6	6	6	6	6
2	Low Dose TA	1E10	WT	6	6	6	6	6	6
2	Low Dose TA	1E10	KO	6	6	6	6	6	6
3	Mid Dose TA	5E10	WT	6	6	6	6	6	6
3	Mid Dose TA	5E10	KO	6	6	6	6	6	6
4	Mid-High Dose TA	1E11	WT	6	6	6	6	6	6
4	Mid-High Dose TA	1E11	KO	6	6	6	6	6	6
5	High Dose TA	4E11	WT	6	6	6	6	6	6
5	High Dose TA	4E11	KO	6	6	6	6	6	6

[a] vg = vector genomes Stock vector concentration will be 5.64E13 gc/ml viral genomic titer by qPCR. Total dosing volume per pup will be 0.02 mL.
[b] Necropsy: At the 30 Day & 90 Day euthanasia timepoints, 3M/3F per genotype will be designated for bioanalysis. Necropsy days at the 30 Day & 90 Day euthanasia timepoints are +/- 3 days. "BioA"/hematology analysis, and 3M/3F per genotype will be designated for serum chemistry "SC" evaluations. Refer to Section 11.4. Clinical pathology analysis will not be performed on 7 Day Necropsy animals, due to low total blood volume.

9 ANIMAL IDENTIFICATION, GENOTYPING, AND SPARE ANIMALS

Mice will be uniquely identified by ear tag, microchips, markings, shaving and/or an alphanumeric number according to *[SOP number and name]*.

All pups will be genotyped (tail or toe snip) per *[SOP number and name]*.

Samples will be sent to Transnetyx for genotyping. The primer sequences to be used for genotyping are as follows: *[sequences of primers]*

Mice will be bred to fulfill study design requirements. Spare animals may be substituted for study animals or replace study animals if animals are found dead or cannibalized following injections. Early postnatal mortality is expected, and replacement animals will be used in these cases or in the event of a mis-dose or an assigned animal needs to be removed from study for health or any other reason through 7 days post-injection. Unused spare animals may be euthanized or used for a different study.

10 TEST ARTICLE ADMINISTRATION

10.1 Formulation

The stock TA will be provided at a concentration of 5.64E13 gc/ml viral genomic titer by qPCR and stored at -70 to -90ºC. The TA will be diluted with the Vehicle to achieve the dosing concentrations described in Table 1. The TA formulation may be prepared in advance of dosing and stored at -70 to -90ºC until the day of use. The stock TA and dosing solution should be mixed manually by inversion or pipetting up and down when needed. On the day of dosing, the dosing TA formulations will be stored on wet ice and/or 2-8°C. Syringes will be filled and will then be held at ambient temperature prior to dosing.

The details for TA preparation will be documented on the formulation forms.

10.2 Intravenous Injection in Facial Vein

The TA at the designated doses per Table 1 will be administered intravenously (IV) by alert temporal (facial) vein injection on P0-1 per *[SOP number and name]*. An insulin syringe attached to a 30- or 31-gauge needle will be used to administer the TA or Vehicle. Following identification of the temporal vein (which appears shadowy, runs dorsal to ventral, feeds into the jugular vein, and remains fixed regardless of skin position), the syringe containing TA or Vehicle will be inserted into the temporal vein, the contents expelled, and the needle allowed to remain in the vein for several seconds to prevent backflow of the injectant. Animals will be recovered and returned to the dam cage. Any abnormal observations will be noted in the study file.

11 OBSERVATIONS AND MEASUREMENTS

Timelines of clinical observations, body weights, blood, and tissue collection are summarized in Table 2.

Table 2. Collection and Measurement Timeline

Analysis	Blood/Tissue Collection [B]	Time Point [D]
Detailed Clinical Observations [A]	N/A	All groups; P1-P4, P7, then weekly until Necropsy
Body Weight	N/A	All groups; P1-P4, P7 then weekly until Necropsy

Analysis	Blood/Tissue Collection [B]	Time Point [D]
Clinical Pathology [C,E]	Clinical chemistry (minimum of 0.5 mL) Hematology (minimum of 0.25 mL)	All groups; 30 Day & 90 Day only: At Necropsy
Metabolite and BioAnalysis [C,E]	Blood (target 0.1-0.2 mL) to plasma	All groups; At Necropsy
1-13C propionate oxidation[F]	Expired gas	All groups (except 7-day) on Day 30 (+/- 3 days)
Histology	See Table 5	Group 1 (Vehicle): 3M/3F per time point per genotype: At Necropsy Group 2 (Low Dose): 3M/3F per time point per genotype: At Necropsy Group 5 (High Dose): 3M/3F per time point per genotype: At Necropsy
Tissue Biodistribution	See Table 6	Group 1 (Vehicle): 3M/3F per time point for WT: At Necropsy Group 2 (Low Dose): 3M/3F per time point per genotype: At Necropsy Group 5 (High Dose): 3M/3F per time point per genotype: At Necropsy

[A] Detailed clinical observations are in addition to standard twice daily AMS observations
[B] Volumes listed are for blood collection.
[C] "Bioanalysis-BioA"-designated animals will have blood collected for bioanalysis applications and for hematology, contingent on sufficient volume. "Serum Chemistry-SC"-designated animals will have blood collected for clinical chemistry.
[D] "Weekly" refers to every 7 days +/- 2 days
[E] Analysis is contingent on available blood volume, and may not be performed if blood volume is insufficient.
[F] Due to the difficulty of performing in vivo 1-13-C propionate oxidation in animals, we will attempt to conduct this evaluation but a final decision will be made at a later time

11.1 Data Collection

General Data Documentation Note: Whenever possible, a validated computerized data acquisition system [e.g., Provantis™ or Animal Management System (AMS)] will be used for data acquisition, recording, and or analysis. If unavailable, data will be recorded on forms for each animal, and then entered retrospectively to the electronic system. Hard copy forms will be prepared as backup to Provantis or AMS and will be used when electronic data collection is not feasible. Excursions in refrigerators and freezers temperature may be expected due to use; excursions per *[SOP number and name]* will be assessed for study impact by Study Director or designee.

11.2 Clinical and Detailed Observations

Clinical observations will be performed twice daily (morning and afternoon) and will be documented in AMS. Observations will be oriented towards (1) identifying dead, weak, or moribund animals, and (2) documenting the onset and progression of any abnormal clinical signs. The Study Director or designee will be notified of animals with questionable health status. Decisions regarding the euthanasia of weak or moribund animals will be made by the Study Director in consultation with a veterinarian and the Sponsor.

Detailed clinical observations will be performed according to Table 2. These observations will be performed once daily on their designated dates according to *[SOP number and name]*. Abnormal observations will be reported to the Study Director, and additional observations may be added in cases of morbidity. Severe clinical signs will warrant veterinary consultation, and the course of action will be determined based on discussions with the Study Director and the Sponsor. Additional observations may be added at the discretion of the Study Director or designee.

11.3 Body Weights

Animals will be weighed according to Table 2 until scheduled euthanasia or declared moribund or found dead per *[SOP number and name]*. The interval and frequency of body weight collections may be changed by the Study Director or designee. A terminal body weight will also be obtained on the day of scheduled euthanasia.

11.4 Blood Collections

Terminal blood collections will be collected as described in Table 2. Blood samples will be collected via cardiac puncture for analyses described in the following sections. *[SOP number and name]* will be followed.

11.4.1 Bioanalysis

Only animals designated for "BioA" analysis will have blood samples collected for metabolite analysis. Note that all 7 Day Necropsy blood samples will be used for bioanalysis.

The priority for blood collections for "BioA" mice is as follows: metabolite analysis, and hematology (refer to Section 11.4.2). If there is insufficient volume for all collections, it will be noted in the study file.

Blood will be collected as described in Table 2 into tubes for metabolite analysis: Blood (target 0.1-0.2 mL) will be collected and transferred into K_2EDTA tubes and kept on wet ice. After blood is added, the K_2EDTA tube will be gently inverted to mix well. After collection, the blood samples will be stored on wet ice and will be processed to plasma by centrifugation (1300 g, 2-8°C, ≥ 10 minutes). Plasma samples will be stored at -70 to -90ºC until analysis.

Plasma samples will be analyzed by LC-MS for the analytes 2-methylcitrate, propionyl-carnitine, and 3-hydroxypropionic acid, per *[method number and name]*.

11.4.2 Clinical Pathology

Only animals designated for "SC" analysis will have blood samples collected for clinical chemistry. Analysis will only be performed if there is sufficient blood volume.

For "BioA" mice, refer to Section 11.4.1 for additional blood collections.

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

Blood samples will be collected as described in Table 2 for the following analysis:

Clinical chemistry, 1x clot/SST tube (minimum 0.5 mL)
Hematology, 1x K_2EDTA tube (minimum of 0.25 mL)

Following collection, all samples will be inverted to ensure proper mixing.

Collected samples will be transferred for analysis per *[SOP number and name]*. Sample analysis will be performed on all study animals for which adequate sample volumes are obtained and for which no analytical problems are encountered. If target collection volumes are not obtained or if evaluations are not performed, a reason and notation will be included in the raw data.

For clinical chemistry analyses, whole blood will be centrifuged and separated into cellular and serum fractions. Serum samples will be analyzed on a Hitachi cobas c501Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN) or Vitros 5600 Integrated Chemistry System (Ortho Clinical Diagnostics, Rochester, NY) per *[SOP number and name]*. The clinical chemistry parameters to be measured or calculated are shown in Table 3.

Table 3. Serum Chemistry Parameters

Analyte	Abbreviation[a]	Units
Alanine Aminotransferase (Alanine Transaminase)-Serum	ALT	IU/L
Albumin	ALB	g/dL
Alkaline Phosphatase	ALP	IU/L
Aspartate Aminotransferase (Aspartate Transaminase)-Serum	AST	IU/L
Bicarbonate	HCO3	mg/dL
Bilirubin (Total)	BILI-T	mg/dL
Blood Urea Nitrogen	BUN	mg/dL
Calcium	CA	mg/dL
Chloride	CL-S	mmol/L
Cholesterol (Total)	CHOL	mg/dL
Creatine kinase	CK	IU/L
Creatinine	CRE-S	mg/dL
Glucose	GLU	mg/dL
Gamma Glutamyltransferase	GGT	IU/L
Lactate Dehydrogenase	LDH	IU/L
Phosphate (Phosphorus)	PHOS	mg/dL
Potassium	K-S	mmol/L
Protein (Total)	TP	g/dL
Sodium	NA-S	mmol/L
Triglycerides	TRIG	mg/dL
Albumin/Globulin[b]	A/G	None
Blood Urea Nitrogen/Creatinine[b]	BUN/CRE	None
Globulin[b]	GLOBN	g/dL

[a]The final results may be reported as described above or using the abbreviations from the chemistry system as appropriate
[b]Calculated parameters and ratios

For hematology analyses, whole blood will be collected into tubes containing anticoagulant. Hematology samples will be analyzed by automated analyses (ADVIA™2120 Hematology System) per *[SOP number and name]*. The parameters for hematology are shown in Table 4. Any residual samples will be discarded after the analyses are completed.

Table 4. Hematology Parameters

Parameter	Abbreviation[a]	Units
Red Blood Cell Count	RBC	$10^6/\mu L$
Hemoglobin	HGB	g/dL
Hematocrit	HCT	%
Mean Corpuscular Volume	MCV	fL
Mean Corpuscular Hemoglobin Concentration	MCHC	g/dL
Mean Corpuscular Hemoglobin	MCH	pg
Platelet Count	PLT	$10^3/\mu L$
Mean Platelet Volume	MPV	fL
Reticulocytes (Absolute)	RETIC	$10^9/L$
Percent Reticulocytes (Relative)	RETIC	% RBC
Red Cell Distribution Width	RDW	%
White Blood Cell Count	WBC	$10^3/\mu L$
Neutrophils	PMN	$10^3/\mu L$ (and %)
Lymphocytes	LYM	$10^3/\mu L$ (and %)
Monocytes	MONO	$10^3/\mu L$ (and %)
Eosinophils	EOS	$10^3/\mu L$ (and %)
Basophils	BASO	$10^3/\mu L$ (and %)
Large Unstained Cells	LUC	$10^3/\mu L$ (and %)

[a]The final results may be reported as described above or using the abbreviations from the hematology system as appropriate.

11.4.3 In vivo 1-13C Propionate Oxidation

Due to the difficulty of performing in vivo 1-13-C propionate oxidation in animals, we will attempt to conduct this evaluation as follows.
In vivo 1-13C-propionate oxidation will be determined by collecting expired gas from mutant and WT mice after the animals are injected by the intraperitoneal route with 200 micrograms of 1-^{13}C-sodium propionate. Within each dose group, expired gas from three male or female animals will be combined to comprise each sample, such that each dose group will have a total of n=4 samples per time point per genotype. Combining expired gas collections from 3 animals will increase the sample signal to noise. Four metabolism style chambers will be implemented to allow the collection of expired breath into an airtight container. The chamber will contain a CO2 probe to allow the direct measurement of CO2 generated by each animal. An aliquot of expired air will be removed from the chamber for analysis of 13C enrichment in CO2. The isotope ratio (13C/12C) of the expired gas will be determined by isotope ratio mass spectrometer using the University of New Mexico Isotopes Analysis Core. The percent dose metabolized at each time point will be calculated as % dose metabolized = total 13C excreted (mmol/dose (mmol) × 100%). Any revisions to the method will be included in the final report.

12 PATHOLOGY

12.1 Euthanasia and Necropsy

At the time of scheduled euthanasia or in cases of moribund euthanasia, mice will be euthanized by intraperitoneal injection of an overdose of a barbiturate-based sedative (Euthasol®) per *[SOP number and name]*. A complete necropsy will be performed on all main study animals. Necropsies will consist of a complete external and internal examination including the injection sites, body orifices (ears, nostrils, mouth, anus, etc.) and cranial, thoracic and abdominal organs

and tissues. All gross findings will be recorded in descriptive terms including locations(s), size, shape, color, consistency, and number. The detailed necropsy and tissue collection procedures will be described in a study specific necropsy guide.

12.1.1 Moribund and Found Dead Animals

Animals showing signs of severe debility, particularly if death appears imminent, and animals for which humane euthanasia is deemed necessary, will be euthanized. Reasons for euthanasia will be documented in the study file. A cause of death will be determined if possible.

Organ weights, clinical pathology, and tissue collection will not be performed on moribund or found dead animals prior to 7 days post-dosing. Moribund animals older than P7 will be euthanized and necropsied within 24 hours of euthanizing. In the case when moribund animals cannot be necropsied immediately following euthanasia, the carcass will be stored at 4-10°C until time of necropsy. Gross necropsy, organ weight, and/or tissue collection will be performed on moribund animals. Gross necropsy, organ weight, and/or tissue collection may not be performed on found dead animals. The Study Director will consult with veterinary and necropsy staff, and the Sponsor, to make this determination for found dead animals.

12.1.2 Tissue Collection and Preservation

A terminal body weight will be recorded for animals undergoing scheduled or moribund euthanasia. No terminal body weight will be collected for animals found dead.

Once an animal has reached a surgical plane of anesthesia after being administered Euthasol®, the thorax will be opened and blood, as much as possible, will be collected by cardiac puncture. Blood will be collected for "BioA" and "SC" animals, with priority, as described in Table 2 and Section 11.4.

For all histopathology mice (see Table 2; Groups 1, 2 & 5, 3M/3F per time point per genotype) at scheduled euthanasia, tissues will be collected and weighed as listed in Table 5. Tissues will be collected and processed for assessment of histopathology, and all tissues will be fixed in 10% NBF or Modified Davidson's solution (testes, epididymis, and eyes with optic nerve). Remaining carcasses will be discarded.

For all remaining mice at scheduled euthanasia, tissues for vector biodistribution, gene expression, and Pcc enzyme activity (Table 6 and Section 12.1.5) will be harvested using clean collection techniques. Tissues will not be weighed. For DNA, RNA, Pcc Enzyme Activity, and Western Blot analysis, samples will be collected, snap-frozen in liquid nitrogen, and stored at -70 to -90ºC until analysis.

For dosed animals found dead or euthanized in a moribund condition past P7, all tissues listed in Table 5 will be collected and fixed in 10% NBF (testes, epididymis, and eyes with optic nerve in Modified Davidson's solution). Tissues, including gross lesions (if applicable), will be examined by microscopic histopathology on these animals in order to identify, if possible, the cause of death or moribund condition. Organ weights will be collected for animals euthanized as moribund, but not for animals found dead.

The detailed procedures on tissue harvest and processing will be described in the study specific necropsy guide. Any missing tissues during necropsy will be noted in the study file.

Table 5. Tissues for Histopathology

Tissue to Harvest	Weigh	Histopathology
Adrenal Gland (pair)		x
Bone Marrow (Femur)		x
Brain	x	x
Coagulating gland and seminal vesicle		x
Duodenum		x
Epididymis		x
Heart	x	x
Eye with optic nerve		x
Jejunum (with Peyer's patch if possible)		x
Kidney (pair)	x	x
Liver (with gall bladder)[A]	x	x
Lung with mainstem bronchi and cauda trachea	x	x
Lymph node[B] (inguinal, mesenteric and central iliac)		x
Ovaries (pair, females)	x	x
Stomach		x
Pancreas		x
Skeletal Muscle (Quadriceps femoris)		x
Spleen	x	x
Testes (pair, males)	x	x
Thymus	x	x
Gross Lesions[C]		x

[A] Weigh liver with drained gallbladder still attached.
[B] If it can be identified and recovered
[C] If present

Table 6. Tissue Collection for Biodistribution

Tissues[A]	DNA (n)	RNA (n)	Pcc Enzyme Activity (n)	Western Blot (n)
Brain	1	1		
Gonads[B]	1	1		
Heart	1	1	1	1
Eye with optic nerve	1	1		
Kidneys	1	1		
Liver	1	1	1	1
Lungs	1	1		
Spleen	1	1		
Stomach	1	1		
Skeletal Muscle (Quadriceps femoris)	1	1		

[A] When collections are designated for all 4 applications and there is insufficient sample, assay priority is as follows: Pcc Enzyme Activity, DNA, RNA, and Western Blot.
[B] Collect 1 gonad for DNA and 1 gonad for RNA.
n, number of samples per animal

12.1.3 Histopathology

The fixed tissues for histopathological examination will be trimmed, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin per *[SOP numbers and names]*.

Tissues from histopathology mice listed in Table 5 will be fixed in NBF (unless otherwise stated in Section 12.1.2). Tissues in Table 5 will be trimmed, paraffin embedded, sectioned at 5 µm and stained with Hematoxylin and Eosin. Slides will be read at by a board-certified veterinary pathologist.

Samples from all of the organs in Table 5 from Groups 1, 2 & 5 dose groups (3M/3F per time point per genotype) at the three euthanasia time points will be evaluated for histological changes attributable to the vector. Microscopic analysis of all listed tissues from unscheduled euthanasia or animals found dead (if collected) will also be performed.

The histopathological findings will be included in the Pathologist's report for inclusion in the Final Report.

12.1.4 Biodistribution

Tissues in Table 6 (3M/3F per time point per genotype for Groups 1, 2 and 5) will be collected for vector DNA biodistribution determination and transgene mRNA expression analysis via qPCR (*[method number and name]*) and RT-qPCR (*[method number and name]*), respectively.

Contributing scientist analysis reports will be included in the Final Report.

12.1.5 PCC Enzyme Activity Assay

Tissues designated in Table 6 will be collected and stored at <-60°C until analyzed for Pcc enzyme activity by LC-MS per *[method number and name]*.

Contributing scientist analysis reports will be included in the Final Report.

12.1.6 Western Blot for PCCA Protein

Tissues designated in Table 6 will be collected for Western Blot and stored at <-60°C until shipment. Frozen samples will be shipped on dry ice to a sponsor-designated laboratory. This laboratory will provide methods and a contributing scientist report for inclusion in the Final Report.

13 STATISTICAL ANALYSES

Data will be analyzed using appropriate techniques for the type of data collected. Analyses will be used upon the approval of the Study Director and Study Statistician and will be included in the Final Report.

All data will be analyzed using Provantis, GraphPad Prism, WinNonLin, or SAS®.

14 SAMPLE SHIPMENT

1. Western Blot samples from Section 12.1.6 will be shipped on dry ice to a sponsor-designated laboratory.

2. Unused Test Article from Section 5 will be shipped on dry ice to the sponsor at the following address: TBD

15 VERSION HISTORY AND CHANGE SUMMARY

Version	Approval Date	Summary of Changes (including sections)	Justification for Changes
01	See SD signature	Original Signed Protocol	N/A

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

AAV9-PCCA: GLP 6-MONTH TOXICITY STUDY WITH BIODISTRIBUTION FOLLOWING A SINGLE INTRAVENOUS INJECTION IN MICE

SPONSOR: *[name and contact information of Sponsor]*

NCATS REPRESENTATIVE: *[name and designation of Sponsor representative]*

CONTRACT NUMBER: *[contract number]*

CONTRACTOR: *[name of contractor]*

PRINCIPAL INVESTIGATOR: *[name of principal investigator]*

STUDY DIRECTOR: *[name of study director]*

PROPOSED IN-LIFE PHASE: *[duration of proposed in-life phase study]*

Start: *[start date of study]*

Finish: *[end date of study]*

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

I. OBJECTIVE

The objective of this study is to determine target organ toxicity and biodistribution of **AAV9-PCCA gene** vector administered once via intravenous injection in mice with a 6-month observation period.

II. MATERIALS AND METHODS

A. Test and Control Article:

1. **Name of Test Article: AAV9-hPCCA**

2. **Name of Control Article:**

3. **Characterization and Documentation of Methods of Synthesis, Fabrication or Derivation:**

   a. **Test Article:** Compound identity, strength, quality, stability and purity as well as documentation of methods of synthesis, fabrication or derivation are the responsibility of the Sponsor. Sufficient quantity of drug shall be reserved for archiving from each lot and shipment used.

   b. **Control Article:** TBD

4. **Stability and Storage:**

   a. **Test Article:** TBD

   b. **Control Article:** TBD

5. **Formulation Preparation, Stability and Storage:**

   a. **Test Article:** Pre-formulated material will be provided.

6. **Dose Concentration:** Test article will be diluted to required dose concentration (vg/mL) immediately prior to delivery to maintain uniform volume of injection. A sample from each dosing mixture will be collected and returned to the manufacturer for analysis. An adequate quantity of each dosing mixture will be retained for possible analysis until the acceptance of the final report on this compound. Syringe and needle compatibility study will be performed to determine 'conditions in-use' stability.

B. Test System:

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

1. Species, Strain Supplier and Test System Justification:

C57BL6 Mice supplied by an approved commercial supplier will be used in this study. This is an accepted species to support studies of compounds used or intended for use in humans.

2. Initial Age, Sex and Weight:

On the day of dosing, the weight ranges of mice will be 6 weeks old and approximately 20 grams.

3. Care and Housing:

General procedures for animal care and housing will be in accordance with the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996 and the U.S. Department of Agriculture through the Animal Welfare Act (Public Law 99-198). Appropriate caging and bedding or cage board (not cedar or pine chips) will be used. No contaminants should be present in the bedding which could interfere and affect the results of the study. Environmental parameters will be set to maintain conditions specified in the facility SOPs. Environmental conditions will be within specified limits of at least 90 percent of scheduled observations.

4. Diet and Water Supply:

Diet is to be certified, commercial, dry rodent chow provided *ad libitum*. Water source will be the public supply given *ad libitum*. No contaminants will be present in the feed or water which could interfere and affect the results of the study.

5. Quarantine:

All animals will be quarantined for a minimum of 7 days prior to baseline measurements. No prophylactic or therapeutic treatment will be administered during the quarantine period. Only healthy animals will be placed on study.

6. Animal Identification:

All animals will be given a unique identification number for this study by ear tag or other approved method.

C. Experimental Design

1. Randomization:

In order to obtain groups that are comparable by weight, all animals will be randomly assigned to their respective treatment groups using a computer-based body weight stratification procedure. The individual body weights required for randomization are to be determined on day -3.

2. Group Assignments:

After randomization, animals will be assigned to three dose groups and a vehicle control group (VCTL) as indicated in following table.

Group	Dose	Day 7[a]	Month 1[a]	Month 3[a]	Month 6[a]
I	0 (Vehicle Control)	20(10+10)	20(10+10)	20(10+10)	20(10+10)
II	Low 1E13 vg/kg/dose	20(10+10)	20(10+10)	20(10+10)	20(10+10)
III	Mid 4E13 vg/kg/dose	20(10+10)	20(10+10)	20(10+10)	20(10+10)
IV	High 3E14 vg/kg/dose or MFD[b]	20(10+10)	20(10+10)	20(10+10)	20(10+10)

[a]Number of animals necropsied (M+F); 5M/5F from each dose group and euthanasia time point will be designated for evaluation of biodistribution and hematology. The remaining 5M/5F will be designated for histopathology and serum chemistry.
[b]MFD, maximum feasible dose (MFD) will be determined based on AAV conc in the 200L tox lot and max volume to be injected per animal (2 mL/kg IV)

3. Route of Administration and Reason for Choice:

The test article will be administered once by intravenous injection because this is the intended route of administration of this test article in humans.

4. Dosing Procedure:

The amount of test article administered to each mouse will be a uniform volume for all dose levels and for all animals in the study. All calculations for dilution of the test article will be performed prior to study commencement and will be checked by a second individual who will initial and date the verification.

5. Measurements:

a. Clinical Signs:

All animals will be observed twice daily during 1st week after dosing and at least once daily thereafter until study ends or more often as clinical signs warrant.

b. Body Weight:

All surviving animals will be individually weighed weekly for first 60 days and biweekly thereafter. The animals should be weighed at approximately the same time each day.

c. Food Consumption

Food consumption will be recorded weekly

d. Clinical Pathology:

Blood should be drawn from each animal for clinical pathology and anti-drug antibody determinations on the days of necropsies prior to termination. A blood sample will be obtained prior to the necropsy of each animal sacrificed in a moribund condition, if possible. Blood will be collected from 5M/5F for hematology and 5M/5F for serum chemistry as noted in the table above.

Hematology:

Erythrocyte count (RBC) - $10^6/mm^3$
Hemoglobin (HGB) - g/dL
Hematocrit (HCT) - %
Mean corpuscular volume (MCV) - fl
Mean corpuscular hemoglobin (MCH) - pg
Mean corpuscular hemoglobin concentration (MCHC) - g/dL
Platelet count (Plate) - $10^3/mm^3$
Reticulocyte count (RETIC) - %RBC
Total leukocyte count (WBC) - $10^3/mm^3$
Differential leukocyte count - $10^3/mm^3$
Nucleated red blood cell count (nRBC) - nRBC/100 WBC

Clinical Chemistry:

Urea nitrogen (BUN) - mg/dL
Serum aspartate aminotransferase (AST) - I.U./L
Serum alanine aminotransferase (ALT) - I.U./L
Alkaline phosphatase (ALP) - I.U./L
Gamma glutamyl transferase (GGT) - I.U./L
Glucose (GLUC) - mg/dL
Creatinine (CREA) - mg/dL

Creatine kinase (CK)-mg/dL
Total protein (TP) -g/dL
Albumin (A) - g/dL
Globulin (G; by calculation) - g/dL
A/G ratio
Sodium (Na) - mEq/L
Potassium (K) - mEq/L
Chloride (Cl) - mEq/L

e. Functional Observation Battery (FOB) A standardized and validated battery of neurobehavioral evaluations, designed to assess central/peripheral nervous system status will be conducted (according to SOPs, examiner blinded to treatment group) on all animals during the study week of month 1 and 6 necropsies.

f. Immunogenicity: Anti-AAV9 and anti-PCCA antibodies will be determined on the day of necropsies.

g. Biodistribution: qPCR and RT-PCR methods for biodistribution will be used and the following tissues will be collected from all animals at necropsy: blood, injection site, heart, brain, liver, lung, spleen, kidneys, and ovary/testis.

All tissues from Day 7 animals in the control and high dose will be tested for qPCR and subsequently RT-PCR in positive tissues. Any tissues positive by qPCR will be tested in lower dose groups and at subsequent timepoints (month 1, 3 and 6). Any gross lesions observed will also be sampled for qPCR analysis.

h. Interferon-gamma enzyme-linked immunospot (ELISPOT) assay:
Spleen samples will be processed for T-cell responses to AAV9 and PCCA by interferon-gamma (IFN-γ) ELISPOT assay on 5 animals/sex/group at each necropsy timepoint.

i. Necropsy Procedure:

All animals from the study will be sacrificed as shown in the above study design table. Tissues from five mice/sex/group will be collected and immersion fixed in formalin for histopathology. Another five mice/sex/group will be used for PCR tissue collection.

Two samples of the tissues listed in section h. above will be collected. One set (100 – 200 mg per sample) for qPCR analysis of viral vector DNA will be placed into sterile tubes, snap-frozen in liquid nitrogen and kept at < -70°C for biodistribution. The other set will be collected using disposable 5-mm biopsy punches and immediately immersed into RNAlater for gene expression. All tissues will be harvested using techniques that avoid the potential for cross contamination among different tissue samples.

Moribund animals should be terminated out of sequence with complete histopathology and clinical pathology performed as for scheduled necropsies. Animals found dead will have a complete necropsy, unless severely autolyzed.

All animals will have final body weights taken and will be bled for clinical pathology determinations prior to termination. A complete necropsy and all antemortem observations will be recorded for each animal and commented on or confirmed at necropsy. Animals which are clinically normal will also be so indicated. A pathologist will be available to examine any unusual findings.

The tissues listed below with the following exceptions will be fixed in cold, buffered neutral 10% formalin. Brain and spinal cord will be fixed in 4% paraformaldehyde. The eye, optic nerve and testes are preserved in modified Davidson's fixative. The animal identification will be retained with tissues taken during necropsy.

List of tissue samples		
adrenal (2) aorta brain (cerebrum, cerebellum) cecum cervix colon duodenum epididymis (2) esophagus eye (2) femur with bone marrow heart ileum injection site jejunum kidney (2) sciatic nerve	Seminal vesicle lacrimal gland liver lung with large bronchi lymph node (mandibular) lymph node (mesenteric) mammary gland (females) optic nerve (2) ovary (2) pancreas pituitary gland prostate rectum salivary gland [mandibular (2)]	Skeletal muscle (biceps femoris) spinal cord (cervical, thoracic and lumbar) spleen sternum with bone marrow femoro-tibial joint stomach testis (2) thymus thyroid (2 lobes) with parathyroid tongue trachea urinary bladder uterus vagina

Organ Weights (animals from histopathology group only)

The following organs, gender appropriate, will be weighed at scheduled necropsy. Organ weight data will not be collected on animals found dead or necropsied prior to scheduled necropsy. Paired organs will be weighed together. Organ-to-body weight ratios and organ-to-brain weight ratios will be calculated.

List of organs	
Adrenal Glands	Ovaries (without oviduct)
Brain	Spleen
Epididymides	Testes
Heart	Thymus
Kidneys	Uterus (with cervix)
Liver	
Lungs	

j. Microscopic Pathology:

Tissue Processing

All fixed tissues from (five mice/sex) control and high dose groups will be processed to slides.

Histopathologic Evaluation

A complete histopathological evaluation of all tissue collected at necropsy (except animal identification) for all terminated or found dead animals will be conducted by a pathologist.

All groups (five mice/sex) –any tissues with gross findings or significant organ weight differences.

In addition, all tissue slides prepared from animals in Groups 1 and 4 will be examined by the study pathologist. Any target tissues identified in Groups 1 and 4 will be examined in Groups 2 and 3.

Any macroscopic lesion identified in unscheduled necropsies (animals that die during the study) will be examined microscopically. An internal peer review will be performed according to SOP.

All lesions will be categorized either as drug-related or non-drug related. Each lesion should be listed and coded by the most specific topographic and morphologic diagnoses, severity and distribution.

III. QUALITY ASSURANCE

A. Type of Study:

As a result of the GLP nature of this study, it will be conducted in strict compliance with FDA Good Laboratory Practice (GLP) Regulations. The study will be conducted using procedures that are consistent with GLP regulations,

and the study protocol, procedures, data and report will be audited by the laboratory's Quality Assurance Unit. Submission ready reports will be provided.

B. Standard Operating Procedures:

All operations pertaining to this study, unless specifically defined in this protocol, will be performed according to the standard operating procedures of the laboratory and any deviations will be documented.

C. Protocol Ammendments:

All changes in or revisions of an approved protocol and the reasons therefore will be documented, signed, and dated by the Principal Investigator, Study Director and the NCATS representative. Amendments will be maintained with the protocol. Verbal approval for a protocol change may be granted by the NCATS representative, but a written amendment will follow.

D. Records:

In view of the GLP nature of the study, data will be audited by the laboratory's Quality Assurance Unit. Study data will be archived in the laboratories archive for a period required by the GLP regulations prior to shipment to an archive designated by the NCATS.

IV. PROTOCOL APPROVALS:

Study Director:_____Date:_____

Principal Investigator:_____Date:_____

NCATS Study Monitor;_____Date:_____

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3.2.S Drug Substance

3.2.S.1 General Information

3.2.S.1.1 Nomenclature

The drug substance name is Adeno-Associated Virus 9 human Propionyl-Coenzyme A (CoA) Carboxylase, alpha subunit (AAV9-hPCCA).

3.2.S.1.2 Structure

AAV9-hPCCA is an Adeno-Associated Virus 9 vector expressing a functional human codon optimized cDNA encoding the Propionyl-CoA Carboxylase, alpha subunit (*PCCA*), under control of the *[specific promoter]*. The AAV9 capsid was selected to further enable hepatic and cardiac transduction. The therapeutic transgene cassette was designed with a *[specific promoter]* to enable wide expression. A schematic of the vector transgene and description of the cassette features are included in Figure 1. For purposes of the GMP manufacturing process, the inverted terminal repeats (ITRs) of the GMP AAV9-hPCCA have been optimized, strictly conserving all other elements of the research grade AAV9-hPCCA-UMMS used in the proof-of-concept (POC) studies.

Figure 1: Vector Transgene Schematic and Description of the Cassette

[Schematic of the vector including length of different cassette elements]

Three separate plasmids are used to generate AAV9-hPCCA. pAAV9-hPCCA is the gene of interest (GOI) plasmid. The pHelper-Kan plasmid provides the adenoviral helper genes, E2A, E4, and VA for AAV packaging. The pRC-9-Kan plasmid provides the AAV rep and cap genes for AAV viral vector packaging.

pAAV9-hPCCA is a *[XX]* bp adeno-associated virus (AAV) serotype 9 plasmid containing the gene of interest (GOI) with 5' and 3' *[AAV serotype]* ITR fragments. The plasmid includes a high copy number ColE1/pMB1/pBR322/pUC origin of replication and *[a specific promoter]*. The plasmid contains an intron downstream to promoter. The plasmid also carries a kanamycin resistance cassette located upstream from the f1 bacteriophage origin of replication. The plasmid encodes the GOI for human propionyl-CoA carboxylase alpha chain enclosed between AAV ITRs. Propionyl-CoA carboxylase is a heterodimeric mitochondrial enzyme that takes part in fatty acid metabolism. Downstream to the GOI there is *[a specific regulatory element]*. The pAAV9-hPCCA plasmid map is provided in Figure 2 and the sequence is provided in Attachment 1.

pHelper-Kan is a 11,569 bp AAV helper plasmid containing adenovirus E2A, E4, and VA helper genes. The plasmid includes a high copy number ColE1/pMB1/pBR322/pUC origin of replication and mammalian promoter sequence. The plasmid encodes a neomycin/kanamycin resistance cassette and an F1 bacteriophage origin of replication. The helper plasmid map (pHelper-Kan) is provided in Figure 3 and the sequence is provided in Attachment 1.

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pRC9-Kan is a 7,264 bp AAV packaging plasmid containing AAV2 replication and packaging proteins (Rep) and AAV9 capsid proteins (Cap). The plasmid includes a high copy number ColE1/pMB1/pBR322/pUC origin of replication. The plasmid also carries a neomycin/kanamycin resistance cassette, an f1 bacteriophage origin of replication and a bacterial lac promoter located upstream of the rep/cap genes. The pRC9-Kan plasmid map is provided in Figure 4 and the sequence is provided in Attachment 1.

Figure 2: pAAV9-hPCCA Plasmid Map

[Graphical plasmid map]

Figure 3: Helper Plasmid Map (pHelper-Kan)

[Graphical plasmid map]

Figure 4: Rep-Cap Plasmid Map (pRC9-Kan)

[Graphical plasmid map]

3.2.S.1.3 General Properties

Propionic acidemia (PA) is a rare inborn error of metabolism resulting from deleterious variants in the PCCA or PCCB genes leading to impaired activity of propionyl-CoA carboxylase (PCC). AAV9-hPCCA, an Adeno-Associated Virus 9 (AAV9) vector expressing a functional human codon optimized cDNA encoding the Propionyl-CoA Carboxylase, alpha subunit (PCCA), was developed as a gene therapy for PCCA-related PA. AAV9-hPCCA will introduce corrected DNA into PA patients having a mutation in the PCCA gene. The gene is delivered using a modified AAV virus, serotype AAV9, which is not infectious in humans but maintains its natural ability to deliver genetic material into cells. The AAV9-hPCCA will introduce a normal copy of the human PCCA gene and help restore the normal function of the PCC enzyme in the catabolism of propionyl-CoA. AAV9-hPCCA was tested in a mouse model of PA (the PCCA knock-out mice, designated *Pcca*-/-), in which it was able to rescue the PA phenotype.

3.2.S.2 Manufacture

3.2.S.2.1 Manufacturer(s)

Table 1 lists the manufacturing and testing sites for the AAV9-hPCCA drug substance.

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Table 1: Drug Substance Manufacturing and Testing Facilities

Manufacturer/Contractor Name and Address	Responsibility
Charles River – Keele [Contractor address]	• Manufacture and testing of pAAV9-hPCCA plasmid, pHelper-Kan plasmid, and pRC9-Kan plasmid Master Cell Banks
Charles River – Alderley Park [Contractor address]	• Manufacture and testing of High Quality (HQ) pAAV9-hPCCA, pHelper-Kan, and pRC9-Kan plasmids
Charles River – Rockville [Contractor address]	• Manufacture and testing of HEK293-S Master Cell Bank and Working Cell Bank • Manufacture, testing, and release of AAV9-hPCCA drug substance

3.2.S.2.2 Description of Manufacturing Process and Process Controls

The AAV9-hPCCA drug substance manufacturing operations will be performed at Charles River *[contractor address]* in the GMP manufacturing facility following approved procedures. The manufacturing process uses polyethyleneimine (PEI)-based triple transfection in HEK293-S cells, which is a commonly used method for AAV production. Information on the HEK293-S Working Cell Bank (WCB) Lot 0108-22001 and the parent Master Cell Bank (MCB) Lot 0071-18002 is included in Section 3.2.S.2.3.2. Information on the pAAV9-hPCCA, helper, and rep-cap plasmids is included in Section 3.2.S.2.3.1.

Figure 5 includes a high-level overview of the proposed 200L manufacturing process that will be used to manufacture material for the GLP tox study and the first-in-human clinical trial. The proposed upstream and downstream processes are briefly summarized following the process flowchart. The proposed manufacturing process at the 200L scale is based on the upstream and downstream runs successfully completed at the 50L scale. A high-level overview of the differences between the 50L material (used in preclinical studies, NHGRI-PCCA-002) and the 200L process intended to manufacture material for the GLP tox study and first-in-human clinical trial is included in Table 2. No differences are anticipated between the 200L GLP tox material and the 200L clinical material. A complete description of the manufacturing process will be included in the IND.

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Table 2: High-Level Differences Between 50L and 200L Process

Material or Parameter	**50L Scale (Used in Preclinical Studies, NHGRI-PCCA-002)**	**200L Scale (Proposed for GLP tox study and First-in-Human Clinical Trial)**
HEK293-S Cells	Research Cell Bank Lot HEK293-S31MAR21 (Derived from Master Cell Bank Lot 0071-18002)	Working Cell Bank Lot 0108-22001 (Derived from Master Cell Bank Lot 0071-18002)
pAAV9-hPCCA	R&D Grade Plasmid (Derived from R&D pMCB)	HQ Plasmid (Derived from GMP pMCB)
Helper Plasmid	R&D Grade Plasmid (Derived from R&D pMCB)	HQ Plasmid (Derived from GMP pMCB)
Rep-Cap Plasmid	R&D Grade Plasmid (Derived from R&D pMCB)	HQ Plasmid (Derived from GMP pMCB)
Upstream Process	50L Scale, XDR-50 Bioreactor Operating conditions: pH 7.1 (using Na_2CO_3), DO 40% (agitation and aeration), and temperature 37°C (auto-control)	200L Scale, XDR-200 Bioreactor Operating conditions: pH 7.1 (using $NaHCO_3$), DO 40% (agitation and aeration), and temperature 37°C (auto-control)
Downstream Process	High-level process indicated in Figure 5	High-level process indicated in Figure 5 (Only anticipated changes are related to scale)
Testing	Data included in Table 9	Proposed release panel in Table 8

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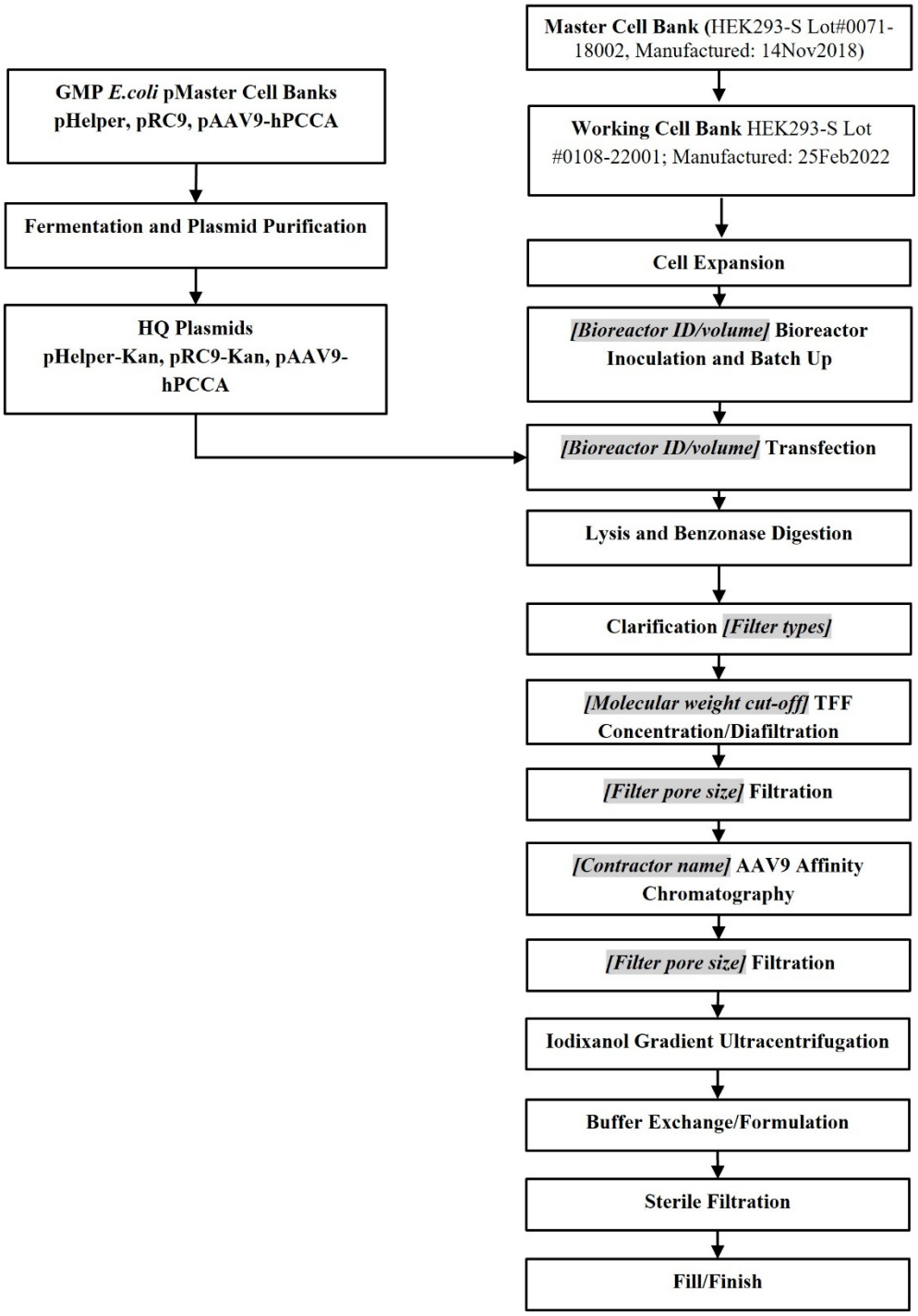
Figure 5: AAV9-hPCCA Manufacturing Process Overview



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3.2.S.2.2.1 Upstream Manufacturing Process

The manufacturing process begins with the thaw of one vial of HEK293-S WCB Lot 0108-22001. The thawed cells are transferred to a centrifuge tube containing pre-warmed *[specific growth media]* growth media *[specific growth media with supplements]*. After centrifugation, the cells are resuspended in fresh media and added to a 125mL shake flask containing warmed *[specific growth media]* media. Samples are removed for viable cell density and viability and the cells are then cultured in an incubator set to 37°C, 5% CO_2, and a shaking speed of 130 RPM with high humidity. The cells are expanded by passaging every few days into progressively larger flasks to generate sufficient inoculum for the 200L bioreactor.

An *[bioreactor ID/volume]* single-use bioreactor bag is set up and probes for pH and dissolved oxygen (DO) detection are autoclaved and calibrated. The bioreactor is batched with *[specific growth media]* media and the agitation, temperature, and air overlay controls are turned on. On the day of inoculation, the pH and DO probes are standardized based on offline measurements and the pH control loop is turned on. An appropriate volume of inoculum from the cell culture flasks is pooled, transferred to a bioprocessing bag and then added to the bioreactor via sterile weld. The DO control loop is activated immediately after inoculation. A sample is removed after inoculation to determine initial viable cell density, viability, and metabolite levels. Samples are taken daily until harvest to assess cell growth, metabolite consumption, and to verify on-line pH and DO measurements. Once the initial viable cell density target is met, a batch up is performed by adding additional pre-warmed media and increasing the agitation speed.

Approximately three days after inoculation, transfection is performed. A transfection cocktail is prepared by combining pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan (*[specific ratio]* molar ratio of each plasmid) with *[specific growth media]* media and *[specific transfection reagent]*. The transfection cocktail is added aseptically to the bioreactor and a cell count is performed approximately 15 minutes after transfection. A glucose feed is initiated approximately *[XX hours]* post transfection. In preparation for harvest (day 6), final cell count and metabolite measurements are performed, and samples are removed for the tests listed in Table 5.

3.2.S.2.2.2 Downstream Manufacturing Process

On day 6, *[buffer concentration]* lysis buffer *[buffer composition]* and 10 U/mL Benzonase are added to the bioreactor, which is held at 37°C at 175 RPM for two hours. To inhibit the Benzonase activity, 5M NaCl is added to a final concentration of 0.5M. A series of depth filters are then used to remove cell debris and recover the AAV9 post cell lysis and digestion. The clarification filtration train is composed of *[filter types/pore sizes]*. The clarified material is concentrated and diafiltered into *[buffer composition]* buffer using tangential flow filtration (*[molecular weight cut-off]*) to remove the Tween-20 added during lysis and to reduce the

loading volume prior to affinity chromatography. Following diafiltration, the retentate is *[filter pore size]* filtered to generate the affinity chromatography load.

[Product Name] AAV9 affinity chromatography is used to capture the AAV9 vector and remove most impurities, such as host cell DNA and host cell protein. Prior to use, the packed column is sanitized with *[reagent and concentration]* followed by washes with water for injection (WFI). *[Buffers]* are passed through sequentially to charge and equilibrate the column. After loading, the column is washed with AAV9 buffer A and wash buffer (*[composition]*). The AAV9 vector is eluted with an elution gradient of wash buffer and AAV9 buffer B and each collected main peak fraction is immediately neutralized with *[buffer composition and pH]*. The main peak fractions are pooled, *[filter pore size]* filtered and stored temporarily at 4°C.

Iodixanol gradient ultracentrifugation is used to enrich the full capsids for the AAV9 vector. This step is limited by volume; therefore, the AAV9 main peak is concentrated using tangential flow filtration *[molecular weight cut-off]* and adjusted to a final concentration of *[buffer concentration]*. Four different concentrations of iodixanol *[concentrations]* are prepared using *[buffer composition]*. Each iodixanol gradient is transferred into an OptiSeal ultracentrifuge tube. The concentrated main peak is overlaid onto each gradient and then centrifuged at *[speed, time, and temperature of centrifugation]*. A sample is taken from each fraction to determine the full capsid content. The full capsid fractions are pooled, diluted with *[buffer name and composition]* and concentrated/diafiltered into *[buffer name]* using tangential flow filtration *[molecular weight cut-off]* for iodixanol removal. The recovered retentate is assayed by qPCR and adjusted as needed to meet the target concentration *[buffer composition and pH]*.

The process continues to drug product manufacturing; refer to Section 3.2.P.3.3.

3.2.S.2.3 Control of Materials

Information related to the starting materials, including the plasmids and HEK293-S cells, is included in this section. A detailed list of raw materials used to manufacture the AAV9-hPCCA drug substance will be included in the IND.

3.2.S.2.3.1 AAV9 Production Plasmids

The three High Quality (HQ) plasmids used to produce the AAV9-hPCCA drug substance (pHelper-Kan, pRC9-Kan and pAAV9-hPCCA) are manufactured by Charles River – Alderley Park (refer to Section 3.2.S.1.2 for the plasmid maps). The starting material for each plasmid is a GMP plasmid Master Cell Bank (pMCB) manufactured by Charles River – Keele. A brief summary of the processes used to manufacture the pMCBs and plasmid DNA (pDNA) is included here. Complete manufacturing information, including manufacturing summary reports and COAs for each pMCB and pDNA, will be provided in the IND.

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3.2.S.2.3.1.1 Plasmid Master Cell Banks

Each pMCB is manufactured by Charles River – Keele under their GMP Quality Management System. No animal-origin materials or reagents are used in the manufacturing process. A high-level overview of the pAAV9-hPCCA MCB manufacturing process is included in Figure 6. The manufacturing processes used for the pHelper-Kan and pRC-9 MCBs are very similar to the process depicted in Figure 6. A complete description of the manufacturing processes used for all three pMCBs will be included in the IND.

The list of typical specifications for each pMCB is included in Table 3; COAs for each pMCB will be submitted in the IND.

Figure 6: pAAV9-hPCCA MCB (GMP) Manufacturing Process

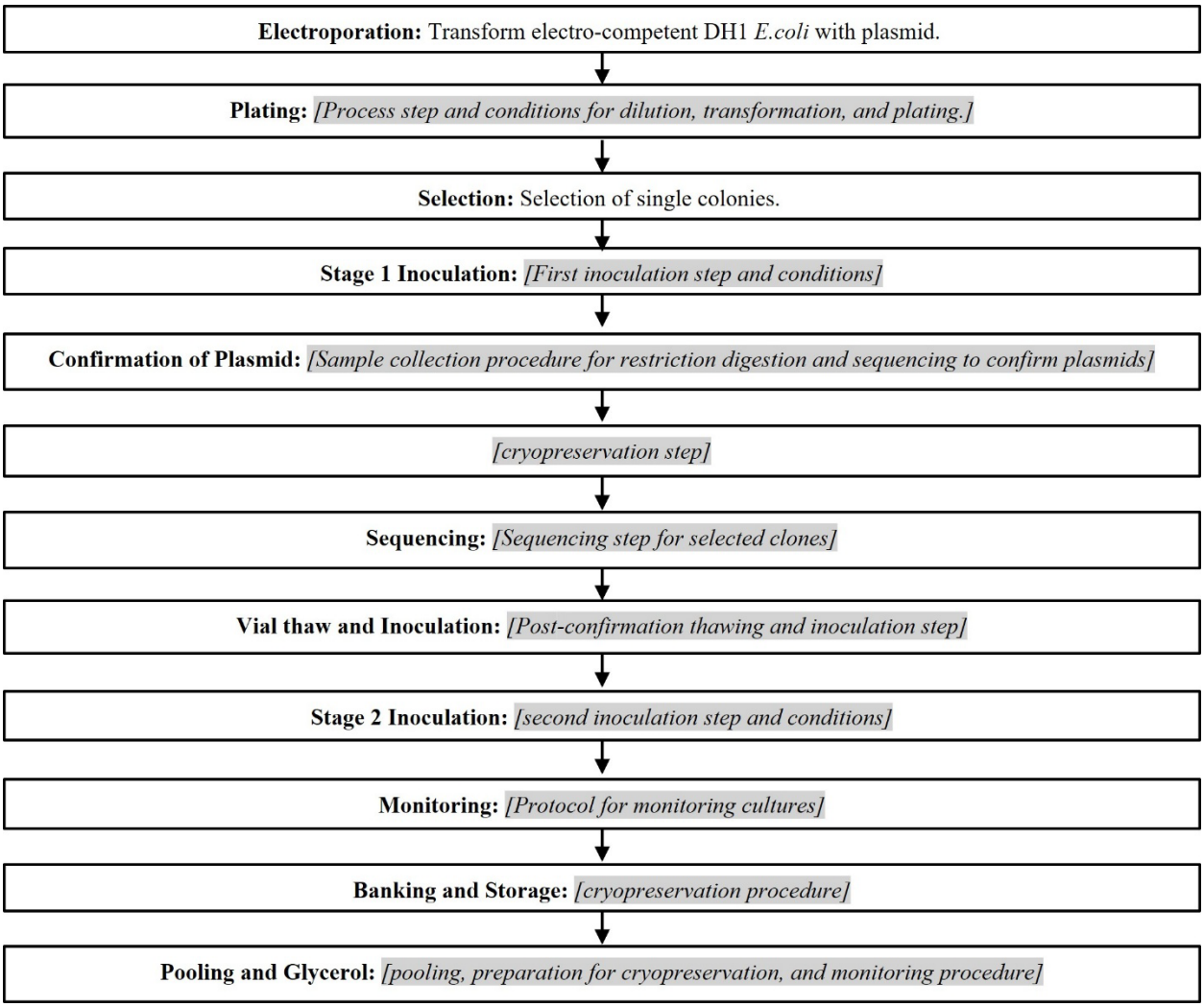

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Table 3: Plasmid Master Cell Bank Release Specifications

Test	Method	Specification
Identity by Restriction Digest	*[SOP number]*	Confirm Plasmid Identity
Microbial Cell Bank Characterization - Confirmation of Species	*[SOP number]*	Report profile and identity Positive for β-galactosidase activity
Microbial Cell Bank Characterization - Confirmation of Phenotype gyrA96	*[SOP number]*	>95% resistance to Nalidixic acid at 20µg/mL
Microbial Cell Bank Characterization - Confirmation of Phenotype recA1	*[SOP number]*	Sensitive to UV radiation
Microbial Cell Bank Characterization - Confirmation of Phenotype thi-1	*[SOP number]*	Thiamine auxotroph
Microbial Cell Bank Characterization - Confirmation of Plasmid Encoded Drug Resistance (Plasmid Retention)	*[SOP number]*	>95% resistance for replica plated colonies to Kanamycin 50µg/mL
Microbial Cell Bank Characterization - Viable Count	*[SOP number]*	Report Result
Microbial Cell Bank Characterization - Colony Morphology	*[SOP number]*	Report Result
Microbial Cell Bank Characterization - Gram Staining	*[SOP number]*	Confirmation of Gram-negative rod form
Microbial Cell Bank Characterization - Purity Test - Plating Method	*[SOP number]*	Absence of bacterial and/or fungal contamination
Sequencing of DNA using ABI 3500 Dx Genetic Analyzer	*[name of contractor] [method number]*	100% match to reference plasmid DNA sequence
Detection of Bacteriophage Contamination in Samples of E.coli	*[name of contractor] [method number]*	Confirm free of bacteriophage
Determination of Plasmid Copy Number by qPCR	*[name of contractor] [method number]*	Report Result

3.2.S.2.3.1.2 Plasmid DNAs

The HQ pHelper-Kan, rep-cap (RC9-Kan) and pAAV9-hPCCA plasmids are manufactured by the Charles River – Alderley Park facility under their HQ Quality Management System. The HQ Quality Management system adopts the "principles of GMP", including full traceability of materials, full room changeover prior to production, production in a segregated and dedicated

space, process and change control, use of master batch records, and aseptic fill finish. HQ plasmids are manufactured with animal origin free materials/reagents using fully single-use technology to prevent cross-contamination. A high-level overview of the HQ pDNA manufacturing process (300-500 mg scale) is included in Figure 7. A complete description will be included in the IND. The list of typical specifications for each pDNA are included in Table 4; COAs for each pDNA will be submitted in the IND.

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Figure 7: HQ Plasmid DNA Manufacturing Process Overview

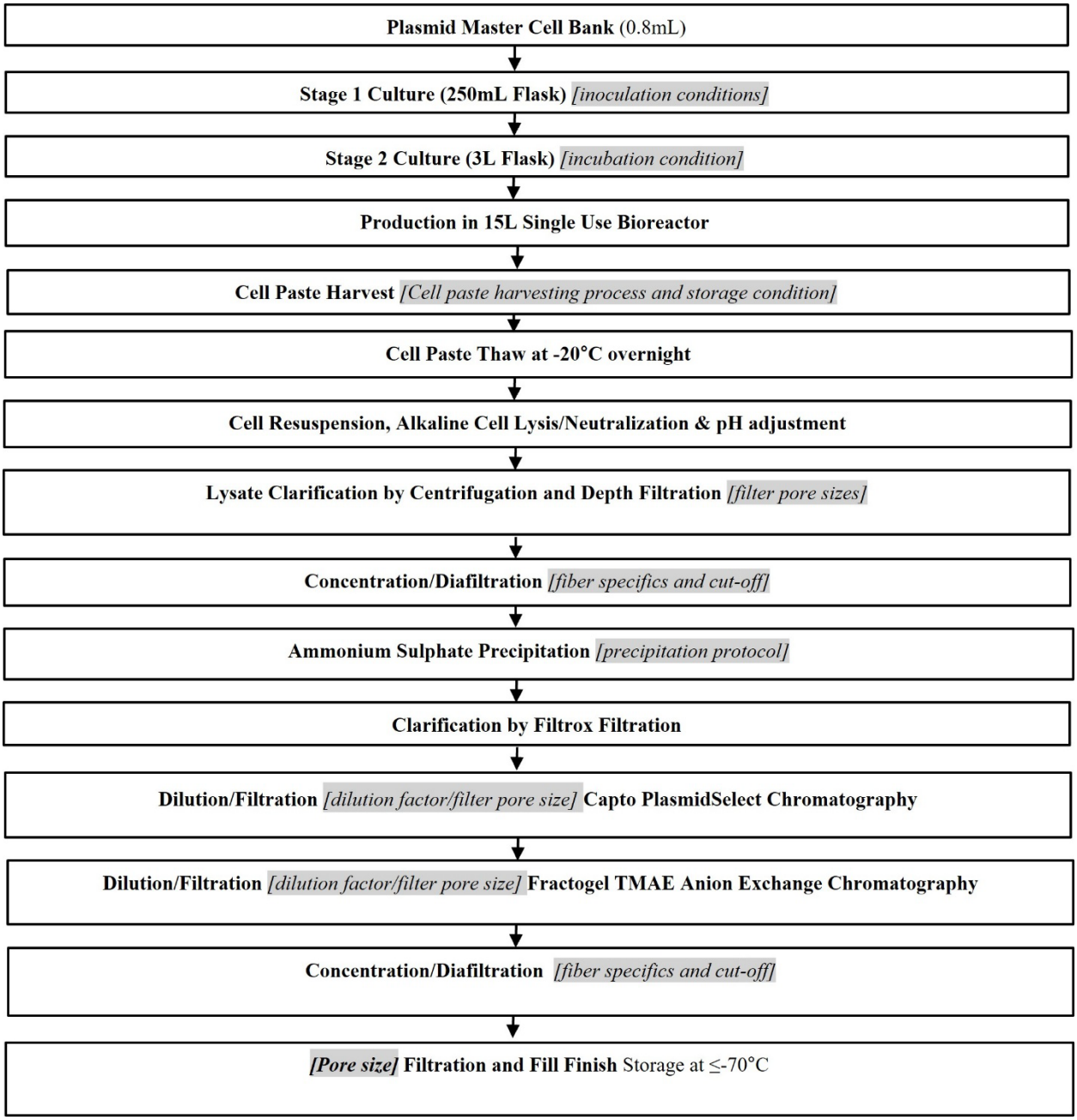

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Table 4: Plasmid DNA Release Specifications

Test	**Method**	**Specification**
Appearance of Solution (Method 2)	*[SOP number]* *[method numbers]*	Clear, colorless solution, free from visible particulate matter Clarity - <I Color - <B9
Potentiometric Measurement of pH of Solution	*[SOP number]* *[method number]*	8.0 ± 0.5
Identity by Restriction Digest	*[SOP number]* *[method number]*	Confirm Plasmid Identity
Total Nucleic Acid by Absorbance Spectroscopy	*[SOP number]*	1.0 ± 0.1 mg/mL
Purity by OD260:280	*[SOP number]*	1.8 – 2.0
Purity by Agarose Gel Electrophoresis	*[SOP number]*	% Total Plasmid: ≥95% % Supercoiled: Report Result % Open Circle: Report Result % Other: Report Result
Endotoxin Determination	*[SOP number]* *[method number]*	≤10EU/mg
Purity by Free Solution Capillary Electrophoresis	*[SOP number]*	≥85% Supercoiled
Residual Kanamycin	*[SOP number]*	<5ppm
Residual gDNA by QPCR	*[SOP number]*	≤5%w/w gDNA
ELISA for the Measurement of Residual *E. Coli* host Cell Proteins	*[SOP number]*	≤2%w/w HCP
Residual RNA by Reverse Phase HPLC	*[SOP number]*	≤5%w/w RNA
Microbial Testing for Bioburden	*[SOP number]* *[method number]*	<1cfu/10mL TAMC and <1cfu/10mL TYMC
Plasmid Identity by Next Generation Sequencing	Outsourced to *[name of contractor]* *[method number]*	100% match to reference plasmid DNA sequence
Detection of mycoplasma and related mollicutes by Real-Time PCR	Outsourced to *[name of contractor]* *[method number]*	Negative for Mycoplasma
Sterility (Direct Inoculation)	Outsourced to *[name of contractor]* *[method number]*	No Growth

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3.2.S.2.3.2 HEK293-S Cells

3.2.S.2.3.2.1 HEK293-S Master Cell Bank Lot 0071-18002

HEK293-S Master Cell Bank (MCB) Lot 0071-18002 was manufactured by Vigene Biosciences (now Charles River, Rockville MD). The COA for HEK293-S MCB Lot 0071-18002 is included in Attachment 2. Raw material information will be included in the IND.

To prepare Charles River's proprietary HEK293-S master cell bank, one vial of adherent HEK293 cells from ATCC (CRL-1573, Lot: 70009859) was expanded by passaging 14 times in T-Flasks (Passages 37 –51). The final five passages were used to adapt the culture to low serum conditions. The cells were then adapted to suspension culture, with the next passage redesignated as HEK293-S (Passage 1). The cells were passaged three additional times, with the last passage being cultured in the absence of serum. The cells were then frozen as a process development (PD) suspension cell bank (VBI HEK293-S, p4, M6712). Subsequently, one vial of the HEK293-S PD bank was thawed in suspension culture in serum-free medium and expanded over 6 additional passages. At Passage 10, the cells were frozen as VBI master cell bank HEK293-S (VBI P0071, Lot 0071-18002). The cells were frozen in aliquots at 1.5e7 cells/mL, with 98.6% viability.

3.2.S.2.3.2.2 HEK293-S Working Cell Bank Lot 0108-22001

HEK293-S Working Cell Bank Lot 0108-22001 was manufactured by Vigene Biosciences (now Charles River, Rockville MD) from MCB Lot 0071-18002. The COA for HEK293-S WCB Lot 0108-22001 is included in Attachment 2. Raw material information will be included in the IND.

On February 10, 2022, one vial of MCB Lot 0071-18002 was thawed inside a BSC using a glass beaker containing prewarmed (37± 1°C) water for injection (WFI). One milliliter from the thawed vial was pipetted into a 15 mL centrifuge tube containing 9 mL prewarmed *[specific culture medium]*. The tube was then centrifuged at 800 RPM for 5 minutes at 20°C. The supernatant was discarded, and the cells were resuspended in 5 mL of prewarmed *[specific culture medium]*. A sample was removed for cell count and viability. The resuspended cells were transferred into a 125 mL flask containing prewarmed *[specific culture medium]* and incubated at 37± 1°C, 7% CO_2 on a shaker platform set to 135 RPM.

Every *[duration between passaging cells]* between February 14 – February 22, 2022, the cells were passaged into progressively larger flasks and incubated under the same conditions. After the final incubation of passage 5 on February 25, 2022, the cells were harvested. The cell culture was split into eight 500 mL centrifuge bottles and centrifuged at 1450 ± 50 RPM for 10 minutes at 20°C. The supernatants were discarded, the cells were resuspended with 25 mL *[specific culture medium]* and then pooled into a 2 L flask. The viable cell density was confirmed to be *[cell density threshold]* with a viability of *[cell viability threshold]* and the 2 L flask containing the pooled cell suspension was incubated briefly during preparation for cryopreservation. Freeze media was prepared by adding 80 mL of DMSO to 320 mL of complete F17 media. The cell suspension *[specific volume]* and freeze media *[specific volume]* were combined into a 1 L

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bottle, mixed gently and then aliquoted into 1.8 mL cryovials at a target fill volume of 1.0 mL. A total of 450 vials were filled. After inspection, each cryovial was labeled and then frozen at -70°C in prechilled Stratacooler benchtop coolers. After approximately 20 hours, vials were removed for release testing and the remaining vials were transferred to liquid nitrogen for long term storage.

3.2.S.4 Control of Drug Substance

3.2.S.4.1 Specification

The proposed AAV9-hPCCA end of cell culture release tests and specifications are included in Table 5. The proposed release tests and specifications for the AAV9-hPCCA drug substance are included in Table 6. Specifications will be established after completion of the first 200L engineering run; target values are included where possible.

Table 5: Proposed AAV9-hPCCA End of Cell Culture Release Tests

Test	Contractor/Test Identification	Specification
Bioburden (Spread Plate Count)	*[name of contractor]* *[method number]*	< 2 CFU/mL
Testing for the Presence of Agar Cultivable and Non-cultivable Mycoplasmas in Accordance with USP and the EP Guidelines	*[name of contractor]* *[method number]*	Mycoplasmastasis: No Inhibition Mycoplasma: Not Detected
In vitro Adventitious Virus Assay (Tissue Culture Safety Testing: MRC-5, Vero, and HEK293)	*[name of contractor]* *[method number]*	Not Detected

Table 6: Proposed AAV9-hPCCA Drug Substance Release Tests and Specifications

Test	Contractor/Test Identification	Specification
Genomic Titer (Gene-Specific qPCR)[1]	*[name of contractor]* *[method number]*	≥ 5E13 vg/mL
Total AAV9 Capsid Titer (ELISA)	*[name of contractor]* *[method number]*	≥ 2E13 vp/mL
Bioburden (Spread Plate Count)	*[name of contractor]* *[method number]*	< 2 CFU/mL
Bacterial Endotoxin per USP<85> (LAL/Chromogenic Method)	*[name of contractor]* *[method number]*	<5 EU/mL

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Test	**Contractor/Test Identification**	**Specification**
Replication Competent AAV (Culture/qPCR)	To be determined	Not detected
Vector Purity (SDS-PAGE and Silver Stain)	*[name of contractor]* *[method number]*	VP1, VP2, and VP3 Present and No Other Apparent Bands
Genome Sequencing of Viral Product (Sanger or NGS)	To be determined	100% Match of GOI Sequence to Reference
Empty/Full Particles (Cryo-TEM)	To be determined	To be determined[2] (Target: ≥ 70% full)
Residual HEK293 Host Cell Protein (ELISA)	*[name of contractor]* *[method number]*	To be determined[2] (Target: ≤ 100 ng/mL)
Residual Host Cell DNA (qPCR)	*[name of contractor]* *[method number]*	To be determined[2]
Residual Benzonase (ELISA)	*[name of contractor]* *[method number]*	To be determined[2] (Target: ≤ 10 ng/mL)
Residual Adenovirus 5 E1A DNA (qPCR)	*[name of contractor]* *[method number]*	To be determined[2]
Residual Plasmid DNA (qPCR)	*[name of contractor]* *[method number]*	To be determined[2]
Residual Tween-20 (UV/Vis Spectrophotometry)	*[name of contractor]* *[method number]*	To be determined[2]
Residual Polyethyleneimine (HPLC)	*[name of contractor]* *[method number]*	To be determined[2]
Residual Iodixanol (HPLC)	HPLC	To be determined[2]
Capillary Gel Electrophoresis	To be determined	For Information Only
Genomic Titer by ddPCR[1]	To be determined	For Information Only

Note: Non-compendial methods will be qualified.
[1]Preclinical and clinical dose calculation based on qPCR titer. ddPCR performed for information only.
[2]Specification to be established prior to manufacturing the GMP clinical lot.

3.2.S.6 Container Closure System

The AAV9-hPCCA drug substance is not stored prior to production of the drug product.

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3.2.S.7 Stability

3.2.S.7.1 Stability Summary and Conclusions

The current manufacturing plan is to fill the drug product the same day the drug substance is formulated; therefore, no drug substance stability study is planned. Stability data to support holding of the drug substance prior to filling the drug product will be included in the IND, if necessary.

3.2.P Drug Product

3.2.P.1 Description and Composition of the Drug Product

The AAV9-hPCCA drug product is a sterile, aqueous buffered solution composed of the AAV9-hPCCA drug substance formulated in *[buffer]*.

3.2.P.3 Manufacture

3.2.P.3.1 Manufacturers

Table 7 lists the manufacturing and testing sites for AAV9-hPCCA drug product.

Table 7: Drug Product Manufacturing and Testing Facilities

Manufacturer/Contractor Name and Address	Responsibility
Charles River – Rockville 5 Research Court Rockville, MD 20850	• Manufacture and testing of the AAV9-hPCCA drug product • Stability testing

3.2.P.3.3 Description of Manufacturing Process and Process Controls

The formulated drug substance is 0.2µm filtered and then aliquoted into cryovials to produce the final drug product. The final drug product vials are labeled and stored at -80°C. The AAV9-hPCCA drug substance will be filled into vials after sterile filtration. Details regarding the drug product manufacturing process will be submitted in the IND.

Samples of the final vialed product will be submitted for final release and stability testing. Refer to Section 3.2.P.5.1 for the proposed release specification and Section 3.2.P.8.1 for the stability testing plan.

3.2.P.5 Control of Drug Product

3.2.P.5.1 Specifications

The proposed release tests and specifications for the AAV9-hPCCA drug product are included in Table 8. Specifications will be established after completion of the first 200L engineering run; target values are included where possible.

DISCLAIMER: *Information in this document is from early process development, and later improvements have been made. Results and processes may not be applicable to other products.*

Table 8: Proposed AAV9-hPCCA Drug Product Release Tests and Specifications

Test	Contractor/Test Identification	Specification
Appearance (Visual Inspection)	*[name of contractor] [method number]*	Clear, Colorless Solution with No Visible Foreign Particulates
Genomic Titer (Gene-Specific qPCR[1])	*[name of contractor] [method number]*	≥ 5E13 vg/mL
Sterility Testing of Final Containers and Biological Products (USP<71> Direct Method)	*[name of contractor] [method number]*	No Growth
Bacteriostasis/Fungistasis (USP<71> Direct Method)	*[name of contractor] [method number]*	No Inhibition
Subvisible Particle Analysis per USP<787>	*[name of contractor] [method number]*	≥ 10 µm particles: ≤ 6000 particles per container ≥ 25 µm particles: ≤ 600 particles per container
Bacterial Endotoxin per USP<85> (LAL/Chromogenic Method)	*[name of contractor] [method number]*	<5 EU/mL
Osmolality per USP<785>	*[name of contractor] [method number]*	To be determined[2]
pH per USP<791>	*[name of contractor] [method number]*	7.4 ± 0.4
Vector Purity (SDS-PAGE and Silver Stain)	*[name of contractor] [method number]*	VP1, VP2, and VP3 Present and No Other Apparent Bands
Aggregation by Dynamic Light Scattering (DLS)	*[name of contractor] [method number]*	To be determined[2]
Potency by Western Blot	*[name of contractor]* (Method Number Pending)	PCCA Expression in HepG2-Knockout Cells

Note: Non-compendial methods will be qualified.
[1]Preclinical and clinical dose calculation based on qPCR titer. ddPCR performed for information only.
[2]Specification to be established prior to manufacturing the GMP clinical lot.

3.2.P.5.4 Batch Analysis

Testing data from AAV9-hPCCA Lot TL-21-001-41 (development lot at 50L scale), which was used in preclinical studies (NHGRI-PCCA-002), are included in Table 9.

Testing data from the toxicology and clinical-grade batches of AAV9-hPCCA will be included in the IND.

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Table 9: Batch Analysis Data for AAV9-hPCCA Development Lot TL-21-001-41 (50L Scale)

Test	Procedure (SOP) Number or Vendor	Specification	Result
Residual Host Cell HEK293 DNA (qPCR)	*[SOP number]*	Report Results	2.5E+06 pg/mL
Residual Host Cell HEK293 Protein (ELISA)	*[SOP number]*	Report Results	Not Detected (LOD 2 ng/mL)
Vector Purity (SDS-PAGE with Silver Stain)	*[SOP number]*	Report Results	VP1, VP3, and VP3 Identified. No other apparent bands.
Residual Benzonase (ELISA)	*[SOP number]*	Report Results	Not Detected (LOD 0.20 ng/mL)
Bacterial Endotoxin (USP<85>)	*[SOP number]*	Report Results	25.4 EU/mL
Viral Genomic Titer (ddPCR)	*[SOP number]*	Report Results	1.07E+14 gc/mL
Viral Genomic Titer (qPCR)	*[SOP number]*	Report Results	5.64E+13 gc/mL
AAV9 Capsid Titer (ELISA)	*[SOP number]*	Report Results	1.45E+14 particles/mL
Percent Full Capsid (Cryo-TEM)	*[name of contractor]*	Report Results	91.6% Full, 8.4% Empty
Potency (Western Blot using Whole Cell Lysate)	*[SOP number]*	Report Results	Shows Dose-Dependent Effects of PCCA Expression in HepG2-Knockout Cells

[1]At the time of testing, the ddPCR genomic titer assay procedure was not effective; however, the assay was developed and fit for purpose.

3.2.P.7 Container Closure System

The AAV9-hPCCA drug product container closure system will be either 2mL or 5mL Daikyo Crystal Zenith® cyclic olefin polymer vials with NovaPure™ 4432/50 chlorobutyl FluroTec™ laminated stoppers and West Ready Pack™ Flip-Off™ aluminum seals (West Pharma). Additional information on the drug product container closure system will be included in the IND.

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3.2.P.8 Stability

3.2.P.8.1 Stability Summary and Conclusion

An accelerated stability study will be conducted according to the high-level design presented in Table 10.

The proposed real-time stability plan for AAV9-hPCCA drug product (stored at -80°C) is provided in Table 11.

Table 10: Proposed AAV9-hPCCA Drug Product Accelerated Stability Study

Attribute	Test	Method	Time Points (Hours)
CONTENT[a]	Genomic Titer	qPCR	0, 8, 24, 48, 96, 144, 192, 240, 288
PURITY[a]	Aggregation	Dynamic Light Scattering	0, 48, 144, 240
POTENCY[a]	AAV9-hPCCA Potency	Western Blot using Whole Cell Lysate	0, 48, 144, 240
CONTENT[b]	Genomic Titer	qPCR	0, 8, 24, 48, 96, 144, 192, 240, 288
PURITY[b]	Aggregation	Dynamic Light Scattering	0, 48, 144, 240
POTENCY[b]	AAV9-hPCCA Potency	Western Blot using Whole Cell Lysate	0, 48, 144, 240
CONTENT[c]	Genomic Titer	qPCR	0, 8, 24, 48, 96, 144, 192, 240, 288
PURITY[c]	Aggregation	Dynamic Light Scattering	0, 48, 144, 240
POTENCY[c]	AAV9-hPCCA Potency	Western Blot using Whole Cell Lysate	0, 48, 144, 240

[a]Storage condition: 40°C
[b]Storage condition: 22°C
[c]Storage condition: 2-4°C

DISCLAIMER: *Information in this document is from early process development, and later improvements have been made. Results and processes may not be applicable to other products.*

Table 11: Proposed AAV9-hPCCA Drug Product Real-Time Stability Plan

Storage Condition: -80°C

Attribute	Test	Method	Specifications	Time Points (Months)
QUALITY	Appearance	Visual Inspection	Clear, Colorless Solution with No Visible Particulates	0, 3, 6, 9, 12, 18, 24, 36, 48, 60
QUALITY	pH	USP <791>	7.4 ± 0.4	0, 3, 6, 9, 12, 18, 24, 36, 48, 60
QUALITY	Osmolality	USP <785>	To be determined[1]	0, 3, 6, 9, 12, 18, 24, 36, 48, 60
CONTENT	Genomic Titer	qPCR	≥ 5E13 vg/mL	0, 3, 6, 9, 12, 18, 24, 36, 48, 60
PURITY	Vector Purity	SDS-PAGE / Silver Staining	VP1, VP2, and VP3 Present and No Other Apparent Bands	0, 3, 6, 9, 12, 18, 24, 36, 48, 60
SAFETY	Sterility	USP <71>	No Growth	0, 12, 24, 36, 48, 60
PURITY	Aggregation	Dynamic Light Scattering	To be determined[1]	0, 3, 6, 9, 12, 18, 24, 36, 48, 60
POTENCY	AAV9-hPCCA Potency	Western Blot using Whole Cell Lysate	PCCA Expression in HepG2-Knockout Cells	0, 6, 12, 18, 24, 36, 48, 60

[1]Specification to be established prior to manufacturing the GMP clinical lot.

ATTACHMENT 1: PLASMID SEQUENCES

pAAV9-hPCCA Sequence *[plasmid sequence]*

pHelper-Kan Sequence *[plasmid sequence]*

pRC9-Kan Sequence *[plasmid sequence]*

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ATTACHMENT 2: HEK293-S CELL BANK CERTIFICATES OF ANALYSIS

The following Certificates of Analysis are included in this Attachment:

HEK293-S Master Cell Bank Lot 0071-18002 *[Certificate of analysis]*

HEK293-S Working Cell Bank Lot 0108-22001 *[Certificate of analysis]*

***DISCLAIMER:* This information is from an earlier stage; updates have been made, so it may no longer be applicable.**

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CERTIFICATE OF TESTING

Sample Name	[redacted] 50L Run	**Sample Type**	End of Cell Culture and Drug Substance
Lot Number	LEI-24JAN22 (EOCC) & TL-21-001-41 (Drug Substance)	**Manufacturer**	Charles River Laboratories, Inc. (CRL-Rockville) (CRL-RKV)

END OF CELL CULTURE			
Assay	**Laboratory / Test Identification**	**Specification**	**Result**
Bioburden (Spread Plate Count)	[redacted]	Report Results	0 CFU / 1 mL
[redacted] **Viral Genomic Titer (ddPCR)**	[redacted]	Report Results	3.5E+11 gc/mL
Mycoplasma Detection using qPCR	[redacted]	Report Results	Negative for Mycoplasma Species Contamination
DRUG SUBSTANCE			
Assay	**Laboratory / Test Identification**	**Specification**	**Result**
Residual Host Cell HEK293 DNA (qPCR)	[redacted]	Report Results	2.5E+06 pg/mL
Residual Host Cell HEK293 Protein (ELISA)	[redacted]	Report Results	Not Detected[2]
Vector Purity (SDS-PAGE with Silver Staining)	[redacted]	Report Results	VP1, VP2, and VP3 Identified and No Other Apparent Bands
Residual Benzonase (ELISA)	[redacted]	Report Results	Not Detected[3]
Bacterial Endotoxin USP <85>	[redacted]	Report Results	25.4 EU/mL
[redacted] **Viral Genomic Titer (ddPCR)**	[redacted]	Report Results	1.07E+14 gc/mL
[redacted] **Viral Genomic Titer (qPCR)**	[redacted]	Report Results	5.64E+13 gc/mL
AAV9 Capsid Titer (ELISA)	[redacted]	Report Results	1.45E+14 particles/mL
Percent Full Capsid (Cryo-TEM)	[redacted]	Report Results	91.6% Full, 8.4% Empty

1. *At the time of testing, the ddPCR genomic titer assay was not an effective SOP however the assay was developed and fit for purpose.*
2. *Detection Limit for the residual host cell HEK293 protein assay is 2 ng/mL.*
3. *Detection Limit for the residual benzonase assay is 0.20 ng/mL.*

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CERTIFICATE OF TESTING

Quality Control Lead Signature [redacted] **Date** 06 Dec 2022

Revision History

Rev. 01 – Gene-specific qPCR result added at client request.
Rev. 00 – New document.

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PROTOCOL

Study Title:	Testing of compatibility, in-use, and stability of the gene therapy product AAV9-hPCCA
Protocol Number:	*[protocol number]*
Sponsor:	*[name and contact information of sponsor]*
Test Facility:	*[address of testing facility]*
Study Director:	*[name of study director]*
Sponsor Representative:	*[name of sponsor representative]*
Version	01

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SPONSOR SIGNATURE

Sponsor Representative Date

SIGNATURES

Study Director	Date	Management	Date

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

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LIST OF FIGURES **Page No.**

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1 OBJECTIVE

The purpose of this study is to evaluate the compatibility of AAV9-hPCCA with materials intended to be used in the efficacy study in mice and in the clinical study (needle/syringe and other delivery systems). Additionally, this study will perform the in-use evaluation of AAV9-hPCCA with materials intended to be used during the thaw, dilution, and infusion of the clinical product. Lastly, this study will evaluate the stability of AAV9-hPCCA to support expected time from thaw to clinical infusion.

2 JUSTIFICATION

Propionic acidemia (PA) is an inherited autosomal recessive metabolic disorder that is characterized by deficiency of propionyl-CoA carboxylase (PCC), which is an enzyme necessary to break down proteins and fats. This disorder presents early in infants and without treatment, coma and death may occur. The sponsor has been developing a new AAV9 gene therapy that replaces the deficit in PCCA, one of two genes that encodes the PCC enzyme. Treatment with this AAV9-hPCCA therapy has shown a reversal in the loss of PCC protein and an increase in survival of PCCA knock out (i.e., PccaQ133Lfs*41/Q133Lfs*41 homozygous) mice.

This study will test the compatibility, in-use, and stability of the gene therapy product AAV9-hPCCA from frozen drug product through simulated administration.

3 REGULATORY COMPLIANCE

This study will not be conducted in accordance with U.S. FDA 21 CFR Part 58 (Good Laboratory Practices for Nonclinical Laboratory Studies, GLP). Standard operating procedures (SOPs) will be followed.

No animal work will be conducted for this study.

4 KEY STUDY PERSONNEL

Changes in the Study Director, if any, will be added by revision. Any additional key study personnel changes or additions will be identified in the final report.

Study Director:	*[name of study director]*
Sponsor Representative:	*[name of sponsor representative]*
Contributing Scientist – Analytical:	*[name of contributing scientist]*
Contributing Scientist Vector Biodistribution and Transgene Expression:	*[name of contributing scientist]*
Contributing Scientist– ELISA:	*[name of contributing scientist]*

5 TEST ARTICLE

The AAV9 test article and diluent will be provided and characterized by the Sponsor or designee. Documentation on, at a minimum, the identity (supplier/manufacturer), viral titer, injection volume, injection titer, batch number and/or lot number, mass/concentration, sterility, and stability for the test and carrier article will be provided, as certificates of analysis or equivalent documents, for inclusion in the final report.

5.1 Test Article

Identity:	AAV9-hPCCA (50L)
Description:	*[description of test article, including promoter, gene of interest, and other key elements]*. The AAV9-hPCCA drug product is a sterile, aqueous buffered solution composed of the AAV9-hPCCA drug substance formulated in *[buffer composition]*.
Supplier/Manufacturer:	Charles River Laboratories (CRL)
Stock Viral Titer:	5.64 e+13 gc/ml viral genomic titer by qPCR
Batch/Lot Number:	TL-21-001-41
Storage Conditions:	-90°C to -70°C

5.2 AAV9-hPCCA Diluent

Identity:	Sterile Saline
Description:	Phosphate Buffer Saline (PBS)
Supplier/Manufacturer:	Will be included in the final report
Batch #/Lot#:	Will be included in the final report
Storage Conditions:	2°C-8°C and ambient, as applicable

6 EXPERIMENTAL DESIGN

Table 1 summarizes the delivery devices that will be used in both the preclinical efficacy studies and the clinical studies. Prepared test article will be passed through individual devices, and samples will be collected for analysis as shown in Figures 1 and 2. Test article will also be analyzed for (partial) stability under different storage conditions.

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Table 1. Experimental Design

Study Type	Description[A]	Formulated Test Article Concentration (gc/mL)	Volume Delivered through Device (mL)
Preclinical	BD Insulin Syringes with 3/10 mL capacity and 30G needle #328431 (plastic)	5E13 vg/mL	20 µL – 1000 µL
Clinical	5 or 10mL Luer-lock (B Braun, #4617053V-02 or #4617100V-02) or 30 or 50mL Luer-lock syringe (BD, #302832, #309653) (Polypropylene / Polyisoprene)	*[starting dose]* *[higher dose]*	~30 mL
Clinical	Carefusion, Alaris PC unit, Model 8015 with the Alaris modules, along with Microbore Tubing extension set, 60 inches, 0.03" (B Braun; #V6223), and 1.2 micron PES in-line filter (Pall, AEF2NT)	*[starting dose]* *[higher dose]*	~30 mL
Clinical	PICC (Peripherally inserted central catheter) line BARD catheter, 4-5 french, double lumen	*[starting dose]* *[higher dose]*	~30 mL
Clinical	Peripheral intravenous line (PIV): B Braun #490102 (15 drops/mL)	*[starting dose]* *[higher dose]*	~30 mL

[A] Actual product numbers and materials used will be included in the final report.

7 TEST ARTICLE FORMULATION

The stock TA will be provided at a concentration of 5.64E13 gc/ml viral genomic titer by qPCR and stored at ≤-80ºC. Test article will be formulated according to *[method number and name]*. The stock TA will be thawed on wet ice or at 2-8°C, thaw time will be noted, and TA will be formulated with the diluent at *[name of contractor]* to achieve the concentrations described in Table 1. On the day of testing, the prepared TA formulation will be stored on wet ice and/or at 2-8°C.

The details for TA preparation will be documented on the formulation forms.

8 STABILITY, COMPATIBILITY TESTING, AND MEASUREMENTS

8.1 Testing Conditions for Stability

Following preparation of test article in diluent, aliquots (approximately 0.3 mL each) of prepared test article will be held at ambient temperature or at 2-8°C for approximately 0, 6, 12, 18, 24, and 48 hours (clinical setting testing) or 0, 0.5, 1, 6, and 12 hours (pre-clinical setting), as summarized in Figure 1. At each time point under different storage conditions, samples will be subsequently transferred to -70 to -90°C for storage until analysis (see Section 8.3). Three aliquots of test article will be evaluated per storage condition and time point for each assay described in Section 8.3.

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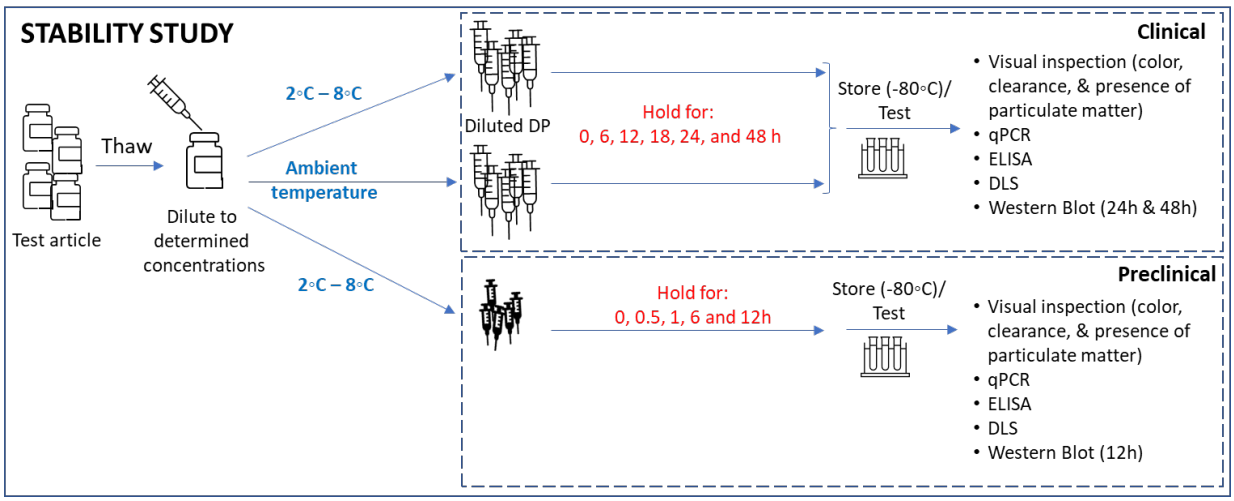
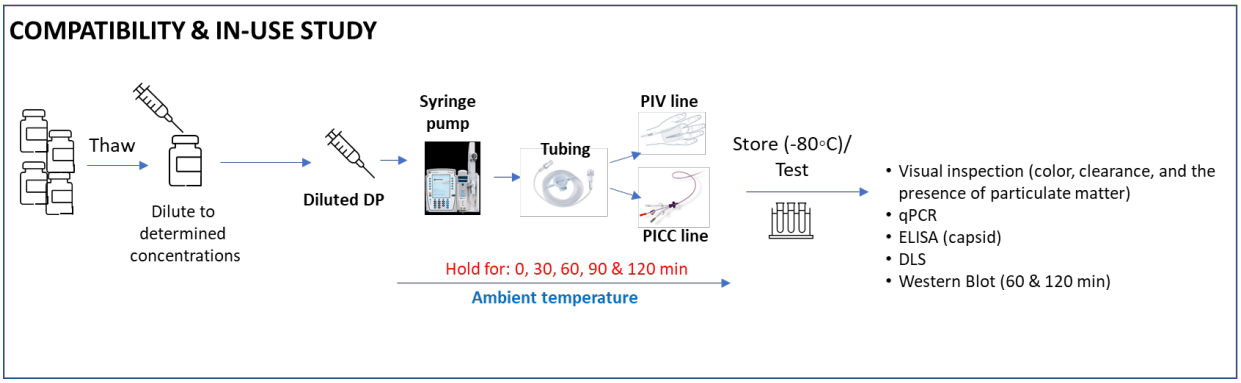
Figure 1. Schematic for evaluation of stability. The rationale for this testing is to obtain partial stability data for the drug product (DP) or TA in the devices (syringes prepared for infusion) up to 48h for clinical and up to 12h for preclinical settings.



8.2 Testing Conditions for Compatibility

Following preparation of test article in diluent, aliquots (volumes described in Table 1) of prepared test article will be loaded into the respective delivery devices and stored at ambient temperature for approximately 0, 30, 60, 90, and 120 minutes as shown in Figure 2 Then samples will be collected into cryovials (identical to the stored DP) before being placed at -70 to -90°C for storage until analysis (see Section 8.3). Three aliquots of test article will be evaluated for each delivery device (PICC and PIV for clinical setting) per storage condition and time point for each assay described in Section 8.3.

Figure 2. Schematic for evaluation of compatibility. The rationale for this testing is to obtain compatibility data after holding the DP in dispensing devices for up to 120 min, twice longer than the expected time to infuse the DP in clinic.



8.3 Measurements

Test article aliquots will be evaluated according to *[method number and name]*. Aliquots will also be visually inspected for color, clearance, and the presence of particulate matter. Three aliquots of test article will be evaluated per storage condition and time point for each assay,

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unless otherwise indicated. Number of samples per assay is summarized in Table 2. Results of these analyses will inform the explicit AAV9-hPCCA delivery mechanisms for the proposed preclinical and clinical studies. Based on the proposed clinical therapeutic window *[proposed dose range]*, a loss of < 15% of material is deemed acceptable as this would still yield a low dose (clinical cohort #1, *[starting dose]*) as potentially efficacious, and accounts for a -9% to +9% interval to account for error in each measurement.

Table 2. Collection and Measurement Timeline

Analysis	Time Point	Number of Samples
qPCR	Partial stability*: Target 0, 6, 12, 18, 24, and 48 hours (clinical) Target 0, 0.5, 1, 6, and 12 hours (pre-clinical) Compatibility: Target 0, 30, 60, 90 and 120 min	Stability at Ambient: 3 x 6 time points x 2 concentrations = 36 samples Stability at 2-8°C: (3 x 6 time points x 2 concentrations) + (3 x 5 timepoints) = 51 samples Compatibility at Ambient: 3 x 5 time points x 3 devices = 45 samples
Enzyme-linked immunosorbent assay (ELISA)	Partial stability*: Target 0, 6, 12, 18, 24, and 48 hours (clinical) Target 0, 0.5, 1, 6, and 12 hours (pre-clinical) Compatibility: Target 0, 30, 60, 90 and 120 min	Stability at Ambient: 3 x 6 time points x 2 concentrations = 36 samples Stability at 2-8°C: (3 x 6 time points x 2 concentrations) + (3 x 5 timepoints) = 51 samples Compatibility at Ambient: 3 x 5 time points x 3 devices = 45 samples
Dynamic Light Scattering (DLS)	Partial stability*: Target 0, 6, 12, 18, 24, and 48 hours(clinical) Target 0, 0.5, 1, 6, and 12 hours (pre-clinical) Compatibility: Target 0, 30, 60, 90 and 120 min	Stability at Ambient: 3 x 6 time points x 2 concentrations = 36 samples Stability at 2-8°C: (3 x 6 time points x 2 concentrations) + (3 x 5 timepoints) = 51 samples Compatibility at Ambient: 3 x 5 time points x 3 devices = 45 samples
Western Blot	Partial stability*: Target 24 and 48 hours (clinical) and 12h (preclinical) Compatibility: Target 60 and 120 minutes	Stability at Ambient: 3 x 2 time points x2 concentrations = 12 samples Stability at 2-8°C: (3 x 2 time points x 2 concentrations) + (3 x 1 time point) = 15 samples Compatibility at Ambient: 3 x 2 time points x 4 devices = 24 samples
Visual inspection for color, clearance, and the presence of particulate matter	Partial stability*: Target 0, 6, 12, 18, 24, and 48 hours (clinical) Target 0, 0.5, 1, 6, and 12 hours (pre-clinical) Compatibility & in-use: Target 0, 30, 60, 90 and 120 min	Stability at Ambient: 3 x 6 time points x 2 concentrations = 36 samples Stability at 2-8°C: (3 x 6 time points x 2 concentrations) + (3 x 5 timepoints) = 51 samples Compatibility at Ambient: 3 x 5 time points x 3 devices = 45 samples

**Testing will focus on critical quality attributes listed in the table, not full panel of stability measurements.*

***DISCLAIMER**: This information may no longer be applicable due to subsequent improvements*

8.3.1 Genomic Titer (qPCR)

Frozen samples designated for genomic titer analysis will be submitted to *[name of contractor]* and will be analyzed via qPCR (*[method number and name]*). A contributing scientist report will be included in the Final Report.

8.3.2 Capsid Titer (ELISA)

Frozen samples designated for ELISA will be submitted to *[name of contractor]* for analysis via *[method number and name]*. Samples will be analyzed using a commercial kit (e.g., AAV9 Titration ELISA; Progen #PRAAV9). A contributing scientist report will be included in the Final Report.

8.3.3 Dynamic light scattering (DLS) for AAV (aggregation)

Frozen samples designated for DLS analysis will be analyzed at *[name of contractor]*. A contributing scientist report will be included in the Final Report.

8.3.4 Potency (Western Blot)

Frozen samples collected at 60 and 120 min (compatibility testing) and 12, 24 and 48 hours (partial stability testing) will be shipped on dry ice to a sponsor-designated laboratory for Western Blot analysis. This laboratory will provide methods and a contributing scientist report for inclusion in the Final Report.

9 STATISTICAL ANALYSES

Data will be analyzed using appropriate techniques for the type of data collected. Analyses will be used upon the approval of the Study Director and/or Study Statistician and will be included in the Final Report.

All data will be analyzed using Provantis, GraphPad Prism, WinNonLin, or SAS®.

10 SAMPLE SHIPMENT

1. Western Blot samples from Section 8.3.4 will be shipped on dry ice to a sponsor-designated laboratory.

2. Unused Test Article from Section 5 will be shipped on dry ice to the sponsor the following address

11 VERSION HISTORY AND CHANGE SUMMARY

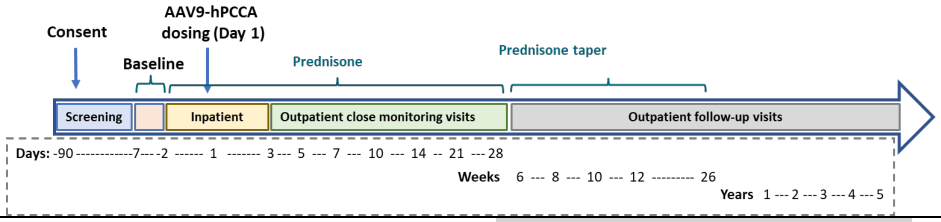
Version	Approval Date	Summary of Changes (including sections)	Justification for Changes
01	See SD signature	Original Signed Protocol	N/A

Appendix: Natural history study protocol

[Natural history study (NCT02890342). Information available on clinicaltrials.gov]

DISCLAIMER: *This clinical trial design has been updated after the pre-IND meeting. This study design is specific to AAV9-hPCCA and may not be applicable to all studies or diseases.*

Proposed Phase 1/2 Clinical Trial Protocol Synopsis

Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Sponsor	NCATS
Study Phase	Phase 1/2
Study Schema	
Study Population	Pediatric and adult patients, ≥3 years of age, *[NIH Clinical Center-specific criteria]* with clinically, biochemically, and/or molecularly confirmed pathogenic variants in *PCCA* that cause PA
Number of Sites and Subjects	One clinical site; approximately 4-9 research participants.
Treatment Groups	• *Cohort 1: [starting dose]* (N=3-6) • *Cohort 2*: *[higher dose]* (N=1-3) In addition, there will be a comparator group (historical control) from the NIH natural history study of PA (NCT02890342) [1,2].
Primary Objectives	1. To assess safety and tolerability of intravenous administration of AAV9-hPCCA in research participants with *PCCA* type PA 2. To assess changes from baseline in response biomarkers, a.k.a., pharmacodynamic (PD) response, to AAV9-hPCCA
Secondary Objectives	1. To assess patient outcomes as measured by the frequency and severity of specified clinical events, including metabolic crises in need of sick-day dietary modification or hospitalization and/or need for referral for liver, kidney or liver and kidney transplantation 2. To assess patient and caregiver reported outcomes (a separate consent will be presented to the caregiver)
Study Design	This is a phase 1/2, open-label, safety, dose-escalation, single-center, clinical study of AAV9-hPCCA gene therapy in research participants with *PCCA*-related PA. The study will be informed with a comparator group from an ongoing natural history study at NIH (NCT02890342; Natural History, Physiology, Microbiome and Biochemistry Studies of Propionic Acidemia). The study will consist of the following 2 cohorts: 1. Cohort 1: approximately 3-6 research participants, treated with a single *[starting dose]* of AAV9-hPCCA 2. Cohort 2: approximately 1-3 research participants, treated with a single *[higher dose]* of AAV9-hPCCA ***[Detailed study schema]***

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Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Study Design (continued)	***Cohort 1. [starting dose]*** The first participant dosed in this cohort will be an adolescent, ≥12 years to <18 years of age. If no eligible participant is identified, we will age de-escalate to the next eligible participant ≥3 and <18 years of age. A Data and Safety Monitoring Board (DSMB) review of available safety data will be performed at the end of 12 weeks after investigational drug administration for the first participant. If the safety review is satisfactory, dosing will proceed to the next participant (≥3 years of age), with at least a 12-week stagger separating dosing between eligible study participants. The cohort intends to enroll up to a total of three participants (age ≥3 years), pending DSMB review. Once the cohort is fully enrolled, the DSMB will review aggregate data and advise on dose escalation to Cohort 2. All participants will be monitored with key metabolic biomarkers and standard clinical and laboratory evaluations for 52 weeks and then followed by collection of safety data for an additional four years. • If clinical assessment and biomarker data do not support the efficacy of this dose (trend of changes in *[disease related biomarkers]* [1,3-5] in the first three participants, we will proceed to Cohort 2, after the DSMB reviews data for these three participants treated in Cohort 1. • If clinical assessment and biomarker data support the efficacy of this dose in the first three participants, three more will be enrolled and treated in Cohort 1. After a total of six participants are treated and the last participant has been followed for 12 weeks, the DSMB will review safety data for the entire Cohort 1. ***Cohort 2. [higher dose]*** The first participant dosed in this cohort will be an adolescent, ≥12 to <18 years of age. If no eligible participant is identified, we will age de-escalate to the next eligible participant ≥3 and <18 years of age. The DSMB will review available safety data at the end of 12 weeks after investigational drug administration for this first participant. If the safety review is satisfactory, dosing of subsequent participants will proceed in an analogous manner to Cohort 1, up to a total of three participants ≥3 years of age (pending DSMB review of data). At least 12 weeks will separate dosing between eligible study participants. All participants will be monitored with key metabolic biomarkers and standard clinical and laboratory evaluations for 52 weeks and then followed by collection of safety data for an additional four years. At the end of Cohort 2, there will be a DSMB review of safety data for the entire cohort.

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Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Study Design: Study timeline and schedule of events	***Study timeline and schedule of events (see Table A.1 below this synopsis)*:** • Study candidates will be evaluated for study eligibility as part of the screening period, ~90 days prior to the anticipated dosing day. Participants may co-enroll, if not already participating, in the Natural History Study of propionic acidemia conducted at NIH (NCT02890342). For screening, study candidates will undergo collection of labs, diagnostic studies, consultation with hepatology, cardiology, nephrology as needed, dietary assessment, and neurocognitive evaluation to determine their eligibility according to the inclusion/exclusion criteria outlined in this clinical synopsis. • Participants who meet the eligibility criteria during the screening will be re-evaluated seven days prior to the anticipated infusion date (+/- 2 days; baseline) to ensure they continue meeting the eligibility criteria. • Participants eligible for AAV9-hPCCA administration will commence prophylactic treatment with oral corticosteroid (prednisone or prednisolone): 1 mg/kg/day (not to exceed a maximum dose of 60 mg/day) started 24±4 hours prior to starting administration of AAV9-hPCCA. This dose will continue for 30 days but could be extended if there is any evidence of immunological response to AAV9, based on elevated liver function tests, and if clinically indicated, other laboratory parameters. Upon completion of the oral 1 mg/kg/day corticosteroid course, the corticosteroid dose will be tapered over the following 28 days. See Appendix A, Table A.1 for management of elevated transaminases and corticosteroid tapering. • Participants will be hospitalized for the administration of AAV9-hPCCA up to three days prior to the anticipated dosing day for baseline evaluations, pre-medications, and placement of the IV and peripherally-implanted central catheter (PICC) line, as applicable. Participants will be observed at the hospital for 72 hours post-dosing prior to their discharge (see Appendix A, Table A.2 for management of infusion-related reactions). They will remain local to NIH at Children's Inn for ~2-3 months and return for monitoring visits every 2-3 days for the first two weeks, then weekly for the first month, biweekly until week 12 and then at 6 months (week 26), and 1, 2, 3, 4, and 5 years post AAV9-hPCCA administration. • Participants will be closely monitored for liver toxicity and metabolic events, such as vomiting/metabolic acidosis (see Appendix A, Table A.3 for management of vomiting & metabolic acidosis). • Participants will be closely monitored for signs of hyperammonemia (see Appendix A, Table A.4). • Participants will be closely monitored for signs of thrombocytopenia, microangiopathic hemolytic anemia, and organ damage (e.g., acute kidney injury, GI issues, or central nervous system [CNS] manifestations) of thrombotic microangiopathy (TMA), including clinical signs, frequent complete blood count (CBC) with peripheral smears/schistocyte counts, platelet counts/D-dimer and complement levels (see Appendix A, Table A.5 for management of TMA). An experienced nephrologist and expert in TMA are part of the clinical team and will guide all assessments. Participants who develop TMA will receive eculizumab or a similar drug, and if TMA progresses to severe kidney failure, hemodialysis will be implemented. Any patient weighing <40kg or aged <13 years will be transferred to a facility that can perform pediatric dialysis, such as Children's National Hospital (CNH).

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Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Study Design: Study timeline and schedule of events (continued)	• Myocarditis will be managed according to grade of severity, per table below and monitoring of troponin levels (See Appendix A, Table A.6). • Participants will be monitored for anaphylaxis.During the first 12-month follow-up after administration of AAV9-hPCCA, participants will continue taking their baseline medications and diet as advised by their healthcare providers, unless a change is warranted based on the clinical findings. • Participants will be followed-up for safety and efficacy of AAV9-hPCCA for five years.
Study Design: Rules for suspending the study (enrollment and investigational product (IP) administration):	The study will be *suspended* if within the study period after the administration of AAV9-hPCCA, any of the following occurs: 1. A participant dies due to a medical event related to AAV9-hPCCA administration. 2. A participant develops a malignancy determined to be related to the drug administration. 3. A participant experiences a Grade 4 or higher adverse event (based on CTCAE v5.0) deemed to be related to AAV9-hPCCA. 4. Any Grade 4 serious adverse event that could be related to study procedures (e.g., complications from corticosteroids). 5. The DSMB chair or sponsor of the study determines that a medical event requires additional evaluation by the full DSMB.
Study Design: Rules for stopping the study	The study will be *stopped* if within the first 12 months after the administration of AAV9-hPCCA, if any of the following occurs: 1. The sponsor determines that an event or data warrant termination of the study for any reason. 2. A participant develops malignancy determined by the sponsor to be related to the AAV9-hPCCA administration. 3. A participant death is determined by the sponsor to be related to the AAV9-hPCCA administration. If the study has been stopped for any reason listed above, participants who had been dosed prior to the qualifying event, will continue to be followed as part of the long-term five -year follow-up.
Estimated Study Duration	Individual research participant involvement in the study is five years plus three months, from screening and baseline assessments to the end of the long-term follow-up. The active phase study duration is estimated to be 24 to 60 months, depending on the rate of enrollment (three vs two participants per year).

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Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Justification for Study Population	PA is a rare, genetically and clinically heterogeneous disease *[number of patients in the NIH NHS study on PCCA-related PA],* with affected individuals displaying a range of clinical severity and no common variant in the *PCCA* gene [6]. Many PA patients receive elective liver transplant (LT) early in life, and median ages have been reported for different patient cohorts: • 2.7 years, with a minimum of 0.6 years and maximum of 23.0 years, as reported in a European cohort using questionnaire-based data collection [7] • 3.2 years, with a minimum of 1.1 years and maximum of 9.0 years, as reported in UK and French cohorts [8] • 1.9 years, with a minimum of 0.4 years and a maximum 9.4 years, as reported in a retrospective study [9] Additional data from several literature reviews on LT in PA [10-13] highlight the need for earlier elective LT in order to avoid recurrent life-threatening metabolic decompensations with hyperammonemia, to prevent long-term complications (cardiomyopathy and end-stage renal disease) and to maximize neurocognitive function. However, it is noted that LT does not guarantee restoration of clinical function. Within the UK and French cohorts, it was noted that seven of 12 patients (58%) died within the first year after LT. Further, out of 17 LT procedures, 13 had early and severe complications [8]. Published literature suggests PA is an ultra-rare disease affecting ~1 in 243,000 individuals in the US with approximately half due to variants in the *PCCA* gene [14-16]. This highlights the challenges of enrolling a homogeneous study population in this first-in-human study. The rationale for including patients ≥3 years of age is based on the following arguments: 1. Older, adult PA patients with severe disease accumulate irreversible end-organ damage and thus are less likely to benefit from the proposed gene therapy trial. 2. Due to the largely irreversible nature of end-organ damage in PA, it is the younger patients who are more likely to benefit from the experimental AAV9-hPCCA therapy. Therefore, the study needs to include a pediatric population. 3. There is a limited number of individuals with *PCCA*-related PA, and many undergo LT in early childhood, excluding them from this AAV trial, which necessitates the inclusion of pediatric patients.

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Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Summary of Eligibility Criteria: Inclusion Criteria	1. Participants ≥3 years of age at the time of consent *[NIH Clinical Center specific criteria]* (whenever applicable, assent will be obtained) 2. Individuals with the diagnosis of *PCCA*-related PA confirmed molecularly (*PCCA* gene analysis). 3. Biochemical evidence of PA (e.g., elevated 2-MC, 3-OHP in body fluids) 4. A clinical history consistent with severe PA, defined as meeting at least two of the conditions listed below: a. A history of neonatal encephalopathy with or without hyperammonemia b. A history of hemodialysis for hyperammonemia after infancy c. One or more hospitalizations, including pediatric intensive care unit (PICU) and/or emergency room (ER) visits or need for sick-day dietary adjustment for metabolic ketoacidosis in the last 24 months prior to study enrollment. d. Complete protein tolerance less than the recommended daily allowance for age and/or gastrostomy feeding-dependance for meeting caloric needs. e. One or more disease-related complications including cognitive impairment, failure to thrive (height, and/or weight, or head circumference falls lower than the third percentile), renal disease, basal ganglia injury, optic nerve disease, history of pancreatitis, bone marrow failure, or cardiomyopathy. 5. Ability and willingness to comply with the scheduled study visits and procedures. 6. Complete vaccination according to the CDC vaccination schedule for age at the time of consent, with exception of live attenuated vaccines with appropriate time intervals post-immunization per accepted recommendations. 7. Females of childbearing potential who are sexually active must use at least one method of contraception. 8. Males who are sexually active must agree to use an effective barrier method (male or female condom) of contraception starting one week before and continuing until six months after gene transfer. If the participant's partner is able to become pregnant, a second form of contraception will be required for the same duration.

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Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Summary of Eligibility Criteria: Exclusion Criteria	1. A molecular genetic diagnosis of *PCCB*-related PA. 2. High propionate oxidative capacity, *[threshold value]* as seen in healthy individuals and individuals with PA after LT. 3. Anti-AAV9 neutralizing antibody titer above *[cut-off value].* 4. Episodes of metabolic decompensation within two months prior to the scheduled dose administration 5. History of the following interventions at any point in the past: a. Gene therapy or mRNA therapy b. Solid organ transplantation c. Cell transfer therapy 6. History of investigational drugs within five half-lives of the drug before the first screening visit, whichever comes first. 7. History of malignancy or immunocompromised state, regardless of etiology 8. Left ventricular ejection fraction *[XX]* by transthoracic ECHO or other comparable diagnostic modality. 9. QTcF *[XX]* for either male or female participants. 10. Creatinine-based estimated glomerular filtration rate (eGFR) of *[XX]*m^2 as estimated by the bedside Schwartz eGFR equation (<chronic kidney disease stage *XX*) or ongoing dialysis for chronic kidney disease. 11. Any of the following laboratory finding at the time of the screening visit: a. Hemoglobin *[XX]* Absolute neutrophil count *[XX]* b. Platelet count *[XX]* c. Alanine transaminase (ALT), aspartate aminotransferase (AST), or total bilirubin *[XX]* times the upper normal limit for age d. Plasma lipase or amylase *[XX]* times the upper normal limit for age e. Plasma ammonia *[XX]* times the upper normal limit for age or clinical symptoms of hyperammonemia (e.g., lethargy, excessive irritability, vomiting) 12. Clinical or non-invasive testing indicative of advanced liver fibrosis or history of liver disease, a pre-existing diagnosis of portal hypertension. 13. History of chemotherapy, granulocyte colony stimulating factor (GCSF) or immunomodulating drugs (e.g., corticosteroid or intravenous immunoglobulin) within six months before the first screening visit. 14. Participant has received a live virus vaccine in the previous six weeks prior to screening (measles, mumps, rubella [MMR], Varicella, Rotovirus, Varivax, oral Polio). Live vaccines should not be administered within the 6 weeks prior or after AAV9-hPCCA administration. 15. Serology positive for hepatitis B virus (HBV), Hepatitis C virus (HCV), or human immunodeficiency virus (HIV), CMV, EBV, or a positive T-spot. 16. Ongoing/active infection (including current COVID-19 infection). Investigator to confirm complete resolution of infection for at least 14 days prior to dosing. 17. Use of concomitant medications to manage chronic condition(s) which interfere with the mechanism of action for AAV9-hPCCA in the opinion of the Investigator and dose(s) must not alter for at least four weeks before screening through to dosing (Day 1). 18. History of anaphylaxis. 19. History of severe allergic reactions to any components of the gene therapy product.

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Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Summary of Eligibility Criteria: Exclusion Criteria (continued)	20. Pregnant or breast-feeding. 21. History of a medical condition or family history of a disorder (e.g., a familial cancer predisposition syndrome) which, in the opinion of Investigator, can exclude a participant from participating in the study.
Concomitant Medications	For the first three months to one year on study, concomitant medication administration is kept unchanged, unless clinically indicated changes are required.
Drug, Drug Dosage, and Formulation	The dose of AAV9-hPCCA for the clinical study is proposed to be *[starting dose],* and, depending on safety and efficacy signals, *[higher dose],* as justified by the rationale described below (see **Dose Justification**). AAV9-hPCCA is a non-pyrogenic solution that will be stored per manufacturer's specifications until ready to be used. It will be thawed prior to administration as a one-time IV infusion given via peripheral intravenous (PIV) or PICC line over 30-60 minutes using a syringe pump (510(k) cleared).
Comparator Group	Historical control from the NIH natural history study of PA, which is ongoing.
Dose Justification	Proof-of-concept results showing effective rescue of the *Pcca*$^{-/-}$ mouse model from neonatal lethality with AAV9-hPCCA inform dose translation. Based on the survival data in the neonatal mouse studies, *[range]* vg/kg is the therapeutic window where we would expect to see therapeutic effects in research participants. To our knowledge, there are no reliable studies to scale AAV9 dosing from mice to humans, but there are several examples of systemic AAV9 gene therapy studies that have achieved clinical translation and are informative for our efforts. Pediatric metabolic disorders include MPSIIIa and MPSIIIb (Transpher A and B), where doses between 0.5E13 to 1.0E14 vg/kg have been proposed, and GM1 gangliosidosis (5.0E13vg/kg) [17.18]. Considering *[XX]* vg/kg to be a high dose for PCCA deficiency, we propose a starting dose cohort of *[XX]* vg/kg *[relation of proposed clinical starting dose to higher dose in mice]* as a starting point to assess safety and explore a biomarker response in PCCA-deficient participants. We propose to study up to six patients, and given the grave nature of this metabolic disorder, advocate for an initial dose of *[XX]* vg/kg that might have efficacy. If the first three participants enrolled in Cohort 1 do not show a biomarker response, including lowering of *[disease related biomarkers],* dose escalation to *[XX]*vg/kg would be proposed and three additional participants would be enrolled, pending a safety assessment and DSMB review. In summary, an initial human dose of *[XX]*vg/kg with escalation to *[XX]* vg/kg of AAV9-hPCCA has a reasonable likelihood to be safe and to have a therapeutic benefit in participants with PA. These doses will be confirmed after the results of the six-month GLP toxicity study in wild-type mice become available.
Route of Administration	A single PIV infusion. In participants with poor or unreliable peripheral IV access, a PICC line will be used.
Dose Escalation	The study is composed of Cohort 1 (*[starting dose]*) and Cohort 2 (*[higher dose]*). Escalation is discussed above in "Study Design."
Procedures	1. PIV or PICC insertion, as needed. 2. Intravenous delivery of AAV9-hPCCA. *3. [biomarker related procedure].*

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Title	A Phase 1/2, Open-Label Safety, Dose-Escalation, Single-Center Clinical Study of AAV9-hPCCA Gene Therapy in Propionyl-CoA Carboxylase Subunit Alpha (*PCCA)*-Related Propionic Acidemia (PA)
Primary Endpoints	• Incidence of treatment-related adverse events, treatment-emergent adverse events, and serious adverse events and their relationship to AAV9-hPCCA administration based on CTCAE v5.0. • Absolute and percent change from baseline in plasma *[disease related biomarker]* in the *[biomarker assay]* at *[XX]* weeks (interim endpoint for DSMB assessment) and at the end of the *[XX]* study period (primary endpoint).
Secondary Endpoint	• Healthcare utilization related to PA (number of ER visits and hospitalizations/year, total days spent in the hospital/year), new or worsening complications and change in dietary regimen • Quality of life of patients and caregivers and neurocognitive outcomes
Exploratory Endpoints	*[Disease specific exploratory endpoints]*
Statistical Considerations	Descriptive statistics

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Schedule of Clinical Activities

[Schedule of activities table]

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Appendix A

Table A.1: Management of Elevated Transaminases.

CTCAE Grade	Corticosteroid treatment
Grade 2 (>2.0-5.0 x ULN) (or elevations over 1.5x baseline value if baseline close to 2.0 ULN or as deemed necessary by the treating team)	*[Management instructions per institutional SOPs/guidance]*
Persistent or Recurrent Grade 2, or Grade 3 (>5.0-20.0 x ULN) that does not plateau or resolve within 7 days of prednisolone to baseline or Grade 1	*[Management instructions per institutional SOPs/guidance]*
Grade 4 (>20.0 x ULN) with or without elevated direct bilirubin and elevated International Normalized Ratio (INR)	*[Management instructions per institutional SOPs/guidance]*

Tapering of prednisolone for subjects weighing <60kg: 1 mg/kg/day x 30 days; 0.8mg/kg/day x1 week; 0.6mg/kg/day x1 week; 0.4mg/kg/day x1 week; 0.2mg/kg/day x1 week; Off; The principal Investigator (PI) will consult with an expert pediatric endocrinologist on the taper protocol.

A three-day course of IV methylprednisolone at 10 to 20 mg/kg/day followed by 10-5-4-3-2-1 mg/kg/day taper on days 4 to 9 will be considered for Grade 4 liver toxicity, outlined for liver toxicity in other gene therapy trials [19, 20]. The intravenous course will be followed by oral prednisolone at 2mg/kg until sustained normalization of AST/ALT is achieved.

If there is no response to high-dose corticosteroid therapy, then T-cell targeted immunosuppressants will be considered at the discretion of the PI.

Synthetic function of the liver and INR will be monitored and treated in consultation with GI/hepatology consultants, who will assist with diagnostic work-up and evaluate the need for a liver biopsy.

Table A.2: Management of Infusion-related Reactions.

CTCAE Grade	Treatment
Grade 1: Mild transient reaction; infusion interruption not indicated; intervention not indicated	*[Management instructions per institutional SOPs/guidance]*
Grade 2: Therapy or infusion interruption indicated but responds promptly to symptomatic treatment and prophylactic medications indicated for ≤24 hours. Investigator discretion will be utilized to determine whether to re-start dosing	*[Management instructions per institutional SOPs/guidance]*
Grade 3: Prolonged (e.g., not rapidly responsive to symptomatic medication and/or brief interruption of infusion); recurrence of symptoms following initial improvement; hospitalization indicated for clinical sequelae. Investigator discretion will be utilized to determine whether to re-start dosing	*[Management instructions per institutional SOPs/guidance]*

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CTCAE Grade	Treatment
Grade 4: Life-threatening consequences; urgent intervention indicated	*[Management instructions per institutional SOPs/guidance]*

Table A.3: Management of Vomiting/Metabolic Acidosis

CTCAE Grade	Treatment
Grade 2, repeat vomiting episodes >3, reduced PO intake, trace or increase from baseline ketonuria	*[Management instructions per institutional SOPs/guidance]*
Grade 3, persistent vomiting, reduced oral intake associated with metabolic acidosis (pH<7.3) and ketonuria (mild to moderate)	*[Management instructions per institutional SOPs/guidance]*
Grade 4, persistent metabolic acidosis with or without elevated lactate not responding to previous interventions	*[Management instructions per institutional SOPs/guidance]*

Table A.4: Management of Hyperammonemia

CTCAE Grade	Treatment
Grade 3 hepatic failure, asterixis; mild encephalopathy, Confirmed elevated ammonia >100umol/L	*[Management instructions per institutional SOPs/guidance]*
Hyperammonemic encephalopathy/coma (Ammonia >200umol/L)	*[Management instructions per institutional SOPs/guidance]*
Hyperammonemic encephalopathy/coma (Ammonia >500umol/L), concern for metabolic stroke	*[Management instructions per institutional SOPs/guidance]*

Most common adverse reactions (≥5%) to N-carbamylglutamate are neutropenia, anemia, vomiting, electrolyte imbalance, decreased appetite, hypoglycemia, lethargy/stupor, encephalopathy and pancreatitis/lipase, amylase increased.

Table A.5: Management of Thrombotic Microangiopathy (TMA)

CTCAE Grade	Treatment
Grade 1, PLT count <150 - 75.0 x 10e9 /L	*[Management instructions per institutional SOPs/guidance]*
Grade 2-3, PLT count <75.0 - 25.0 x 10e9 /L	*[Management instructions per institutional SOPs/guidance]*
Grade 4, PLT count <25.0 x 10e9 /L	*[Management instructions per institutional SOPs/guidance]*

DISCLAIMER: *This clinical trial design has been updated after the pre-IND meeting. This study design is specific to AAV9-hPCCA and may not be applicable to all studies or diseases.*

Table A.6: Management of Kidney Dysfunction

CTCAE Grade	**Treatment**
Grade 1, Creatinine increase <1.5 x baseline	*[Management instructions per institutional SOPs/guidance]*
Grade 2, Creatinine increase >1.5 - 3.0 x baseline	*[Management instructions per institutional SOPs/guidance]*
Grade 3, Creatinine increase >3.0 x baseline	*[Management instructions per institutional SOPs/guidance]*
Grade 4, Creatinine increase >6.0 x ULN	*[Management instructions per institutional SOPs/guidance]*

Table A.7: Management of Myocarditis

CTCAE Grade	**Treatment**
Grade 1, Asymptomatic changes in ECG or routine echocardiogram	*[Management instructions per institutional SOPs/guidance]*
Grade 2, Symptoms with moderate activity or exertion, chest pain; elevation of heart rate above expected; ST segment changes; elevations of troponin; atrial or ventricular ectopy including single beats or couplets; or any combination of these findings	*[Management instructions per institutional SOPs/guidance]*
Grade 3, Severe with symptoms at rest or with minimal activity or exertion; atrial or ventricular tachycardia that is hemodynamically tolerated and self-terminating; mildly or moderately reduced ejection fraction of left ventricle as measured by any imaging technique; new onset pericardial effusion	*[Management instructions per institutional SOPs/guidance]*
Grade 4, Hemodynamic instability requiring more than a single inotropic agent; severely reduced ejection fraction of left ventricle; atrial or ventricular tachycardia; end-organ injury due to shock	*[Management instructions per institutional SOPs/guidance]*

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References

1. Shchelochkov, O.A., et al., *Severity modeling of propionic acidemia using clinical and laboratory biomarkers.* Genet Med, 2021. **23**(8): p. 1534-1542.
2. Shchelochkov, O.A., et al., *Chronic kidney disease in propionic acidemia.* Genet Med, 2019. **21**(12): p. 2830-2835.
3. Vockley, J., et al., *Challenges and strategies for clinical trials in propionic and methylmalonic acidemias.* Mol Genet Metab, 2023. **139**(3): p. 107612.
4. Longo, N., et al., *Biomarkers for drug development in propionic and methylmalonic acidemias.* J Inherit Metab Dis, 2022. **45**(2): p. 132-143.
5. Manoli, I., et al., *1-(13)C-propionate breath testing as a surrogate endpoint to assess efficacy of liver-directed therapies in methylmalonic acidemia (MMA).* Genet Med, 2021. **23**(8): p. 1522-1533.
6. Shchelochkov, O.A., N. Carrillo, and C. Venditti, *Propionic Acidemia*, in *GeneReviews((R))*, M.P. Adam, et al., Editors. 1993: Seattle (WA).
7. Molema, F., et al., *Liver and/or kidney transplantation in amino and organic acid-related inborn errors of metabolism: An overview on European data.* J Inherit Metab Dis, 2021. **44**(3): p. 593-605.
8. Charbit-Henrion, F., et al., *Early and late complications after liver transplantation for propionic acidemia in children: a two centers study.* Am J Transplant, 2015. **15**(3): p. 786-91.
9. Pillai, N.R., et al., *Liver transplantation in propionic and methylmalonic acidemia: A single center study with literature review.* Mol Genet Metab, 2019. **128**(4): p. 431-443.
10. Chakrapani, A., et al., *Safety, efficacy, and timing of transplantation(s) in propionic and methylmalonic aciduria.* J Inherit Metab Dis, 2023. **46**(3): p. 466-481.
11. Sen, K., et al., *Solid organ transplantation in methylmalonic acidemia and propionic acidemia: A points to consider statement of the American College of Medical Genetics and Genomics (ACMG).* Genet Med, 2023. **25**(2): p. 100337.
12. Vara, R., et al., *Liver transplantation for propionic acidemia in children.* Liver Transpl, 2011. **17**(6): p. 661-7.
13. Yap, S., R. Vara, and A. Morais, *Post-transplantation Outcomes in Patients with PA or MMA: A Review of the Literature.* Adv Ther, 2020. **37**(5): p. 1866-1896.
14. Kraus, J.P., et al., *Mutation analysis in 54 propionic acidemia patients.* J Inherit Metab Dis, 2012. **35**(1): p. 51-63.
15. Almasi, T., et al., *Systematic literature review and meta-analysis on the epidemiology of propionic acidemia.* Orphanet J Rare Dis, 2019. **14**(1): p. 40.
16. Chapman, K.A., et al., *Incidence of maple syrup urine disease, propionic acidemia, and methylmalonic aciduria from newborn screening data.* Mol Genet Metab Rep, 2018. **15**: p. 106-109.
17. Flanigan KF, S.N., Couce ML, Escolar M, Truxal KV, McBride KL, de Castro MJ, Fuller M, Paneda A, Ruiz J. *Updated Results of Transpher A, a Multicenter, Single-Dose, Phase 1/2 Clinical Trial of ABO-102 Gene Therapy for Sanfilippo Syndrome Type A (Mucopolysaccharidosis IIIA) [abstract #200].* in *American Society for Gene and Cell Therapy*. 2021.
18. KL McBride, N.S., ML Couce, KM Flanigan, KV Truxal, TR Simmons, MJ de Castro, F Rinaldi, MT Oreiro, L Jaensch, M Fuller, J Ruiz, *Safety, Tolerability, and Preliminary Evidence of Biopotency in Transpher B, a Multicenter, Single-dose, Phase 1/2 Clinical Trial of ABO-101 Gene Therapy for Sanfilippo Syndrome Type B (Mucopolysaccharidosis IIIB) (5175).* . Neurology, 2020. **94**.
19. Chand, D., et al., *Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy.* J Hepatol, 2021. **74**(3): p. 560-566.
20. Feldman, A.G., et al., *Subacute Liver Failure Following Gene Replacement Therapy for Spinal Muscular Atrophy Type 1.* J Pediatr, 2020. **225**: p. 252-258 e1.

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Table of Significant Changes to the Protocol Synopsis

No	INTERACT	Pre-IND
1	Study population (age): 2 -18 years of age	≥3 year of age and *[NIH Clinical Center-specific criteria]*
2	~10 pts Dose escalation design, 3 dose levels: 1-3 patients in cohort 1 *[low dose]*, approximately 4 patients in cohort 2 *[medium dose]*, and approximately 4 patients in the optional cohort 3 *[high dose]*.	~6-9 pts Two dose levels: • *Cohort 1: [starting dose]* (N=3-6) • *Cohort 2*: *[higher dose]* (N=1-3) In addition, there will be a comparator group (historical control) from the NIH natural history study of PA (NCT02890342).
3	Primary objective & endpoint: safety & tolerability	Primary objectives & endpoints: safety & **efficacy** - Added efficacy objective: "To assess changes from baseline in response biomarkers, aka, pharmacodynamic (PD) response, to AAV9-hPCCA" - Endpoint: Absolute and percent change from baseline in *[two disease related biomarkers]* at [*XX*] weeks (interim endpoint for DSMB assessment) and at the end of [*XX weeks*] study period (primary endpoint).
4	Exploratory endpoints: - Absolute and percent change from baseline of *[disease related biomarker]* at multiple time points as part of the short-term 12-month and long-term 5-year follow-up.	Added: - Measurement of AAV9 neutralizing antibodies (NAb) and anti-PCCA binding antibodies and T cell responses - Absolute and percent change from baseline of biomarkers such as *[other disease related biomarkers]* Removed: - *[other exploratory endpoints]*
5	Dosing time between patients: 8 weeks	12 weeks
6	DSMB review: after treatment of each patient	After treatment of the first and third patient in Cohort 1, and if decision is made not to proceed to Cohort 2, at the end of Cohort 1; after the first patient and third patient of Cohort 2, if study proceeds to the higher dose/Cohort 2 (see schema in Study Design of the protocol synopsis)

DISCLAIMER: *The clinical trial design has been updated after the Pre-IND meeting; this design may not be applicable to all studies or diseases.*

No	INTERACT	Pre-IND
7	Efficacy measurements enabling a decision to proceed to Cohort 2 or Cohort 3: not defined	Efficacy of each dose evaluated based on: trend of changes in *[disease related biomarkers]*
8	Justification for target population: not included	Included
9	Eligibility	1. Expanded on clinical history consistent with severe PA for inclusion in the study 2. Refined and expanded exclusion criteria based on INTERACT comments (e.g., exclusion of ongoing/active infection (including current COVID-19 infection).
10	Follow-up: 1 year and then patients would be rolled into a long-term study for 4 years	5 years of follow-up
11	Screening period: 1 month	3 months
12	Close monitoring for thrombocytopenia, microangiopathic hemolytic anemia, and organ damage (e.g., acute kidney injury, GI issues, or CNS manifestations) of thrombotic microangiopathy (TMA), signs of TMA: not discussed	Addressed: Participants will be closely monitored for signs of thrombocytopenia, microangiopathic hemolytic anemia, and organ damage (e.g., acute kidney injury, GI issues, or CNS manifestations) of thrombotic microangiopathy (TMA), including clinical signs, frequent CBC with peripheral smears, platelet counts/D-dimer and complement levels. An experienced nephrologist and expert in TMA is part of the clinical team and will guide all patient assessments. Participants who develop TMA will receive eculizumab, and if TMA progresses to severe kidney failure, hemodialysis will be implemented.
13	Close monitoring for signs of hyperammonemia; liver toxicity and metabolic events, such as vomiting/metabolic acidosis; Myocarditis, and anaphylaxis: not discussed	Addressed, and appendix with management guidelines included

Appendix: Test method - mitochondrial metabolites

[Commercially available assay]

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

INTERACT RESPONSE MATRIX

Nonclinical

INTERACT Question 1: Does the Agency agree that the efficacy observed in the proof of concept (POC) studies in the Pcca-/- neonatal mouse model (Section 2.1.3) is sufficient to support proposed clinical testing?

FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
Your proposed first-in-human (FIH) clinical trial will enroll individuals with an age range from 2 to 18 years old. This trial represents more than a minor increase over minimal risk. Therefore, per 21 CFR 50.52, prior to initiating a study in children, in accordance with Subpart D, you must provide evidence that administration of the study agent provides a prospect of direct benefit (PDB). Based on the information provided in Section 2.13 (pages 16-21) of your INTERACT meeting package, we tentatively agree that the summarized data generated following retro-orbital injection of AAV9-hPCCA in the Pcca-/- neonatal mice are sufficient to support a PDB. However, please note that a final determination as to the adequacy of these data will be made following our review of comprehensive material, including complete study reports, that you should provide in your IND. Please also address the following comments in your pre-IND and IND submissions:	The clinical trial design has been updated. We plan to enroll patients who are at least three years old, *[NIH Clinical Center specific criteria].* We plan to perform an efficacy study in the Pcca-/- neonatal mouse model with AAV9-hPCCA (50L batch) delivered via facial vein injection. This route of administration more closely resembles the one planned for delivery of the investigational product in the first-in-human (FIH) clinical trial. Similarly, the age of the mice corresponds to the age of the patients most likely to be treated in the clinical trial and to benefit from this gene therapy. In addition, we will perform a GLP toxicity study with AAV9-hPCCA (200L engineering lot) in wt animals. Protocols for both studies are included in this submission. In the IND, we will provide the study reports in support of the clinical trial design	NHGRI-PCCA-001 Study Report, NHGRI-PCCA-002 Study Report, 6 M GLP Tox Study Protocol, *[protocol number]* Efficacy Protocol
1.Please provide a comprehensive discussion, with accompanying data, of the biological relevancy of the Pcca-/- neonatal mouse model to the proposed patient population, including: a) progression of the abnormal phenotype observed in this model (i.e., biochemical, morphological, functional), b) the similarities and differences in this model and the disease phenotype (e.g., pathophysiology, biochemistry, functional changes, etc.), and c) the timing of AAV9-hPCCA administration in this model relative to the disease status in the proposed patient population.	Information regarding the generation of the Pcca-/- mouse model, along with its relevance are provided within the briefing package.	Briefing Package Section 15.2.2, NHGRI-PCCA-01 Study Report, NHGRI-PCCA-02 Study Report
2.Please provide a summary table with the similarities and differences between the preclinical lot(s) of AAV9-hPCCA administered to the mice compared to the clinical lot(s).	Relevant information is provided in the briefing package, and contains similarities and differences for early stage feasibility lots, as well as non-GMP and GMP lots produced (and to be produced) at Charles River Laboratories.	Briefing Package Section 15.1.1, PCCA pre-IND_CMC_200L

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
3.Please confirm that the assay that will be used to determine the vector titers for the clinical lots of AAV9-hPCCA are also used for the lots administered in the POC study. If the same assay was not used, the vector titer for the preclinical lot(s) should be re-determined using the planned clinical assay and this result should be used to re-calculate the vector dose levels administered in your POC study.	The POC study will be repeated using the 50L lot material (efficacy study in Pcca-/-animals). The titer for this material and for the 200L engineering lot as well as the clinical 200L lots will be measured using the same method: qPCR. The COA for each of these lots will include titer measurements via ddPCR, as FIO. The previous research lots had the titer measured by qPCR and/or ddPCR; vendor specific (UMMS and CRL).	Briefing Package Section 15.1.2
4.Please provide data from bench testing that confirm the compatibility of the preclinical lot(s) of AAV9-hPCCA with the vector delivery system (e.g., needle/syringe system) used in the POC study. Your evaluation should include the ability to consistently deliver accurate pre-specified dose levels of AAV9- hPCCA (e.g., assessment of vector genomes after passage of the preclinical lot through the needle/syringe delivery system used in the preclinical studies). If vector loss is observed, please provide, in the study report and in data tables, the actual vector dose level administered.	Full information will be provided in the IND. The proposed study plan is included in the pre-IND package, but compatibility and stability studies will be performed after the pre-IND meeting.	*[protocol number]*
5.You described (Appendix A, page 37) that Pcca+/- mice exhibit a wild type phenotype; and genotypes and sexes for mice were not found after injection or not available for those that died before genotype and sex could be established. Please confirm that you only included Pcca-/- neonatal mice in the study (Table 1, page 16).	The results in table 1 referred only to the Pcca-/- mice, based on Appendix A table. Due to mouse pup fragility, all animals were injected with AAV9-hPCCA upon birth prior to genotyping, and thus the study overall included heterozygote animals, too. The survival curve and other measurements show the comparison between WT and Pcca -/-	NHGRI-PCCA-001 Study Report
6.Please specify a) the method used to randomly assign animals to all study groups, and b) the method for staggering dosing of animals. Please discuss the effect of these study design elements on the potential for study bias.	Briefly, animals were injected with vehicle or AAV9-hPCCA in the first 24h after birth. Pups could not be genotyped until weaning, and thus there was no randomization.	NHGRI-PCCA-001 Study Report, NHGRI-PCCA-002 Study Report
7.Please explain the procedure used to collect blood from the mice at multiple intervals (Days 30, 90, and 101 post-dose) for plasma levels of 2-methylcitrate (2-MC) and provide the determination method for 2-MC levels. Please confirm that the plasma 2-MC levels depicted in Figure 3 (page 19) represent the mean±SD from individual animals, and not from pooled samples for each group.	Procedures for collection of blood are included in the PoC study reports. Briefly, this is based off a method developed at the University of Colorado where labeled [methyl-D3]2-methylcitric acid is added to samples in a known amount, followed by extraction, evaporation and silylation. Prepared samples are analyzed by gas chromatography/mass spectrometry with a mass selective detector. Going forward this assay will be moved to Mayo Clinic Laboratories.	Briefing Package Section 15.3.2, NHGRI-PCCA-001 Study Report, NHGRI-PCCA-002 Study Report, 2MC_Clinical_Method

INTERACT Question 2: Does the agency agree with our proposal of establishing the FIH dosing based on the Pcca-/- neonatal mouse model studies (Section 2.1.3)?

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
We cannot yet agree to your proposal to establish the clinical starting dose level based only on the POC data in the Pcca-/- neonatal mouse model provided in Section 2.1.3. These POC data in combination with the safety data from the GLP toxicology study should inform the starting dose level for your planned FIH clinical trial.	We are in full agreement and will follow this guidance and will include the GLP tox plan in the briefing package	GLP 6 M mice protocol. PCCA_31MAY2023

INTERACT Question 3a: Does the Agency agree with the proposed toxicology study design in WT mice, specifically: Does the Agency agree with the proposed toxicology dose of up to 1 x 10^{14} vg/kg in support of the proposed FIH doses?

FDA Response	Sponsor Response	Relevant Document(s)/Section(s)
Discussion of definitive toxicology study designs is outside the scope of an INTERACT meeting. Please refer to CBER's SOPP 8214, titled 'INTERACT Meetings with Sponsors for Drugs and Biological Products' for the details. Thus, we recommend that you refrain from initiating this study until you receive our feedback in a future pre-IND meeting. We have the following general comments regarding this study. However, we emphasize that these comments are not all-inclusive, and that you should provide a detailed study protocol in your pre-IND submission.	N/A	N/A
1.The lot(s) of AAV9-hPCCA administered in this definitive study should be identical to the intended clinical product in terms of manufacturing process, product identity, and final formulation.	We do not anticipate any changes to be made terms of manufacturing process, product identity, and final formulation between the 200L engineering lot, which will be used in the GLP toxicity study, and the 200L clinical lots that will be manufactured later.	PCCA pre-IND_CMC_200L
2.Please note that the POC data provided in Section 2.1.3 (pages 16-21) show that, following AAV9-hPCCA IV administration, Pcca-/- neonatal mice have a dose-dependent improvement of the disease phenotype, including increased survival and reduction of plasma 2-MC levels, with the highest dose level of 3.3 x 1014 vg/kg resulting in the most significant benefit. Please note that the dose levels specified in Table 4 (page 22) for your toxicology study will not support administration of AAV9-hPCCA at a clinical dose level higher than 1 x 10^{14} vg/kg.	We agree with the Agency and will use the maximum feasible dose in the GLP tox study as the highest dose.	Briefing Package Section 15.2.4, GLP 6 M mice protocol. PCCA_31MAY2023

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INTERACT Question 3b: Does the Agency agree with the proposed toxicology study design in WT mice, specifically: does the Agency agree that the GLP toxicology study ROA, tail vain injection in 8-week WT mice, is sufficient to support our proposed FIH human trial using either the IV or PICC line ROA?

FDA Response	Sponsor Response	Relevant Document(s) / Section(s)
The proposed clinical route of administration is via intravenous (IV) injection. Thus, IV administration of AAV9-hPCCA in the GLP toxicology study is appropriate. In your pre-IND and IND submissions, please provide an explanation regarding the age of the wild-type mice at the time of dosing. We acknowledge that you plan to include the peripherally inserted central catheter (PICC) route of administration in your clinical trial if peripheral IV access in a subject is poor or unreliable (page 33). Please provide a table that compares administration of AAV9-hPCCA via the IV and PICC routes, to include parameters such as, vector concentration, infusion volume, flow rate, and any anticipated differences in the biodistribution (BD) profile.	PICC line and PIV will be used for IP delivery intravenously. A syringe pump will be used (Carefusion, Alaris PC unit, Model 8015 pump with the Alaris modules).	Briefing Package Section 15.2.5, GLP 6 M mice protocol. PCCA_31MAY2023

INTERACT Question 4: For the immunogenicity assessment portion of the planned toxicology study, we plan to only perform in vivo anti-AAV9 capsid antibody testing. We will not conduct any additional immunogenicity assessments, such as ELISpot assays in WT animals or mouse antibodies against PCCA, since the mouse immune responses to the encoded transgene may not be informative towards human translation. Does the Agency agree?

FDA Response	Sponsor Response	Relevant Document / Section
We do not agree with your position to not evaluate potential immunogenicity to the AAV9-hPCCA transgene product in your proposed GLP toxicology study. The murine immune response to the transgene product is an important factor in overall interpretation of the resulting data. Please include a plan to assess humoral and cellular immunogenicity to the PCCA transgene product in your GLP toxicology study protocol.	These assessments have been added to the study protocol.	GLP 6 M mice protocol. PCCA_31MAY2023

Clinical

INTERACT Question 5a: Regarding the proposed clinical synopsis (Section 2.3.1), does the Agency concur with the proposed study design?

INTERACT Response Matrix

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	**Sponsor Response**	**Relevant Document(s) /Section(s)**
No, we cannot agree with your current study design. You propose, page 29, an open-label Phase 1/2 dose-escalation study (three dose cohorts) of systemic AAV9- hPCCA gene therapy in up to 10 pediatric and adult PCCA patients, aged 2-18 years, with "clinically, biochemically, and/or molecularly confirmed PCCA genetic mutations that cause PA."	N/A	N/A
1.Given the inherent risks with gene therapy, in addition to clinical evidence, you must demonstrate that each subject has: (1) documented biochemical evidence of a deficiency in propionyl-CoA carboxylase (PCC) enzyme; and (2) pathogenic biallelic mutation(s) in the gene coding for this enzyme.	Based on INTERACT discussions, none of the *[XX]* patients seen at NIH have had enzymatic diagnosis. PA is diagnosed by metabolites, and it is exceedingly rare to not find biallelic pathogenic mutations in PCCA or PCCB. Enzyme assays that exist are neither validated nor specific for PCC activity. Standard of care and diagnostic protocols recommended by experts do not recommend any kind of enzymatic assays. We propose to diagnose participants based on triorthogonal evaluation (clinical, biochemical, and molecularly confirmed PCCA PA).	PCCA Protocol Synopsis
a.Please explain whether you plan to use laboratory developed procedures for these critical eligibility decisions. We are concerned that these in vitro diagnostic (IVD) devices may represent significant risk devices under 21 CFR 812.3(m)(3) requiring an IDE.	1. PCCA genetic analysis: test performed by GeneDx: https://www.genedx.com/tests/detail/pcca-gene-sequencing-and-del-dup-563 2. Mayo Clinic Laboratories, CLIA certified, *[disease related biomarkers]* tests. https://www.mayocliniclabs.com/test-catalog/overview/616610	2MC_Clinical_Method
b.Regarding gene mutation and PCC assessments, please submit to your IND: i.Name, location, and CLIA certification of the diagnostic laboratory(ies) ii.Detailed test methodologies and/or SOP(s), and information regarding these in vitro devices and reagent qualification iii.Validation level of the diagnostic test(s) for suitability (i.e., accuracy, sensitivity, specificity, reproducibility iv.Information regarding QA/QC systems in place, and diagnostic test report sign-off procedure(s). v. Risk assessments detailing the risk to the study population in case of false positive results leading to recruitment of study subjects who may not otherwise be eligible for enrollment in the study.	This information will be provided in the IND.	N/A
c.Regarding gene mutation assessments, please justify the mutations of the population you intend to include in your study.	AAV9-hPCCA targets the mutation in PCCA only. No PCCB-related PA patients will be included in the study.	Briefing Package Section 15.3
d.Regarding PCC assessments, please: (1) clarify the threshold for PCC deficiency with justification; and (2) include PCC reference ranges with units of measure.	This information will be provided in the IND.	N/A

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	Sponsor Response	Relevant Document(s) /Section(s)
2.The branched chain acidurias, including PA, have three clinical presentations: (1) a severe neonatal-onset form with acute metabolic decompensation and neurological distress; (2) an acute, intermittent, late-onset form also with recurrent episodes of metabolic decompensation; and (3) a chronic, progressive form presenting as hypotonia, failure to thrive, and developmental delay. There are also various forms of disease, e.g., neurologic, hepatic, hematological, and immunological. Your briefing package does not provide enough information to determine whether the subpopulation(s) for your FIH study are appropriate. Since this first study is dose ranging, we recommend studying a homogeneous population of PA subjects to have interpretable results. When selecting your desired population, please ensure subjects have a favorable benefit-risk profile. 3.Your proposed study in children involves more than a minor increase over minimal risk, as stated above in our response to Question # 1, the additional safeguards under 21 CFR 50, Subpart D, and in particular 21 CFR 50.52 applies. Such clinical investigations must offer the PDB at all dose levels to pediatric subjects as demonstrated in animal models or adult subjects. For early phase human studies, it is especially important to enroll at least an initial cohort of adult subjects to obtain preliminary data on safety and feasibility, bioactivity, and preliminary efficacy to support enrollment of pediatric subjects. Because there are adults with the acute intermittent form of PA, please provide your rationale, with any available data, explaining why it would not be possible to achieve the primary study objectives by enrolling adult subjects.	PA is a rare, heterogeneous disease (only *[XX]* PCCA-related PA patients participate in the NIH NHS study), with patients displaying a range of clinical severity, from mild to severe, and no common mutation in the PCCA gene (ref. 2). This presents the difficulty of enrolling a homogeneous study population in this first-in-human study. The rationale for including patients ≥3 years of age is based on the following arguments: 1. Older, adult, PA patients with severe disease accumulate irreversible end organ damage and are thus less likely to benefit from the proposed gene therapy trial. Due to the largely irreversible nature of end organ damage in PA, it is the younger patients who are more likely to benefit from the experimental AAV9-hPCCA therapy. With the limited number of PCCA-related PA patients, restricting eligibility to 3 to 18 years of age has the risk of not enrolling. 2. Furthermore, liver transplant (LT) for PA most commonly occurs in the first 2 two decades of life, and although LT patients have fewer hospitalizations, their other organs (e.g., brain, heart, and kidney) remain at risk for long-term complications even after transplant. LT patients require life-long immunosuppressive treatment, which adds to the burden of their disease. We therefore believe that our primary target group in each cohort should be patients under age 18. In addition, the first participant in each cohort is proposed as an adolescent, not adult, because of organ damage in adults with severe disease. If an adult meeting all eligibility criteria is discovered, they may be enrolled but are not targeted specifically as sentinel participants.	Briefing Package Section 15.3, PCCA Protocol Synopsis

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	Sponsor Response	Relevant Document(s) /Section(s)
4.You have proposed an open-label single-arm study without a comparator. While an open-label, single-arm study may be acceptable for an early-phase study, a concurrent control group will facilitate data interpretability with regard to safety, bioactivity and preliminary efficacy.	Study design has been modified to include a concurrent control group with PA patients enrolled in the NHS study for whom the investigators have a large body of PA data.	Briefing Package Section 15.3.3, PCCA Protocol Synopsis
Due to rarity of PA and the substantial unmet medical need, we recommend that this early phase clinical trial should be adequately designed to demonstrate preliminary effectiveness to support marketing approval. To achieve this goal, we recommend that you modify your study design to employ a randomized control design, if feasible. If not feasible, please consider a prospective concurrent control with patients on standard of care who are not interested in gene therapy. If you ultimately determine that only a natural history control is feasible, please note that control patients should be as similar as possible to the population that will receive the experimental therapy with respect to disease sub-type, genotype, demographics, baseline functional status, and concomitant therapy with the exception of receiving the investigational product. Additionally, study observations should be performed using the same methodology and timing in the controls and the experimental subjects.	We have included an efficacy objective and corresponding endpoints, and escalation from Cohort 1 to Cohort 2 will also be based on safety and efficacy evaluations.	Briefing Package Section 15.3.2, PCCA Protocol Synopsis

INTERACT Question 5b: Regarding the proposed clinical synopsis (Section 2.3.1), does the Agency concur with the safety and sufficiency of a proposed initial human dose of 1 x 10^{13} vg/kg for FIH testing?

FDA Response	Sponsor Response	Relevant Document / Section
No, we do not agree. Please see our response to Question #2 above.	Please see the current dosing plan and associated justification.	PCCA Protocol Synopsis, GLP 6 M mice protocol. PCCA_31MAY2023

INTERACT Question 5c: Regarding the proposed clinical synopsis (Section 2.3.1), does the Agency concur with the proposed three dose cohorts and dose escalation plan?

INTERACT Question 5d: Regarding the proposed clinical synopsis (Section 2.3.1), does the Agency concur with the FIH population with an age range of participants from 2 to 18 years?

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	Sponsor Response	Relevant Document / Section
Response for 5c: We agree with the importance of dose exploration, however it is premature to comment on the acceptability of your proposed dose cohorts. Please see our response to Question #2 above. Regarding dose escalation, you propose a staggering interval of at least 8 weeks between study participants. A DSMB review of available safety data will commence at the end of 8 weeks after dosing of each subject. The staggering interval between cohorts is unspecified. Regarding the within-cohort staggering interval, it is premature to comment on its appropriateness because you did not provide justification. As a general rule, the staggering interval should be sufficient to monitor for acute and subacute adverse events. We also recommend that you specify a minimum period of safety follow-up for a cohort that will be reviewed prior to beginning the next dosing cohort. The observation periods within and between cohorts should be justified based on your preclinical experience and any other relevant scientific data. Response for 5d: No, we do not agree. Please see our response to Question #1.	Based on the updated protocol design, we plan to stagger all patients by 12 weeks, and at the end of Cohort 1, there will be a DSMB review. If clinical assessment and biomarker data do not support the efficacy of this dose in the first 3 participants, we will proceed to Cohort 2. If clinical assessment and biomarker data support the efficacy of this dose, three more participants will be enrolled and treated in Cohort 1. After a total of 6 participants are treated and the last participant has been followed for 12 weeks, the DSMB will review safety data for the entire cohort, and there will be no escalation to Cohort 2.	PCCA Protocol Synopsis

INTERACT Question 5e: Regarding the proposed clinical synopsis (Section 2.3.1), does the Agency concur that the oral prednisone (1 mg/kg/day) for 4-6 weeks is appropriate to mitigate possible immune response to administration of AAV9-hPCCA?

FDA Response	Sponsor Response	Relevant Document(s) / Section(s)
This may be appropriate. Please justify the dose regimen for the population you wish to study. Please include a maximum non-weight-based dose. Hyperammonemia is a characteristic feature of organic acidemias, particularly PA. We are concerned that prednisone administration may increase catabolism and potentially trigger a hyperammonemic crisis. In your protocol, please address how you will monitor for hyperammonemic crises. Please include instructions to subjects and caregivers in the event of suspected hyperammonemia. With prolonged steroids, subjects are at risk for suppression of the hypothalamic pituitary adrenal (HPA) axis. In your protocol, please include and justify: (1) the use of stress dose steroids; (2) processes to test HPA axis recovery; and (3) steroid tapering plan.	The clinical study design has been updated to address these comments from the FDA as follows: 1. Patients with PA who have plasma ammonia >3 times the upper normal limit for age or clinical symptoms of hyperammonemia (e.g., lethargy, excessive irritability, vomiting) are excluded from participating in the trial. 2. During the study period, we are monitoring the ammonia levels at baseline and 4, 8, 12, and 26 weeks post infusion of the investigational product. In addition, the clinical protocol will include instructions for patients to identify potential signs of hyperammonemia, which will then trigger their review by the clinical team and testing of ammonia level, as determined necessary by the investigators.	PCCA Protocol Synopsis

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

INTERACT Question 5f: Regarding the proposed clinical synopsis (Section 2.3.1), does the Agency concur with the primary and secondary endpoints in the proposed clinical study?

FDA Response	Sponsor Response	Relevant Document / Section
You propose a primary endpoint of the incidence of treatment-related adverse events, treatment-emergent adverse events, and serious adverse events for each cohort. You propose a secondary endpoint of absolute and percent change from baseline of in vivo *[disease specific biomarkers]* at Week *[XX]*. The primary safety endpoint is appropriate for a FIH study with gene therapy. However, given the rarity of this disease, we recommend that you also include an appropriate primary efficacy endpoint that reflects how a patient with PA will feel, function, or survive if they benefit from therapy with your product. Results from *[disease specific biomarker assay]* assessments are not sufficient as a primary efficacy endpoint because this is a surrogate marker. If you plan to use *[disease specific biomarker assays]* in your study, please provide detailed descriptions of these tests and address whether you will develop them as in vitro diagnostic devices. For further information, please see FDA Guidance documents on in vitro companion diagnostics.	Regarding "an appropriate primary efficacy endpoint that reflects how a patient with PA will feel, function, or survive " we propose to use NIH Toolbox: https://www.nia.nih.gov/research/resource/nih-toolbox Regarding the other tests, *[disease specific biomarkers]* are performed in CLIA labs at Mayo Clinic Laboratories https://www.mayocliniclabs.com/. The device we use to evaluate the *[disease specific biomarker assay]* (breath test) is Exalenz BreathID®, cleared by the FDA for non-invasive diagnosis of *H. pylori* infection using 13C-urea: https://pdf.medicalexpo.com/pdf/exalenz/breath-id-breath-test-system/101034-134443.html	Briefing Package Section 15.3.2, PCCA Protocol Synopsis

INTERACT Question 5g: Regarding the proposed clinical synopsis (Section 2.3.1), does the Agency concur with the study duration and the proposed intervals for biomarker and preliminary efficacy testing?

FDA Response	Sponsor Response	Relevant Document(s) / Section(s)
You propose a study duration of 52 weeks. Subjects who complete the 12-month short-term study will roll into the long-term study, which will include interval assessment of safety and efficacy of AAV9-hPCCA for up to 5 years of follow-up. The active phase of your study is likely insufficient given patients with PA may have a slowly progressive decline in function. We recommend that you consider a longer period of study to determine preliminary effectiveness of your product. In future submissions, please provide more details regarding your long-term follow-up (LTFU) plans. At a minimum, all subjects who receive a partial or full dose should be followed for collection of safety data for at least five years. Please note, because of the high-risk nature of gene therapy, you should not withdraw subjects from this study or the LTFU study unless the subject or legally authorized representative withdraws consent. As a resource, FDA has a helpful guidance on long-term follow-up after gene therapy.	The proposed protocol has been updated to include safety visits yearly up to year 5	PCCA Protocol Synopsis

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

INTERACT Question 6: In light of recent safety signals related to the subacute complement cascade activation in recent trials of systemic high dose AAV9 mediated gene transfer in boys with DMD, does the Agency have any concerns and/or feedback regarding the proposed clinical protocol synopsis, risk mitigation strategy and AAV9-hPCCA development plan in the planned FIH study?

FDA Response	Sponsor Response	Relevant Document / Section
We are unable to comment on programs in clinical development. Please refer to the "Dear Health Care Professional Letter" issued March 18, 2021 by Novartis regarding onasemnogene abeparvovec—an AAV9 based therapy for spinal muscular atrophy.5 Please note the warnings and precautions in the current label related to thrombotic microangiopathy (TMA) as information in this section may be applicable for your program.6 To ensure subjects safety, you may also consider incorporating the following suggestions in your protocol to mitigate complement mediated toxicity:	N/A	N/A
1.Design your product to minimize exposure to total viral particles. From the outset of your study, the percent of empty capsids delivered per dose should be as low as possible. Include a maximum ceiling for the total dose of viral particles administered.	The study is designed to include two doses within the anticipated therapeutic window as based on results from the PoC studies, and may be subject to change pending toxicity study results.	Briefing Package Section 15.2.4, PCCA Protocol Synopsis
2.Provide details on your Nab AAV9 assay, and justify the threshold for neutralizing antibodies as an eligibility criterion. It is possible that reactions from neutralizing antibodies (Nab) to the investigational product may contribute to the occurrence of TMA.	In the clinical protocol synopsis, we state that patients with anti-AAV9 neutralizing antibody titer above *[cut-off threshold value]* are not eligible for the study. We are working on selecting a CRO that can help us develop, and then qualify and validate the assay, so it can be used in the clinical trial. We plan to have the assay performed in a CLIA Lab for the clinical trial.	PCCA Protocol Synopsis
3.Please do not administer gene therapy to subjects who have intercurrent infections.	We have included the following exclusion criterion: ongoing/active infection (including current COVID-19 infection). Investigator to confirm complete resolution of infection for at least 14 days prior to dosing.	PCCA Protocol Synopsis

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	Sponsor Response	Relevant Document / Section
4.Please develop clear rules with justification for monitoring subjects for TMA and other safety signals related to the subacute complement cascade activation within your study. We recommend that you have a nephrologist, experienced in the management of TMA, to consult with Investigators on all cases. 5.Provide laboratory and clinical parameters with justification that will trigger treatment with eculizumab. Follow the labeled instructions for dosing and monitoring subjects on eculizumab. There is a boxed warning for life-threatening and fatal meningococcal infections after treatment with eculizumab. Please consider vaccinating subjects against meningococcal infections well before gene therapy administration, e.g., 6 weeks.	This has been addressed in the current protocol synopsis. Participants will be closely monitored for signs of thrombocytopenia, microangiopathic hemolytic anemia, and organ damage (e.g., acute kidney injury, GI issues, or CNS manifestations) of thrombotic microangiopathy (TMA), including clinical signs, frequent CBC with peripheral smears, platelet counts/D-dimer and complement levels. An experienced nephrologist and expert in TMA is part of the clinical team and will guide all patient assessments. Participants who develop TMA will receive eculizumab, and if TMA progresses to severe kidney failure, hemodialysis will be implemented.	PCCA Protocol Synopsis
6.It may be helpful if your DSMB includes clinicians with expertise in gene therapy and a physician who has expertise in diagnosis, treatment, and clinical consequences of TMA. Please consider including a physician-expert in TMA as a member of your DSMB.	This will be implemented at the time the DSMB is formed.	N/A

Regulatory

INTERACT Question 7: Based on the presented POC animal studies and supporting rationale, outlined under Section 2.5, Planning for Expedited Pathways, does the Agency agree with our proposal to submit a Fast Track (FT) designation request at time of IND submission?

FDA Response	Sponsor Response	Relevant Document / Section
It is premature to comment on the appropriateness of a Fast Track designation. If you wish to submit a Fast Track Designation Request, please refer to FDA's Guidance on Expedited Programs for Serious Conditions.	N/A	N/A

INTERACT Question 8: We are proposing submission of a Regenerative Medicine Advanced Therapy Designation (RMAT) designation request within the first two months of the Phase 1/2 FIH study utilizing response biomarker data (Section 2.5). Does the Agency agree with the proposed timing of RMAT designation request?

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	Sponsor Response	Relevant Document / Section
It is premature to comment on the appropriateness of an RMAT designation request. Please see our Guidance on Expedited Programs for Regenerative Medicine Therapies for Serious Conditions. Please note that RMAT designation requires preliminary clinical evidence that the product will meet an unmet medical need. Evidence from biomarkers is not sufficient for the purpose of RMAT designation.	N/A	N/A

Nonclinical/Regulatory

INTERACT Question 9: As per our understanding from our meeting with the Agency on July 2, 2019 and FDA Guidance for Industry, "Preclinical Assessment of Investigational Cellular and Gene Therapy Products", November 2013, is the Agency still in agreement that the planned biodistribution studies for AAV9-hPCCA would be sufficient to support preclinical development programs for subsequent gene targets, utilizing our established AAV9 delivery capsid platform (i.e., different gene target and intended population)? We acknowledge that the Agency would like us to submit a separate IND for each of the four rare disease indications discussed above.

FDA Response	Sponsor Response	Relevant Document(s) / Section(s)
We tentatively agree that the resulting BD data for AAV9-hPCCA from a well-designed study can be leveraged to support a BD platform for the same AAV9 delivery capsid with a different gene target. However: 1) the BD data for AAV9-hPCCA will need to be sufficient to support the proposed FIH clinical trial; 2) the same route of administration (IV injection) should be used; and 3) supplemental data for transgene expression in tissues positive for vector presence may be needed to support clinical trials with AAV9 vector products that include different promoters, target tissues, etc.	For the current PCCA program, the GLP tox study is performed in C57/BL6 mice and includes the evaluation of biodistribution for AAV9-hPCCA. In addition, the efficacy study in neonatal Pcca-/- animals will evaluate transgene expression (by RT-qPCR) and PCCA Protein expression (by Western Blot). We will implement the FDA's advice and outcomes from these two studies when planning the biodistribution studies for the next AAV9-based product that is part of the PaVe-GT platform.	GLP 6 M mice protocol.PCCA_31MAY2023, Protocol *[protocol number]* Efficacy Protocol

INTERACT Question 10: Does the Agency agree that the planned preclinical anti-drug antibody (ADA) measured against the AAV9 capsid in the AAV9-hPCCA toxicology study, would be sufficient to support subsequent planned gene therapy development programs utilizing the same AAV9 capsid under a different IND (i.e., different gene target and intended population)?

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FDA Response	Sponsor Response	Relevant Document(s) /Section(s)
We tentatively agree that ADA data for AAV9-hPCCA can be leveraged to support other products using the same AAV9 vector construct. However, please see our comment on your Question #4 regarding evaluation of potential immunogenicity to the expressed transgene product.	N/A	N/A

INTERACT Question 11: Can the Agency provide feedback on whether a drug master file/s (DMF) would be recommended for the PaVe-GT project?

FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
Please note that as the IND sponsor you are ultimately responsible for providing all the CMC information necessary to assess product safety for the planned Phase 1 trial either as part of the original submission or via a cross-referenced Master File (MF) or IND. For our comprehensive recommendations on the CMC content of an IND to initiate clinical trials, please refer to the FDA guidance "Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)", dated January 2020, (https://www.fda.gov/media/113760/download). Please also note the following:	N/A	N/A
1. If you intend to reference CMC information in your IND that was previously submitted to the Agency under another IND or MF, then you should clearly specify (preferably in a tabular format) the information to be referenced, including the nature of the information (e.g., reagents, testing, manufacturing, etc.), file name, reference number, eCTD module, and page number where the information can be found. This information should also be clearly stated in the Letter of Authorization (LOA) provided by the cross-referenced IND sponsor or MF holder.	N/A	N/A
2. A MF can allow you (the sponsor) to incorporate, by reference, information contained in the MF without the MF holder [e.g., the Contract Manufacturing Organization CMO)] having to disclose proprietary information. The FDA will not discuss the details of your IND submission with the MF holder or IND sponsor without written authorization to do so.	N/A	N/A
3. If the CMO elects to use a MF to document CMC information that will be needed for IND review, we recommend communicating with them prior to submission of your IND to the FDA. Particularly, we recommend submitting the MF well in advance of the IND to allow adequate time for the agency to review the CMC information (that will support the IND) within the 30-day review cycle (for the IND).	N/A	N/A

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FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
4. In the MF, product-specific information (e.g., testing and/or manufacturing details, stability) should be submitted under distinct Sections (for each product), e.g., Section 3.2.S – DS for Product A (in this case, AAV9-hPCCA), Section 3.2.S DS for Product B – Product B, Section 3.2.S DSfor Product C and so on.	N/A	N/A
5. Please note that technical contents that describe Drug Substance (DS)/Drug Product (DP) manufacture (including reagents, manufacturing processes, testing, and manufacturing facilities) would be submitted as a type 2 Master File (MF2). For additional guidance on the type of facility-related information that should be submitted in the MF2 submission (that you would otherwise submit in the IND), please refer to Sections V.A.1 and V.A.2, and Section V.5.C.1 (Appendices) in the following guidance document: Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs), 2020. Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) | FDA. Please note that additional information for the manufacturing facilities can also be separately submitted in a type 5 MF (MF5). In which case, this MF5 should also be cross-referenced in your IND submission.	N/A	N/A
6. Please be advised that the product(s) you are proposing to study under an IND are considered biologics regulated by the Center for Biologics Evaluation and Research (CBER); therefore, it is critical that any MF you and your CMO elect to utilize must be submitted to CBER's electronic submission gateway (ESG). Please find additional information here: https://www.fda.gov/vaccines-blood-biologics/development-approval-process-cber/master-files-cber-regulated-products	N/A	N/A
7. Please be advised that MFs should be submitted through CBER's ESG in eCTD format instead of PDF format.	N/A	N/A
8. Please be aware that an applicant cannot cross-reference MFs for the information on DS/DP/DS intermediate manufacture to support a Biologics Licensure Application (BLA). Information on product manufacturing and testing must be provided in the BLA.	N/A	N/A
9.Please note that MFs are submitted solely at the discretion of their holders (e.g., CMOs) and are not required by statute or regulation.	N/A	N/A
10.Detailed information on the contents and format of MFs can be found in the FDA guidance "Drug Master Files", dated November 2020.	N/A	N/A
We agree that Pharmacology/Toxicology data used to support the PaVe-GT project and intended to be used to support multiple IND submissions, can be submitted under a Drug Master File.	N/A	N/A

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ADDITIONAL CBER COMMENTS

(I) GENERAL

FDA Comments	Sponsor Response	Relevant Document(s)/ Section(s)
1.We recommend that you request a pre-IND meeting with CBER/OTAT when ready, to obtain formal nonbinding comments regarding your product development plan from the three CBER/OTAT review disciplines, consisting of product manufacturing (CMC), pharmacology/toxicology (P/T), and clinical. Please be advised that you should consider and address all recommendations provided in these INTERACT comments when you submit a pre-IND meeting package.	N/A	N/A
2.We refer you to OTAT Learn, a series of online presentations provided by the Office of Tissues and Advanced Therapies (OTAT) which address important topics in the development of products regulated by OTAT. You may find some of these presentations useful in your preparation of regulatory submissions and briefing materials for meetings with FDA. OTAT Learn is available at https://www.fda.gov/vaccines-blood-biologics/news-events-biologics/otp-learn	N/A	N/A
3.FDA has published the following guidance documents that may be helpful when drafting and revising your clinical protocols: a.Guidance for Industry: Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products; and b.Rare Diseases: Common Issues in Drug Development.	N/A	N/A
4.Drug development is a collaboration of diverse stakeholders, such as the pharmaceutical industry, academicians, patients, advocacy groups, and regulators. Transparency in the regulatory process can facilitate that collaboration. Therefore, please consider inviting one or more patients, representatives of patient advocacy groups, and/or investigators to your future interactions such as meetings or teleconferences with the FDA. We recognize that, depending on the nature of the topics under discussion and the time available, it may not be feasible for all such stakeholders to actively participate in the discussion. However, listening to the proceedings of such meetings can provide a valuable opportunity for patient stakeholders to contribute to drug development.	N/A	N/A
5.In accordance with provisions of the 21st Century Cures Act, FDA intends to utilize patient experience data and related information in regulatory review and decision-making. Patient experience data provide information about the impact of a medical condition or a therapy on a patient's life, and information about the patient's preferences for treatment. Data may be collected by any persons (including patients, family members and patients' caregivers, patient advocacy organizations, disease research foundations, researchers, and drug manufacturers). We encourage you to incorporate patient experience data as supportive information to guide medical product development. Additional information can be found at: https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM610442.pdf	N/A	N/A

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(II) CHEMISTRY, MANUFACTURING AND CONTROLS

FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
Please consider the following recommendations for the CMC information that should be submitted to the IND:	N/A	N/A
1. Please note that plasmids used in the manufacturing of AAV vectors are considered critical starting materials. In your IND submission, please include the following:	N/A	N/A
a. Certificates of Analysis (COAs) for the plasmids used in the manufacturing process that includes the grade of plasmid. Please note that research grade plasmids should not be used for the manufacture of AAV gene therapy vectors used in clinical studies.	Research grade plasmids will not be used for the manufacture of AAV9-hPCCA intended for use in clinical studies. The plasmids will be High Quality (HQ)-grade, manufactured by Charles River Laboratories (CRL) at their Alderley Park site. Certificates of Analysis for the plasmids that include the grade of plasmid (HQ) will be submitted in the IND.	PCCA pre-IND_CMC_200L
b. Annotated maps for each plasmid indicating the origin, function, and derivation of each genetic component (i.e., promoters, introns, known coding sequences, polyadenylation signals, and untranslated regions).	Annotated plasmid maps for the three separate plasmids used in the manufacture ofAAV9-hPCCA are included in the preIND briefing package (Module 3.2.S.1.2). The pHelper-Kan plasmid provides the adenoviral helper genes, E2A, E4, and VA for AAV packaging. The pRC9-Kan plasmid provides the AAV rep and cap genes for AAV viral vector packaging. pAAV9-hPCCA is the gene of interest (GOI) plasmid. A vector transgene schematic for the GOI, which indicates the source of each genetic component, and descriptions of the genetic components for the pHelper-Kan and pRC9-Kan plasmids are included in the preIND briefing package and will also be included in the IND.	PCCA pre-IND_CMC_200L
c. Summary reports of sequence analysis for each plasmid used in the manufacture of the product, including sequence analysis of all elements in the plasmid that are key to vector production. The summary should include an explanation of any discrepancies with the expected/reference sequence.	Sequencing reports for the three plasmids used in the manufacture of AAV9-hPCCA (pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan) will be included in the IND. Any discrepancies between the expected sequence and the reference sequence will be explained.	N/A

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FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
d. A description of the procedures put in place by the manufacturer of the plasmids to prevent cross-contamination from other plasmid lots made at the facility in prior campaigns (i.e., segregation, tracking, and changeover systems for manufacturing of the plasmid DNA lots).	A description of the segregation, tracking, and changeover procedures put in place by the plasmid manufacturer (Charles River Laboratories – Alderley Park) to prevent cross-contamination will be included in the IND. Briefly, the production suites are fully segregated and dedicated, with positive pressure cascade between suites. All open manipulations are performed in a class II biological safety cabinet. Prior to manufacture, production suites and associated changing rooms undergo line clearance where all documentation and materials are removed, with campaign clean and room release by the Batch Supervisor. A daily clean is performed at the end of each production day once all processing has been completed. Control of staff cross over between products is achieved through scheduling of production activities. Staff must only work on one live organism type and one plasmid per working day. All biological waste is decontaminated within the suite prior to removal. PPE requirements for entering the manufacturing facility include safety glasses, hairnet, laboratory shoes or overshoes, beard snood (where applicable) and fabric laboratory coat. PPE requirements when entering the production suites include hairnet, beard snood (where applicable), change in safety glasses, overshoes over laboratory shoes, disposable laboratory coat and sterile gloves. Disposable laboratory coats are changed daily for each operator. All batches are assigned a unique batch number for full traceability of the product.	N/A
e. A complete list of raw materials and manufacturing equipment used to make the plasmids (please denote which materials are single-use and which are reused).	A complete list of raw materials and major equipment used to manufacture the pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids will be included in the IND. All equipment and materials used during plasmid manufacturing are single use.	N/A
f. A complete list of the animal-derived products used to make the plasmids.	No animal-derived products are used in the manufacturing of the pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids.	N/A
g. A summary report for cleaning validation studies for any equipment or materials that are not single use.	All equipment and materials used to manufacture the pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids are single use.	N/A

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FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
h. Description of the procedures/testing put in place at the GMP product manufacturing facility to qualify the incoming plasmids as critical starting material.	The HQ plasmids will be sent from the CRL- Alderley Park site to the CRL-Rockville site with a Certificate of Analysis. A description of the procedures put in place at the CRL-Rockville site to receive the incoming plasmids will be included in the IND. Briefly, the CRL-Rockville site will receive the HQ plasmids in accordance with their standard procedures to ensure the plasmids are acceptable starting materials for the manufacture of the AAV9-hPCCA product. The procedure includes inspection to verify the integrity of the shipment contents and a review of the plasmid Certificate of Analysis.	N/A
2. Regarding the assay for measurement of vector strength/concentration (commonly referred to as the vector genome titer assay, e.g., qPCR or ddPCR) used for dose calculation, please note the following: a. The assay must be qualified (suitable for the intended purpose) prior to Phase 1 clinical studies. Failure to submit adequate information supporting assay qualification will result in your IND being placed on clinical hold. b. Please collect assay qualification data for the product under the study and include appropriate reference standards of consistent quality and nature, (e.g., linear DNA vs circular plasmids as standards used in the qPCR assay) and product-specific controls/reference material. c. In the IND, please provide a detailed protocol for the qualification study or the SOP used to qualify your assay, including information on the reference standards, controls, and assay optimization. d. Please provide the study report with data documenting assay qualification, including accuracy, precision (inter-assay and intra-assay precision), specificity, range, and linearity. We recommend that the precision of the qualified/validated assay be less than 15% coefficient of variation (CV). e. Please describe any deviations that occurred during the qualification study. f. Please plan to validate the assay prior to the conduct of clinical studies that will assess product efficacy for licensure. g. For additional recommendations on assay qualification/validation, please refer to ICH Q2R1 “Validation of Analytical Procedures: Text and Methodology” (https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf); and FDA guidance “Analytical Procedures and Methods Validation for Drugs and Biologics”, dated July 2015, (https://www.fda.gov/media/87801/download).	Qualification of the qPCR assay used for dose calculation will be completed prior to the manufacture and testing of the AAV9-hPCCA lot intended for clinical use. The qualification protocol and report, which will include all of the information recommended by FDA, will be submitted in the IND.	N/A

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FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
3. To ensure consistent dosing between clinical and preclinical studies, we recommend using the same qualified assay for calculating the vector strength/titer (i.e., vector genome concentration) of preclinical and clinical lots. In instances where this is not possible, you should plan to conduct a bridging study that will assess the vector strength/titer of the product of the preclinical lot(s) and clinical lot (planned for Phase 1 study) in a side-by-side manner, with the assay that will be used for measuring vector strength/titer of the clinical lots. Please plan to demonstrate a tight correlation between the two different assays using multiple product lots (development, engineering, preclinical lots, etc.). This may require retaining sufficient quantities of the product lots.	To ensure consistent dosing between studies, the same qualified qPCR assay will be used to determine vector titer for AAV9-hPCCA lots to be used in the definitive toxicology study and the clinical trial. The same qPCR assay was used to test the 50L material that was used in preclinical studies (NHGRI-PCCA-002) and subsequent efficacy and toxicology studies.	PCCA pre-IND_CMC_200L
4. In your pre-IND briefing package, please provide your proposed lot release testing plans for DS and DP. We recommend that you set specifications for process-related impurities (process residuals such as host cell protein, host cell DNA, plasmid DNA. nuclease, bovine serum albumin, etc.) and product-related impurities (e.g., empty-to-full capsid [E/F] ratio, aggregates etc.) based on your experience from manufacturing of development lots, preclinical lots, and clinical lots of AAV9-hPCCA, as well as data from manufacturing other AAV vectors using the same manufacturing platform, if available.	The proposed lot release testing plans for Drug Substance and Drug Product are included in the pre-IND briefing package. Broad specifications will be established prior to the manufacture of the GMP lot of AAV9-hPCCA intended for clinical use. Specifications will continue to be refined as additional manufacturing experience is gained.	PCCA pre-IND_CMC_200L
5. You should also submit a tabular listing of all the in-process testing planned for your manufacturing platform and the 'go-no go' criteria/limits set for further processing.	In-process testing for the manufacture of AAV9-hPCCA, including any "go-no go" criteria, will be included in the IND.	N/A

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FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
6. Because you are developing products for rare diseases, it is likely that your clinical plan will involve a small trial to support a marketing application, which may require only a few clinical lots. Considering that, we recommend that you develop and implement a quantitative and biologically relevant potency assay for release testing and stability testing of clinical lots. We have the following recommendations on potency assay development: a. Potency assay should be qualified before initiation of clinical trials intended to provide primary evidence of effectiveness to support a marketing application and validated prior to submission of a marketing application (BLA). b. You may consider developing a matrix of assays to measure the product potency, considering the mechanism of action of the product. In this approach you will measure different aspects of the biological activity of the product (e.g., infectivity, transgene expression, enzymatic activity) as part of the potency matrix. c. We recommend collecting potency data early in clinical development. d. In our experience, potency testing can involve large volumes of sample material; therefore, we recommend that you plan for adequate number of retains from each lot (IND-enabling lots and clinical lots). This can be useful for future testing, assay development, and comparability studies, etc. e. For additional guidance and relevant information on potency assay development, please refer to the following FDA/CBER guidance document "Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products", dated January 2011.	A preliminary potency assay based on Western Blot using whole cell lysate has been developed for the AAV9-hCCA product. The Western Blot potency assay was used to test AAV9-hPCCA manufactured at the 10L and 50L scale. This Western Blot potency assay will be qualified and used to test the 200L GLP tox material and the 200L clinical material for both release and stability testing. As product development advances, the Sponsor will consider the Agency's comments and recommendations regarding the potency assay.	PCCA pre-IND_CMC_200L
7. Considering your plan to develop a platform approach, we recommend that you develop sensitive and precise assays as part of heightened product characterization to evaluate product quality attributes such as the empty-to-full capsid (E/F) ratio, vector particle aggregation, post-translation modifications of the capsid, etc., using more than one approach (assay). You should develop these assays at the early stages of the product development to identify those suitable for lot release testing and product characterization. Please note that all assays used in the analytical release testing plan should be qualified (suitable for the intended purpose). The qualification data (for assay performance: accuracy, precision, linearity, sensitivity and specificity, as applicable to each assay) should be submitted in the IND.	The Sponsor acknowledges the Agency's comments regarding product characterization as part of the platform approach and will take these comments into consideration during platform development. All assays used in the analytical release testing plan for AAV9-hPCCA are qualified or verified (for compendial assays). Assay qualification information will be submitted in the IND.	N/A
8. Please optimize the formulation of final DP taking into consideration product stability during manufacturing (in process stability for example during hold steps), under the conditions of storage, shipping and handling in the clinic (including during administration in the device). Please provide the rationale for the final formulation with supporting data from formulation development studies.	Data to support the stability of the final DP, including during administration in the device, will be submitted in the IND. The formulation development report will also be included in the IND.	N/A
9. Please submit (in your IND) a stability study protocol and any available data supporting the stability of the Drug Substance and Drug Product for the duration of storage. Please note the following recommendations:	N/A	N/A

DISCLAIMER: *This information may no longer be applicable due to subsequent improvements.*

FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
a. Please note that stability protocols should include, but are not limited to, an analysis for product potency, evaluation of product quality, and confirmation of sterility. Please note that, in lieu of sterility testing beyond T=0, you may consider container closure integrity testing according to FDA's 2008 guidance "Container and Closure System Integrity Testing in Lieu of Sterility Testing as a Component of the Stability Protocol for Sterile Products" (https://www.fda.gov/media/76338/download).	A draft drug product stability protocol is included in the pre-IND briefing package (Module 3.2.P.8.1). The final drug product stability protocol and all available stability data will be included in the IND. No storage of the drug substance prior to drug product is currently anticipated.	PCCA pre-IND_CMC_200L
b. For description of each product attribute measured in the stability plan, please include the test method, sampling time points (there should be a zero-time point), and acceptance criteria. Please note that under 21 CFR 312.23(a)(7)(ii), you must conduct stability testing in all phases of the IND, to demonstrate that the product is within acceptable chemical and physical limits for the planned duration of the proposed clinical investigation.	Stability testing will be performed for the planned duration of the proposed clinical investigation. A draft drug product stability protocol is included in the pre-IND briefing package (Module 3.2.P.8.1). The final drug product stability protocol and available stability data will be included in the IND.	PCCA pre-IND_CMC_200L
c. Please consider placing preclinical/engineering lots and all clinical lots on the stability plan.	The lots of AAV9-hPCCA intended for use in the definitive toxicology study and the clinical trial will be placed on stability. Available stability data on each lot will be included in the IND.	N/A
d. In addition to the real-time stability under normal storage conditions, please also assess the stability of the DP under accelerated storage and forced degradation conditions to allow for the identification of stability-indicating assays early in the clinical development.	A preliminary accelerated stability study to begin identification of stability-indicating assays is planned; refer to the study plan included in Module 3.2.P.8.1.	PCCA pre-IND_CMC_200L
e. For recommendations on stability testing, please refer to ICH Q5C: "Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products," (https://www.fda.gov/media/71441/download), ICH Guideline Q1A(R2): "Stability Testing of New Drugs and Products" (https://www.fda.gov/media/71707/download), and ICH Guideline Q1E: "Evaluation of Stability Data" (https://www.fda.gov/media/71722/download).	N/A	N/A
10. Please establish a product specific reference standard material for assays that measure critical quality attributes such as vector genome titer, infectious unit titer, E/F capsid ratio, etc. Please note:	N/A	N/A
a. In the IND/MF, please provide a detailed description of how the reference standard material was derived, manufactured and qualified.	A product-specific reference standard will be established. Details on how the reference standard was derived, manufactured, tested will be included in IND.	N/A
b. Please test the reference material (lot) according to the product release testing plan and product characterization plan. Please submit the COA for the reference lot.	The COA for the reference standard, which includes the results of all release and characterization testing, will be included in the IND.	N/A
c. Please monitor the stability of this reference lot.	Available stability data for the reference standard will be included in the IND.	N/A
d. Please provide your justification for why this reference lot is relevant and acceptable.	Rationale for the acceptability of the reference lot will be included in the IND.	N/A

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FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
11. Please collect device compatibility data for AAV9-hPCCA showing the stability of the product in the intended clinical formulation and clinical delivery device. Please ensure that the product lot used in the compatibility study is manufactured and formulated in a manner comparable to that for the clinical lots. The supporting manufacturing, qualification and testing information for the product lot used in the compatibility study should be submitted in the IND. We have the following additional comments regarding the device compatibility study: a. Please assess the amount (vector genomes) and activity (infectious units or potency) of the product following exposure to the clinical delivery device. b. Please be aware that the study should include tests conducted over the planned dose-range and should take into account the expected time between thaw of the product and infusion. Please perform device compatibility testing for the product under conditions that mimic the clinical scenario (i.e., hold time, formulation/concentration, temperature, presence of contrast agent, etc.); the study design should consider the worst-case scenario (e.g., low product concentration, maximum hold time, time and temperature excursions). c. The data should support the post-thaw product handling instructions provided in the "Instruction to Pharmacy" or "Pharmacy Manual" document that is supplied with the product to all the clinical sites. d. Failure to submit sufficient information supporting product (vector) stability in the delivery device will result in your IND being placed on clinical hold.	Compatibility and stability of the product intended for clinical delivery are going to be evaluated and the plan is included in the pre-IND briefing package for the FDA's review.	Protocol [protocol number]
12. If you intend to use an assay/test to make treatment decisions (e.g., for the selection of subjects using a screening assay), such an assay may be regulated as a companion diagnostic, in which case, we encourage you to request a Pre- Submission meeting with Office of In Vitro Diagnostics and Radiological Health within CDRH's Office of Product Evaluation and Quality (OPEQ) to get advice on the development plan for this assay. Please note that participation of the OTAT review team (for the IND) can be requested at that meeting and would be helpful in ensuring contemporaneous development of both the therapeutic and the diagnostic device/assay. Furthermore, we encourage you to submit the same information (as that submitted with the Pre-Submission package to CDRH) as an amendment to the IND to document assay development. For additional guidance, please refer to the following guidance documents: a. The 2014 FDA guidance document on "In Vitro Companion Diagnostic Devices" (https://www.fda.gov/media/81309/download) b. The 2021 FDA guidance document on "Requests for Feedback and Meetings for Medical Device Submissions: The Q-Submission Program" (https://www.fda.gov/media/114034/download)	N/A	N/A

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(III) PHARMACOLOGY AND TOXOCOLOGY

FDA Comments	Sponsor Response	Relevant Document(s)/ Section(s)
13. For a comprehensive summary regarding the preclinical assessment of cell and gene therapy products, we refer you to the document titled, Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products (November 2013), available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/preclinical-assessment-investigational-cellular-and-gene-therapy-products.	N/A	N/A
14. The preclinical program for any investigational product should be individualized with respect to scope, complexity, and overall design to maximize the contribution and predictive value of the resulting data for clinical safety and therapeutic activity. As recommended in Section III.B.8 of the Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products we encourage you to explore opportunities for reducing, refining, and replacing animal use in your preclinical program. For example, it may be appropriate to use in vitro or in silico testing to complement or replace animal studies. We encourage you to submit proposals and justify any potential alternative approaches.	Please refer to Question #7 in the pre-IND briefing package, regarding the Sponsor's position on animals utilized for pivotal biodistribution data.	Briefing Package Section 14.2

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(III) CLINICAL

FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
15. You propose to include neurocognitive assessments at baseline and *[XX]* weeks. These assessments are performed with Clinical Outcomes Assessments (COA) tools, e.g., Bayley, Vineland. For all COA tools that are intended to support regulatory decision-making and labeling claims, we recommend that you provide the following for FDA review and comment prior to initiating your study: a.Conceptual framework of the instrument; b.Evidence of content validity obtained for the tool, e.g., qualitative research with caregivers, obtained for this specific context of use; c.Exact copy of the instrument as it will be administered during the clinical trial and any training materials and user manuals; d.Proposed scoring algorithm(s) with rationale for any weighting of items or response options in the domain scoring and corresponding information on how the instrument's scores will be analyzed as part of an endpoint; e.Plans for, and results from, evaluation of the psychometric properties and performance of the instrument (i.e., reliability, validity, and ability to detect change) after content validity has been established; f.Pre-specified plans for handling missing data; and g.A priori thresholds (or range of thresholds) representing clinically meaningful within-patient improvement in instrument's scores.	*[Secondary and exploratory endpoints]*	PCCA Protocol Synopsis
16. You state, page 11, that "[s]tandard of care includes life-long dietary protein restriction, supplementation with L-carnitine and supportive treatment." We are concerned that "dietary changes can affect efficacy results and pose significant interpretability challenges, particularly when the clinical trial design did not anticipate or appropriately account for the confounding effect of diet." In drafting your protocol with regard to diet, please see FDA's guidance on optimizing, standardizing, and maintaining diet stability in clinical trials.	Patients with PA need adjustments very frequently based on clinical status. The clinical team will attempt to keep baseline management, treatments, and diet consistent, and only change if clinically indicated for the first year of the study to best compare biomarker responses. Subsequent to that, adjustment/increase to the protein intake may occur based on growth and plasma amino acids, per the standard of care by the expert metabolic team and dietitian.	N/A

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FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
17. Your study has Data and Safety Monitoring Board to review safety data and make recommendations on pausing the study or modifying the protocol. Please provide the DSMB's Charter for our review when you submit your IND. At a minimum, the Charter should include the following information: a. Qualifications of DSMB members b. Roles and responsibilities of members c. Detailed explanation for decision-making d. Meeting schedule, i.e., time interval from detection of serious adverse event to DSMB notification, e.g., 24 hours, and adjudication, e.g., 72 hours e. DSMB discussions should take place at a live meeting (telephone, videoconference) to ensure views of all members can be heard collectively. Prior to the meeting, however, members should independently determine their recommendation(s), and disclose them to other members, to diminish the possibility that a member could dominate the discussion and overly influence the decision f. Voting rules, e.g., whether unanimity or two-thirds majority is required for decision-making g. Written rules on procedures to ensure minimization of bias, such as maintaining confidentiality of the interim data h. Documentation of decisions, e.g., where summary of adjudication discussions and recommendations are memorialized, documentation of all communications with sponsor i. Training of DSMB members j. Kick-off meeting to ensure that all members understand the protocol and decision-making rules prior to first subject enrollment; k. Ongoing assessment of DSMB members for conflicts of interest, including concurrent service on other DSMBs of related or competing products; and l. Replacement of members upon resignation or conflict of interest.	Requested information will be provided in the IND.	N/A
18. In addition to the protocol and Informed consent Document, we recommend that you include the following documents in your IND submission.	N/A	N/A
a. LTFU protocol	Patients will be followed for 5 years as part of this Phase 1/2 clinical protocol. They will not be transferred to a LTFU protocol.	PCCA Protocol Synopsis
b. LTFU Informed Consent Document;	N/A	N/A
c. Investigator Brochure as required by 21 CFR 312.55(a);	The proposed clinical trial will be performed at a single site at the NIH, and as such the Sponsor intends to add pertinent risk information to the clinical protocol. An IB will be generated if a second site is added to the study.	N/A
d. Committee Charters, e.g., DSMB Charter;	Information will be provided in the IND.	N/A

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FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
e. Pharmacy Manual inclusive of dose calculation worksheets;	Information will be provided in the IND.	N/A
f. Administration Manual, if applicable;	Information will be provided in the IND.	N/A
g. Any documents with an internal reference in the protocol, e.g., study manual, study SOPs, etc.	Information will be provided in the IND.	N/A

Sponsor's Post-INTERACT Follow-up CMC Question

Follow-up INTERACT Question 1: Does the Agency agree that GMP-Ready™ grade plasmids are acceptable for the manufacture of the AAV9-hPCCA investigational product proposed for use in a Phase 1/2 clinical study?

FDA Response	Sponsor Response	Relevant Document(s)/ Section(s)
The GMP-ready grade plasmid may be acceptable for manufacture of AAV9-hPCCA investigational product proposed for use in a Phase 1/2 clinical study. However, please address the following additional comments in your IND:	N/A	N/A
a. You have not described in detail the procedures in place in the plasmid manufacturing facility to prevent cross-contamination from other plasmids produced in the same production suite. Please describe the segregation and tracking systems for manufacturing of the GMP-ready grade plasmid DNA.	Segregation and tracking systems for manufacturing the pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids will be included in the IND and are briefly described in Sponsor Response to Additional Comment #1d. Please note that since the submission of the question, manufacturing of the plasmids has been changed to the Alderley Park site within Charles River Laboratories. Accordingly, “GMP-Ready grade plasmids” are now referred to as “HQ Plasmids”.	PCCA pre-IND_CMC_200L
b. Please clarify whether all plasmid manufacturing equipment used for production of the GMP-ready grade plasmid are single-use. If shared equipment is used, please provide a summary report for cleaning validation studies.	All equipment and materials are used to manufacture the pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids are single use.	N/A
c. We recommend that you include a sensitive test for potential contamination from other plasmids that are manufactured in the same suite as part of the qualification for the GMP-ready grade plasmid.	Charles River Laboratories Alderley Park, the manufacturer of the pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids, has a robust system in place to prevent cross contamination, as described in Sponsor Response to Additional Comment #1d. In addition, all equipment and materials are used to manufacture the pAAV9-hPCCA, pRC9-Kan, and pHelper-Kan plasmids are single use to ensure robust cross-contamination control.	N/A
d. The acceptability of the GMP-ready grade plasmid for manufacturing of clinical product will depend on the review of the information submitted in the IND.	Acknowledged.	N/A

Literature Included Within Briefing Package Materials

The table below lists the citations for all literature included within this pre-IND briefing package, and denotes the utility of each for Agency reviewers, by discipline.

Background

Literature Citation	Specific Supportive Information Provided
Allen, R.H., et al., Elevation of 2-methylcitric acid I and II levels in serum, urine, and cerebrospinal fluid of patients with cobalamin deficiency. Metabolism, 1993. **42**(8): p. 978-88.	Information regarding the original method for the 2-methylcitrate biomarker testing.
Forny P, Hörster F, Ballhausen D, et al. Guidelines for the diagnosis and management of methylmalonic acidaemia and propionic acidaemia: Firstrevision. *J Inherit Metab Dis.* 2021;44:566–592. https://doi.org/10.1002/jimd.12370	General background regarding the diagnosis of and treatment for patients with MMA and PA, per a panel of healthcare experts.
Manoli, I., A. R. Pass, E. A. Harrington, J. L. Sloan, J. Gagne, S. McCoy, S. L. Bell, J. D. Hattenbach, B. P. Leitner, C. J. Duckworth, L. A. Fletcher, T. M. Cassimatis, C. I. Galarreta, A. Thurm, J. Snow, C. Van Ryzin, S. Ferry, N. A. Mew, O. A. Shchelochkov, K. Y. Chen and C. P. Venditti (2021). "1-(13)C-propionate breath testing as a surrogate endpoint to assess efficacy of liver-directed therapies in methylmalonic acidemia (MMA)." Genet Med **23**(8): 1522-1533.	Information regarding the method for the 1-(13)C-propionate breath test using the Exalenz Bioscience BreathID device.
Shchelochkov OA, Carrillo N, Venditti C. Propionic Acidemia. 2012 May 17 [Updated 2016 Oct 6]. In: Adam MP, Mirzaa GM, Pagon RA, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2023.	General background of PA including diagnosis, clinical care, and genetics.
Vockley, J., B. Burton, A. Jurecka, J. Ganju, B. Leiro, R. Zori and N. Longo (2023). "Challenges and strategies for clinical trials in propionic and methylmalonic acidemias." Mol Genet Metab **139**(3): 107612.	Current discussion of clinical trial development in PA and other organic acidemias.

Nonclinical

Literature Citation	Specific Supportive Information Provided
Chandler, R.J., et al., Adeno-associated virus serotype 8 gene transfer rescues a neonatal lethal murine model of propionic acidemia. Hum Gene Ther, 2011. **22**(4): p. 477-81.	Description of the *Pcca*$^{-/-}$ mouse pup as a model for PA.

Clinical

Literature Citation	Specific Supportive Information Provided
Longo, N., et al., Biomarkers for drug development in propionic and methylmalonic acidemias. J Inherit Metab Dis, 2022. **45**(2): p. 132-143.	Discussion of relevant biomarkers for patients with PA.
Shchelochkov, O. A., I. Manoli, P. Juneau, J. L. Sloan, S. Ferry, J. Myles, M. Schoenfeld, A. Pass, S. McCoy, C. Van Ryzin, O. Wenger, M. Levin, W. Zein, L. Huryn, J. Snow, C. Chlebowski, A. Thurm, J. B. Kopp, K. Y. Chen and C. P. Venditti (2021). "Severity modeling of propionic acidemia using clinical and laboratory biomarkers." Genet Med **23**(8): 1534-1542.	Background information on data from the Propionic Acidemia Natural History study at NIH, with information regarding clinical biomarkers.

Our Reference: *[FDA-assigned Pre-IND application and reference numbers]*

MEETING SUMMARY
Date: August 9, 2023

National Center for Advancing Translational Sciences (NCATS)
Attention: *[Name, designation and address of sponsor's representative]*

Dear *[Name of sponsor's representative]*:

Attached is a copy of the memorandum summarizing your July 10, 2023, teleconference with CBER. This memorandum constitutes the official record of the meeting teleconference. If your understanding of the meeting teleconference outcomes differ from those expressed in this summary, it is your responsibility to communicate with CBER as soon as possible.

Please include a reference to *[FDA-assigned Pre-IND application and reference numbers]* in your future submissions related to the subject product.

If you have any questions, please contact *[name and contact information of regulatory program manager (RPM)]*.

Sincerely,

[name, designation, and affiliation of the Branch Chief of the Division of Regulatory Review at CBER]

***DISCLAIMER**: This information is specific to AAV9-hPCCA and does not apply directly to other investigational products.*

Meeting Summary
Includes Preliminary Meeting Responses

Meeting ID #:	*[Meeting ID number]*
Submission type & #:	*[Pre-IND reference number]*
Product name:	Adeno-Associated Virus 9 vector expressing a functional human codon optimized cDNA encoding the Propionyl-CoA Carboxylase, alpha subunit (PCCA), under control of the *[specific]*promoter/ **Product Name:** AAV9-hPCCA
Indication:	Treatment of PCCA-related propionic acidemia (PA).
Sponsor:	National Center for Advancing Translational Sciences
Meeting type:	Type B
Meeting category:	Pre-IND
Meeting date & time:	July 10, 2023, 1:00PM – 2:00PM
Meeting format:	Teleconference
RPM:	*[name of RPM]*
Preliminary Meeting Responses:	July 7, 2023
FDA Attendees:	*[Names and designations of FDA attendees]*
Sponsor Attendees:	*[Names and designations of sponsor attendees]*

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Background and Objectives:
National Center for Advancing Translational Sciences (NCATS) submitted a meeting request on May 10, 2023, to discuss CMC, non-clinical, and clinical aspects of NCATS' development program for AA V9-hPCCA for treatment of PCCA-related PA. The pre-meeting materials were submitted on June 9, 2023.

FDA provided its preliminary meeting responses to National Center for Advancing Translational Sciences' questions on July 7, 2023. After reviewing the preliminary meeting responses, National Center for Advancing Translational Sciences notified FDA on July 9, 2023 of its decision to limit the meeting to discuss only question 4 (responses 2 and 3), question 4 (response 5a), question 7 (response 2a), question 8, and question 1 (responses 2 and 4).

Sponsor Question 1:
Does the Agency agree with the release specifications for the drug substance and drug product?

FDA Preliminary Meeting Response to Sponsor Question 1:
Regarding your proposed Drug Substance (DS) and Drug Product (DP) lot release specifications for AAV9-hPCCA, based on the information provided in your briefing document we have the following comments:

1. Because AAV9-hPCCA is developed to treat a rare disease, you may need to leverage the proposed Phase 1/2 clinical study for efficacy data to support a license application. In this case the CMC information, including the potency assay(s) and assay qualification results, submitted to support the clinical study should be appropriate for late-stage clinical development.

2. We note that you include a Western Blot assay for hPCCA expression as the potency assay and have set an acceptance criterion (AC) of "PCCA Expression in HepG2-Knockout Cells". It appears that this assay is a qualitative assessment of transgene expression. This approach may be acceptable for an early phase clinical trial designed to evaluate product safety and tolerability; however, if you intend to leverage the proposed Phase 1/2 clinical study to provide the primary evidence of effectiveness to support a marketing application, you should develop a quantitative, accurate, and precise potency assay early in clinical development. If available, you should submit the assay qualification protocol and report in your IND submission.

3. We recommend that you develop a quantitative measure of the biological function(s) of your product related to the mechanism of action and use it for lot release and stability testing.

4. We note that you include a Next Generation Sequencing (NGS) assay as a transgene identity test for DS lot release testing. An NGS assay that can

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ensure the DS sequence is correct, used in conjunction with a quantitative measure of transgene expression (mRNA or protein), may be adequate to ensure product potency. Please provide a detailed NGS protocol and a description of how you plan to assess the sensitivity, accuracy, and precision of the NGS assay to detect mutant vector sequences in the DS.

5. Please set appropriate acceptance criteria for impurities and residuals based on your experience from manufacture of preclinical, engineering, and clinical lots. The initial lot release acceptance criteria may be set relatively wide based on your limited process development and manufacture experience to begin with and can be tightened as you gain additional platform manufacturing experience.

6. We note that the amount of residual Host Cell DNA (HCD) in the 50L scale preclinical lot (TL-21-001-41) is 2.5x106 pg/mL, which is significantly higher than the World Health Organization (WHO) recommended limit of 10 ng per dose. Because the 50L scale and 200L scale lots are manufactured using a similar process, it is expected that the residual HCD in the 200L scale GMP clinical product will also be significantly higher than the WHO recommended limit. In your IND submission, please justify your proposed limit(s) with manufacturing data that applies to your process or to the manufacturing platform. Accordingly, you should provide a comprehensive risk assessment that takes into consideration the highest dose that will be administered, levels and size of residual DNA in the product lots, the patient population, target tissue, and route of administration. You should also discuss process optimization plans to reduce the level of residual host cell DNA in the commercial product.

7. In your IND submission, please provide a detailed description for each lot release assay (including assay procedures, controls, sensitivity, etc.,), the assay qualification status, and an assay qualification report, if available.

Meeting Discussion for Sponsor Question 1:
Responses 2 and 4

The Sponsor confirmed that data from the proposed Phase 1/2 clinical study may need to be leveraged to support a license application. Two possible approaches for assurance of product potency were discussed. The first approach being the development of a quantitative potency that reflect the product's mechanism of action (e.g., an enzymatic potency assay). FDA clarified that if an enzymatic assay is sufficiently quantitative and precise then additional potency assays such as transgene expression assay would not be required.

The second approach to ensuring product potency that was discussed was development of a quantitative and precise transgene protein expression assay used in conjunction with an NGS assay capable of quantitating vector genomes

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with mutations in the transgene sequence. FDA emphasized the importance of the transgene expression assay being sufficiently precise and ICH Q2(R2) was given as a resource for designing an assay qualification study. FDA emphasized that for this second potency approach the NGS assay is not simply being used as an identity test, but must be sufficiently sensitive to detect mutations in transgene sequence. An example was given where sensitivity is tested by spiking known quantities of mutant sequence into the Drug Substance to demonstrate NGS assay sensitivity.

Sponsor Question 2:
Does the Agency agree with the proposed storage, preparation, and stability testing plan for drug product and infusion formulation/procedure?

FDA Preliminary Meeting Response to Sponsor Question 2:
Based on the information provided in your briefing document, your proposed storage, preparation, and stability testing plan for the drug product and infusion procedure is reasonable. However, we have following recommendations:

1. Regarding your DP stability plan:

    a. You include the Western Blot assay for PCCA expression with the AC of "PCCA Expression in HepG2 Knockout cells" in the stability testing plan. We recommend that you develop a quantitative potency assay and include it in the stability plan as soon as possible.

    b. You plan to conduct sterility testing on the AAV9-hPCCA DP annually. Please be advised that you may consider performing a container closure integrity test in lieu of sterility between t=0 and the last timepoint of your stability testing plan. Please refer to FDA guidance "Container and Closure System Integrity Testing in Lieu of Sterility Testing as a Component of the Stability Protocol for Sterile Products", dated February 2008, for additional recommendations.

2. Regarding your proposed DP storage, preparation, and infusion formulation procedures:

    a. Please provide a description of your established shipping procedures and how you will ensure product stability during shipping of the DP from the storage site to the clinical site.

***DISCLAIMER**: This information is specific to AAV9-hPCCA and does not apply directly to other investigational products.*

b. Please provide a detailed description of the procedures in place (i.e., in the Pharmacy Manual) to ensure chain of custody, product quality, and product stability.

c. Please be advised that the product storage, shipping, handling, preparation, and administration procedures should be supported by your stability study and device compatibility study results.

Meeting Discussion for Sponsor Question 2:
There was no discussion of this question during the meeting.

Sponsor Question 3:
Does the Agency agree that the Sponsor's proposed plan for assessing compatibility of the AAV9-hPCCA drug product with the preclinical and clinical administration devices is acceptable?

FDA Preliminary Meeting Response to Sponsor Question 3:
Your overall device compatibility study plan appears reasonable. However, we have the following additional recommendations:

1. Please provide the Pharmacy Manual in your IND submission.

2. In your design of the study to assess device compatibility (*[protocol number],* Figure 2), you have not specified the hold time of the diluted DP in the syringe prior to holding it for 0, 30, 60, 90, and 120 min in the tested administration device. Please indicate the hold condition (temperature and duration) for the diluted DP in the syringe prior to being held in the administration device (i.e., tubing connected with PIV line or PICC line). Please be advised that the hold condition of the diluted DP in the syringe should represent a worst-case condition as described in the Pharmacy Manual.

Meeting Discussion for Sponsor Question 3:
There was no discussion of this question during the meeting.

Sponsor Question 4:
Depending on whether the 4E13 vg/kg/dose (mid-dose in the GLP toxicity study) or the highest tested dose, 3E14 vg/kg/dose, is found to be the No Observed Adverse Effect Level, does the FDA agree that these doses support the proposed starting clinical dose

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[starting dose] and the proposed maximum clinical dose *[higher dose]*, respectively?

FDA Preliminary Meeting Response to Sponsor Question 4:
Based on the information provided in Section 15.2 (pages 26-41), we cannot yet agree with your proposed dose levels in your planned first-in-human (FIH) clinical trial. The results of your proposed definitive proof-of-concept (POC) and biodistribution/toxicology studies are pending; thus, we do not have adequate data to evaluate your proposed dose levels.

We have the following comments regarding your preclinical development program to support your proposed clinical trial. Please address each comment in your IND submission:

1. You plan to enroll pediatric subjects (3 years of age and older) in your proposed clinical trial. Please note that this trial represents more than a minor increase over minimal risk. Therefore, per 21 CFR 50.52, prior to initiating a study in children, in accordance with Subpart D, you must provide evidence that administration of AAV9-h*PCCA* via the intended intravenous route of administration provides prospect of direct benefit (PDB). Thus, your IND submission should contain sufficient data generated from nonclinical POC studies in animal model of PA to support administration in your proposed pediatric clinical population. We also refer you to FDA Response to Question #7 regarding PDB.

2. Your *Pcca*-/- mouse, generated with a CRISPR-induced Pcca$^{p.Q133LfsX41}$ mutation in exon 5 of *Pcca* gene, represents a severe model of propionic acidemia (PA). Although your completed POC studies described in Sections 15.2.2.1.2.4 and 15.2.2.1.2.5 (page 37), and in Report Nos. NHGRI-PCC-001 and NHGRI-PCC-002, showed AAV9-hPCCA related improvement of animal survival, the other study endpoints (e.g., body weights, clinical pathology, metabolites and bioanalysis, 1-^{13}C-propionate oxidation, organ weights, gross and histopathology) are challenging to interpret due to the early deaths prior to Day 20 of concurrent control *Pcca*-/- mice. Therefore, we encourage you to explore the use of a less severe mouse model of PA disease that may be more informative for assessing these other parameters and allow for timing of product administration that is more representative of the clinical scenario.

Meeting Discussion for Sponsor Question 4
Responses 2 and 3
FDA tentatively agreed with the Sponsor's position of conducting the proposed pivotal POC study in the originally selected *Pcca-/-* mouse model based on its ability to recapitulate features of severe pediatric PA and use of concurrent untreated wild type (WT) controls to aid data interpretation. FDA noted that: i) the sponsor should discuss the dose response relationship of AAV9-hPCCA and the levels of improvement in endpoints observed in AAV9-hPCCA treated *PCCA-/-* mice (compared to the untreated

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WT mice) in the report of the completed pivotal POC study, and ii) justification to support the selection of the *Pcca-/-* mouse model should be provided in the IND submission.

3. In Module 2 of your IND submission, please provide a comprehensive discussion, along with supporting data, of the biological relevance of each selected mouse model of PA to the target clinical population, including: i) life span; ii) disease progression (biochemical, pathophysiological, morphological, and functional); and iii) disease state at time of AAV9-h*PCCA* administration in the model relative to the age of the planned clinical population.

4. Regarding your proposed definitive POC study described in Sections 15.2.1.2.6 and 15.2.3.3 (page 37 and 39, respectively) and outlined in *[protocol number]* in the neonatal *Pcca*-/- mouse model:

    a. Please refer to our comment #2 regarding the use of a less severe model of PA.

    b. Please perform analysis to confirm the genotype of each *Pcca*-/- mouse.

    c. In addition to the proposed in-life assessments, please include daily cageside observations for morbidity and clinical signs.

    d. Please assess the plasma levels of 2-methylcitrate (2-MC), propionylcarnitine/acetylcarnitine (C3/C2) ratio, 3-hydroypropionate (3-HP), and plasma levels of ammonia in your metabolite bioanalysis.

    e. Please note that if you cannot collect an adequate volume of blood from each animal to obtain data for all protocol-specified parameters (hematology, serum chemistry, metabolites bioanalysis), the number of animals should be increased accordingly.

    f. In addition to heart and liver, please include brain and kidney for assessment of enzyme activity and protein expression of the h*PCCA* transgene.

    g. Please assess 2-MC levels in the liver, kidney, heart, and brain.

    h. Please perform histopathology of heart, liver, kidney, and brain in all animals in Groups 1-5.

    i. For any unscheduled deaths, please perform comprehensive clinical pathology, gross and histopathology on a complete list of tissues, and other analyses as appropriate to determine the potential cause of death.

5. Regarding the proposed GLP BD/toxicology study described in Sections

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15.2.3.4, 15.2.4.1, and 15.2.6.5 (pages 39-41) and outlined in the attached protocol of 'GLP 6-month toxicity study' in C57BL/6 mice, please provide:

a. Your rationale for conducting this study in adult mice instead of neonatal mice.

Meeting Discussion for Sponsor Question 4
Response 5(a):
FDA tentatively agreed with the sponsor's rationale for conducting the proposed GLP toxicology study in adult WT mice due to technical challenges of using neonatal mice (e.g., injection procedure, limited blood volumes for toxicity endpoints, etc.), given that both WT adult and neonatal mice are being used for safety evaluation across the proposed pivotal efficacy and toxicity studies to collect robust toxicology data. FDA noted that the sponsor should provide their justification to support their position in the IND submission.

a. Your rationale for the proposed dose levels at 1 x 10^{13}, 4 x 10^{13}, or 3 x 10^{14} vg/kg. The selected dose levels should include potentially biologically active dose levels based on your POC studies and bracket the proposed clinical dose levels.

b. Your rationale for the proposed scheduled sacrifice time points at 7-, 30-, 90-, and 180-days post-administration. In general, we recommend that the selected sacrifice time points coincide with onset, peak, and plateau of h*PCCA* transgene expression.

c. The scoring system of the proposed functional observational battery (FOB) assessment and ensure that qualified personnel perform the assessment and data analysis are masked to control and experimental study groups.

d. Regarding the proposed biodistribution (BD) assessment:

   i. Please collect a complete list of tissues from all animals; however, analysis can be initially limited to the control and the high-dose groups. In addition to the proposed tissues (injection site, heart, brain, liver, spleen, kidney, ovary/testis), please include other major PA-affected tissues (e.g., eyes, pancreas, skeletal muscle, etc.)

   ii. Please archive all tissues, whether evaluated or not, for potential analyses if safety issues arise during the clinical trial.

   iii. For tissues that are positive for vector DNA, please determine h*PCCA* transgene expression.

   iv. The qPCR assay used to evaluate BD should be qualified, to include the

establishment of a standard curve and determination of detection sensitivity, precision, and efficiency (i.e., by spiking a known amount of virus DNA into the blood and tissue samples to be assayed). Please refer to the guidance document, *Long Term Follow-Up After Administration of Human Gene Therapy Products: Guidance for Industry* (January 2020) for further discussion regarding tissue collection and the qPCR assay methodology, including the current CBER/OTP standard for PCR sensitivity (:550 copies/µg genomic DNA), available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/long-term-follow-after-administration-human-gene-therapy-products.

e. In addition to the tissues listed on page 8 of the protocol for histopathology assessment, please include any gross lesions and the dorsal root ganglia.

f. For any unscheduled deaths, please perform comprehensive clinical pathology, gross and histopathology on a complete list of tissues and other analyses as appropriate to determine the potential cause of death.

6. We have the following general comments regarding all in vivo nonclinical studies:

a. Please provide a tabulated summary of the similarities and differences between the various nonclinical lots and the clinical lot(s) of AAV9-h*PCCA* in Module 2 of your IND submission. Parameters to be compared should include but should not be limited to: i) the manufacturing process; ii) vector genome copy titer; iii) infectious titer; iv) full to empty vector ratio; and v) final product formulation.

b. Please use the same assay for vector titer determination for the nonclinical lots of AAV9-h*PCCA* administered in the nonclinical studies and the clinical lot(s) to be administered in your proposed clinical trial. The dose levels of AAV9-h*PCCA* should be calculated based on this analysis. We also recommend that you retain adequate materials from each nonclinical lot so that it can be retested if the assay for the future clinical lot(s) is modified in some manner.

c. Please provide data from bench testing that confirm the compatibility of the nonclinical lots of AAV9-h*PCCA* with the nonclinical delivery system (needle/syringe) used in each animal study. Your evaluation should verify the ability to consistently deliver prespecified does levels of the product. If vector loss is observed, please provide the actual dose level(s) administered in the study reports and data tables.

d. Please ensure that all attempts are made to avoid potential bias in each in vivo study. This includes i) randomized assignment of animals to study

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groups, ii) appropriate staggered dosing of animals across all groups, iii) masked assessment of study parameters and data analysis by qualified personnel.

e. Please ensure that the technical personnel tasked with dosing the mice are appropriately trained. All instances of suspected mis-dosing should be documented in the raw data and in the final study report.

f. Please provide a comprehensive discussion of the risk of complement activation and subsequent potential thrombotic microangiopathy following administration of AAV9-h*PCCA* in patients with PA, who may have compromised liver and cardiac function. Please include in this discussion how your studies in a murine model(s) of PA and healthy mice inform this risk.

g. Please use the clinically proposed dosing unit (vg/kg), in your nonclinical mouse studies and provide the dose levels based on this dosing unit.

Sponsor Question 5:
Does the Agency agree that the combination of the data collected from the 6-month GLP toxicology study using slow bolus tail vein injection in adult mice and the toxicity data from the non-GLP efficacy study using facial vein injection in mouse pups, along with the proposed clinical age de-escalation, support the minimum age of three years for clinical study participants?

FDA Preliminary Meeting Response to Sponsor Question 5:
Pharmacology/Toxicology Response:
Please see our Comments above to your Question #4 regarding the proposed POC study in neonatal mice and the GLP toxicology study in adult mice.

Clinical Response:
Please refer to our Comment 2 of Question 7 regarding the proposed ages for inclusion into your first-in-human study.

Meeting Discussion for Sponsor Question 5:
There was no discussion of this question during the meeting.

Sponsor Question 6:
Does the Agency concur that, pending acceptable results in the planned preclinical studies, the proposed preclinical development program is IND-enabling for a clinical study in patients with PCCA-related PA, starting with adolescent or pediatric patients and then opening enrolment to eligible patients older than 3 years?

FDA Preliminary Meeting Response to Sponsor Question 6:
Pharmacology/Toxicology Response:
Please see our Comments to your Question #4 regarding your nonclinical

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development program.

Clinical Response:
Please refer to our Comment 2 of Question 7 regarding the initiation of your first-in-human study in pediatric subjects as you have proposed.

Meeting Discussion for Sponsor Question 6:
There was no discussion of this question during the meeting.

Sponsor Question 7:
Does the Agency agree with the proposed first-in-human study design, including participant inclusion/exclusion criteria, dosing rationale, study population rationale, staggering of IP administration, stopping rules, safety oversight, and safety and efficacy endpoints?

FDA Preliminary Meeting Response to Sponsor Question 7:
Based on the available information you provided in the meeting package, we cannot yet agree with your proposed first-in-human (FIH) study. You have proposed an open-label, externally controlled study to assess the safety and preliminary efficacy of 2 doses of your gene therapy in children with PCCA-related PA. We have the following comments about your study design, eligibility criteria, dosing rationale, study endpoints, staggering, stopping rules, and safety monitoring plans:

1. Study Design: You have proposed an open-label, externally controlled study with a low-dose cohort of 3-6 subjects and a high dose cohort with 1-3 subjects.

    a. We agree with conducting dose-exploration in your proposed FIH study, but as discussed in 4, Dosing Rationale, below, there is insufficient pre-clinical data to support the specific proposed doses at this time.

    b. While your proposed study design could be acceptable for an early-phase study, we are concerned that you are not maximizing the knowledge that could be gained from this study. Given the rarity of the condition, we highly recommend that you maximize the data interpretability from each subject enrolled. We are concerned that your proposed comparator will limit the interpretability of efficacy data generated from this study. (See our response to question 8 for additional details). If you are able to develop appropriate product and release testing to support a pivotal study, we recommend that you consider designing this study as a larger randomized control trial or at least include a concurrent control group.

2. Study Population: You propose to enroll an adolescent subject aged 12-18 years in each of the two study cohorts prior to enrolling children aged 2: 3 to < 12 years. However, if an eligible adolescent participant is not able to be identified,

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"enrollment will proceed with an eligible participant 2: 3 to <18 years of age, after which the subsequently enrolled participants would be of any age 2: 3 years."

a. Although there are adults with PA, you have proposed to only study children. Your rationale is that "adult patients with PA [propionic acidemia] who have not received elective LT [liver transplant] would be less likely to derive clinical benefit, including but not limited to reasons such as already having accumulated potentially irreversible organ damage. Given this, utilization of adults as sentinels could be considered unethical, given the questionable risk/benefit profile." We note that there is a wide phenotypic spectrum of PA and suspect that there may be adults who have not reached end-stage disease and may benefit from your product. Because this is a first-in-human experience with your investigational product (IP), it is preferable initially to obtain preliminary safety and tolerability data in adults because they can provide informed consent. Please explore the possibility of initiating your study in adults in whom there would be favorable benefit/risk profile and, if you deem this strategy is impracticable, please provide further justification in your IND to support your position.

b. You intend to enroll children, and your clinical investigation is associated with more than a minor increase over minimal risk, and therefore, these risks must be justified by the anticipated direct clinical benefit for each child (21 CFR 50.52). Such prospect of direct benefit (PDB) must be evidence-based (e.g., from adult humans or from appropriate animal models). Please see our response to question 4, for additional information on animal studies to support PDB. Within your IND, please provide evidence to justify your proposal to enroll children when you submit a protocol that will include children. Please ensure that the data supports the lower age-range of children you intend to enroll.

3. Eligibility Criteria

a. You plan to enroll subjects who *[meet NIH Clinical Center specific criteria].* However, you did not provide a rationale for the specified *[NIH Clinical Center specific criteria]*. This information is needed to assess risks to subjects and to ensure subject safety. Please provide your rationale for the *[NIH Clinical Center specific criteria]*.

b. You plan to enroll subjects who have "biochemical evidence of PA (e.g., elevated *[disease related biomarkers]*in body fluids)." However, you did not provide the thresholds for the biomarkers that will be used to determine eligibility. To ensure a favorable benefit/risk and consistency in enrollment of subjects, please provide thresholds for *[disease related biomarkers]* for inclusion into the study in your protocol.

c. You plan to include subjects who have "diagnosis of PCCA-related PA confirmed molecularly (PCCA gene analysis)." To ensure a comprehensive FDA review of the benefit/risk profile, please include the following information regarding the genetic testing in the IND submission:

   i. Name, location, and certification (e.g., a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory) of the genetic diagnostic laboratory

   ii. Detailed test methodology(s) and/or standard operating procedures, and information regarding device and reagent qualification.

   iii. Validation of the diagnostic test(s) for suitability (i.e., accuracy, sensitivity, specificity, and reproducibility).

   iv. A list of reported disease-causing mutation in the PCCA gene obtained from the laboratory.

   v. Information regarding quality control systems in place, and diagnostic test report sign-off procedure(s) (e.g., by an MD, or PhD who was certified with the American Board of Medical Genetics on Clinical Molecular Diagnosis)

d. You plan to enroll subjects who meet at least two of the proposed clinical criteria for severe PA. However, your protocol does not stipulate that eligible subjects must have clinical evidence of severe PA despite optimal standard of care therapy. In general, for a FIH study, as the benefits and risks are unclear, it is preferable to enroll a population who has more limited therapeutic options and thus have a favorable benefit-risk profile despite the uncertainty of the novel therapy. Please limit the initial population in this FIH study to subjects who are unresponsive to optimal standard of care therapy or justify a broader FIH study population.

e. You plan to exclude subjects who have anti-AAV9 titer > *[cut-off value]*. We acknowledge that you are working on developing, qualifying, and validating the assay. However, you did not provide a justification for the specified threshold. Please provide a data-driven scientific rationale and justification for the proposed anti-AAV9 titer of > *[cut-off value]*.

f. You plan to include subjects who have had confirmed complete resolution of infection for at least 14 days prior to dosing. However, because your IP

may induce an immunological response, subjects may be at risk for adverse events if the IP is given in close proximity to an illness or vaccination. To ensure subject safety, please do not administer AAV9-hPCCA within four weeks of an illness (febrile or non-febrile). Also, due to concern for immunologic response, please refrain from administering your product within 4 weeks of a vaccination.

g. Your protocol does not specify the laboratory value parameters for international normalized ratio (INR) and partial thromboplastin time (PTT) that will be used to determine study eligibility. To ensure subject safety and consistent enrollment of subjects, please provide the laboratory parameter range required for inclusion of subjects.

4. Dosing Rationale

a. You plan to enroll four to nine subjects in two dose cohorts. Three to six subjects in Cohort 1 will receive a single dose of *[starting dose]* of AAV9-hPCCA. One to three subjects in Cohort 2 will receive a single dose of *[higher dose]*. AAV9-hPCCA will be administered intravenously over 30-60 minutes using a syringe pump. It is premature for us to agree with the proposed dose levels given that you have not yet completed your nonclinical studies. In your IND, please provide data from your nonclinical studies to justify the proposed dose levels.

b. The Data Safety Monitoring Board will review the study data after the first three subjects are treated in Cohort 1. If there are signs of efficacy and an "acceptable safety profile," then three additional subjects will be treated in Cohort 1. However, if there are no signs of efficacy and no serious adverse events, then you will escalate the dose and one to three subjects will be treated in Cohort 2. Efficacy will be based on "clinical assessment and biomarker data (i.e., changes in *[disease related biomarkers]* at Day *[XX]* vs baseline)."

i. To ensure subjects safety, please clarify what you mean by "acceptable safety profile" and provide objective criteria that will be used to guide dose escalation or expansion. Please define the criteria using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), version 5.0.

ii. The *[proposed biomarkers]* have not been established as validated biomarkers for PA. Determining dose based on pharmacodynamic markers rather than Maximum Tolerated Dose (MTD) can result in the selection of an inadequate dose. Although your proposed approach is reasonable, you may wish to consider using MTD to

determine the dose to take forward into your Phase 2/3 study(ies).

5. Study Endpoints: Your proposed safety endpoint is "incidence of treatment-related adverse events, treatment-emergent adverse events, and serious adverse events (SAEs) and their relationship to AAV9-hPCCA administration based on CTCAE v5.0. These safety endpoints appear reasonable.

6. Your proposed efficacy endpoints are:
    a. Annualized frequency of hospitalizations
    b. Total days spent in the hospital/year
    c. Severity of metabolic acidosis and/or hyperammonemia
    d. Change in dietary regimen
    e. Need for modification to a sick-day diet
    f. New or worsening complications
    g. Quality of life of patient and caregivers
    h. Neurocognitive outcomes
    i. Growth parameters (linear growth, height, and head circumference)
    j. Vineland adaptation behavior score

    We have the following comments regarding your proposed preliminary efficacy endpoints:

    a. "Annualized frequency of hospitalization/ER visits" and "total days spent in the hospital/year" may be impacted by local hospital practices, family preferences, concomitant medical problems, and timing in which medical care is obtained. These confounding variables may make your study data uninterpretable. Please justify the proposed efficacy endpoints of annualized frequency of hospitalization/ER visits and total days spent in the hospital/year.

    b. "Severity of metabolic acidosis and/or hyperammonemia" may be impacted by timing in which "sick day" diets are initiated and medical care is obtained. These confounding variables may make your study data uninterpretable. Please provide further justification for the proposed efficacy endpoint of severity of metabolic acidosis and/or hyperammonemia.

    c. Because dietary changes can affect efficacy results and pose substantial interpretability challenges, subjects should have optimized and stable diets before entering the trial and follow the same principles of dietary management across study groups. To ensure consistency, the trial protocol should define clearly both the dietary goals and management. Also, we recommend that you consider a run-in period for optimizing and stabilizing the patient's diet and to ensure selection of a compliant group

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of patients. For further recommendations regarding optimization and standardization of dietary management in clinical trials, please refer to FDA Guidance, "Inborn Errors of Metabolism that Use Dietary Management: Considerations for Optimizing and Standardizing Diet in Clinical Trials for Drug Product Development Guidance for Industry at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/inborn-errors-metabolism-use-dietary-management-considerations-optimizing-and-standardizing-diet.

d. You plan to collect information regarding diet by having the subject or caregiver complete a diet diary. However, the method by which the subject or caregiver records the food intake may vary and make it difficult to calculate the exact content in the food. For instance, if the subject records "yogurt," there are many different brands available, and all have various protein and sugar content. Quantity should also be considered. Missing dietary data would make it difficult to make comparisons between study groups. Additionally, it will be important to standardize decisions for changes to diet and analysis will need to account for growth and other clinical changes that could account for treatment decisions related to diet. Please provide further justification for change in dietary regimen as a secondary efficacy endpoint and address how you will ensure interpretable data based on subject or caregiver completed diet diaries.

e. Management of "sick day" diet may vary across healthcare providers, and therefore, would make the data uninterpretable. We recommend that you ensure standardized procedures are in place to ensure consistency in "sick day" diet initiation. Please revise your protocol accordingly.

f. Growth may be challenging to assess as growth rates are impacted by multiple factors unrelated to PA, including genetic potential (e.g. parental height), age and timing of puberty. In a small study, this will be difficult to analyze. We recommend that you choose clinical meaningful outcomes that can be reliably interpreted. If you decide to pursue growth as an efficacy endpoint, please refer to FDA Guidance "Measuring Growth and Evaluating Pubertal Development in Pediatric Clinical Trials" at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/measuring-growth-and-evaluating-pubertal-development-pediatric-clinical-trials-draft-guidance for the most appropriate methods for measuring and recording growth.

g. You propose to include neurocognitive assessments such as the NIH Toolbox. As stated in the INTERACT meeting, for all COA tools that are intended to support regulatory decision-making and labeling claims, we recommend that you provide the following for FDA review and comment prior to initiating your study:

i. Conceptual framework of the instrument
ii. Evidence of content validity obtained for the tool, e.g., qualitative research with caregivers, obtained for this specific content of use;
iii. Exact copy of the instrument as it will be administered during the clinical trial and any training materials and user manuals;
iv. Proposed scoring algorithm(s) with rationale for any weighing of items or response options in the domain scoring and corresponding information on how the instrument's cores will be analyzed as part of an endpoint;
v. Plans for, and results from, evaluation of the psychometric properties and performance of the instrument (i.e., reliability, validity, and ability to detect change) after content validity has been established;
vi. Pre-specified plans for handling missing data; and
vii. A threshold (or range of thresholds) representing clinically meaningful within-patient improvement in instrument's scores.

When we have this information, we may be able to provide further feedback on whether the tool is an acceptable efficacy endpoint.

h. In the context of an open-label study, observer-reported and patient-reported clinical outcome assessments (COAs), such as the Vineland and other quality of life measures, are not considered interpretable given caregiver and/or patient knowledge of treatment assignment which can introduce bias. We recommend that you consider caregiver-and patient-reported outcomes as exploratory endpoints which may contribute to the totality of evidence. Please revise your protocol accordingly.

7. Staggering: You plan to stagger the first two subjects within a cohort by 12 weeks. You did not propose a staggering interval between dose cohorts. For subject safety, it is important to have sufficient opportunity to monitor and investigate any acute or subacute severe adverse events, prior to exposing subsequent subjects to your product. Therefore, to ensure subject safety,

a. Please provide a data-driven rationale and justification for the proposed intra-cohort staggering interval of 12 weeks. As a general rule, the length of the staggering interval should be based on timing of adverse events in pre-clinical or clinical experience with your product or related products.

b. Please stagger at least the first 3 subjects in each dose cohort instead of only the first subject. Please revise your protocol accordingly or provide further justification for why your approach provides adequate safeguards to subjects.

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c. Please specify an inter-cohort staggering interval (i.e., staggering interval after treatment of subjects in Cohort 1 and before treating subjects in Cohort 2) and a data-driving justification for your proposal.

8. Stopping Rules: You propose the following "rules for suspending the study (enrollment and investigational product (IP) administration):"
    - A participant dies due to a medical event related to AAV9-hPCCA administration.
    - A participant develops a malignancy determined to be related to the drug administration.
    - A participant experiences a Grade 4 or higher adverse event (based on CTCAE v5.0) deemed to be related to AAV9-hPCCA.
    - Any Grade 4 serious adverse event that could be related to study procedures (e.g., complications from corticosteroids)
    - The DSMB chair or sponsor of the study determines that a medical event requires additional evaluation by the full DSMB.

    Additionally, you propose the following "rules for stopping the study" if they occur "within the first 12 months after the administration of AAV9-hPCCA:"
    - The sponsor determines that an event or data warrant termination of the study for any reason.
    - A participant develops malignancy determined by the sponsor to be related to the AAV9-hPCCA administration.
    - A participant death is determined by the sponsor to be related to the AAV9-hPCCA administration.

    a. We do not agree with your proposed rules for suspending the study as they are only triggered in the event that attribution is made to your product, its administration or other study related procedures. It takes time to investigate relatedness, while this happens the study should be paused to enrollment and treatment of additional subjects.

    b. As your pre-clinical development is not complete, it is premature to determine the adequacy of your pausing/stopping criteria. Please update your stopping criteria based on totality of your non-clinical studies and safety data from clinical studies of related products.

    c. You are enrolling a vulnerable population. Therefore, we recommend that you have an independent data monitoring committee (iDMC). When a stopping rule is triggered, the enrollment and treatment of new subjects in a trial should be paused to enable a comprehensive evaluation by an independent data monitoring committee (iDMC) prior to trial termination or resumption [with or without protocol, informed consent document (ICD) or investigator brochure (IB) changes]. The iDMC can adjudicate causality and advise on study stopping, protocol, ICD and IB revisions. If you elect

to utilize an iDMC, please submit the Charter in your IND.

d. We are unclear about your "additional rules for study stopping." We do not agree that death or malignancy attributed to the product should be limited to 1-year to qualify as a study stopping rule. We recommend removing any time bounds for serious safety signals attributed to your product.

9. Safety Monitoring Plans
    a. AAV9-hPCCA will be given via intravenous (IV) administration over 30-60 minutes. Your protocol does not include a peri-infusion monitoring plan. Absence of a peri-infusion monitoring plan would place subjects at unreasonable risk. To ensure subject safety, please specify the frequency and duration of vital signs and other assessments (as necessary) that will occur during the infusion.

    b. Your protocol does not include infusion starting, slowing and stopping rules. Lack of defined infusion rules may expose subjects to significant risks during the infusion.
        i. Please provide specific criteria, including vital signs ranges and clinical status, that will be used to determine if it is safe to start an infusion (i.e. they still meet eligibility criteria).
        ii. Please provide specific criteria including vital signs ranges and clinical signs or symptoms, that would trigger the decision to slow the infusion.
        iii. Please provide specific infusion stopping criteria, including vital signs and ranges and clinical signs or symptoms, that will trigger the decision to stop the infusion.
        iv. Please provide criteria that will be required before an infusion would be restarted, if applicable.
        v. Please define infusion slowing and stopping rules based on the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), Version 5.0.

    c. Subjects will be hospitalized for 72 hours after AAV9-hPCCA administration. However, you have not provided discharge criteria or discharge instructions. The absence of discharge criteria and discharge instructions could place subjects at increased risk. To ensure subject safety:
        i. Please provide specific discharge criteria, including vital signs and clinical status, that will be used to determine when it is safe to discharge a subject home.
        ii. Please propose discharge instructions to ensure that subjects understand when to seek urgent or emergent medical care.

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Meeting Discussion for Sponsor Question 7
Response 2a:
Sponsor stated that initiating the first-in-human trial in adults is not favorable, because adults with severe disease may have irreversible manifestations of the disease, they are not likely to benefit from the investigational product (IP). The sponsor also stated that adults with severe disease have moderate to severe cognitive impairments and would therefore lack capacity to provide informed consent. FDA expressed that propionic acidemia (PA) is a heterogenous disease and there is a wide phenotypic spectrum, such that there may be an adult population with the intermediate form of the disease who are able to provide informed consent and have not developed irreversible manifestations of the disease, such that they would have potential to benefit from the IP. The Sponsor responded that adults with the intermediate form of the disease have a good quality of life and inclusion into the trial would not represent a favorable benefit-risk profile. FDA asked the sponsor to provide this rationale with supportive evidence in their IND for FDA's review.

Sponsor Question 8:
Does the Agency agree with the plan to use historical and concurrent data from the Natural History study (with or without liver transplant, considering the very small number of patients affected by this rare disease) as a comparator arm for the FIH clinical trial?

FDA Preliminary Meeting Response to Sponsor Question 8:
Your plan is acceptable for an early phase study that is primarily focused on assessing safety and tolerability. However, for interpretable efficacy data an appropriate comparator is needed. An external control can sometimes be used as a comparator. An external control is most likely to be appropriate when the course of untreated disease in a patient population is uniform (i.e. outcomes can be predicted reliably), the study endpoint is objective, and when the outcome on treatment is markedly different. In addition, the external control group should be as similar as possible to the single arm study with respect to all relevant variables including disease subtype/severity, demographics, baseline age, functional status, standard of care, concomitant therapies, and other prognostic or predictive features. Additionally, the types of assessments, methodology for performing assessments and schedule of assessments should be the same in both your single arm study and your external control study. We note the following in the natural history protocol:

1. The subjects' dietary management and concomitant therapy is dictated by healthcare professionals outside of the natural history study and "no study treatments or interventions are offered, other than those medically indicated."
2. The study tests, procedures and clinical outcome measures may vary from one subject to another as "the study team will determine which tests/procedures need to be performed at each visit based on patient history."
3. Study visits may occur at the National Institute of Health (NIH) Clinical Center

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or be performed via telehealth, and occur every 1-3 years, varying from one subject to another.

4. Subjects may have received liver transplant.

The differences in baseline disease characteristics, dietary management, concomitant therapies, types of assessments, methodology of performing assessment and schedule of assessments between subjects within the natural history study as well as between the natural history study and FIH study may confound comparisons on efficacy and safety outcomes and limit data interpretability.

Furthermore, with the use of an external control cohort, we are concerned that the safety and efficacy data may not be interpretable due to selection bias and bias related to knowledge of treatment assignment (i.e., assessor bias and performance bias leading to subjects' changes in behaviors, such as dietary adherence, medication compliance, and emergency diet initiation, that can impact clinical outcomes and confound results).

Therefore, we reiterate our recommendation that you conduct a double-blinded, randomized placebo-controlled trial wherein the control arm would receive no treatment other than optimal standard of care. To retain subjects in the control arm, you might consider offering the product to subjects in the control arm at the completion of the study period, provided the preliminary safety and efficacy data are favorable. Randomization would not fully ensure the absence of bias, but it would remove the introduction of selection bias and allow for standardization of assessments and standard of care between the active treatment and control groups. Blinding of the subjects and assessors may minimize the introduction of detection bias that may result from knowledge of treatment assignment. We strongly encourage you to explore the feasibility of conducting a randomized, controlled trial (RCT) even for an early-phase trial. This design would enable you to obtain robust safety and efficacy data that could be used to guide optimal dose selection and to support your later-phase trial(s).

We do acknowledge the potential challenges of conducting such a trial given the rarity of PA and possible reluctance from caregivers to enroll their child or maintain participation in a trial where their child may not initially receive experimental treatment. Therefore, if you find the above recommended study design not to be feasible, you may consider utilizing an external control group in which the external control group and treatment group are similar in baseline characteristics, standard of care, types of assessments, methodology of preforming assessments, and schedule of assessments (as stated above). Ideally, the data should be generated prospectively to help minimize selection bias and better ensure comparability between the external control study and FIH study. To minimize bias related to knowledge of treatment, we recommend that the assessors be blinded to treatment. We also recommend that you standardize management of subjects' diet, concomitant therapies, and the criteria that trigger the initiation of an emergency diet.

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Meeting Discussion for Sponsor Question 8:
Sponsor stated they do not believe that a randomized control trial (RCT) is feasible because of the rarity of the disease, potential challenges in enrolling a trial with a placebo arm, and invasive nature of the gene therapy protocol (e.g., PICC line insertion for repeat blood draws, steroid prophylaxis, prolonged visit to NIH for safety evaluation). However, the sponsor expressed understanding that an appropriate external control group is needed. Sponsor requested feedback regarding how to best ensure the external control data is adequate. Sponsor stated that in the natural history study that Sponsor plans to use as a comparator for the treatment study, study participants receive a standard workup though additional evaluations may be added if clinically indicated. Also, the same group of investigators in the natural history study will be involved with the performance of assessments during the Phase 1/2 trial.

FDA re-emphasized the external control group and Phase 1/2 group should be similar in baseline characteristics, management of diet and concomitant therapies, types of assessments, methodology of performing assessments, and schedule of assessments. Within the natural history protocol, it is unclear if aside from the *[disease-related biomarker assay],* the assessments in the natural history study are the same as the assessments proposed in the Phase 1/2 study. For instance, it is not clear if the same clinical outcome assessments (COAs) proposed in the Phase 1/2 study are the same as the COAs used in the natural history study. Also, it is unclear if the timing of assessments are similar between the external natural history study and planned Phase 1/2 study.

In response, Sponsor states there may need to be differences in the timing of the assessments between the natural history cohort and the Phase 1/2 cohort and asked FDA for feedback for these instances. FDA stated the determination regarding the appropriateness of differences in the timing of assessments between the two studies will depend on the specific endpoint of interest and extent of the differences. FDA recommended that if Sponsor feels the need to have different timing of assessments between the natural history study and the Phase 1/2 study, the Sponsor should provide their rationale and justification for interpretability of each assessment where timing will differ in the IND.

Sponsor Question 9:
Does the Agency agree with the proposed safety endpoint, and use of the described surrogate endpoints *[proposed disease-related biomarkers]* as the primary efficacy endpoint for the FIH study?

FDA Preliminary Meeting Response to Sponsor Question 9:
No, at this time, we do not agree with the use of a surrogate endpoint as a primary efficacy endpoint. Please provide additional rationale to support why it is necessary to use a surrogate endpoint. Additionally, you have not provided an adequate data-driven justification for the use of *[proposed disease-related biomarkers]* as surrogate

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biomarkers that are reasonably likely to predict clinical benefit. We refer you to the document entitled, "Considerations for Discussion of a New Surrogate Endpoint(s) at the Type C PDUFA Meeting Request" at https://www.fda.gov/drugs/cder-small-business-industry-assistance-sbia/early-engagement-fda-discuss-novel-surrogate-endpoints-november-27-2018-issue. If you can justify why a study based on a clinical endpoint is not feasible, the document still invites questions about the choice of *[proposed disease-related biomarkers]* as surrogate endpoints. While the document does not identify all of the "considerations" as requirements, we note that you have not characterized the following for *[proposed disease-related biomarkers]*:

1. Threshold for change in *[proposed disease-related biomarkers]* required to demonstrate a clinically meaningful effect
2. Consistency of response under various conditions
3. Reliability of *[proposed disease-related biomarkers]* for quantifying changes in the clinical outcome before and after treatment

We recommend the following:

a. Discuss the quantitative relationship between a change in *[proposed disease-related biomarkers]* and a change in clinically meaningful endpoints
b. Describe the reliability and consistency of *[proposed disease-related biomarkers]* measurements under variable circumstances.
c. Study *[proposed disease-related biomarkers]* as exploratory pharmacodynamic markers.
d. Explore clinically meaningful endpoints throughout your development program
e. Consider and incorporate patient experience data in your developmental program and determination of clinically meaningful endpoints. Patient experience data provide information about the impact of a medical condition or a therapy on a patient's life, and information about the patient's preferences for treatment. Data may be collected by any persons (including patients, family members, patients' caregivers, patient advocacy organization, disease research foundations, researchers, and drug manufacturers). For additional information, please refer to FDA Guidance, "Patient-Focused Drug Development: Collecting Comprehensive and Representative Input" at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/patient-focused-drug-development-collecting-comprehensive-and-representative-input.
f. Should you decide to pursue *[proposed disease-related biomarkers]* as surrogate endpoints, please address whether the expected clinical treatment effect size can be demonstrated in a feasible confirmatory trial given the size of the patient population.

We have the following additional comments regarding the use of *[proposed disease-*

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related biomarker] as a surrogate endpoint.

1. Gut bacteria produces *[proposed disease-related biomarker]* from the breakdown of dietary fiber. We are concerned that this natural gut production of *[proposed disease-related biomarker]* may change depending on the subjects' diet and impact the *[proposed disease-related biomarker assay]* results which will then confound the data. In your protocol, please address how you will mitigate the impact of gut bacterial *[proposed disease-related biomarker]* production in the interpretation of *[proposed disease-related biomarker assay]* results.

2. The *[proposed disease-related biomarkers]* utilizes the Exalenz Breath ID, a 510(k) cleared device that is used to aid the diagnosis and post-treatment monitoring of *H. pylori* infection in adults. You state, "it is believed the device's utilization falls within its intended use to 'continually and non-invasively measure changes in the 13CO2/12CO2 ratio of exhaled breath.'" Also, you state, "Previous clinical use of this device was performed in a NH [natural history' study for a related methylmalonic acidemia (MMA) patient population…In this study, 83 breath test measures were performed across *[XX]* MMA patient participants, along with measures for a control arm comprised of healthy adult volunteers and MMA heterozygote parents."

    a. To better understand *[proposed disease-related biomarker assay]*, please provide the step-by-step procedures involved in evaluating *[proposed disease-related biomarker assay]*.

    b. To ensure the Exalenz BreathID can produce reliable and reproducible results, please provide device validation data for use of the device to measure 13CO2/12CO2 ratio in subjects with MMA or PA. Please include demographic data on the subjects who were used to test the reliability and reproducibility of Exalenz BreathID.

    c. As part of the *[proposed disease-related biomarker assay]* test, subjects are given an enteral bolus of *[proposed disease-related biomarker]*. We are concerned that *[proposed disease-related biomarker assay]* may induce a metabolic decompensation event. To ensure subject safety, please provide demographic and safety data to support the safety of *[proposed disease-related biomarker assay]* in subjects with PA.

Meeting Discussion for Sponsor Question 9:
There was no discussion of this question during the meeting.

Sponsor Question 10:
Does the Agency agree with the proposed immunosuppressive regimen?

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FDA Preliminary Meeting Response to Sponsor Question 10:
Based on the limited information you provided in your meeting package, we cannot yet agree with your proposed immunosuppressive regimen. You plan to treat subjects with 1mg/kg/day of oral corticosteroid 24 hours before and for 30 days after AAV9-hPCCA administration, followed by a 28-day taper. The corticosteroid course may be extended if there is "any evidence of immunological response to AAV9, based on elevated liver function tests, and if clinically indicated, other laboratory parameters." We have the following comments regarding your immunosuppressive therapy plans:

1. Hyperammonemia is a characteristic feature of organic acidemias, particularly PA. We are concerned that corticosteroid administration may increase catabolism and potentially trigger a metabolic decompensation event.

    a. To ensure subject safety, please address in your IND how you will mitigate the risks of metabolic decompensation event in the setting of corticosteroid therapy.
    b. You plan to measure ammonia levels at baseline and not again until Day 28 post-treatment with AAV9-hPCCA. Because of the potential risk of corticosteroid-induced metabolic decompensation events, to ensure subject safety, while subjects are receiving corticosteroids, please increase the frequency at which ammonia levels are assessed.

2. There is a risk of adrenal insufficiency following tapering of steroids below physiologic levels after prolonged high-dose corticosteroids. Within your IND study protocol, please address how you will assess suppression of the Hypothalamic-Pituitary-Adrenal (HPA) axis and how you will manage this to avoid adrenal insufficiency. Please discuss when children will be referred to endocrinology for further assessment, management and counseling.

3. To ensure subjects are well-informed of the risks associated with study participation, please discuss risks of prolonged corticosteroid therapy in the ICD.

Meeting Discussion for Sponsor Question 10:
There was no discussion of this question during the meeting.

FDA Questions/Comments sent in Preliminary Meeting Response:

Chemistry, Manufacturing, and Controls

1. You indicate that the AAV9-hPCCA DP will be formulated with a diluent at the clinical site prior to being administered to subjects. In your proposed approach to device compatibility testing, you plan to use Phosphate-buffered Saline (PBS) as the diluent for the DP. Please be advised that the diluent used in your device

compatibility testing study should be the same as that used to formulate the DP in the clinical site pharmacy.

2. If you intend to use a non-compendial diluent in the pharmacy to formulate the final dose that will be administered to the subjects, please:

    a. Describe the manufacturing and release testing plan for the non-compendial diluent, i.e., composition of the diluent and specifications for diluent release.

    b. Document it under a Module 3.2 P (e.g., 3.2 P-Diluent) that is distinct from the DP information (e.g.,3.2 P-Vector).

    c. Provide a stability monitoring plan for the diluent during storage and shipping. Indicate if the diluent will be supplied with the product or provided separately by the pharmacy.

3. It appears that your pHelper-Kan plasmid may contain adenovirus sequences that are not needed for AAV production. In your IND submission, please provide a complete list of all genetic elements in each plasmid used for AAV production.

In your IND submission, please provide a description of each non-compendial test performed for qualification of the master cell bank (MCB), including the assay procedure, controls and standards, and sensitivity, as applicable.

Pharmacology/Toxicology

1. Statements regarding the adequacy of any nonclinical study to support a particular clinical trial or fulfill a specific regulatory requirement are made based solely on the information provided in your pre-IND meeting package and are considered preliminary. A final determination regarding the adequacy of the studies cannot be made without CBER review of complete materials that should be submitted in the IND.

2. In your IND submission, please provide complete study reports for all nonclinical studies used to support the safety and rationale of your proposed clinical trial. These reports should include, but should not be limited to: a) a prospectively written protocol and all protocol amendments or a detailed methodology; b) a detailed description of the study design (e.g., description of the test system used, animal species/animal models, control and test articles administered, dose levels, detailed procedures for test article administration (including delivery device description), and collection of all study protocol parameters, etc.); c)

results for all parameters evaluated for each animal on study; and d) your analysis and interpretation of the study data.

3. For each toxicology study performed, please provide documentation showing that the study was conducted in compliance with Good Laboratory Practice (GLP) as per 21 CFR Part 58. If the study was not GLP-compliant, as directed by 21 CFR Part 312.23(a)(8)(iii), you should provide a brief statement of the reason for the non-compliance in your IND submission. In addition, please specify in the study report any areas that deviate from the prospectively written protocol and the potential impact of these deviations on study integrity. Each study should: a) be conducted according to a prospectively written protocol, b) performed in as nonbiased a manner as possible, and c) have appropriate record keeping and documentation of all data.

4. We strongly recommend oversight of the conduct of all non-GLP toxicology studies and each resulting final study report by a Quality Assurance (QA) unit/person that is independent of the personnel responsible for the conduct of this study, as per 21 CFR Part 58.35. This QA oversight is important to assure study conduct according to sound procedures and to ensure the quality and integrity of the resulting data.

5. Please note that for applicable nonclinical studies initiated after March 15, 2023, standardized datasets in the Standard for the Exchange of Nonclinical Data (SEND) format will be required to be submitted to your CBER IND and BLA. Further details on this requirement, including the types of nonclinical studies and exceptions from this requirement can be found at: https://www.fda.gov/industry/study-data-standards-resources/study-data-submission-cder-and-cber and in the "Study Data Technical Conformance Guide" at https://www.fda.gov/industry/fda-data-standards-advisory-board/study-data-standards-resources. Questions regarding SEND should be directed to CBER-edata@fda.hhs.gov.

6. In Module 4 of your IND, please include copies of publications that are directly supportive of your proposed clinical trial. In Module 2 of your IND, please provide a comprehensive discussion of each cited publication and explain how the data support your clinical trial (e.g., comparison of the product used in the publication to your intended clinical product, route of administration, dose levels, etc.).

7. Please respond to each Pharmacology/Toxicology comment and specify the relevant sections in the IND that contain the supporting data.

8. If this clinical study will not be conducted by a sponsor-investigator, please provide an Investigator Brochure (IB) in the IND submission. For additional

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recommendations on the preparation and content of your IB, please refer to Section 7 of the document titled, *E6(R2) Good Clinical Practice: Integrated Addendum to ICH E6(R1) - Guidance for Industry* (March 2018), available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/e6r2-good-clinical-practice-integrated-addendum-ich-e6r1

9. For a comprehensive summary regarding the nonclinical assessment of cell and gene therapy products, please refer to: a) the document titled, *Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products* (November 2013), available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/preclinical-assessment-investigational-cellular-and-gene-therapy-products and b) the *OTAT Learn* Webinar Series, available at: https://www.fda.gov/vaccines-blood-biologics/news-events-biologics/otat-learn.

10. The nonclinical program for any investigational product should be individualized with respect to scope, complexity, and overall design, to maximize the contribution and predictive value of the resulting data for clinical safety and therapeutic activity. As recommended in Section III.B.8 of the *Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products*, we encourage you to explore opportunities for reducing, refining, and replacing animal use in your nonclinical program. For example, it may be appropriate to use *in vitro* or *in silico* testing to complement or replace animal studies. We encourage you to submit proposals and justify any potential alternative approaches.

END