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Center for Cellular and Molecular Therapeutics

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Meeting Request

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Sponsor Information:

Rebecca Ahrens-Nicklas, MD, PhD
Assistant Professor of Pediatrics
Director, Gene Therapy for Inherited Metabolic Disorders Frontier Program
The Children's Hospital of Philadelphia

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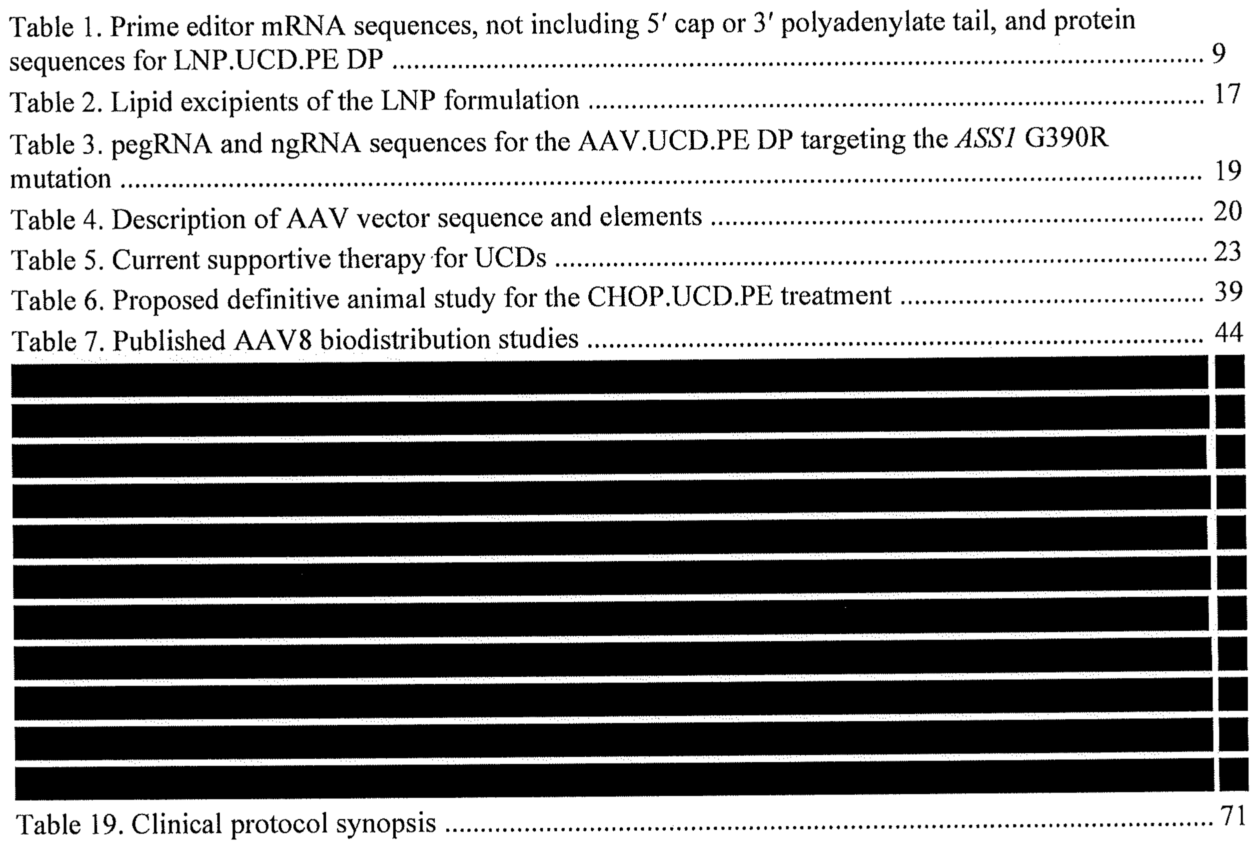

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1. PRODUCT NAME

CHOP.UCD.PE

2. CHEMICAL NAME AND STRUCTURE

The CHOP.UCD.PE treatment comprises two drug products (DPs), LNP.UCD.PE and AAV.UCD.PE, that when used together can effectuate *in vivo* prime editing in hepatocytes for correction of one or two pathogenic mutations in a urea cycle disorder (UCD) gene.

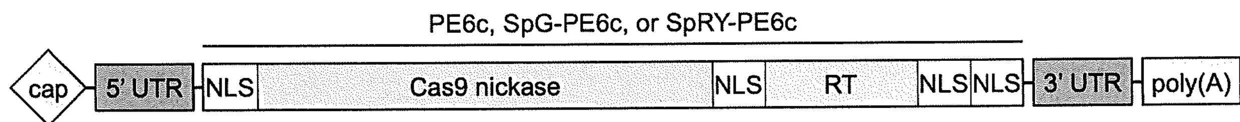
The LNP.UCD.PE DP is a lipid nanoparticle (LNP) formulation comprising lipid excipients and a messenger RNA (mRNA) drug substance (DS) that encodes a prime editor (PE) protein, which has a *Streptococcus pyogenes* Cas9 (SpCas9) nickase and a reverse transcriptase (RT) domain derived from the yeast Tf1 retrotransposon, and that otherwise is >99% identical among all variants of the DP, with the PE varying in its protospacer-adjacent motif (PAM) specificity.

The AAV.UCD.PE DP is an adeno-associated viral (AAV) vector encoding two prime editing guide RNAs (pegRNAs) and two nicking guide RNAs (ngRNAs), all expressed from RNA polymerase III (Pol III) promoters. Portions of these guide RNA (gRNA) sequences vary across variants of the AAV.UCD.PE DP, which are otherwise identical.

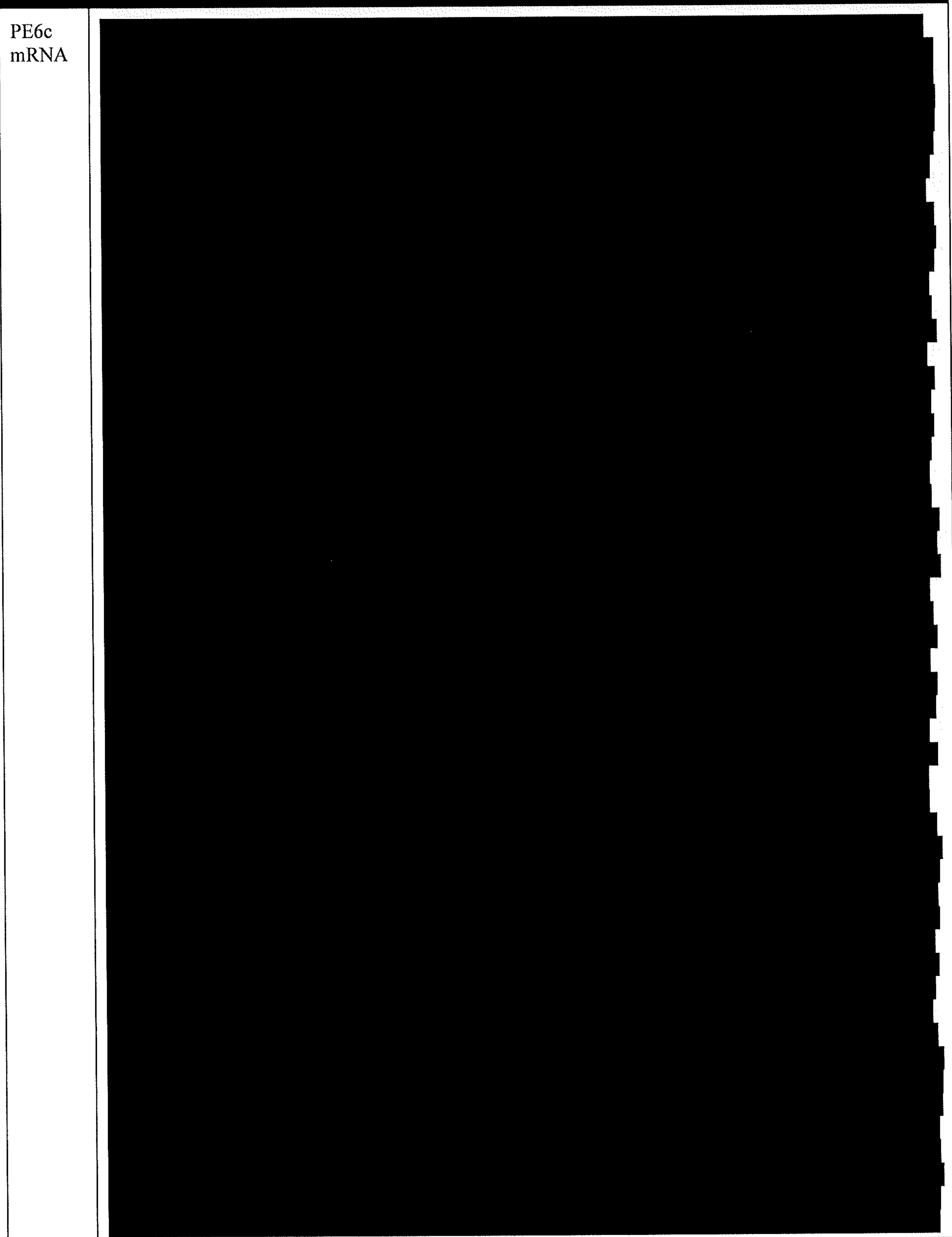
mRNA Drug Substance

Each variant of the LNP.UCD.PE DP has one of several highly similar mRNA DSs. The initial mRNA to be used in the LNP.UCD.PE DP encodes a prime editor 6c (**PE6c**) protein with an SpCas9 nickase, with the H840A mutation in its HNH domain and with a preference for the protospacer-adjacent motif (PAM) NGG, and an evolved and engineered reverse transcriptase (RT) domain derived from the yeast Tf1 retrotransposon (Doman et al., 2023). Other mRNA DSs encode essentially the same editor, with the identical RT domain and with a limited number of amino acid substitutions in the SpCas9 nickase that modify its PAM preference. One such mRNA DS encodes **SpG-PE6c**, which has a more relaxed NGN PAM preference (Walton et al., 2020); another mRNA DS encodes **SpRY-PE6c**, which is near-PAMless, i.e., accommodates almost all PAM sequences (Walton et al., 2020). Each mRNA comprises the same 5′ cap, 5′ untranslated region (UTR), 3′ UTR, and 3′ polyadenylate tail (**Figure 1**). In each mRNA, the PE coding sequence is codon-optimized with uridine minimization and has substitution of all uridines with the modified nucleotide N1-methylpseudouridine. The sequences of the mRNA DSs are ≥99.6% identical, with no more than 22 nucleotides differing between any two of the sequences; the encoded amino acid sequences differ by no more than 11 amino acids, exclusively in the SpCas9 nickase. The mRNA and encoded protein sequences and the limited number of positions that differ among the sequences (indicated in bold underline) are shown in **Table 1**.

Figure 1. Structure of mRNA DS.



Abbreviations: Cap = methylated 5′-Cap-1; UTR = untranslated region; NLS = nuclear localization signal; Cas9 = clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9; RT = reverse transcriptase; poly(A) = polyadenylic acid. Not to scale.

Table 1. Prime editor mRNA sequences, not including 5′ cap or 3′ polyadenylate tail, and protein sequences for LNP.UCD.PE DP.	
PE6c mRNA	

Complete information about the PE6c, SpG-PE6c, SpRY-PE6c, and potentially other highly similar mRNA DSs may be provided in a Type II Master File that will be cross-referenced by the primary CHOP.UCD.PE IND application and subsequent gene-specific IND applications.

Lipid Nanoparticle Drug Product

The LNP.UCD.PE DP comprises the mRNA DS and four lipid excipients: [redacted]. The structures of these components and the target composition of lipid excipients in the DP are provided in **Table 2** below. [redacted]

Table 2. Lipid excipients of the LNP formulation.

[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

[redacted]

Adeno-Associated Viral Vector Drug Product

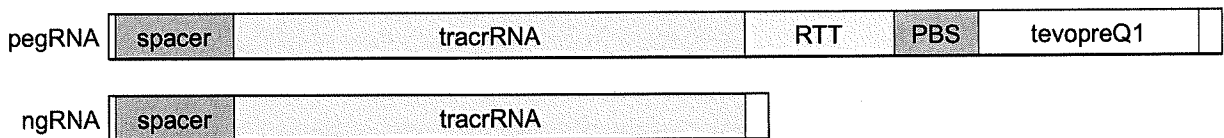
The AAV.UCD.PE DP comprises an adeno-associated viral (AAV) vector encoding prime editing pegRNAs and ngRNAs. Specifically:

- AAV.UCD.PE is a single AAV vector with serotype 8, with the capsid from AAV8 and inverted terminal repeats (ITRs) from AAV2.
- The AAV vector will contain four guide RNA (gRNA) expression cassettes:
  - pegRNA expressed from the human U6 Pol III promoter
  - pegRNA expressed from the mouse U6 Pol III promoter
  - ngRNA expressed from the human 7SK Pol III promoter
  - ngRNA expressed from the human H1 Pol III promoter
- In some AAV.UCD.PE variants, two copies of the same pegRNA will be expressed from the human U6 and mouse U6 promoters, and two copies of the same ngRNA will be expressed from the human 7SK and human H1 promoters.
- In some AAV.UCD.PE variants, two different pegRNAs will be expressed from the human U6 and mouse U6 promoters, and two different ngRNAs will be expressed from the human 7SK and human H1 promoters. One pegRNA and one ngRNA will constitute a prime editing solution for one pathogenic mutation in a UCD gene, and the other pegRNA and the other ngRNA will constitute a prime editing solution for a second pathogenic mutation in the same UCD gene (i.e., for a compound heterozygous patient).
- The initial pegRNA and ngRNA to be used in the AAV.UCD.PE DP constitute a prime editing solution for correction of the *ASS1* c.1168G>A mutation (p.Gly390Arg, or G390R). There are two copies of the pegRNA and two copies of the ngRNA in the vector.
- The expressed pegRNA sequence for correction of the *ASS1* G390R mutation comprises a 5′ guanine leader nucleotide (to facilitate efficient Pol III transcription), a 20-nucleotide spacer sequence, an 86-nucleotide trans-activating CRISPR RNA (tracrRNA) sequence, a 25-nucleotide reverse transcriptase template (RTT), a 14-nucleotide primer binding site (PBS), and a 37-nucleotide tevopreQ1 structured RNA motif, followed by a few 3′ uridine nucleotides resulting from termination of Pol III transcription with a poly(T) termination sequence (**Figure 2**, **Table 3**). Prime editing with this optimized pegRNA corrects the *ASS1* G390R mutation and introduces up to three synonymous edits.
- The expressed ngRNA sequence for correction of the *ASS1* G390R mutation comprises a 5′ guanine leader nucleotide, a 20-nucleotide spacer sequence, and the 86-nucleotide

trans-activating CRISPR RNA (tracrRNA) sequence, followed by a few 3′ uridine nucleotides resulting from termination of Pol III transcription with a poly(T) termination sequence (**Figure 2**, **Table 3**).

- Across different AAV.UCD.PE variants, the ngRNA varies in its sequence in only 20 of 111 positions at most. The pegRNA varies in 20 positions in the spacer sequence at most; the PBS varies in sequence as well as in length, typically ranging from 9 to 15 nucleotides though shorter or longer lengths are possible; the RTT varies in sequence as well as in length, typically ranging from 10 to 30 nucleotides though shorter or longer lengths are possible; and the tracrRNA and tevopreQ1 sequences do not change. **The remainder of the AAV plasmid sequence** (a total of ≈4.1 kilobases between the ITRs, including the pegRNA/ngRNA expression cassettes) **is identical** (**Figure 3**, **Table 4**).
- The full AAV plasmid sequence will be provided in the primary IND application.

Figure 2. Structures of pegRNA and ngRNA.



Abbreviations: pegRNA = prime editing guide RNA; ngRNA = nicking guide RNA; tracrRNA = trans-activating CRISPR RNA; RTT = reverse transcriptase template; PBS = primer binding site.

Table 3. pegRNA and ngRNA sequences for the AAV.UCD.PE DP targeting the *ASS1* G390R mutation.

pegRNA	5′-G**AUUGAUGUUGAUGAACCUGG**GUUUGAGAGCUAUGCUGGAAACAGCAUAGCAAGU UCAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCGAUUA UGAGCCAACGGAUGCGACUG**GGUUCAUCAACAUC**CGCGGUUCUAUCUAGUUACGCGU UAAACCAACUAGAAUUUU-3′
ngRNA	5′-G**AUGAGCCAACGGAUGCGACU**GUUUGAGAGCUAUGCUGGAAACAGCAUAGCAAGU UCAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU-3′

Abbreviations: A = adenosine; C = cytidine; G = guanosine; U = uridine. Bold underline = spacer sequence; underline = reverse transcriptase template (RTT) sequence; bold = primer binding site (PBS) sequence.

It is anticipated that, after the initial clearance of a **primary IND application** for the CHOP.UCD.PE treatment for the correction of the *ASS1* G390R mutation, additional variants of the AAV.UCD.PE DP targeting other pathogenic mutations in the *ASS1* gene will be added by **amendments to the primary IND**. Moreover, it is anticipated that **subsequent gene-specific IND applications** for the CHOP.UCD.PE treatment will add variants of the AAV.UCD.PE DP targeting pathogenic mutations in other UCD genes, namely *CPS1*, *OTC*, *ASL*, *ARG*, *NAGS*, and *SLC25A15*. The pegRNA and ngRNA sequences encoded in each variant of the AAV.UCD.PE DP cannot be specified beforehand, as each new variant of the AAV.UCD.PE DP will be designed **in real time** based on a pathogenic mutation(s) that is: (1) identified upon genetic testing of a patient diagnosed with an infantile-onset UCD; and (2) demonstrated to be amenable to corrective editing by a PE encoded by an mRNA DS specified in **Table 1**.

Figure 3. Vector map.

Table 4. Description of AAV vector sequence and elements.		
Description	**Start**	**Stop**

3. PROPOSED INDICATION FOR USE

CHOP.UCD.PE is proposed for the treatment of hyperammonemia in patients with deficiencies in enzymes of the urea cycle who are homozygous or compound heterozygous for a pathogenic mutation(s) in any urea cycle disorder (UCD) gene, including *ASS1*, *CPS1*, *OTC*, *ASL*, *ARG*, *NAGS*, and *SLC25A15*, that can be efficiently corrected by a prime editor (PE) with a *Streptococcus pyogenes* Cas9 (SpCas9) nickase and a yeast Tf1 reverse transcriptase (RT) domain.

4. DOSAGE FORM, ROUTE OF ADMINISTRATION, AND DOSING REGIMEN

The CHOP.UCD.PE treatment comprises two DPs, AAV.UCD.PE and LNP.UCD.PE, that will be administered at different times—the latter at least two weeks after the former—via intravenous (IV) infusions at doses based on body weight. There is the potential for repeat dosing of the LNP.UCD.PE DP to achieve the desired therapeutic effect.

5. LIST OF SPONSOR ATTENDEES

Attendee	Affiliation	Title

6. HISTORY OF THE PROJECT/BACKGROUND

Introduction

Hepatic inborn errors of metabolism (IEMs) are individually rare but collectively affect 1:1000–1:2500 births (Applegarth et al., 2000; Sanderson et al., 2006). Most arise from recessive loss-of-function mutations in genes encoding key enzymes in hepatic biochemical pathways. Loss of enzyme activity results in accumulation of upstream toxic metabolites and/or insufficient production of downstream products. In many cases, abnormal liver biochemistry induces secondary organ dysfunction, especially in the brain. Each disorder has a distinct molecular etiology, with more than 140 hepatic IEMs cataloged to date. However, many hepatic IEMs share cardinal features that make them ideal candidates for a platform-based gene editing approach including: (1) the molecular etiology (i.e., editing target) is unambiguous; (2) accumulated metabolites are well-established disease and therapeutic biomarkers, allowing for patients to serve as their own controls; (3) studies demonstrate the clinical benefit of liver correction (an organ that is accessible with current delivery technologies); (4) restoring 10-20% of hepatic enzyme activity often corrects disease phenotypes; and (5) many patients in the U.S. are identified as neonates through universal newborn screening.

Humans ingest protein to support growth and the synthesis of a number of key macromolecules. Nitrogen waste generated from protein catabolism is converted to ammonia, which under normal physiologic conditions is converted to urea via the urea cycle (**Figure 4**). Urea is then excreted in urine to maintain whole-body nitrogen homeostasis. Loss of function of any of the six enzymes of the urea cycle—encoded by *CPS1* (carbamoyl phosphate synthetase 1), *OTC* (ornithine transcarbamylase), *ASS1* (argininosuccinate synthetase), *ASL* (argininosuccinate lyase), *ARG* (arginase), and *NAGS* (*N*-acetylglutamate synthetase)—results in a urea cycle disorder (UCD). In

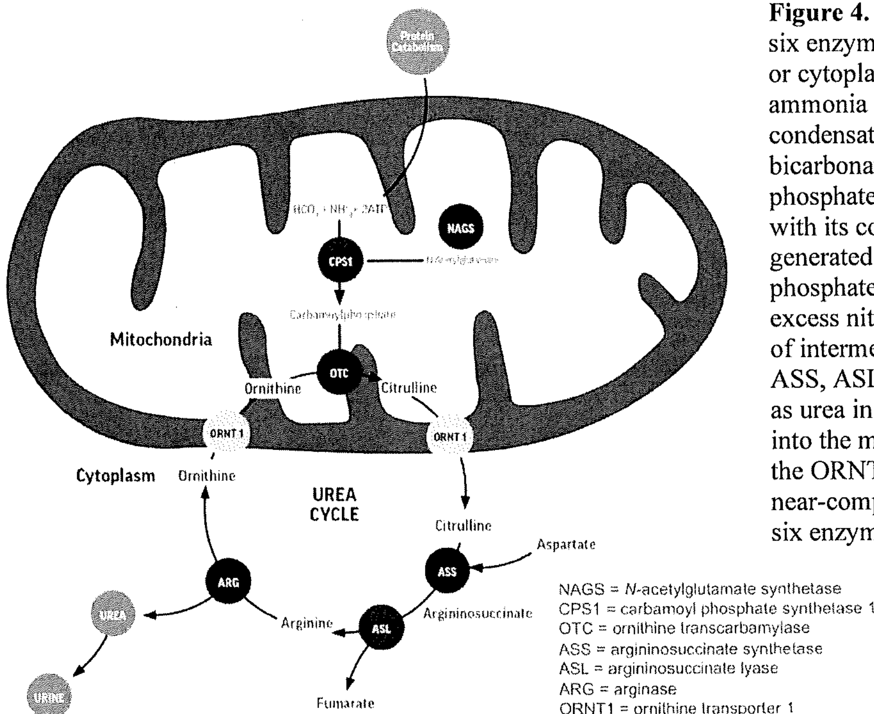


Figure 4. The urea cycle. In the liver, six enzymes either in the mitochondria or cytoplasm enable the conversion of ammonia into urea, starting with the condensation of ammonia (NH_4^+) and bicarbonate to form carbamoyl phosphate, a reaction catalyzed by CPS1 with its cofactor *N*-acetylglutamate, generated by NAGS. Carbamoyl phosphate enters the urea cycle, with the excess nitrogen passed through a serious of intermediates via the actions of OTC, ASS, ASL, and ARG and then excreted as urea in urine. Ornithine is shuttled into the mitochondria via the action of the ORNT1 transporter. A complete or near-complete deficiency of any of the six enzymes or the transporter can result in hyperammonemia and its clinical sequelae. Adapted from https://ravicti.eu/this-is-a-ucd/.

addition, a UCD can arise from defects in the mitochondrial ornithine transporter (ORNT1), encoded by *SLC25A15*.

Unmet clinical need

Severe UCD patients typically present as neonates and have a profound decrease in enzymatic function in any one of the six enzymes of the urea cycle, or a lack of function of a transporter that carries urea cycle intermediates. This results in toxic accumulation of ammonia in the blood and accumulation of specific urea cycle amino acids that aid in diagnoses and therapeutic monitoring. Patients are at risk of developing extreme hyperammonemia that can lead acutely to coma and death (Blair et al., 2015) and chronically to profound neurologic dysfunction. Current UCD medical management includes lifelong dietary protein restriction with administration of medical formulas and nitrogen scavenger medications (**Table 5**). While these standard-of-care medical interventions reduce the risk of dangerous hyperammonemic crises, they are insufficient to protect most UCD patients, who experience recurrent episodes with any minor physiologic stressors (Choi et al., 2022). Each hyperammonemic episode is associated with a high risk of permanent neurologic damage and death. It is estimated that 50% of infants with infantile-onset UCDs die in early infancy (Nettesheim et al., 2017).

Table 5. Current supportive therapy for UCDs.

Therapeutic aim	Chronic management	Emergent management during decompensations
Reduce toxic ammonia accumulation through scavenger therapy or direct removal	• Oral glycerol phenylbutyrate (Ravicti) • Liver transplantation after patient is large enough	• Intravenous (IV) sodium phenylacetate / sodium benzoate (Ammonul) • Dialysis (if needed)
Prevent fasting or inadequate caloric intake, which triggers catabolism of endogenous protein	• Frequent feeds, avoid fasting • Closely monitor caloric need and provide fats and carbohydrates as extra calories as needed	• High-dextrose IV fluids • IV lipids
Limit protein intake while avoiding any essential amino acid deficiencies, which trigger catabolism of endogenous protein	• Carefully provide non-essential amino-acid-free medical formula (i.e., "incomplete protein") to maintain normal essential amino acid levels • Strictly limit intake of natural (i.e., "complete") protein • Supplement specific urea cycle amino acids that are lacking in certain UCDs, such as citrulline in OTC deficiency	• Stop all protein intake for a limited period of time • Provide IV arginine (for all UCDs except arginase deficiency), an essential amino acid generated in the urea cycle

Given that medical measures are not very effective, liver transplantation has become the standard of care for severely affected patients at many institutions (Leonard & McKiernan, 2004; Pritchard et al., 2022; García Vega et al., 2023). As the urea cycle occurs entirely within the liver, transplantation can normalize the metabolic defect. However, transplantation is often delayed by donor availability and the need for neonates to grow to an appropriate size for

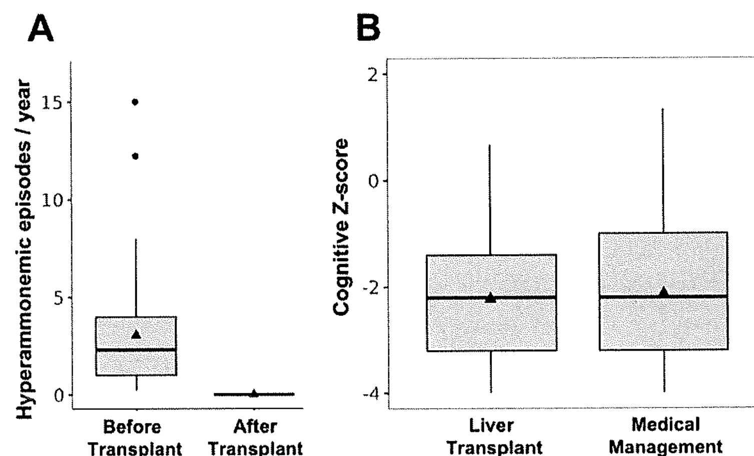


Figure 5. Severe urea cycle disorder patients have a high risk of repeat hyperammonemia and neurologic injury despite the standard of care. **(A)** Hyperammonemic episodes occurred frequently in severe UCD patients before liver transplantation but resolved after transplantation. **(B)** Transplantation did not improve cognitive outcomes as compared to medical management, likely because of neurologic injury that occurred prior to transplantation. Data are shown as medians (thick line) and means (triangle). The length of the box represents the interquartile range. Each point represents an outlier. Adapted from Posset et al., 2024.

transplantation (Yamamoto et al., 2019). Patients often are not large enough to be transplant candidates until after their first birthday. During this first year of life, most patients experience irreversible neurologic damage, even with close medical management (Posset et al., 2024) (**Figure 5**). Liver transplantation is also associated with acute and chronic risks and challenges—perioperative and postoperative complications including mortality, risk of rejection of the transplanted organ, and increased risk of infections and cancer due to the lifelong need for immunosuppressive therapy.

Treatment with CHOP.UCD.PE may result in the same metabolic correction as liver transplantation, but without the risks of transplantation. It may also be given earlier in the disease course, resulting in much better long-term outcomes.

Population with potential for benefit

While all UCDs can present as late-onset, attenuated forms, the most severe infantile-onset patients have the greatest potential for benefit from the CHOP.UCD.PE treatment, given their high risk of neurologic injury and death. Hundreds of mutations in UCD genes annotated as pathogenic or likely pathogenic have been cataloged in UCD patients, with a large subset being single-nucleotide changes or small indel mutations. Each of these single-nucleotide and indel mutations is potentially amenable to correction by prime editing due to the versatility of the technology (Anzalone et al., 2019). In the vast majority of UCD patients, the disease-causing mutations are relatively unique, i.e., *N*-of-1 or *N*-of-few, which requires a high degree of **personalization** to individual patients. Notwithstanding the challenge of deploying bespoke variants of the CHOP.UCD.PE treatment in a short enough timeframe to be of maximal benefit for patients with infantile-onset UCDs—ideally, within several months after birth or the initial diagnosis—many UCD patients stand to benefit from **personalized** corrective editing treatments. The seven diseases that are the focus of the primary IND application for the CHOP.UCD.PE treatment and the subsequent gene-specific IND applications are:

- Citrullinemia type 1, which has an estimated incidence of 1:250,000 (Summar et al., 2013). In the cytoplasm, ASS catalyzes the third step of the urea cycle, conversion of citrulline and aspartate into argininosuccinate. There is a recurrent pathogenic mutation in

the *ASS1* gene that has been reported in multiple populations around the world, the G390R mutation (Engel et al., 2009). For the initial IND application, the Sponsor proposes a variant of the CHOP.UCD.PE treatment that can correct the *ASS1* G390R mutation, and representative patient cases are presented below.

- Carbamoyl phosphate synthetase 1 (CPS1) deficiency, which has an estimated incidence of 1:1,300,000 (Summar et al., 2013). In the mitochondria, CPS1 catalyzes the first rate-limiting step of the urea cycle, the condensation of ammonia and bicarbonate to form carbamoyl phosphate. There are no highly recurrent pathogenic mutations in *CPS1*.
- Ornithine transcarbamylase (OTC) deficiency, which has an estimated incidence of 1:56,500 (Summar et al., 2013). In the mitochondria, OTC catalyzes the second step of the urea cycle, conversion of carbamoyl phosphate and ornithine to citrulline, which is then transported out of the mitochondria. There are no highly recurrent pathogenic mutations in *OTC*. Unlike the other UCD genes—which cause disease in an autosomal recessive fashion—*OTC* resides on the X chromosome, and male patients with a single pathogenic allele manifest the disease, whereas female patients with a single pathogenic allele have variable penetrance.
- Argininosuccinate lyase (ASL) deficiency, also known as argininosuccinic aciduria, which has an estimated incidence of 1:218,750 (Summar et al., 2013). In the cytoplasm, ASL catalyzes the fourth step of the urea cycle, conversion of argininosuccinate into arginine and fumarate. There are no highly recurrent pathogenic mutations in the *ASL* gene, although several local founder mutations have been reported (Balmer et al., 2014).
- Arginase deficiency, which has an estimated incidence of 1:950,000 (Summar et al., 2013). In the cytoplasm, arginase catalyzes the fifth step of the urea cycle, conversion of arginine into urea and ornithine, the former of which is excreted in urine and the latter of which is transported into the mitochondria. While at a population level arginase deficiency patients have a lower risk of hyperammonemic crises, there are reports of severe neonatal-onset cases. There are no highly recurrent pathogenic mutations in *ARG*.
- *N*-acetylglutamate synthetase (NAGS) deficiency, which has an estimated incidence of <1:2,000,000 (Summar et al., 2013). In the mitochondria, NAGS synthesizes *N*-acetylglutamate, which is an essential cofactor of CPS1. There are no highly recurrent pathogenic mutations in *NAGS*.
- Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome, also known as ornithine translocase deficiency, which has an estimated incidence of <1:2,000,000 (Summar et al., 2013). HHH arises from pathogenic mutations in *SLC25A15*, which encodes the transporter that shuttles ornithine from the cytoplasm into the mitochondria.

The Sponsor is proposing to treat patients [redacted] with infantile-onset UCDs presenting with hyperammonemic crises.

Therapeutic Rationale

Rationale for the liver as the target organ

The urea cycle is largely active in hepatocytes, especially periportal hepatocytes, and correction of the primary genetic defect in a UCD patient solely within the liver via organ transplantation is curative for hyperammonemia (see **Figure 5**). Natural history studies of UCD patients demonstrate two clinical presentations: infantile-onset (often neonatal-onset) disease, in which hyperammonemia that can cause irreversible brain injury can occur within a few days of birth, with high morbidity and mortality; and late-onset disease, with moderate or mild symptoms that emerge later in childhood or adulthood and are elicited by protein-rich meals or situations of high metabolic demand.

Analyses of correlations between residual enzyme activity and severity of disease, performed by the Urea Cycle Disorders Consortium (UCDC) and the European Registry and Network for Intoxication Type Metabolic Diseases (E-IMD) Consortia Study Group, have been informative

for several UCDs. **For patients with citrullinemia type 1, a threshold of 8.1% residual ASS enzymatic activity distinguishes severe disease from attenuated disease** (Zielonka et al., 2019). For male patients with OTC deficiency, a threshold of 4.3% residual OTC enzymatic activity distinguishes severe disease from attenuated disease (Scharre et al., 2022). For patients with ASL deficiency, a threshold of 7.9% residual ASL enzymatic activity distinguishes severe disease from attenuated disease (Zielonka et al., 2020).

Infantile-onset CPS1 deficiency is generally associated with less than 5% of the normal CPS1 activity in liver, whereas late-onset CPS1 disease is associated with higher residual activity (Martínez et al, 2010). Among a cohort of Japanese patients with CPS1 deficiency, one patient with late-onset disease (13 years of age) was observed to have 4.8% residual hepatic enzymatic activity, and another patient with genetically confirmed late-onset disease (13 years of age) had 17% residual activity (Kurokawa et al., 2007). In the same cohort, three patients with genetically confirmed infantile-onset disease had 6%, 6.25%, and 11% residual activity, respectively. By extrapolation, achieving correction of ≈10-15% of the *CPS1* alleles in the hepatocytes of infantile-onset CPS1 deficiency patients with no residual activity (i.e., complete loss of function) should ameliorate disease phenotypes in these patients.

An independent body of evidence for therapeutic editing thresholds in UCDs comes from preclinical studies, i.e., functional rescue of phenotypes in UCD mouse models. Using a floxed *Ass1* mouse model in which liver-directed AAV-Cre was used to tune the level of gene knockdown, the Sponsor found that 5% residual expression of *Ass1* is lethal, whereas 10-15% residual expression keeps some mice alive (**Figure 6**), **suggesting a therapeutic hepatocyte allelic editing threshold of ≈10-15% to ameliorate disease phenotypes in citrullinemia type 1 patients**.

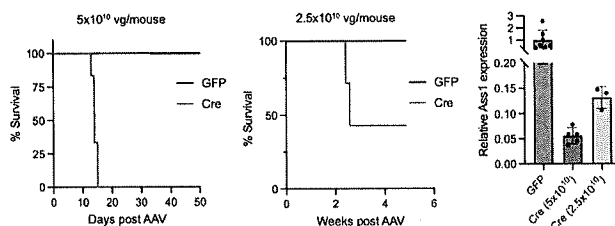


Figure 6. *Ass1* expression and mouse survival. 5% expression is lethal; 10-15% keeps some mice alive.

In a floxed *Cps1* mouse model in which liver-directed AAV-Cre was used to achieve gene knockdown, resulting in hyperammonemia and death soon after treatment, liver-directed AAV expression of CPS1 that achieved 15% of the wild-type level of protein activity resulted in reduced plasma ammonia levels and promoted survival (Nitzahn et al., 2020). These observations are concordant with the therapeutic editing threshold of ≈10-15% extrapolated from human genetics.

In the *spf^ash* mouse model, which has a hypomorphic *Otc* allele and which experiences hyperammonemia and death when challenged with a high-protein diet, liver-directed AAV-mediated homology-directed repair (HDR) corrective editing of the pathogenic allele in neonatal mice resulted in reduced plasma ammonia levels and promoted survival on a high-protein diet (Yang et al., 2016). The mean HDR editing of 10% resulted in ≈15-20% of the wild-type level of OTC protein activity, with the baseline level of enzymatic activity in *spf^ash* being ≈5%, suggesting a therapeutic hepatocyte allelic editing threshold of ≈15% to ameliorate disease phenotypes even in patients with complete OTC deficiency.

In an *Asl* mouse model that dies shortly after birth, liver-directed AAV expression of ASL that achieved 5-10% of the wild-type level of enzymatic activity significantly improved life

expectancy (Ashley et al., 2018), suggesting a therapeutic hepatocyte allelic editing threshold of ≈10% to ameliorate disease phenotypes in ASL deficiency patients.

In an *Arg* knockout mouse model of arginase deficiency that has hyperammonemia and dies shortly after birth, adenoviral expression of arginase normalized plasma ammonia levels and promoted survival when the liver arginase enzymatic activity was ≈20% that of control heterozygous mice but not when the activity was ≈10% that of heterozygous mice (Gau et al., 2009). These observations suggest a therapeutic hepatocyte allelic editing threshold of ≈10% to ameliorate disease phenotypes in arginase deficiency patients.

In summary, the clinical and preclinical evidence point to **≈10-15% corrective editing in hepatocytes generally being of therapeutic value across the UCDs**.

Rationale for a prime editing strategy

With the CHOP.UCD.PE treatment, the Sponsor plans to use prime editing that introduces a corrective change in the genome, in a highly specific manner, at the site of any of a collection of pathogenic mutations in genes encoding enzymes involved in the urea cycle, namely *ASS1*, *CPS1*, *OTC*, *ASL*, *ARG*, *NAGS*, and *SLC25A15*. The effect of reverting one of these mutations to wild-type would be to restore functionality to the protein product, i.e., the enzyme or transporter, durably reducing and even normalizing blood ammonia levels in a patient with at least one copy of the mutation in question.

The CHOP.UCD.PE treatment will comprise two drug products (DPs). The first DP, LNP.UCD.PE, will comprise lipid nanoparticles (LNPs) encapsulating an mRNA encoding a PE6c prime editing protein, in liquid form for intravenous (IV) infusion and delivery to the hepatocytes in the liver. The second DP, AAV.UCD.PE, will comprise an adeno-associated viral (AAV) vector encoding prime editing guide RNAs (pegRNAs) and nicking guide RNAs (ngRNAs) targeting the site(s) of either one or two pathogenic mutations in the *ASS1*, *CPS1*, *OTC*, *ASL*, *ARG*, *NAGS*, or *SLC25A15* gene, in liquid form for IV infusion and delivery to the hepatocytes in the liver.

The mechanism of action of the CHOP.UCD.PE treatment is as follows:

(1) The AAV vector (AAV.UCD.PE DP) will be administered via IV infusion and internalized by the hepatocytes, leading to the entry of vector genomes into the nucleus, synthesis of second DNA strands, formation of stable episomes, and expression of the encoded pegRNA/ngRNA cassettes. This process takes roughly two weeks after administration of the AAV vectors to achieve full expression. The pegRNA/ngRNA expression will persist as long as the episomes are present in the hepatocytes, the durability of which is dependent on the rate of proliferation of the hepatocytes in the treated subject but is expected to be at least a few months if not years. Comprehensive nonclinical data have shown that **the AAV characteristics of the DP drive the biodistribution profile of the DP**.

(2) The LNPs (LNP.UCD.PE DP) will be administered via IV infusion at least two weeks following the administration of the AAV vectors. Because of the persistence of pegRNA/ngRNA expression from AAV episomes, there is the possibility of administering repeat LNP doses to increase the overall proportion of corrective editing in the hepatocytes in the treated subject.

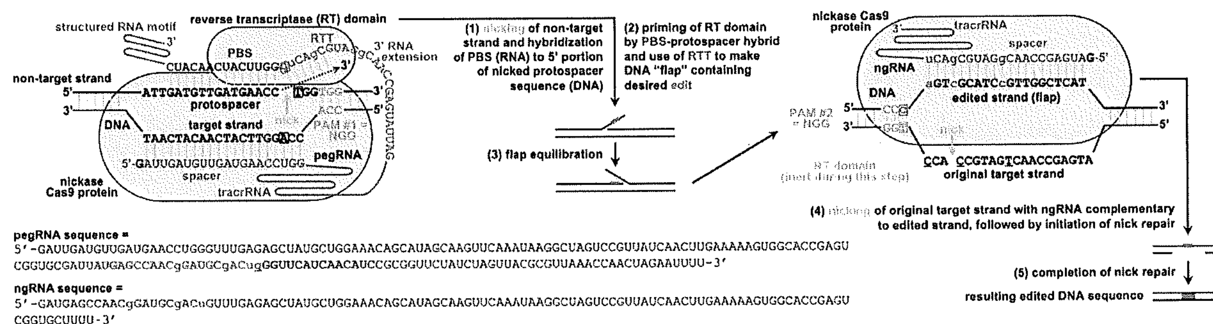


Figure 7. Schematic of the stepwise mechanism of prime editing. As an illustrative example, prime editing with a pegRNA and ngRNA optimized for correction of the *ASS1* G390R mutation is shown. Lowercase letters indicate the intended edits (corrective edit and additional synonymous edits), and boxes indicate the position of the pathogenic variant intended to be corrected to wild-type. Purple text = spacer sequence, magenta text = protospacer-adjacent motif (PAM) sequence, red text = reverse transcriptase template (RTT) sequence, blue text = primer binding site (PBS) sequence, and orange arrows = nick sites.

(3) The LNPs will be internalized by the hepatocytes following binding of endogenous apolipoprotein E (apoE) to the LNP, leading to engagement of low-density lipoprotein receptor (LDLR) on the plasma membrane and subsequent receptor-mediated endocytosis of the LNP carrying the mRNA. Comprehensive nonclinical data have shown that **the LNP characteristics of the DP drive the biodistribution profile of the DP**.

(4) After LNP-bound apoE engages with LDLR, endocytosis of the LNP occurs. The subsequent reduction in endosomal pH results in a charge-based interaction of the ionizable lipid with the endosomal membrane, which ultimately results in the release of the mRNA cargo into the cytoplasm (Akinc et al., 2010; Kulkarni et al, 2018).

(5) The mRNA will be translated into a prime editor protein (e.g., PE6c), which comprises a catalytically impaired Cas9 nuclease domain (that functions as a single-strand nickase and minimizes the production of double-strand breaks) fused with a reverse transcriptase (RT) domain (Anzalone et al., 2019) (**Figure** 7).

(6) The pegRNA comprises a tracrRNA domain that complexes with the Cas9 nickase domain and a spacer sequence that corresponds to a protospacer DNA sequence matching the region of the UCD gene spanning the targeted mutation. The protospacer is located immediately upstream of the PAM, which is required for prime editing activity. **Each protospacer is chosen to be unique in the genome, enabling a highly specific pegRNA that would not bind efficiently elsewhere in the genome and that would thus minimize off-target editing.**

(7) Base pairing between the spacer sequence of the pegRNA and the target DNA sequence will result in displacement of the PAM-containing genomic DNA strand to form a single-stranded DNA R-loop, which is nicked by the Cas9 domain (**Figure** 7).

(8) Base pairing of the primer binding site (PBS) of the pegRNA and the 5′ portion of the nicked protospacer sequence will result in a double-strand structure that "primes" the RT domain for reverse transcription, positioning the adjacently located reverse

transcriptase template (RTT) of the pegRNA to serve as the template for synthesis of a new DNA "flap". The flap spans the entirety of the RTT sequence, after which the RT domain runs into the tracrRNA portion of the pegRNA complexed to the Cas9 protein, preventing further reverse transcription (**Figure 7**).

(9) By design, the RTT contains the desired edit, i.e., correction of the pathogenic mutation to wild-type. The RTT may also contain additional edits, i.e., synonymous changes, that inhibit mismatch repair (MMR) at the next stage of the process.

(10) The new edited DNA flap can compete with the single-strand DNA with the 3′ portion of the nicked protospacer sequence, displacing the latter from hybridization with the target strand. The edited DNA flap can itself hybridize with the target strand, albeit with a mismatch(es). If there is minimal mismatching, the cell's MMR mechanism will favor retention of the original unedited single-strand DNA and elimination of the edited DNA flap. With additional mismatches, MMR is inhibited, which increases the retention of the edited DNA flap in the double-strand DNA and elimination of the original single-strand DNA (Chen et al., 2021) (**Figure 7**).

(11) The presence of the ngRNA further favors the retention of the edited DNA strand. The ngRNA comprises a tracrRNA domain that complexes with the Cas9 nickase domain and a spacer sequence that is perfectly complementary to the edited DNA strand, but not to the original unedited single-strand DNA. **The protospacer is chosen to be unique in the genome, enabling a highly specific ngRNA that would not bind efficiently to the unedited UCD gene locus or elsewhere in the genome and that would thus minimize off-target editing.**

(12) Once engaged by the ngRNA, the Cas9 domain will nick the original target strand to induce a DNA repair mechanism that uses the edited strand as the template for DNA repair, resulting in both DNA strands now having the desired corrective edit (**Figure 7**).

(13) The AAV.UCD.PE DP could potentially encode two different pegRNA/ngRNA pairs that, in combination with the same prime editing protein, each correct a distinct pathogenic allele, allowing for the **simultaneous correction of both pathogenic alleles in a compound heterozygous patient**.

(14) Corrected alleles will restore activity of the urea cycle, which will convert ammonia to urea and would be expected to reduce fasting plasma ammonia levels.

Rationale for clinical trial design

As the study design and major endpoints would be identical for an individual trial of any of the variants of the CHOP.UCD.PE treatment, regardless of the UCD gene mutation targeted for correction, the Sponsor is proposing an **umbrella clinical trial design** (Woodcock & LaVange, 2017; Ahrens-Nicklas & Musunuru, 2025) with the CHOP.UCD.PE treatment. Specifically, the Sponsor's proposed Phase I/II first-in-human (FIH) clinical study plans to enroll infantile-onset UCD patients [redacted], with at least one copy of one of the targetable UCD gene mutations (e.g., *ASS1* G390R mutation) resulting in hyperammonemia, in an umbrella trial design. The proposed design is provided in **Section 12, Clinical Program Overview**.

Status of Product Development

The Sponsor has established that a certain combination of PE6c, a pegRNA, and a ngRNA can efficiently and specifically correct an exemplary pathogenic UCD gene mutation—namely, the *ASS1* G390R mutation—to wild-type in human hepatocytes *in vitro* and in mutation-humanized mice *in vivo*. A description of the studies performed to date are provided in this section, below. The design of the proposed IND-enabling studies, including the definitive biodistribution and toxicology animal study and the off-target analyses, are provided in **Section 10, Proposed Nonclinical Studies**. The planned chemistry, manufacturing, and controls (CMC) are described in **Section 11, Chemistry, Manufacturing, and Controls**. The proposed clinical study design is described in **Section 12, Clinical Program Overview**.

Prime editor screening in a cell model with UCD gene mutations

A challenge in developing corrective editing therapies is the lack of readily available *in vitro* models harboring rare patient-specific mutations in which to test the efficacy of drug candidates. Accordingly, the Sponsor sought to generate human hepatocyte cell lines bearing patient-specific mutations, using cultured HuH-7 hepatoma cells, a commonly used proxy for primary human hepatocytes (which can only be maintained in culture for several days).

The Sponsor found that techniques like nuclease-mediated HDR editing and prime editing do **not** reliably allow for generation of such cell lines for all mutations. Moreover, one disadvantage of using clonal cell lines derived from single cells of a transformed cell line like HuH-7 is that there can be substantial heterogeneity among different clonal cell lines in various characteristics, including transfectability with either plasmids or LNPs. Thus, the ability to make head-to-head comparative assessments of editing efficiencies for different mutations, across different cell lines, is compromised. (For example, it would not be possible to reliably compare the efficiency of an editing solution for mutation #1 in one cell line with the efficiency of an editing solution for mutation #2 in another cell line, to determine which editing solution is more potent.) Another disadvantage is that even when successful, generating a clonal edited HuH-7 cell line can take several months, time that cannot be afforded for a patient with an infantile-onset UCD who is at ongoing risk for permanent neurologic injury and even death with any hyperammonemic crisis.

Accordingly, the Sponsor has adopted an alternative approach, taking only a few weeks, in which multiple mutations are introduced into the same HuH-7 cells. This is achieved using a lentiviral vector with genomic sequences spanning individual mutations (**Figure 8**). For example, the lentiviral vector might have a ≈100-bp *ASS1* genomic sequence spanning the G390R mutation, a ≈100-bp genomic sequence spanning a second *ASS1* mutation, and ≈100-bp genomic sequences spanning additional UCD gene mutations. The lentivirus is used to transduce HuH-7 cells.

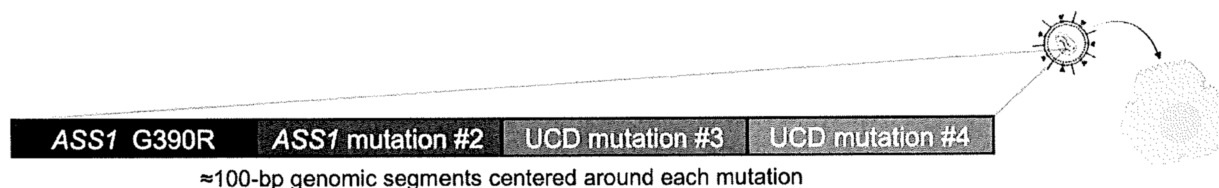


Figure 8. Lentivirus-transduced HuH-7 cell line. Schematic showing how a prototypic lentiviral vector might be used to transduce human HuH-7 hepatoma cells with a cassette comprising four adjacent ≈100-bp genomic segments with four UCD gene mutations including *ASS1* G390R.

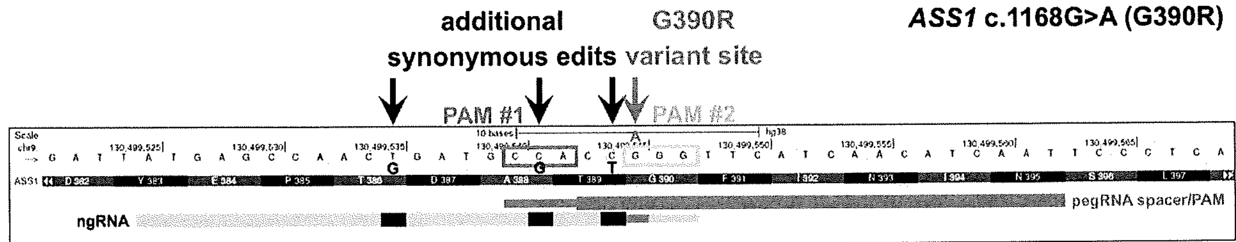


Figure 9. Schematic of the genomic site of the *ASS1* G390R mutation. Adapted from the UCSC Genome Browser (GRCh38/hg38). The red arrow and the vertical yellow bar indicate the position of the G altered to A (letter in red) by the G390R variant on the sense strand. The black arrows indicate the sites of additional synonymous edits introduced by the intended prime editing (letters in black). The red horizontal bar indicates the protospacer (thick) and PAM (thin) sequences targeted by pegRNA. The grey horizontal bar indicates the protospacer (thick) and PAM (thin) sequences targeted by ngRNA, with matches to the edits introduced by the pegRNA indicated by the black and red portions of the bar (i.e., the ngRNA does not match the wild-type sequence at the black positions). The red box indicates the PAM sequence of the pegRNA (oriented on the antisense strand), and the grey box indicates the PAM sequence of the ngRNA (oriented on the sense strand).

To expeditiously identify a prime editing solution for the *ASS1* G390R mutation (**Figure 9**), the Sponsor generated a lentivirus-transduced HuH-7 cell line harboring the mutation. (Multiple attempts to use prime editing to cleanly introduce the mutation into the endogenous *ASS1* locus

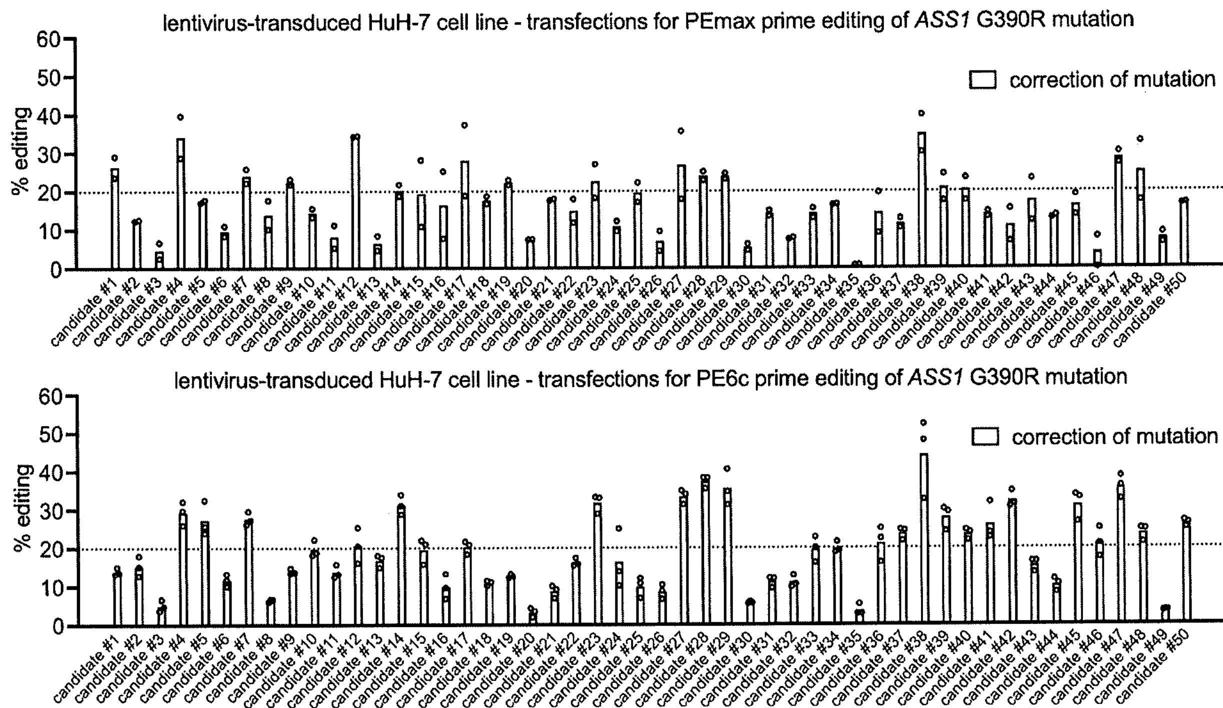


Figure 10. Screening of candidate prime editing solutions for the correction of the *ASS1* G390R mutation. Corrective editing (determined from NGS of genomic DNA) upon transfection of G390R lentivirus-transduced HuH-7 cells with plasmids encoding PEmax or PE6c prime editor and the candidate pegRNA/ngRNA pairs, calculated as the proportion of aligned sequencing reads with the corrective edit ($n = 2$ to 3 biological replicates). The dotted lines are set at 20%, a threshold consistent with the amount of corrective editing in hepatocytes projected to have a therapeutic effect in a patient with citrullinemia type 1 and a single *ASS1* G390R allele.

in HuH-7 cells were unsuccessful.) Using the G390R lentivirus-transduced HuH-7 cell line, the Sponsor screened a variety of pegRNA/ngRNA pairs in combination with either the PEmax prime editor or the PE6c prime editor in plasmid transfection experiments. The candidate pegRNAs were nominated by a machine learning algorithm, with the pegRNAs varying by the PBS length, RTT length, and the number and identity of synonymous edits that would be introduced by the intended prime editing. For the top 50 candidate pegRNAs, ngRNAs were designed against sequences with the intended prime editing. In general, prime editing was more efficient with PE6c compared to PEmax (**Figure 10**). With either prime editor, candidate #38 had the most efficient corrective editing and was chosen for further development—although other candidates had substantial editing and would also be credible therapeutic solutions.

Validation in a humanized mouse model

To perform *in vivo* testing of the CHOP.UCD.PE strategy, the Sponsor wished to generate humanized mice with the *ASS1* G390R mutation so that the lead PE6c/pegRNA/ngRNA set and, ultimately, **<u>the investigational DPs can be directly tested *in vivo*</u>**, rather than using mouse-specific surrogate products.

The Sponsor generated a humanized knock-in mouse model in the C57BL/6J background by knocking human *ASS1* exon 14 with the c.1168G>A (G390R) point mutation and approximately 250 bp flanking intronic genomic sequence (150 bp in the 5′ direction, 100 bp in the 3′ direction) in place of the corresponding orthologous genomic region of the murine *Ass1* gene (exon 15 and flanking intronic genomic sequence) via gene targeting in C57BL/6J mouse embryonic stem (ES) cells (**Figure 11**). Upon generating and confirming correctly targeted ES cells, the ES cells were injected into blastocysts, resulting in male chimeric mice that were bred to generate F1 offspring. F1 offspring with confirmed germline transmission, i.e., heterozygous for the knock-in

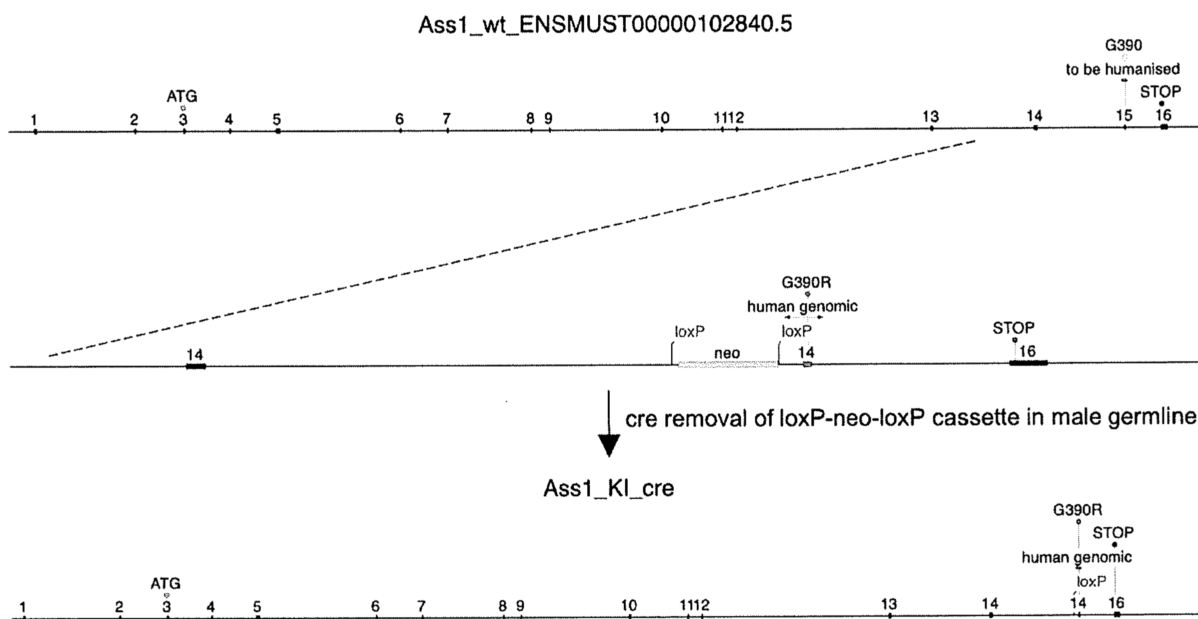


Figure 11. Generation of $ASS1^{G390R}$/+ mice. Schematic of the strategy used to generate humanized knock-in mice with the *ASS1* G390R mutation, via homologous recombination in mouse ES cells, blastocyst injections, and breeding of male chimeric mice to obtain heterozygous $ASS1^{G390R}$/+ mice.

allele, were bred to expand the colony. The Sponsor observed that no mice homozygous for the knock-in allele survived longer than 24 hours after birth, consistent with past efforts to generate *Ass1* knockout mice (Patejunas et al.,1994) as well as other mouse models of neonatal-onset UCDs (Schofield et al., 1999; Reid Sutton et al., 2003; Senkevitch et al., 2012; Wang et al., 2017; Khoja et al., 2019). This phenomenon of perinatal lethality across UCD genes **makes it prohibitive to use UCD mice to model biological responses to DPs** with respect to functional endpoints (i.e., prolonged survival, reduced blood ammonia levels, etc.). Accordingly, the Sponsor holds that the only use of mouse models would be to demonstrate ***in vivo* hepatic editing efficiency** of DPs. For this reason, the Sponsor used heterozygous knock-in mice (designated $ASS1^{G390R}$/+ mice) for studies of *in vivo* prime editing.

The Sponsor subsequently performed dose-response studies of the AAV/LNP treatment in $ASS1^{G390R}$/+ mice. The AAV vector encoded two copies each of the lead pegRNA and lead ngRNA (candidate #38 in **Figure 10**), and the LNPs contained PE6c mRNA. **The AAV vector and mRNA/LNPs were research-grade versions of the intended AAV.UCD.PE and LNP.UCD.PE DPs.** The AAV vector was administered first in mice approximately six weeks of age, followed by the LNPs two weeks later. In the initial study, the AAV dose was varied, and the LNP dose was held constant at 3 mg/kg. The mean whole-liver corrective editing of the *ASS1* G390R mutation in each dose group was ≈30–40% (**Figure 12A**), suggesting that the LNPs were the limiting factor. Next-generation sequencing (NGS) demonstrated precise prime editing, with the expected additional synonymous edits observed along with the desired corrective editing (**Figure 13**). In the next study, the LNP dose was varied, and the AAV dose was held constant at 1×10^{11} vg (the lowest dose tested in the initial study). There was an unambiguous LNP dose dependence for corrective editing efficiency (**Figure 12B**). In all the conditions, editing at or above the estimated editing threshold needed for clinical benefit for a patient with citrullinemia type 1 and homozygous for the *ASS1* G390R mutation (such as **Representative Cases 1 and 2** described in **Section 6, History of the Project/Background, Patient Cases Amenable to Treatment Via Corrective Editing by CHOP.UCD.PE**) was observed.

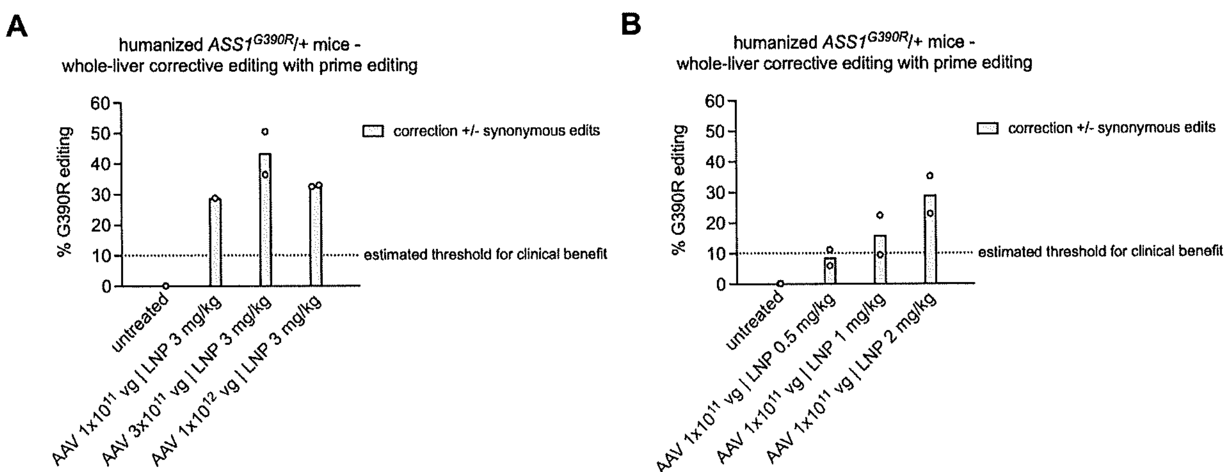


Figure 12. Whole-liver corrective editing in $ASS1^{G390R}$/+ mice. Corrective editing with or without synonymous editing of the endogenous G390R mutation sequence in $ASS1^{G390R}$/+ mice, upon an initial single treatment with the AAV vector at the specified dose, followed by a single treatment with the LNPs at the specified dose 2 weeks after the AAV treatment (*n* = 2 liver samples per mouse in mice initially treated at ≈6 weeks of age, with necropsy 1 week after the LNP treatment). In **(A)** the AAV dose was varied and the LNP dose was held constant; in **(B)** the LNP dose was varied and the AAV dose was held constant.

Editing in whole liver in mouse treated with AAV-pegRNA/ngRNA +LNP-PE6c to correct *ASS1* G390R

T386 D387 A388 T389 390R baseline/reference identity of *ASS1* codons

T386 D387 A388 T389 G390 codons resulting from prime editing

T G A G C C A A C T G A T G C C A C C A G G T T C A T C A A C A T C A A T T C C - Reference (G390R)

sense strand of *ASS1* — pegRNA spacer/PAM

– T G A G C C A A C T G A T G C C A C C A G G T T C A T C A A C A T C A A T T C C - 43.90% (2155 reads)
+ T G A G C C A A C G G A T G C G A C T G G G T T C A T C A A C A T C A A T T C C - 43.19% (2120 reads)
+ T G A G C C A A C T G A T G C G A C T G G G T T C A T C A A C A T C A A T T C C - 7.60% (373 reads)
+ T G A G C C A A C G G A T G C T A C T G G G T T C A T C A A C A T C A A T T C C - 0.37% (18 reads)
– T G A G C C A A C T G A T G C G A C T A G G T T C A T C A A C A T C A A T T C C - 0.29% (14 reads)
– T G A G C C A A C G G A T G C C A C C A G G T T C A T C A A C A T C A A T T C C - 0.22% (11 reads)

+ = **reads with** ☐ corrective edit +/– ☐ synonymous bystander edit(s)
× = **reads with** ☐ corrective edit + ☐ **nonsynonymous bystander edit (no examples observed here)**
– = **reads with no corrective edit +/–** ☐ synonymous bystander edit(s)

Figure 13. Corrective prime editing of the *ASS1* G390R mutation after AAV/LNP treatment of *ASS1*^{G390R}/+ mice. Standard CRISPResso (http://crispresso2.pinellolab.org/submission) NGS output for the editing in the liver sample from the AAV/LNP-treated mouse that displayed the highest level of editing in **Figure 12A** (in the next-to-last bar in the graph). The codons in the vicinity of the G390R site are indicated; the top-listed amino acid is the baseline/reference identity of the codon, and the bottom-listed amino acid is the one that results from prime editing. The red horizontal bar indicates the spacer/protospacer sequence of the pegRNA, and the adjacent thin red box indicates the PAM sequence.

7. MEETING PURPOSE

The purpose of the meeting is to seek input on the summarized nonclinical proof-of-concept and efficacy data; the proposed definitive animal study; the proposed assessment of potential off-target editing; the proposed chemistry, manufacturing, and controls; and the proposed clinical study. The objectives of the meeting are to receive advice from the Agency as summarized in the enclosed questions.

8. PROPOSED AGENDA

The Sponsor's proposed agenda is presented below.

Topic	Estimated Duration
Introductions	5 minutes
Discussion of questions	50 minutes
Summary and review of action items	5 minutes

9. LIST OF QUESTIONS, GROUPED BY DISCIPLINE

Nonclinical

Question #1: Does the Agency agree that cellular studies, rather than humanized mouse studies, will provide sufficient proof-of-concept (POC) data to support the administration of each variant of the CHOP.UCD.PE treatment to infantile-onset UCD patients?

Question #2: Does the Agency agree that the proposed definitive toxicology study of one variant of the CHOP.UCD.PE treatment (AAV.UCD.PE and LNP.UCD.PE DPs) in wild-type nonhuman primates (NHPs) and existing biodistribution data from prior studies of AAV8 and the proposed LNP formulation will provide sufficient data to support IND applications for all variants of the CHOP.UCD.PE treatment?

Question #3: Does the Agency agree that the proposed definitive toxicology study of one variant of the CHOP.UCD.PE treatment in wild-type NHPs will provide sufficient data to support re-dosing of patients with the LNP.UCD.PE DP component?

Question #4: Does the Agency agree that the proposed off-target editing studies of a given variant of the CHOP.UCD.PE treatment will provide sufficient data to support the administration of that variant of the CHOP.UCD.PE treatment to infantile-onset UCD patients?

Question #5: Does the Agency agree that the overall nonclinical development plan is sufficient to support IND applications for all variants of the CHOP.UCD.PE treatment?

Chemistry, Manufacturing, and Controls (CMC)

Question #6: Does the Agency agree that the proposed quality of materials and specifications for release testing of each mRNA DS, LNP.UCD.PE DP, and AAV.UCD.PE DP are acceptable to support IND applications for the CHOP.UCD.PE treatment?

Question #7: Does the Agency agree that the overall chemistry, manufacturing, and controls plan could be sufficient to support an accelerated approval of the CHOP.UCD.PE treatment?

Question #8: Does the Agency agree that the proposed potency assays for the CHOP.UCD.PE treatment are acceptable to support IND applications for all variants of the CHOP.UCD.PE treatment?

Clinical

Question #9: Does the Agency agree that the general design, including the proposed safety and exploratory efficacy outcome measures, enrollment criteria, data capture and monitoring plans, and long-term follow-up plan are appropriate for the umbrella trial protocol outlined in the protocol synopsis?

Question #10: Does the Agency agree that if there is a uniform response across subjects, the proposed umbrella trial in a limited number of subjects could be sufficient for an accelerated approval of the CHOP.UCD.PE treatment?

10. PROPOSED NONCLINICAL STUDIES

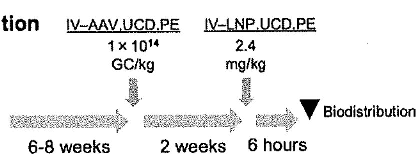
Question #1: Does the Agency agree that cellular studies, rather than humanized mouse studies, will provide sufficient proof-of-concept (POC) data to support the administration of each variant of the CHOP.UCD.PE treatment to infantile-onset UCD patients?

Question #2: Does the Agency agree that the proposed definitive toxicology study of one variant of the CHOP.UCD.PE treatment (AAV.UCD.PE and LNP.UCD.PE DPs) in wild-type nonhuman primates (NHPs) and existing biodistribution data from prior studies of AAV8 and the proposed LNP formulation will provide sufficient data to support IND applications for all variants of the CHOP.UCD.PE treatment?

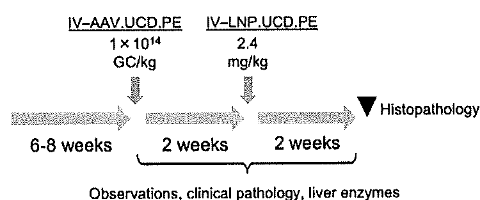
Question #3: Does the Agency agree that the proposed definitive toxicology study of one variant of the CHOP.UCD.PE treatment in wild-type NHPs will provide sufficient data to support re-dosing of patients with the LNP.UCD.PE DP component?

Non-GLP toxicology / biodistribution study in nonhuman primates
n **= 12**

Single dose, biodistribution
n **= 3**



Single dose, toxicology
n **= 6**



Repeat dose, toxicology
n **= 3**

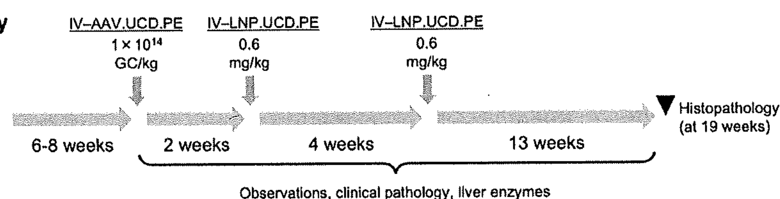


Figure 16. Schematic of proposed definitive NHP biodistribution and toxicology study. Green arrows indicate dosing; black arrowheads indicate necropsy. Not to scale.

Sponsor Position: The proposed definitive animal study (**Figure 16**, **Table 6**, and **Appendix 1 – Definitive Animal Study Synopsis**) has been designed specifically in accordance with the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)**, particularly **Section IV: Considerations for Nonclinical Studies.** In light of the Agency's feedback for a Pre-IND meeting for another of the Sponsor's corrective editing DPs [redacted], the Sponsor is proposing to assess the biodistribution and toxicology of the DP in a Good Laboratory Practice (GLP)-like definitive study in nonhuman primates (NHPs) (**Figure 16** and **Table 6**).

Table 6. Proposed definitive animal study for the CHOP.UCD.PE treatment.

Study Design	Dose Groups	Primary Assessments
GLP-like biodistribution/ toxicology study in wild-type NHPs, with a variant of the CHOP.UCD.PE treatment matched to the *ASS1* G390R mutation, using the AAV.UCD.PE DP and LNP.UCD.PE DP in tandem	Single 1×10^{14} AAV dose and single 2.4 mg/kg LNP dose biodistribution and toxicology groups, with doses separated by 2 weeks; single 1×10^{14} AAV dose, followed by two repeat 0.6 mg/kg LNP doses 4 weeks apart; vehicle control group; $n = 3$ animals per group	General safety, clinical observations including cardiac and respiratory rates, clinical pathology including liver function tests, lipid excipient levels, and anti-PEG and anti-Cas9 antibodies in blood at various timepoints up to necropsy; gross and histological pathology, and lipid excipient and mRNA quantification in liver and other organs at necropsy and for unscheduled deaths

A more comprehensive summary of the proposed definitive NHP biodistribution/toxicology study is available in **Appendix 1 – Definitive Animal Study Synopsis**.

Because there are no rat or NHP models of UCDs, particularly models with patient-specific UCD gene mutations, studies with wild-type rats or NHPs would have limited utility for assessing on-target editing efficiency of the CHOP.UCD.PE treatment. The Sponsor proposes to assess **biodistribution and toxicology in NHPs only**. Limiting the proposed biodistribution/toxicology study to a single species **respects the Replacement, Reduction, and Refinement framework to minimize animal use**.

As the biodistribution and toxicology of AAV8 vectors have been thoroughly characterized in past preclinical studies (see **Table 7** below) and AAV8 vectors have been used in a variety of clinical DPs (Nathwani et al., 2011b; Nathwani et al., 2014; Konkle et al., 2021; D'Antiga et al., 2023), the Sponsor holds that assessment of the AAV.UCD.PE DP **alone** would be redundant. Rather, the focus of the proposed definitive biodistribution and toxicology study is on the LNP.UCD.PE DP, in combination with the AAV.UCD.PE DP.

Furthermore, the Sponsor holds that performing the proposed definitive NHP biodistribution/ toxicology study with **one variant** of the CHOP.UCD.PE treatment—with the variant of the

treatment (AAV.UCD.PE and LNP.UCD.PE) matched to the *ASS1* G390R mutation—is sufficient to support the primary IND application for **all variants** of the CHOP.UCD.PE treatment targeting pathogenic *ASS1* mutations and to support subsequent gene-specific IND applications for **all variants** of the CHOP.UCD.PE treatment targeting pathogenic mutations in the other UCD genes.

All variants of the LNP.UCD.PE DP will be nearly identical. All will be formulated in the same way using **identical lipid excipients, which drive the biodistribution and toxicology**. The only distinction will be in the mRNA component. The PE6c, SpG-PE6c, and SpRY-PE6c mRNA components, shown in **Table 1**, are very similar. The full-length mRNAs, spanning the coding sequence, the 5′ and 3′ untranslated regions, and the 3′ polyadenylate sequence, are identical in length at about 5.2 kilobases. These mRNAs differ within the Cas9 coding portion by at most 22 bases distributed throughout the Cas9 sequence; the remainder of the sequence, including the RT portion, is identical. Thus, a maximum of 22 out of ≈5200 positions in the mRNAs differ (≥99.6% identity). The difference in the mRNAs is not expected to affect the toxicology of the DP. **The Sponsor will confirm that the physiochemistry of the LNP DP is not affected by a change in the mRNA component.**

Across different AAV.UCD.PE DP variants, each ngRNA varies in its sequence in only 20 of 111 positions at most. Each pegRNA varies in 20 positions in the spacer sequence at most; the PBS varies in sequence as well as in length, typically ranging from 9 to 15 nucleotides though shorter or longer lengths are possible; the RTT varies in sequence as well as in length, typically ranging from 10 to 30 nucleotides though shorter or longer lengths are possible. The remainder of the AAV vector sequence (a total of ≈4.1 kilobases between the ITRs, including the pegRNA/ngRNA expression cassettes) is unchanged (**Figure 3**, **Table 4**), meaning that variants of the AAV.UCD.PE DP will be ≥96% identical in sequence. **This small amount of variation is not expected to affect the biodistribution and toxicology.**

The variant of the CHOP.UCD.PE treatment matched to the *ASS1* G390R mutation, which will use the PE6c mRNA DS in the LNP.UCD.PE DP and two copies each of the pegRNA and ngRNA sequences in **Table 3**, will be the only variant tested in the definitive NHP biodistribution/toxicology study. Because the minor differences described above are not expected to affect the biodistribution and toxicology of the DPs, the Sponsor holds that it is unnecessary to test additional variants of the CHOP.UCD.PE treatment. In addition, the Sponsor holds that **the experimental design of the definitive NHP biodistribution/toxicology study respects the Replacement, Reduction, and Refinement framework to minimize animal use**.

The Sponsor anticipates that, after the initial clearance of the primary IND for the CHOP.UCD.PE treatment, **additional CHOP.UCD.PE variants targeting pathogenic *ASS1* mutations will be added by amendment to the IND in real time as new subjects are diagnosed and screened**. Each subsequent gene-specific IND for the CHOP.UCD.PE treatment will include at least one defined CHOP.UCD.PE variant for the given UCD gene, after which additional CHOP.UCD.PE variants targeting pathogenic mutations in that gene will be added by amendment to the gene-specific IND in real time as new subjects are diagnosed and screened.

The definitive NHP study (**Figure 16** and **Table 6** above) comprises two treatment groups that will undergo initial dosing with the AAV.UCD.PE DP (1×10^{14} vg/kg [redacted]) followed 2 weeks later by **single dosing** with the LNP.UCD.PE DP (2.4 mg/kg) and then a necropsy endpoint either 6 hours later or 2 weeks later,

with the latter matched to a vehicle control group, and a treatment group that undergoes initial dosing with the AAV.UCD.PE DP (1×10^{14} genome copies/kg) followed by **repeat dosing** with two consecutive treatments with the LNP.UCD.PE DP, with the repeat doses separated by 4 weeks, followed by a long-term necropsy endpoint (13 weeks following the second treatment). For the repeat dosing group, the first dose is 0.6 mg/kg , and the second dose is 0.6 mg/kg. Additional goals of this nonclinical study design are to (1) demonstrate ≥95% clearance of LNP components from the blood within 2 weeks after each dosing, (2) establish that the expected sequelae of LNP treatment, e.g., ALT elevations, are either absent or rapidly self-resolve within the 2-week intervals following each of the two doses, (3) evaluate if repeat dosing generates an anti-Cas9 or anti-PEG antibody response, and (4) document that long-term toxicological effects with repeat dosing are absent.

In regard to specific subsections of the FDA Guidance:

"The use of in vitro models ... should be considered for evaluating the activity of a human GE product in the target cell type(s) for genomic modification."

Going forward, the Sponsor will use lentivirus-transduced HuH-7 cells harboring the patient's disease-causing UCD gene mutation(s), with the *ASS1* G390R mutation as a positive reference control, as outlined in **Figure 8**, to assess for on-target editing activity by variants of the CHOP.UCD.PE treatment, as exemplified in **Section 6, History of the Project/Background, Status of Product Development**, especially **Figure 10** and **Figure 12**. The Sponsor holds that the correlation between *in vitro* and *in vivo* editing efficiency demonstrated for the *ASS1* G390R mutation is sufficient to justify the use of ***in vitro* studies**, **without the need for additional *in vivo* studies**, to design and evaluate additional variants of the CHOP.UCD.PE treatment.

The Sponsor wished to assess for activity of the research-grade versions of the AAV.UCD.PE variant and LNP.UCD.PE variant (the same batches used for the mouse studies in **Figure 12**) *in vitro*. The Sponsor made use of the same lentivirus-transduced HuH-7 cell line used in the initial plasmid-based prime editing screening experiments (**Figure 10**). Using a fixed LNP.UCD.PE dose and varying AAV.UCD.PE doses, dose-dependent corrective prime editing was observed (**Figure 17**). The expected correction of the *ASS1* G390R mutation and introduction of synonymous edits (to inhibit mismatch repair and increase the overall efficiency of prime editing) were observed, with no bystander editing.

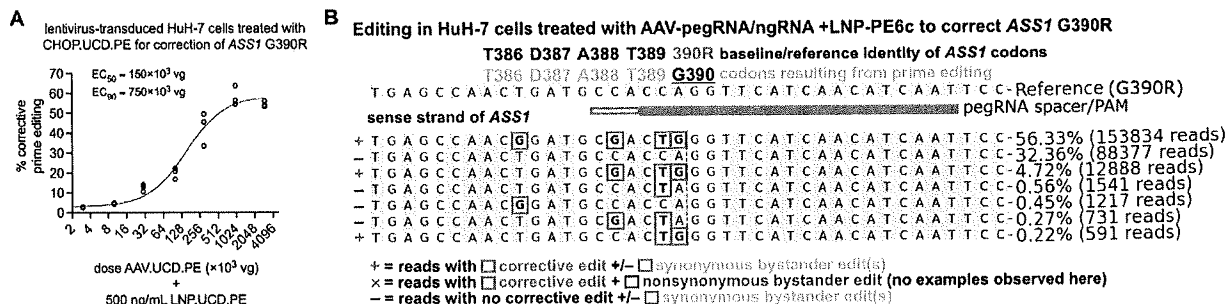


Figure 17. Corrective prime editing of the *ASS1* G390R mutation after AAV/LNP treatment of lentivirus-transduced HuH-7 cells. (A) On-target corrective prime editing (by NGS) with research-grade versions of AAV.UCD.PE and LNP.UCD.PE, via treatment of lentivirus-transduced HuH-7 cells with varied AAV doses, followed two days later by treatment with a fixed LNP dose, followed two days later by extraction and analysis of genomic DNA. **(B)** Standard CRISPResso NGS output for the editing in the HuH-7 sample that displayed the highest level of editing in (A). The codons in the vicinity of the G390R site are indicated; the top-listed amino acid is the baseline/reference identity of the codon, and the bottom-listed amino acid is the one that results from prime editing. The red horizontal bar indicates the spacer/protospacer sequence of the pegRNA, and the adjacent thin red box indicates the PAM sequence.

In principle, this experiment could serve as the basis for a **potency assay** for the AAV.UCD.PE DP, with the EC_{50} for corrective editing of the target mutation (as measured by NGS), in combination with a predetermined fixed dose of a validated reference batch of LNP.UCD.PE, serving as a quantitative measure of potency. An alternative configuration of this experiment, in which varying doses of LNP.UCD.PE are tested in combination with a predetermined fixed dose of a validated reference batch of AAV.UCD.PE in a lentivirus-transduced HuH-7 cell line with the mutation targeted by that batch of AAV.UCD.PE, could serve as the basis for a potency assay for the LNP.UCD.PE DP.

Specifically, the Sponsor proposes to use lentivirus-transduced HuH-7 cells harboring target UCD gene mutations in addition to the *ASS1* G390R mutation (which would serve as the reference control for all future patient-specific mutations under consideration) as the basis of the potency assays. (Refer to **Section 11, Chemistry, Manufacturing, and Controls, CHOP.UCD.PE Treatment, Potency Assays** for a comprehensive description of the proposed potency assays.) This potency assay would allow for determination of whether particular clinical batches of the DPs meet minimum potency thresholds that would make them appropriate for use in the patient dosing scheme laid out in the clinical protocol (**Section 12, Clinical Program Overview**).

"The animal species and/or models selected for in vivo studies should demonstrate a biological response to the human GE product ... Given the differences in the genomic sequences between humans and animals, analysis of the biological activity may be done in a species-specific context (e.g., using a surrogate product), as appropriate."

The Sponsor holds that, for several reasons, humanized animal models are **not** suitable for demonstration of biological responses to variants of the CHOP.UCD.PE treatment:

- Because of the high degree of **personalization** that will be needed to make different versions of the DP suitable for individual patients with unique/near-unique UCD gene mutations, and the **limited timeframe** to administer the treatment (ideally within several

months after the birth or initial diagnosis of a patient), it will **not** be possible to generate a new homozygous knock-in mouse model—with a patient's mutation in the endogenous UCD gene locus—needed to demonstrate a biological response to the treatment.

- Even if a new homozygous knock-in UCD mouse model could be fully generated in a reasonable timeframe, the high likelihood of **perinatal lethality** (Schofield et al., 1999; Khoja et al., 2019; Patejunas et al., 1994; Reid Sutton et al., 2003; Senkevitch et al., 2012; Wang et al., 2017) would make it prohibitive to demonstrate a biological response to the treatment.
- The utility of a heterozygous UCD mouse model (whether knock-in of the mutation into the endogenous locus or into a safe harbor locus such as *Rosa26*) would be limited to demonstrating *in vivo* hepatic editing efficiency of the treatment—which can instead be modeled with *in vitro* studies (e.g., **Figure 17**).
- The **Replacement, Reduction, and Refinement framework to minimize animal use** should be respected as much as possible.

"In vivo nonclinical safety studies for a human GE product (or surrogate product) should incorporate elements of the planned clinical trial (e.g., dose level range, ROA, delivery device, dosing schedule, study endpoints, concomitant therapies, etc.), to the extent feasible."

The definitive NHP biodistribution/toxicology study (**Figure 16** and **Table 6**) has been designed with the planned clinical trial in mind. As described above, the dosing protocol of AAV.UCD.PE and LNP.UCD.PE parallels the protocol clinical protocol, with matching of the two-week interval between dosing of the two DPs. **The Sponsor will measure the concentrations of the AAV.UCD.PE and LNP.UCD.PE DPs after passage through the needle and syringe system used in the toxicology study to ensure device compatibility.**

"Assessment of biodistribution should be conducted to characterize the distribution, persistence, and clearance of the GE product, any expressed GE components in vivo, editing activity in target and non-target tissues, and the potential for inadvertent germline modification. These evaluations may be conducted independently or in conjunction with POC and/or safety studies."

Biodistribution following IV administration of AAV8 has been well characterized in small and large animal models. Intravenous delivery of AAV8 leads to robust distribution in the liver (Wang et al., 2011; Chen et al., 2013; Wang et al., 2014; Ferla et al., 2017; Greig et al., 2017; Collaud et al., 2019). Compared to some other AAV serotypes, AAV8 has demonstrated superior transduction of hepatocytes (Davidoff et al., 2005; Wang et al., 2010; Wang et al., 2015). Additionally, AAV8 vectors are widely used clinically and have demonstrated safety (Nathwani et al., 2011b; Nathwani et al., 2014; Konkle et al., 2021; D'Antiga et al., 2023) in humans following systemic administration of the range of doses anticipated for clinical translation of our work (1×10^{11} to 5×10^{12} vg/kg) (Nathwani et al., 2011b; D'Antiga et al., 2023). Since AAV8 biodistribution studies have been conducted by several groups—a list of studies is included in **Table 7**—the Sponsor proposes to not do a rigorous AAV biodistribution study. Instead, the Sponsor will focus on LNP biodistribution, since the LNP formulation used for LNP.UCD.PE is less well-characterized, has less published data, and has had more limited clinical use than AAV8 to date.

Table 7. Published AAV8 biodistribution studies.		
Species	**AAV dose (vg/kg)**	**Reference**
Mice	$0.4–4 \times 10^{12}$	Monahan et al., 2015
	1.3×10^{13}	Wang et al., 2014
	2×10^{12}	Jeyakumar et al., 2023
	7.5×10^{12}	Chen et al., 2013
	2×10^{12}	Ferla et al., 2017
	$0.5–5 \times 10^{13}$	Chen et al., 2020
Rats	2.5×10^{13}	Collaud et al., 2019
Dogs	$0.3–5 \times 10^{14}$	Mack et al., 2017
Nonhuman primates (NHPs)	3×10^{12}	Wang et al., 2011
	1.25×10^{13}	Greig et al., 2017
	1×10^{12}	Nathwani et al., 2007
	2×10^{12}	Nathwani et al., 2011a

In prior studies with LNPs, it has been observed that LNPs predominantly distribute to the liver, with minor distribution to the spleen and adrenal glands (Gillmore et al., 2021; Musunuru et al., 2021; Lee et al., 2023; see **Figure 15C**). The Sponsor will assess distribution, persistence, and clearance of GE components in NHPs (**Figure 16** and **Table 6**). The Sponsor proposes a tiered approach, wherein lipid excipients [redacted] will be assessed in a broad selection of tissues first, and the expressed GE component (PE6c mRNA) will then be assessed only in tissues that are positive for lipid excipients.

"The intended clinical GE product should be evaluated in the definitive POC and safety studies, as feasible."

Engineering batches of the AAV.UCD.PE and LNP.UCD.PE DPs, [redacted] with the intended clinical manufacturing processes (as described in **Section 11, Chemistry, Manufacturing, and Controls**) will be used for the definitive NHP biodistribution/toxicology study.

"Editing efficiency required to achieve the desired biological activity or therapeutic effect."

The necessary editing threshold (≈10-15% whole-liver corrective editing) has been established by prior human genetic studies and mouse studies (summarized in **Section 6, History of the Project/Background, Therapeutic Rationale**).

"Assessment of immunogenicity of the GE components and expressed transgene(s)."

The definitive NHP biodistribution/toxicology study (**Figure 16** and **Table 6**) will monitor the development of anti-drug antibodies, specifically against the Cas9 component of the prime editor and the PEG-lipid component of the LNP. The AAV vector will expresses no proteins, but only gRNAs—which are not intrinsically immunogenic compared to proteins.

"Evaluation of the potential for inadvertent germline modification."

The definitive NHP biodistribution/toxicology study (**Figure 16** and **Table 6**) will assess lipid excipients [redacted] and the expressed GE component (PE6c mRNA) of the

LNP.UCD.PE DP in the gonads. However, even if the proposed definitive NHP biodistribution/ toxicology study documents mRNA delivery in gonads, the Sponsor holds that a germline transmission study with the CHOP.UCD.PE treatment is unnecessary, because editing would require **both** the presence of the PE6c mRNA from the LNP.UCD.PE DP and pegRNA/ngRNA expression from the AAV.UCD.PE DP **in the same germ cells**; either one alone cannot effect editing. It has been well documented by prior studies that AAV8 does not distribute into the germline (Favaro et al., 2009; Ferla et al., 2017). Thus, the Sponsor judges the risk of inadvertent germline modification to be negligible.

Question #4: Does the Agency agree that the proposed off-target editing studies of a given variant of the CHOP.UCD.PE treatment will provide sufficient data to support the administration of that variant of the CHOP.UCD.PE treatment to infantile-onset UCD patients?

Sponsor Position: The Sponsor notes the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)** related to off-target editing, contained in **Section IV: Considerations for Nonclinical Studies**.

In regard to specific subsections of the FDA Guidance:

"Identification of on- and off-target editing events, including the type, frequency, and location … Multiple methods (e.g., in silico, biochemical, cellular-based assays) that include a genome-wide analysis are recommended to reduce bias in identification of potential off-target sites."

The Sponsor is planning to use **three orthogonal methods** to **nominate** candidate sites of **off-target editing** for all variants of the CHOP.UCD.PE treatment. Of note, these same methods were similar to the methods used to support a recent single patient expanded access IND ███ ███ (Musunuru et al., 2025).

Due to its mechanism of action (see **Figure 7**), prime editing requires Cas9 nicking as the first step—without that event, no on-target editing or off-target editing could occur. Nicking of one DNA strand (with activity from only one intact cleavage domain) requires the same engagement of Cas9 with a target DNA strand as full nuclease activity (i.e., nicking of both strands with two intact cleavage domains). Accordingly, the Sponsor holds that **Cas9 nuclease-based** nomination techniques generate an appropriate list of **candidate** off-target sites for a prime editor.

The **first nomination method** is Circularization for High-throughput Analysis of Nuclease Genome-wide Effects by sequencing (CHANGE-seq) (Lazzarotto et al., 2020), a **homology-independent** biochemical assay that provides an **unbiased genome-wide analysis** (**Figure 18**). CHANGE-seq uses Tn5 tagmentation to fragment genomic DNA obtained from cells (e.g., human hepatocytes), followed by circularization of the DNA fragments via intramolecular ligation. After enzymatic degradation of any remaining linear DNA, the circular DNA will be mixed *in vitro* with a ribonucleoprotein (RNP) comprising a recombinant Cas9 protein (standard *S. pyogenes* Cas9 with an NGG PAM preference, SpG-Cas9 with an NGN PAM preference, or near-PAMless SpRY-Cas9, depending on the PAM preference of the prime editor used) complexed with the mutation-specific chemically synthesized pegRNA or ngRNA. The RNP will cleave certain oligonucleotide sequences with a double-strand break that linearizes the circular DNA molecule. After blunting, end-repair, and adaptor ligation to the ends of linearized DNA molecules, next-generation sequencing (NGS) will identify the sequences that were edited *in vitro* and the frequency of editing, generating a rank-ordered list of candidate (i.e., potential) off-target sites.

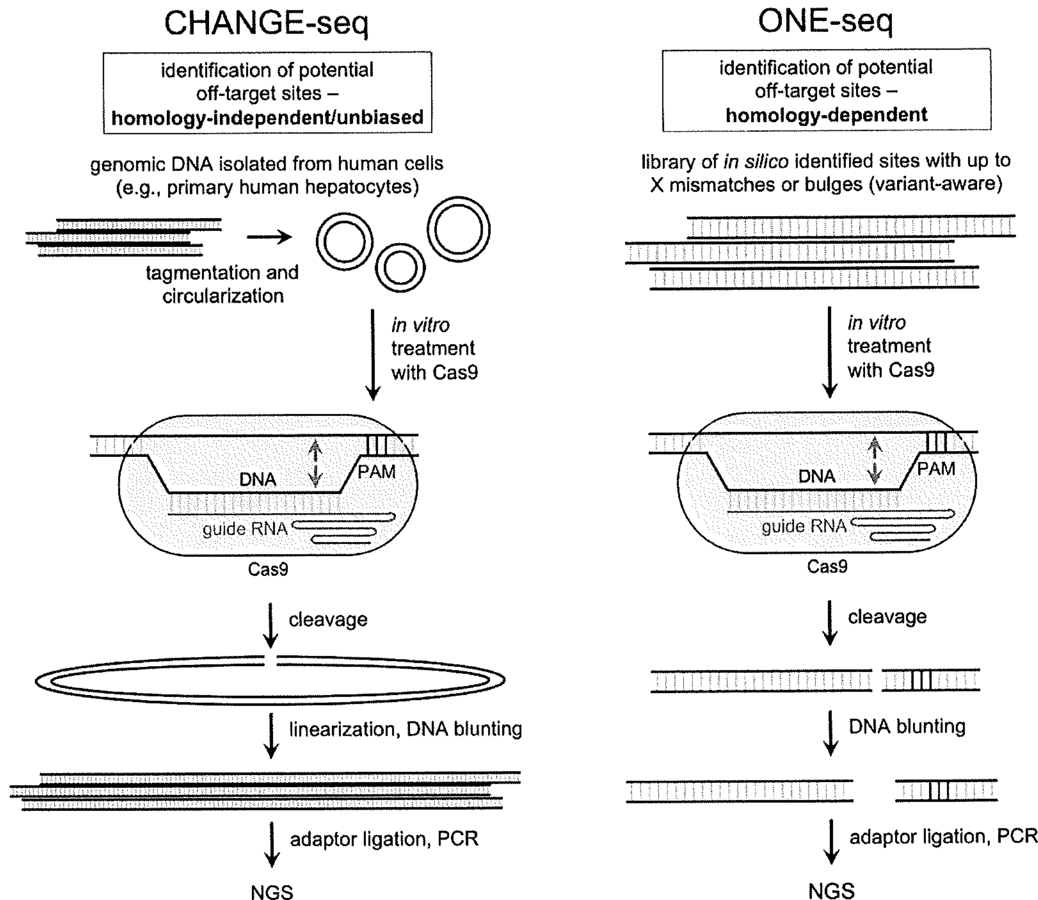


Figure 18. Off-target nomination methods. These methods nominate candidate off-target sites, which are subsequently evaluated to verify whether or not off-target editing genuinely occurs at the sites in target cells.

The **second nomination method** is bioinformatic prediction, which relies on sequence similarity between genomic sites and the protospacer/PAM sequence specified by either a pegRNA or a ngRNA. This method uses bioinformatic tools such as Cas-OFFinder and GuideScan (Bae et al., 2014; Perez et al., 2017) to identify sites in the reference human genome with up to three mismatches, or up to two mismatches plus up to one RNA or DNA bulge. These sites will automatically be regarded as candidate (potential) off-target sites.

The **third nomination method** is OligoNucleotide Enrichment and sequencing (ONE-seq), a **homology-dependent** biochemical assay that uses a synthetic human genomic library selected by sequence similarity to the protospacer/PAM sequence specified by either a pegRNA or a ngRNA (Petri et al., 2021) (**Figure 18**). Thus, ONE-seq is an extension of the second method (bioinformatic prediction). The Sponsor will design a library with sites in the reference genome with up to five mismatches, or up to three mismatches plus up to one RNA or DNA bulge, to each on-target protospacer/PAM sequence. After synthesis by a commercial vendor, the library will be mixed *in vitro* with an RNP comprising recombinant Cas9 protein (with the appropriate PAM preference) complexed with the chemically synthesized pegRNA or ngRNA. The RNP will cleave certain oligonucleotide sequences with a double-strand break. After blunting, end-repair, and adaptor ligation, NGS will quantify the frequency with which each unique oligonucleotide

sequence was edited *in vitro*, generating a rank-ordered list of candidate (potential) off-target sites (typically, the on-target site is at or near the top of the list).

Standard off-target assessment techniques share a critical limitation: each is tied to the specific individual genome represented by the cells or the genomic DNA sample used for analysis. For this reason, most off-target analyses have overlooked the potential for naturally occurring human genetic variation to create novel off-target editing sites in some patients. Furthermore, even if one were to predict that a common or rare genetic variant might create an off-target editing site, it can be challenging to evaluate whether editing actually occurs at that site in the therapeutically relevant cells (e.g., hepatocytes) if there is no way to obtain such cells from a patient with that genetic variant. The Sponsor proposes to use the ONE-seq methodology to empirically identify candidate off-target sites created by genetic variation. **Variant-aware** ONE-seq uses oligonucleotide libraries designed not just using the reference human genome but also incorporating data from the 1000 Genomes Project, the Human Genome Diversity Project, etc., with bioinformatic tools like CRISPRme (Cancellieri et al., 2023).

"Verification of off-target sites should be conducted using methods with adequate sensitivity to detect low frequency events … For in vivo GE products, the analysis should also include the major cell types in which editing events are detected. Appropriate controls should be included to confirm the quality of the assay and to assure interpretability of the results and its suitability for the intended use."

The Sponsor is planning to use **a primary approach** and, if needed, **a secondary approach** to **verify** candidate sites as *bona fide* off-target sites, i.e., sites where off-target editing genuinely occurs in hepatocytes.

The **primary verification approach** is the rhAmpSeq system (Integrated DNA Technologies), which uses highly multiplexed, targeted amplicon sequencing. rhAmpSeq can readily accommodate hundreds or even thousands of candidate sites. Any sites that either (1) are flagged by rhAmpSeq as having off-target editing, or (2) are dropouts with rhAmpSeq, can be reassessed with individual targeted amplicon sequencing (PCR followed by NGS). Regarded as the gold standard, this approach typically has a lower limit of detection of $\approx$0.1% editing.

The Sponsor proposes to assess off-target editing by the CHOP.UCD.PE treatment in three groups of cell types:

- Lentivirus-transduced HuH-7 cell line bearing the targeted UCD gene mutation (as shown in the example in **Figure 8**), untreated vs. treated with saturating doses of the AAV.UCD.PE and LNP.UCD.PE DPs (e.g., the EC_{90} value calculated from a dose-response study of the combined treatment in the HuH-7 cell line, as in **Figure 17**)
- Primary human hepatocytes (PHHs) from at least three donors (obtained via a commercial vendor), untreated vs. treated with saturating doses of the AAV.UCD.PE and LNP.UCD.PE DPs, with PE/pegRNA/ngRNA expression confirmed via comparison of treated PHHs to treated lentivirus-transduced HuH-7 cells by quantitative reverse transcription PCR (qRT-PCR) of the PE mRNA and gRNAs
- Additional cultured or primary cell types nominated by the proposed definitive NHP biodistribution/toxicology study (**Figure 16** and **Table 6**), due to substantial mRNA delivery, and untreated vs. treated with saturating doses of the AAV.UCD.PE and LNP.UCD.PE DPs, with PE/pegRNA/ngRNA expression confirmed with qRT-PCR

The lentivirus-transduced HuH-7 cells have the advantage that they can be used to directly assess for on-target editing efficiency **simultaneously** with off-target editing, confirming that the CHOP.UCD.PE treatment has successfully transfected the cells and has exposed them to saturating amounts of the mRNA and gRNA components. HuH-7 cells are highly proliferative, reflecting a distinct cellular state from the quiescent PHHs.

The **secondary verification approach**, termed Lenti-seq, would involve only high-priority candidate off-target sites that are created by human genetic variation and are not present in readily available hepatocytes. HuH-7 cells will be transduced with a lentivirus bearing a concatenated sequence bearing ≈100-bp fragments spanning (1) the on-target UCD gene mutation sequence and (2) each of the high-priority candidate variant off-target sites, like the scheme showed in **Figure 8**. Treatment of these off-target lentivirus-transduced HuH-7 cells with saturating doses of the AAV.UCD.PE and LNP.UCD.PE DPs will be followed by genomic analysis for on-target corrective editing of the UCD gene mutation and for off-target editing in each of the candidate variant off-target sites.

"Assessment of genomic integrity, including chromosomal abnormalities, insertions or deletions, and potential oncogenicity or insertional mutagenesis."

To assess genomic integrity, WGS will be performed in PHHs treated with saturating doses of the AAV.UCD.PE and LNP.UCD.PE DPs. Aligned WGS reads from pre- and post-treated PHH genomic DNA will be analyzed with the bioinformatics tool Manta (Chen et al., 2016) to detect rare **structural variants**. Briefly, Manta identifies split reads (i.e., single read that spans a structural variation breakpoint such as in an inversion or translocation) to precisely locate the breakpoints of SVs and then performs local *de novo* assembly of the regions surrounding the breakpoints. The use of an AAV vector entails the risk of **insertional mutagenesis**, particularly at sites of double-strand breaks (which are not expected to occur at high frequency with prime editors, unlike with nucleases). AAV insertion analysis will be conducted by the Penn Viral Molecular High Density Sequencing Core using their internally developed integration site pipeline for paired-end reads (INSPIIRED) (Sherman et al., 2016) (**Figure 19**). Insertion analysis will be completed with genomic DNA from PHHs treated with saturating doses of the AAV.UCD.PE and LNP.UCD.PE DPs.

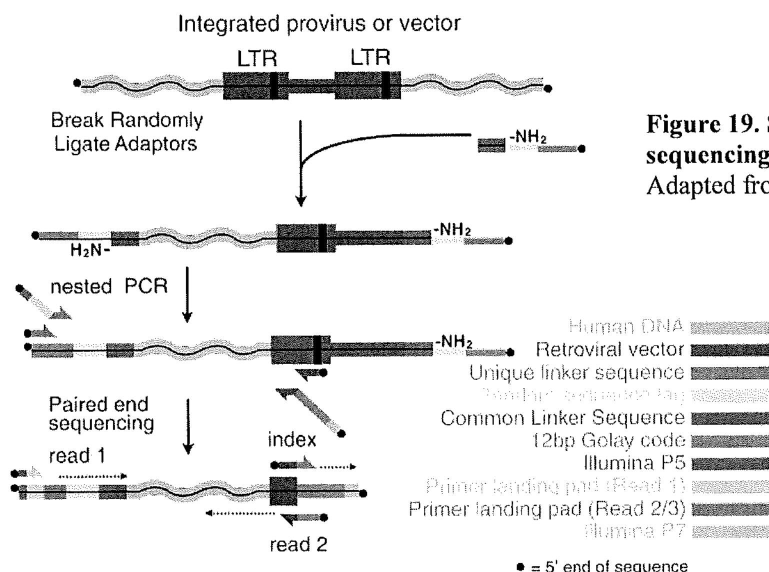


Figure 19. Schematic of a method for isolating and sequencing sites of viral vector integration. Adapted from Sherman et al., 2016.

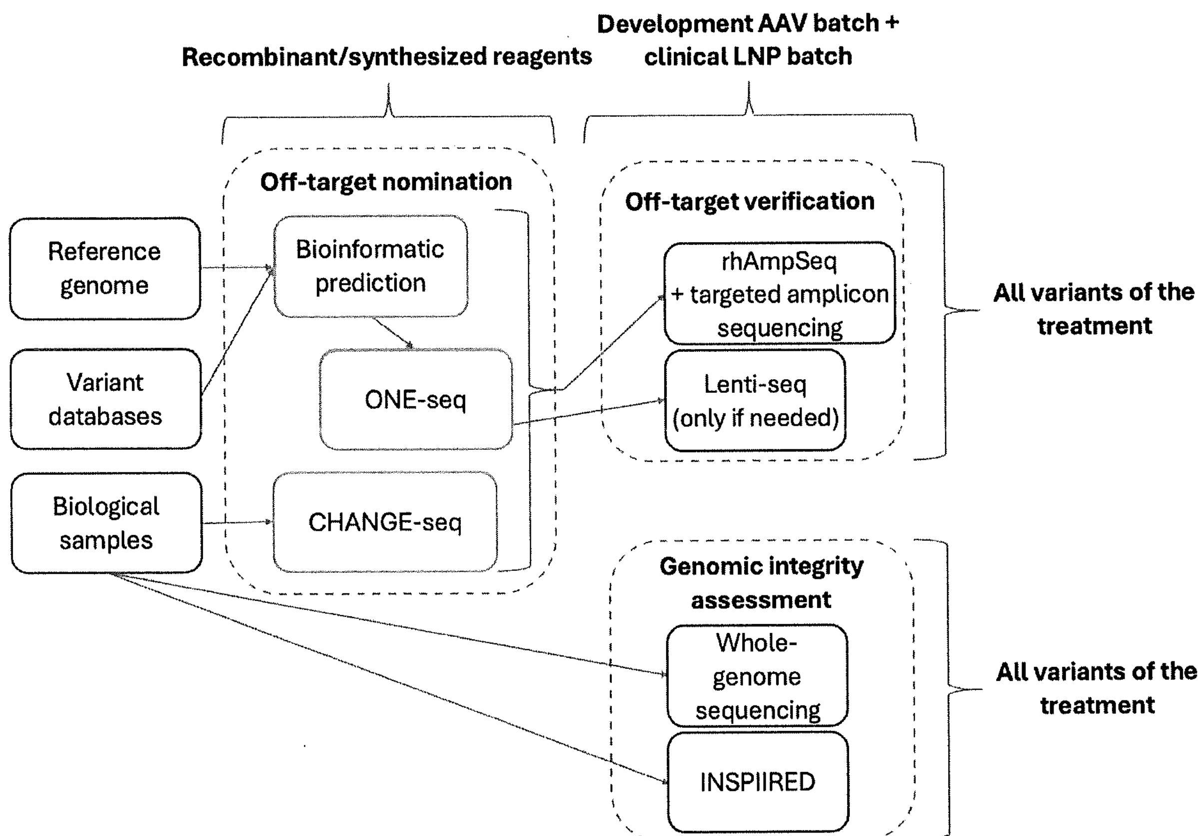


Figure 20. Overview of testing plan for off-target analyses.

For the **overall testing plan** (**Figure 20**) the Sponsor proposes to do the CHANGE-seq and ONE-seq **nomination methods** with recombinant Cas9 protein and chemically synthesized gRNAs. **Verification approaches** will be undertaken with a **clinical batch** of the LNP.UCD.PE DP and a **development batch** of the variant of the AAV.UCD.PE DP to be used to correct a subject's mutation(s). This takes advantage of the fact that a single clinical batch of the LNP.UCD.PE DP could be used for many subjects, whereas the AAV.UCD.PE DP would be individualized for each subject; the Sponsor wishes to undertake the off-target verification analyses **prior** to committing to production of a clinical batch of the AAV.UCD.PE DP. For any verified site of off-target editing with the development batch of the AAV.UCD.PE DP, the Sponsor will re-verify the off-target editing with the clinical batch of the AAV.UCD.PE DP.

The Sponsor will also assess the effect of **repeat** LNP treatment of cells with select variants of CHOP.UCD.PE (e.g., *ASS1* G390R mutation) on on-target editing and off-target editing.

"Evaluation of the biological consequences associated with on- and off-target editing, including, but not limited to, viability and function of the edited cells (e.g., differentiation capacity of progenitor cells)."

Because on-target editing entails the correction of a **mutation** to wild-type, it is expected to have only favorable or neutral biological effects on the edited cells, even non-target cells.

For any site for which off-target editing by the CHOP.UCD.PE treatment has been **verified** by rhAmpSeq and/or targeted amplicon sequencing, the Sponsor will apply a **risk assessment framework** to assess the biological risk of the edit(s) at the site:

(1) Is the edit in or near a cancer gene, e.g., in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Tate et al., 2019)?

(2) Does the edit affect a genomic site that is likely to have functional impact: e.g., coding versus non-coding, Ensembl Variant Effect Predictor analysis (McLaren et al., 2016), and Combined Annotation-Dependent Depletion (CADD) score (Rentzsch et al., 2019)?

(3) Is the edit likely to affect gene expression in the target tissue (hepatocytes) or other tissues in which on-target editing is evident?

(4) Do structural variants involving the site of the edit occur?

(5) Is the edit likely to occur at pharmacological doses of AAV.UCD.PE and LNP.UCD.PE DPs administered to patients (rather than the saturating doses used in off-target assays)?

Should any off-target sites be identified in the studies described above with any variant of the CHOP.UCD.PE treatment, and if a particular off-target edit is deemed to be of high risk by the framework above, the Sponsor will seek guidance from the Agency on the proposed experimental path to address this risk directly.

Additional genotoxicity assessment.

With respect to the AAV.UCD.PE DP, the genotoxicity of the AAV8 vector has been extensively characterized in past studies (see **Table 7**).

Question #5: Does the Agency agree that the overall nonclinical development plan is sufficient to support IND applications for all variants of the CHOP.UCD.PE treatment?

Sponsor Position: The overall nonclinical development plan is outlined in this section, above, and addresses each relevant recommendation of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024).**

<u>Question #7:</u> Does the Agency agree that the overall chemistry, manufacturing, and controls plan could be sufficient to support an accelerated approval of the CHOP.UCD.PE treatment?

Sponsor Position: The Sponsor notes that the plausible mechanism pathway may be relevant to the CHOP.UCD.PE development program, and that guidance on this new pathway may be forthcoming from the Agency later in 2026. In the interim, the Sponsor proposes the following.

The Sponsor intends to enroll 5 subjects who collectively represent at least 3 UCDs (recognizing that this will entail submission and clearance of the primary IND as well as at least two subsequent gene-specific INDs) in the proposed Phase I/II study. The Sponsor proposes that upon enrollment of the full cohort of 5 subjects and completion of the Phase I/II study, the Sponsor will request an end of Phase 2 (EOP2) meeting with the Agency. If it is determined by the Agency that the primary safety endpoint is acceptable, and that the secondary endpoint demonstrates a consistent therapeutic effect of CHOP.UCD.PE across subjects, the Sponsor and the Agency would discuss the possibility of accelerated approvals under the plausible mechanism pathway for Biological License Applications for the 3+ UCD genes represented by the 5

subjects, based on the data from the Phase I/II study alone, i.e., with no need for data from additional subjects.

The Sponsor holds that the proposed CMC plan is appropriate for a Phase I/II study. In a scenario where the clinical DP batches made for the Phase I/II subjects demonstrate consistent critical quality attributes, and the clinical responses among subjects are consistently favorable, the Sponsor proposes that the Phase I/II data be sufficient to support Biological License Applications even if the process performance qualification standards ordinarily expected for commercial manufacturing were not undertaken for the Phase I/II study.

The Sponsor notes that in a context in which small, individualized DP batches are manufactured on a per-patient basis (particularly for the AAV.UCD.PE DP), some of the general principles of process validation may not be as relevant as they would be for multiple-large-batch manufacturing that provides DP for a large cohort of patients. This is particularly true for ultra-rare diseases like the UCDs, for which a commercial program may not ever supply DPs for more than a few dozen patients per year. Production of multiple batches of the same variant of the CHOP.UCD.PE solely for the purpose of process validation, when that CHOP.UCD.PE variant would treat at most one patient, seems unwarranted. Instead, the Sponsor seeks flexibility from the Agency in this regard, for example, allowing data from the production of multiple individualized CHOP.UCD.PE variants, manufactured specifically for the treatment of subjects in the clinical trial, to support the product release specifications.

Potency Assays

Question #8: Does the Agency agree that the proposed potency assays for the CHOP.UCD.PE treatment are acceptable to support IND applications for all variants of the CHOP.UCD.PE treatment?

Sponsor Position: The Sponsor notes the recommendations of the **FDA Guidance for Potency Tests for Cellular and Gene Therapy Products (2011)** and the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)** related to potency assays, contained in **Section III.B.3.i: In vivo-administered Human Genome Editing Drug Products**, specifically the following points:

- *"For early phase studies, potency assays evaluating the ability of the GE components to perform the desired genetic sequence modification may be adequate."*
- *"We recommend that, whenever possible, the potency assays be performed in the target cells or tissues (or a representative surrogate)."*
- *"We also recommend inclusion of such a potency assay in the DP stability studies."*

The Sponsor proposes a potency assay for both the **mRNA DS** and the **LNP.UCD.PE DP**, using a lentivirus-transduced HuH-7 cell line harboring a cassette with a ≈100-bp *ASS1* genomic sequence spanning the G390R mutation, a human U6 promoter driving expression of the pegRNA that corrects the G390R mutation (see **Table 3**), and a human U6 promoter driving expression of the ngRNA that corrects the G390R mutation (see **Table 3**). This cell line would constitutively express the pegRNA/ngRNA pair and harbor the prime editing target sequence (which otherwise would not be present in the cell line, since the G390R sequence differs from the endogenous wild-type *ASS1* locus). Upon establishing this cell line, the Sponsor can use it in

two ways, in each case assessing potency by evaluating the efficiency of corrective prime editing of the *ASS1* G390R mutation:

- First, the cell line can be transfected with an **mRNA DS** using the Lipofectamine MessengerMax reagent and, ultimately, the level of corrective prime editing can be measured by amplicon-based sequencing, i.e., NGS, with an eight-point dose range to establish an EC_{50} (see **Figure 17** for an example of this kind of assay), using a pre-validated mRNA batch as a reference standard. This same cell line can be used to assess potency of PE6c mRNA, SpG-PE6c mRNA, or SpRY-PE6c mRNA, since the pegRNA/ngRNA set for targeting the *ASS1* G390R mutation uses NGG PAMs that are compatible with all three prime editors.
- Second, the cell line can be treated with an **LNP.UCD.PE DP** via direct addition to the media and, ultimately, the level of corrective prime editing can be measured by amplicon-based sequencing with an eight-point dose range to establish an EC_{50}, using a pre-validated DP batch as a reference standard.

Upon developing and qualifying these potency assays, the Sponsor plans to establish a Master Cell Bank to provide a long-term source of the HuH-7 cell line, which would be used for potency assays for all variants of the mRNA DS and all variants of the LNP.UCD.PE DP.

For the **AAV.UCD.PE DP**, which is the personalized component of the CHOP.UCD.PE treatment, no single cell line can support a generalized potency assay. Accordingly, the Sponsor proposes a potency assay using a lentivirus-transduced HuH-7 cell line harboring an individual subject's target mutation(s) along with the *ASS1* G390R mutation, which will serve as a **positive reference control**. (Refer to **Figure 8** for a schematic of an example of this kind of lentivirus-transduced HuH-7 cell line.) The version of the cell line tailored to a subject's mutation(s) will be used to assess for the desired genetic sequence modification(s)—namely, the on-target corrective prime editing activity appropriate to the variant of the AAV.UCD.PE DP manufactured for the subject, as determined by amplicon-based sequencing.

In the potency assay, the lentivirus-transduced HuH-7 cells will be treated with the subject's AAV.UCD.PE DP, followed two days later by treatment with a fixed dose of a pre-validated batch of the appropriate variant of the LNP.UCD.PE DP. Ultimately, the level of corrective prime editing will be measured by amplicon-based sequencing with an eight-point dose range to establish an EC_{50} (see **Figure 17** for an example of this kind of assay). In parallel, the HuH-7 cells will also be treated with a reference standard, i.e., a pre-validated batch of the AAV.UCD.PE DP variant that corrects the *ASS1* G390R mutation, followed by the same LNP.UCD.PE DP. The editing efficiency of the subject's AAV.UCD.PE DP will be reported in relation to the G390R reference standard.

The HuH-7 cell-based assays are now being optimized, and for the proposed Phase I/II clinical trial to be conducted under the master protocol in the primary IND, the Sponsor will use these assays, with minimum thresholds of editing efficiency—vis-à-vis the reference standards, with **modeling data and calculations** to be provided and justified in the IND applications—serving as acceptance criteria for DS and DP release and for stability testing [redacted]. The Sponsor will continue to develop and qualify the assays as quantitative potency assays that are predictive of *in vivo* potency.

12. CLINICAL PROGRAM OVERVIEW

Question #9: Does the Agency agree that the general design, including the proposed safety and exploratory efficacy outcome measures, enrollment criteria, data capture and monitoring plans, and long-term follow-up plan are appropriate for the umbrella trial protocol outlined in the protocol synopsis?

Sponsor Position: The Sponsor notes the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024),** particularly **Section V: Considerations for Clinical Studies**: "*Clinical trial design should include an appropriately-defined patient population, an efficient and safe approach to product administration (including data-based dosing, dose schedule, and treatment plan), adequate safety monitoring, and appropriate safety and efficacy endpoints.*"

In accordance with the Guidance, the Sponsor proposes a Phase I/II open-label umbrella clinical trial designed to evaluate the safety and efficacy of CHOP.UCD.PE (**Table 19**). The full clinical protocol and informed consent form will be included in the **master protocol** in the primary IND application for the CHOP.UCD.PE treatment.

Table 19. Clinical protocol synopsis.	
Title:	A Phase I/II open-label safety and efficacy study of CHOP.UCD.PE in patients with urea cycle disorders due to variants amenable to corrective editing by CHOP.UCD.PE
Study description:	To date, there are no one-time, disease-modifying medical therapies that durably correct neurotoxic ammonia elevations in patients with severe urea cycle disorders (UCDs). The goal of this study is to restore expression and activity of a deficient urea cycle enzyme or related transporter and reduce ammonia levels through corrective prime editing of a disease-causing variant(s) in a UCD gene.
Objectives:	**Primary objective:** To evaluate the safety and tolerability of a single intravenous (IV) treatment with CHOP.UCD.PE in subjects with UCDs **Secondary objectives:** To evaluate the clinical efficacy of a single IV treatment with CHOP.UCD.PE in subjects with UCDs
Inclusion criteria:	

	[redacted]
Exclusion criteria:	[redacted]
Primary endpoint:	[redacted]
Secondary endpoint:	[redacted]
Exploratory endpoints:	[redacted]

Study population:	
Phase:	
Description of sites/ facilities enrolling participants	
Description of study intervention	
Study duration:	
Participant duration:	

Prescreening period to assess amenability of a mutation to corrective editing by the CHOP.UCD.PE treatment

CHOP.UCD.PE is designed to treat patients with severe infantile-onset UCDs who are homozygous or compound heterozygous for a mutation(s) targeted by a variant of the treatment that specifically targets the subject's mutation(s). While it is possible that a subject will present with the *ASS1* G390R mutation, it is likely that most subjects will have mutations other than the *ASS1* G390R mutation, and some subjects will have mutations not previously cataloged.

If a subject's mutation(s) has not already been evaluated for amenability to corrective editing by CHOP.UCD.PE, a referring provider can request a prescreening evaluation in which the provider will give the investigators the following de-identified information about the potential subject:

- Disease-causing mutation(s)

The investigators will then complete *in vitro* analysis to assess the amenability of the mutation(s) to corrective editing by CHOP.UCD.PE as exemplified in **Section 6, History of the Project/Background, Status of Product Development**, especially **Figure 10**.

If the mutation(s) is found to be amenable, the investigators will provide this information to the subject's referring provider, and the subject will be eligible for screening.

If a subject's mutation(s) has previously been adjudicated to be amenable to corrective editing by CHOP.UCD.PE, a prescreening period is not required.

Subject screening

At screening, all subjects will have confirmatory review of their clinical UCD gene sequencing results by a clinical geneticist. If there is any uncertainty regarding the molecular diagnosis, subjects may undergo repeat confirmatory molecular testing.

If a subject is compound heterozygous for two alleles that are amenable to corrective editing by CHOP.UCD.PE, an AAV.UCD.PE DP may be produced to target both alleles (one copy of a pegRNA and one copy of a ngRNA targeting the first allele, and one copy of another pegRNA and one copy of another ngRNA targeting the second allele). If only one allele is amenable to CHOP.UCD.PE, two copies of the pegRNA and two copies of the ngRNA targeting that allele will be included in the AAV.UCD.PE DP.

To ensure there is an appropriate benefit-to-risk ratio for each subject, during screening the data and safety monitoring board (DSMB) (see details below) will review the data needed to support the determination of inclusion/exclusion criteria for each subject that passes the investigator screening. In addition, the DSMB will review the *in vitro* data to support the amenability of the mutation(s) to corrective editing by CHOP.UCD.PE, as well as the mutation-specific off-target assessments.

Once the DSMB has confirmed that a subject has successfully passed screening, the subject will be eligible for dosing if the mutation(s) is already included in the IND. If the mutation(s) is not included in the IND, an IND amendment will be submitted to the Agency that includes:

- The certificate of release for the subject-specific variant of the AAV.UCD.PE DP
- *In vitro* data supporting the amenability of the mutation(s) to corrective editing by the subject-specific variant of the CHOP.UCD.PE treatment
- *In silico* and *in vitro* off-target editing data for the subject-specific variant of the CHOP.UCD.PE treatment

The subject will be eligible for dosing after the Agency has approved the IND amendment.

Data and safety monitoring board and subject enrollment timeline

A DSMB will be established, comprising at least 4 people who are experts in UCDs, gene editing therapies, and safety/pharmacovigilance. The DSMB will review screening data, safety data, and exploratory efficacy data from all study participants at predetermined intervals that will be specified in the full clinical protocol provided with the primary IND application. They will also meet as any concerns arise. Communications or meetings will occur at a minimum of quarterly intervals each year, including the occasions of the following milestones for each subject:

Rationale for dose selection and dosing plan

To prioritize the safety of subjects, the dosing plan will begin with a low-dose regimen that is still predicted to provide benefit. This regimen will be finalized after completion of the proposed definitive NHP biodistribution/toxicology study (**Figure 16** and **Table 6**).

Immunosuppression plan

Plan for liberalization of dietary protein and nitrogen scavenger medications

Question #10: Does the Agency agree that if there is a uniform response across subjects, the proposed umbrella trial in a limited number of subjects could be sufficient for an accelerated approval of the CHOP.UCD.PE treatment?

Sponsor Position: The Sponsor notes that the plausible mechanism pathway may be relevant to the CHOP.UCD.PE development program, and that guidance on this new pathway may be forthcoming from the Agency later in 2026. In the interim, the Sponsor proposes the following.

The Sponsor intends to enroll 5 subjects who collectively represent at least 3 UCDs (recognizing that this will entail submission and clearance of the primary IND as well as at least two subsequent gene-specific INDs) in the proposed Phase I/II study. The Sponsor proposes that upon enrollment of the full cohort of 5 subjects and completion of the Phase I/II study, the Sponsor will request an end of Phase 2 (EOP2) meeting with the Agency. If it is determined by the Agency that the primary safety endpoint is acceptable, and that the secondary endpoint demonstrates a consistent therapeutic effect of CHOP.UCD.PE across subjects, the Sponsor and the Agency would discuss the possibility of accelerated approvals under the plausible mechanism pathway for Biological License Applications for the 3+ UCD genes represented by the 5 subjects, based on the data from the Phase I/II study alone, i.e., with no need for data from additional subjects. If the Agency feels accelerated approvals are not warranted by data from the Phase I/II study alone, the Sponsor and the Agency would discuss a Phase III extension of the umbrella clinical trial and the number of additional subjects and UCD genes that should be included in the Phase III extension in order to support accelerated approvals.

The Sponsor acknowledges that the initial dosing regimen for the CHOP.UCD.PE treatment may not have the desired therapeutic efficacy. If this should prove the case [redacted] in the Phase I/II study, the Sponsor will propose amending the master protocol in the primary IND to allow for a higher dose of either the LNP.UCD.PE DP or the AAV.UCD.PE DP or both to be administered to subsequent subjects in the Phase I/II study. The Sponsor will also propose amending the master protocol so that the initial subjects can receive a second dose of the LNP.UCD.PE DP, with the rationale that the AAV8 vector administered via the AAV.UCD.PE DP will still be present in the hepatocytes and expressing the gRNAs, allowing for a second round of prime editing activity. The proposed definitive NHP biodistribution/toxicology study (**Figure 16** and **Table 6**) includes a repeat dosing group to allow for this contingency.

13. A LIST OF FDA STAFF ASKED TO PARTICIPATE IN THE REQUESTED MEETING

The participation of members of the Center for Biologics Evaluation and Research is requested.

14. TELECONFERENCE DATE/TIME

February 27, 2026, at 11:00 am ET.

15. FORMAT OF THE MEETING

A face-to-face webinar has been requested and scheduled.

16. REFERENCES

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