

MEETING TYPE:

INTERACT Meeting Request and Briefing Package

SPONSOR INVESTIGATOR:

[REDACTED]
[REDACTED]

INVESTIGATIONAL PRODUCT:

Recombinant Adeno-Associated Virus 8 Human Solute Carrier Family 4 Member
11 (rAAV8-hSLC4A11)

INDICATION:

Congenital hereditary endothelial dystrophy (CHED)

DATE:

December 6, 2024

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1. INTRODUCTION

1.1 Overview

The investigators propose the use of recombinant Adeno-Associated Virus 8 Human Solute Carrier Family 4 Member 11 (rAAV8-hSLC4A11) for the treatment of infants and children with Congenital hereditary endothelial dystrophy (CHED) associated with biallelic mutations in *SLC4A11*. *SLC4A11*, which is used as a positive marker for ex vivo expanded corneal endothelial cells, plays an essential role in the corneal endothelium by enabling energy-producing glutaminolysis through maintaining ammonia homeostasis, mitigating oxidative stress associated with glutaminolysis, sustaining antioxidant responses, and preventing apoptosis.

CHED-associated missense and nonsense mutations in *SLC4A11* lead to loss of function or expression of the encoded protein, with secondary dysfunction of the corneal endothelial “pump” activity and development of the progressive corneal edema that characterizes CHED. The bilateral, diffuse corneal edema that characterizes CHED is associated with significantly increased corneal thickness, up to twice normal, leading to bilateral visual impairment and secondary deprivational amblyopia and nystagmus.

CHED is a rare pediatric disease with a currently unmet medical need. There is no effective pharmaceutical treatment for CHED, and thus the only treatment option for vision restoration in affected infants and children is corneal transplantation, which is associated with an increased risk of intraoperative and postoperative complications in pediatric patients, including higher rates of transplant rejection and failure. Additionally, as the mean survival of initial corneal transplants for CHED is only approximately 10 years and given a decreasing duration of graft survival for each successive corneal transplant, multiple corneal transplants are typically needed to maintain vision for individuals with CHED. Given the increasing risk of permanent vision loss secondary to complications associated with repeated corneal transplantation, such as glaucoma, there is a need for alternative, more efficacious and sustainable treatment options for treating CHED.

As CHED is a monogenic disorder associated with loss of function of the SLC4A11 protein in the avascular, immune-privileged cornea, it represents an ideal disorder to treat using a gene therapy approach utilizing a viral vector targeting the corneal endothelium. rAAV8-hSLC4A11 is a recombinant adeno-associated virus (rAAV) vector engineered to restore wild type human SLC4A11 protein in the non-dividing corneal endothelial cells. Non-replicative rAAV8-hSLC4A11 vector is packaged in an AAV serotype 8 (AAV8) capsid and contains single-stranded cDNA encoding SLC4A11 protein variant B (NM_032034), the most abundantly expressed SLC4A11 transcript variant in the human cornea.

The administration of rAAV8-hSLC4A11 via a single injection into the anterior chamber of *Slc4a11*-deficient mice restores corneal endothelial function and reverses or halts the progression of corneal edema in the treated eye compared to the non-injected contralateral eye.

Administration of rAAV8-hSLC4A11 via a single injection into the corneal stroma of one eye of *Slc4a11*-deficient mice similarly resulted in a sustained reduction or stabilization of corneal thickness compared to the pre-injection baseline in a dose-dependent manner, while untreated contralateral corneas showed a progressive increase in thickness. Significant differences in

central corneal thickness were observed in treated eyes compared to control eyes within six weeks at each dosage tested, with persistent effects observed 26 weeks post-injection.

Preclinical investigations involving the corneal intrastromal delivery of rAAV8 vectors in large animal models, such as rabbits¹ and dogs², have shown promising safety profiles. These profiles are marked by minimal to no immunological reactions in both ocular and non-ocular tissues, the absence of transgene expression in non-corneal ocular and extraneous tissues (e.g. liver, kidney, spleen, heart), and minimal to no detectable neutralizing antibodies against the AAV8 capsid in the aqueous humor, vitreous humor or serum fluids.

Additional preclinical studies have demonstrated that intrastromal injection of rAAV8-hSLC4A11 in ex vivo donor human corneas effectively transduces the corneal endothelium, that rAAV8-hSLC4A11 produces SLC4A11 protein and that *SLC4A11* replacement restores SLC4A11-mediated NH₃ and H⁺ permeability in *SLC4A11*^{-/-} human endothelial cells. Collectively, these studies support the feasibility and potential of corneal intrastromal delivery of rAAV8-hSLC4A11 to improve vision in infants and children with CHED associated with biallelic mutations in *SLC4A11*.

1.2 Need for INTERACT Meeting

The investigators are requesting an INTERACT meeting to pose several important questions to better inform the development and conduct of appropriate preclinical studies. Specifically, the investigators are seeking comment of the selection of Gottingen mini pigs as an appropriate large animal model for the toxicology and biodistribution studies; of the dose translation strategy from the small rodent disease model to the proposed large animal toxicology study and from the proposed large animal toxicology study to the first-in-human clinical trial; that the preclinical data provide sufficient evidence of a direct benefit for the subjects to be enrolled in the first-in-human trial; to limit the enrollment to a pediatric population; and to gain alignment on the strategy for potency assurance and the stability study.

1.3 Bespoke Gene Therapy Consortium (BGTC)

The lead investigator, Dr. Anthony Aldave MD, from the University of California Los Angeles, is working in collaboration with the Bespoke Gene Therapy Consortium (BGTC) on the development of rAAV8-hSLC4A11, one of eight investigational gene therapies in the BGTC portfolio.

The BGTC is managed by the Foundation for the National Institutes of Health (FNIH) and is a public-private partnership between the NIH, FDA, industry and nonprofit partners with experience in designing, developing and manufacturing AAV-based gene therapies. It was formed to address the challenge of developing AAV gene therapies for rare monogenic diseases. The BGTC's objectives are to streamline and accelerate clinical development, support first-in-human clinical trials for several rare diseases, and to increase knowledge about AAV transduction, production and gene expression through grants that support research. By reducing the cost and challenges for the development of AAV gene therapies, BGTC's efforts may change what diseases are of commercial interest, with rare diseases becoming more mainstream for investment by the pharmaceutical industry and other stakeholders.³

The aim of the BGTC is to facilitate drug development for rare diseases using a platform based approach where appropriate. While this meeting request is specific to rAAV8-hSLC4A11, some referenced methods may be relevant across the BGTC portfolio, and these will be noted where applicable. Agency feedback obtained through this meeting may be used to inform other BGTC gene therapies as the context is deemed scientifically appropriate. The BGTC and collaborating sponsors may refer to the information contained in this briefing package along with Agency feedback obtained from this meeting in future BGTC meetings, applications or other correspondence. Please note that Agency feedback from two informal meetings of the BGTC with FDA/CBER/OTAT on September 29, 2022 (CMC topics) and June 27, 2023 (preclinical topics) have been referenced in this briefing package. In the informal meetings with FDA, the BGTC received general, non-binding feedback from the Agency. This is the first INTERACT meeting request for a specific gene therapy in development with BGTC.

1.4 Prior Regulatory Interactions

rAAV8-hSLC4A11 received Rare Pediatric Disease Designation (RPD-2024-897) on August 14, 2024 and Orphan Drug Designation (DRU-2024-10269) on September 19, 2024, both for the treatment of congenital hereditary endothelial dystrophy. No other applications or meeting requests have been submitted to the Agency for this gene therapy.

2. PRODUCT NAME OR CODE

Recombinant Adeno-Associated Virus 8 Human Solute Carrier Family 4 Member 11 (rAAV8-hSLC4A11)

3. PROPOSED INDICATIONS

Intrastromal corneal injection of rAAV8-hSLC4A11 vector is proposed to manage corneal opacification in infants and children affected with congenital hereditary endothelial dystrophy (CHED, OMIM# 217700, ORPHAcode 293603) with homozygous or compound heterozygous pathogenic mutations in the solute carrier family 4 member 11 gene (*SLC4A11*).

4. GENE THERAPY OVERVIEW

4.1 Construct

AAV8-hSLC4A11 is a non-replicating, rep/cap-deleted, rAAV vector containing single stranded cDNA encoding wild type human SLC4A11 protein. The vector contains AAV serotype 2 inverted terminal repeats (ITRs) and an expression cassette consisting of a eukaryotic translation elongation factor 1 α promoter (EF1 α), a Kozak sequence, the wild type human SLC4A11 variant B (NM_032034) cDNA and a bovine growth hormone polyadenylation sequence packaged in the AAV serotype 8 (AAV8) capsid (**Figure 1**). The human EF1 α promoter was selected because of the ubiquitous expression of SLC4A11 in human cornea as well as other organs and tissues. SLC4A11 variant B (NM_032034) was selected as the transgene variant as it is the most abundantly expressed transcript variant in the human corneal endothelium. A single-stranded design was used given the 3089 bp size of human SLC4A11 variant B (NM_032034) cDNA (**Figure 2**).

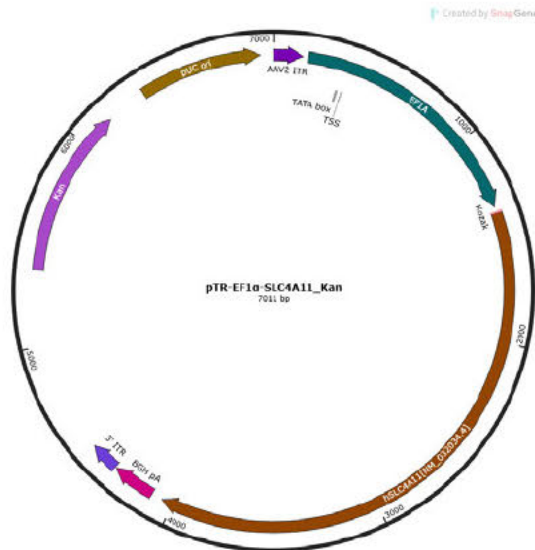


Figure 1. Schematic of the plasmid vector used to produce single stranded AAV-hSLC4A11. ITR2 = inverted terminal repeat serotype 2, EF1 α = eukaryotic translation elongation factor 1 α promoter, hSLC4A11 = human solute carrier family 4 member 11, bGH pA = bovine growth hormone polyadenylation signal.



Figure 2. Organization of the AAV8-hSLC4A11 vector. ITR2 = inverted terminal repeat serotype 2, EF1 α = eukaryotic translation elongation factor 1 α promoter, hSLC4A11 = human solute carrier family 4 member 11, bGH pA = bovine growth hormone polyadenylation signal.

4.2 Completed And Planned Nonclinical Studies

4.2.1 Completed Nonclinical Studies

The administration of recombinant adeno-associated virus (rAAV) gene therapy vectors containing the wild-type *Slc4a11* transgene via a single injection into the anterior chambers of *Slc4a11*-deficient mice has exhibited significant restoration of corneal endothelial function and the reversal of corneal edema.⁴ To evaluate the *in vivo* efficacy of corneal intrastromal delivery of a rAAV8 vector encoding human SLC4A11 cDNA, we performed a single corneal intrastromal injection of 3 μ L of an unpurified rAAV8-hSLC4A11 vector preparation in one eye of *Slc4a11*^{-/-} mice at 4-5 weeks of age. Five doses were tested: 1 x 10⁹, 3.3 x 10⁸, 1 x 10⁸, 1 x 10⁷ and 1 x 10⁶ vg/eye. For each mouse, the eye receiving the rAAV injection was randomized, and the non-injected contralateral eye served as the control. rAAV-injected eyes showed a sustained reduction/stabilization of central corneal thickness (CCT) from baseline in a dose-dependent manner, whereas untreated contralateral eyes showed a progressive increase in CCT (**Figure 3**).

A significant difference in CCT was observed in the treated eye compared with the control eye at week 6 following the injection at each of the dosages tested, with the effect observed through the most recent assessment 26 weeks after the injection.

A dose-dependent corneal subepithelial opacification and vascularization was also observed in AAV-injected eyes that was associated with the quality of the unpurified AAV vector solution used. The toxicity of AAV8-hSLC4A11 will be thoroughly evaluated in the planned toxicology studies using a clinically relevant drug product.

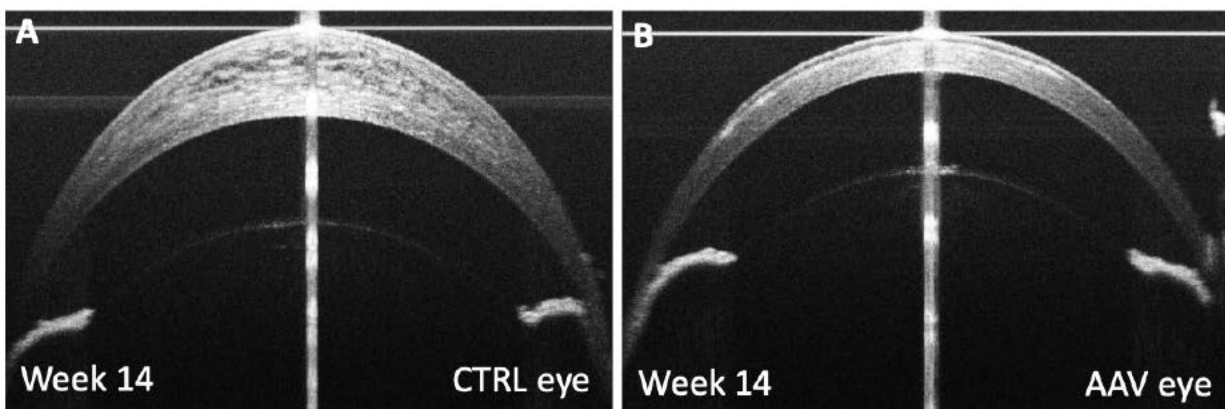


Figure 3. Intrastromal AAV8-mediated SLC4A11 delivery rescues corneal edema in *Slc4a11* knock out mouse. Anterior segment ocular coherence tomography (AS OCT) images of a *Slc4a11*-deficient mouse 14 weeks after corneal intrastromal injection of 1×10^9 vg of the rAAV8-hSLC4A11 vector in one eye (AAV eye). A significant difference in corneal thickness is seen when compared with the untreated eye (CTRL eye).

The investigators' preclinical findings involving the intrastromal administration of rAAV8 vectors harboring various transgenes in the corneas of large animal models, including rabbit,¹ canine,² and feline (unpublished data), have demonstrated excellent safety profiles. These profiles are characterized by minimal to no ocular and non-ocular immunologic reactions, absence of vector-mediated transgene expression in non-corneal ocular tissues or extraneous tissues (such as liver, kidney, spleen, heart, and contralateral non-injected corneal tissue), and minimal to no detectable neutralizing antibodies against the AAV8 capsid in the aqueous, vitreous humor or serum. Additionally, the investigators' unpublished studies in human donor corneas demonstrated that a single deep intrastromal injection of rAAV8-GFP achieved GFP fluorescence in more than 37% of corneal endothelial cells after 15 days, indicating the ability to deliver SLC4A11 cDNA to the corneal endothelial cells that are affected in patients with CHED. These studies indicate the feasibility and provide preliminary safety information on corneal intrastromal delivery of a rAAV8 vector expressing human SLC4A11 (rAAV8-hSLC4A11). Further, unpublished preclinical studies of bilateral sequential corneal intrastromal injection of rAAV8 support the feasibility of sequential treatment of both eyes using the same AAV8 vector, offering the possibility of treating both eyes of individuals with CHED at different timepoints using the same rAAV8-hSLC4A11. Please see [Section 8.2](#) for more details on completed nonclinical studies.

4.2.2 Planned Nonclinical Studies

Pending completion of research grade and subsequently, toxicology grade rAAV8-hSLC4A11 vector (GMP quality) by the contract manufacturer, the investigators plan to perform the following nonclinical studies: administration of research grade rAAV8-hSLC4A11 vector via a single injection into the corneal stroma of one eye of *Slc4a11*-deficient mice; a pilot 3-month study on the pharmacology, biodistribution, immunogenicity, and toxicity of the toxicology grade rAAV8-hSLC4A11 vector; and an IND enabling GLP-compliant safety, toxicology and biodistribution study of the toxicology grade rAAV8-hSLC4A11 vector, the details of which will be provided in a future Pre-IND Meeting Information Package.

Dose optimization and biodistribution studies in *Slc4a11*^{-/-} mice

The investigators will perform a single corneal intrastromal injection in one eye of 10 *Slc4a11*^{-/-} mice (5 male, 5 female) at 4 weeks of age with the research grade rAAV8-hSLC4A11 vector at five dose group (1 x 10⁹ vg/eye, 3.3 x 10⁸ vg/eye, 1 x 10⁸ vg/eye, 1 x 10⁷ vg/eye and 1 x 10⁶ vg/eye). For each mouse, the eye receiving the AAV injection will be randomized, with the contralateral eye serving as the control. Anterior segment OCT will be performed in both eyes before injection to measure baseline CCT (mean ± SEM) and will be performed bi-weekly after injection until post-injection week 26. Slit-lamp examination of the anterior ocular segment and retinal OCT will also be performed at each time point to monitor for evidence of adverse reactions. After the week 26 time point, immunohistochemistry will be performed on tissue sections from each AAV8-hSLC4A11 injected mouse eye to assess the expression and distribution of exogenous SLC4A11. Biodistribution assays will also be conducted for each dose group to determine the viral vector distribution in non-ocular tissue (e.g. liver, blood, heart, kidneys).

The minimal effective dosage (MED) and the maximum tolerated dosage (MTD) in the *Slc4a11*^{-/-} mice and the results of the non-GLP pilot toxicology and biodistribution studies will be determined to inform the initial dosages to be used in the IND enabling GLP-compliant safety, toxicology and biodistribution studies. MED is the lowest dosage that achieves a statistically significant reduction in corneal edema progression. The progression of corneal edema will be measured by the change in CCT. MTD is the highest dosage that shows no signs of toxicity.

Non-GLP pilot toxicology and biodistribution study

As toxicology studies take time to plan and conduct, the investigators are seeking early feedback of the preferred animal species to be used for the non-GLP pilot toxicology and biodistribution study. Gottingen minipigs have emerged as a prominent large animal model in ocular research due to their well-documented genetics and manageable size.^{5,6} Given this, as well as the fact that pigs are the laboratory animal species that has a corneal volume closest to that of humans,⁷ the investigators propose to use the Gottingen minipig as the large animal in which to perform a pilot 3-month study on the pharmacology, biodistribution, immunogenicity, and toxicity of toxicology grade rAAV8-hSLC4A11 vector (GMP quality) from our CDMO.

Slit lamp ophthalmoscopy, tear collection, intraocular pressure (IOP) measurement, AS OCT, endothelial cell imaging with specular or confocal microscopy, hematology, coagulation, and

clinical chemistry will be performed on each minipig prior to a single administration of one of three doses of the rAAV8-hSLC4A11 vector and at Weeks 2, 4, and 13 (prior to necropsy). At Week 13 (terminal endpoint), minipigs will be sacrificed and eyes will be enucleated for ocular tissue and aqueous fluid collection and assessment. Samples of non-ocular tissues and blood will be used for biodistribution studies and histopathology (see [Section 8.2](#)).

4.3 Manufacturer, Manufacturing Process and Schematic

Andelyn Biosciences (Andelyn) is contracted to develop and manufacture both the drug substance (plasmid) and the rAAV8-hSLC4A11 drug product (vector). Andelyn (FDA Establishment Identifier Number 3026042970) is a full-service cell gene therapy CDMO focused on the development, characterization and production of viral vectors for gene therapy. Andelyn has produced cGMP material for more than 450 clinical batches and 75 global clinical trials in the last 20 years. Andelyn's capabilities include plasmid development and manufacturing, process development and cGMP clinical and commercial manufacturing, with a cGMP manufacturing capacity for both adherent and suspension processes up to a 2,000-liter capacity.

Andelyn will be generating good manufacturing practice (GMP) master cell banks (MCB) of the pTR-EF1-SLC4A11_Kan plasmid and will manufacture research grade and toxicology grade rAAV8-hSLC4A11 vector to be used in the planned preclinical studies described in [Sections 4.2](#) and [8.2](#). A Letter of Authorization will be provided in future regulatory submissions (e.g. Pre-IND and IND) to permit cross reference to Andelyn's Master File #28320. To facilitate review in this meeting request, Andelyn has shared summary descriptions of development and production stages.

Gene of interest (GOI) plasmid feasibility assessment

The sponsor has provided Andelyn with the GOI plasmid, pAAV-kan-EF1A-hSLC4A11, which will be used for the production of the rAAV8-hSLC4A11 vector. To evaluate the yield performance of the pAAV-kan-EF1A-hSLC4A11 plasmid in the Andelyn plasmid production platform, a 1 x 12 L production run of GOI plasmid was performed and tested for DNA homogeneity, identity, and purity. The Post-Harvest Yield (mg/L) and (mg/gm pellet) of the 12 L production was 4.3mg/L and 0.18 mg/gm pellet. The Final Post-Purification Yield of the 12 L production was 1.7 mg/ L and 0.07 mg/ gm pellet. Andelyn will provide the sponsor with pAAV-kan-EF1A-hSLC4A11 plasmid research grade material.

GOI plasmid MCB generation

A schematic of the master cell banking process is depicted in [Figure 4](#). Standard testing will include quantification (e.g. concentration, purity), identity (e.g. restriction digest, DNA sequencing 4X coverage), lytic and lysogenic bacteriophage testing, and bacterial characterization and viability. Andelyn will provide the sponsor with approximately 140 vials GMP plasmid master cell bank.

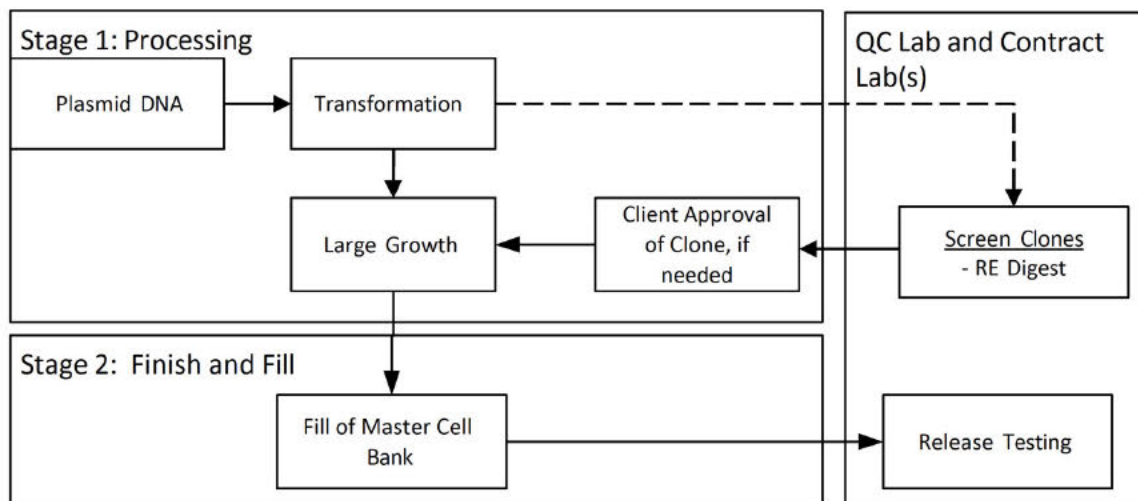


Figure 4. Generation of GMP master cell bank of the pTR-EF1-SLC4A11_Kan plasmid. Schematic of the master cell banking process by Andelyn.

TOX grade 72L production of the AAV8 helper plasmid and TOX grade 72L production of the GOI plasmid

Andelyn will perform 1 x 72L flask TOX grade production of the AAV8 helper plasmid and 1 x 72L flask TOX grade production of the pTR-EF1-SLC4A11_Kan plasmid. Standard testing panel includes quantification (concentration), safety (endotoxin and bioburden), identity (sequencing and restriction digest), and purity (plasmid DNA homogeneity). Andelyn will provide the investigator with AAV8 and GOI pDNA TOX grade material for the planned GLP study (for discussion in the future Pre-IND meeting).

AAV upstream production yield assessment

The rAAV8-hSLC4A11 vector plasmid was evaluated in terms of AAV8 vector production. Three 125 mL flasks containing 50mL media were seeded with Andelyn's suspension AAV Curator™ cell line and transfected using Andelyn's FectoVIR-mediated transfection platform. Four days post transfection, the culture was harvested separately from each flask. The crude culture was sampled to determine cell density and viability at harvest. The culture was centrifuged, and the supernatant was sampled to determine AAV titer by ddPCR. The assessment yielded an average titer of 5.40×10^{10} vg/mL at harvest, exceeding the typical minimum threshold for further manufacturing of 2.5×10^{10} vg/mL.

AAV downstream process (DSP) optimization

Given confirmation of expected harvest titers from the yield assessment, Andelyn will perform 3 x 2L flask productions, which will be used for downstream purification evaluation using Andelyn's all-column platform. Andelyn will provide the investigator with a DSP optimization report including genomic titer and vector material.

Research grade AAV production

2L scale production of research grade rAAV8-hSLC4A11 will be performed at Andelyn's Development Center using Andelyn's AAV suspension platform. Standard testing of research grade AAV8-hSLC4A11 includes titer by ddPCR, infectious unit titer, potency, purity (SDS-PAGE, full to empty ratio, AAV particle size and aggregation, rcAAV, sub-visible particulate), safety (endotoxin, bioburden, bacteriostasis & fungistasis, mycoplasma), identity (DNA and protein), osmolarity, pH, and total protein quantification. Andelyn will provide the investigator with research grade rAAV8-hSLC4A11 vector.

Toxicology grade AAV production.

AAV TOX grade 50L scale manufacturing of rAAV8-hSLC4A11 will be performed at Andelyn's Development Center using Andelyn's AAV suspension platform in a Pall® bioreactor. Standard testing of TOX grade rAAV8-hSLC4A11 includes titer by ddPCR, infectious unit titer, potency, purity (SDS-PAGE, full to empty ratio, AAV particle size and aggregation, rcAAV, sub-visible particulate), safety (endotoxin, bioburden, bacteriostasis & fungistasis, mycoplasma), identity (DNA and protein), osmolarity, pH, and total protein quantification. Andelyn will provide the investigator with TOX grade rAAV8-hSLC4A11 vector (for discussion in the future Pre-IND meeting).

A schema of the general manufacturing steps is provided in **Figure 5**.

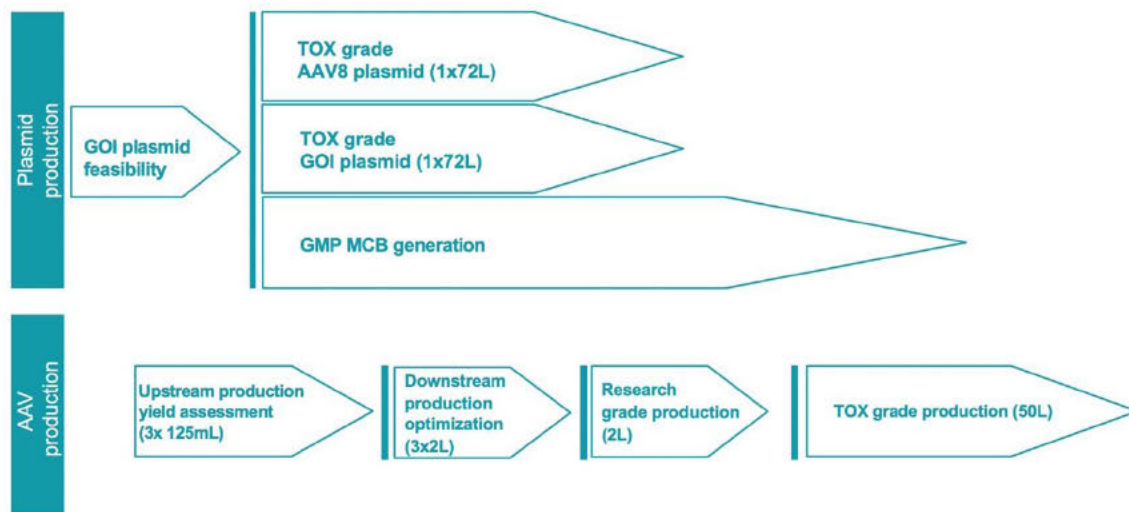


Figure 5. Andelyn manufacturing steps. Schematic diagram of the proposed Andelyn manufacturing stages to produce toxicology (TOX) grade pTR-EF1-SLC4A11_Kan plasmid, TOX grade AAV8 helper plasmid, and GMP master cell banks (MCB), and to manufacture research grade and TOX grade rAAV8-hSLC4A11 vector to be used in the proposed preclinical studies.

5. DOSAGE FORM, ROUTE OF ADMINISTRATION AND DOSING REGIMEN

AAV8-hSLC4A11 is a non-replicating, rep/cap-deleted, rAAV vector containing wild type human single stranded cDNA encoding SLC4A11 variant B protein. The formulation relies on Andelyn Biosciences' proprietary buffer composition and the AAV8 capsid, which has been reported to efficiently achieve corneal gene delivery in many species including human corneas ex vivo.^{1,2}

The route of administration is a single corneal intrastromal injection of a volume of 100 µL, which is consistent with the 50 –100 µL volume of voriconazole and amphotericin B injected into the cornea when treating fungal keratitis, and thus is an appropriate and clinically utilized volume for injection into the human cornea.⁸⁻¹⁰ This dose is also consistent with the 100 µL dosage volume planned in the toxicology and biodistribution studies in Gottingen minipigs, which is based on the fact that the posterior corneal surface area in the pig is approximately 25 times that of the *Slc4a11*^{-/-} mice, in which a 3 µL dose of rAAV8-hSLC4A11 vector preparation was injected into the cornea in one eye. The cellular target of AAV8-hSLC4A11 is the corneal endothelium, which consists of a monolayer of non-dividing endothelial cells, representing a suitable target for a primarily episomal AAV vector.

Vector dosing will be based on completed and planned dose-response studies and planned safety, toxicology and biodistribution studies.

6. PURPOSE OF THE MEETING

The purpose of this meeting is to obtain Agency reviewer guidance and feedback on early development CMC, nonclinical and clinical plans in preparation for a Pre-IND and eventual IND application. Specifically, the investigators wish to seek agreement and input from the Agency for specific questions listed in this meeting request.

7. PROPOSED AGENDA

If a teleconference is granted, we are proposing the following agenda.

Topic	Duration
Introductions	5 minutes
Discussion of prioritized questions (to be provided after written response)	40 minutes
Meeting conclusions and summary of discussion	15 Minutes

8. AGENCY QUESTIONS GROUPED BY DISCIPLINE

8.1 Chemistry, Manufacturing and Controls

8.1.1 Question 1 and 2: Measurement of Potency

See [Section 4.3](#) for manufacturing process overview.

The investigator, in collaboration with the BGTC, intend to develop and qualify a potency assay that measures transgene (hSLC4A11) mRNA expression following transduction into a cell line known to be susceptible to the AAV8 serotype. The investigator believes that measurement of hSLC4A11 mRNA expression, combined with Sanger sequencing of the transgene, provides an adequate measure of potency of the rAAV8-hSLC4A11 vector. Once these two methods are qualified, they will be used to measure potency in all Phase 1 clinical lots intended for patient administration.

1) Does the Agency agree that measurement of hSLC4A11 mRNA expression in a serotype-susceptible (AAV8) cell line, coupled with Sanger sequencing, is an appropriate measure of potency for release of Phase 1 clinical drug product?

The scope of the BGTC is to facilitate first-in-human trials for genetic diseases where there is no current commercial interest. However, BGTC is considering a potency assurance strategy for late-phase development and potential commercial licensure in the event that initial trials yield promising clinical results.

2) Once validated, the investigator/BGTC believe that these same mRNA quantification and Sanger assays described in Question 1 would be an adequate and reproducible measure of potency for release of Phase 3 and commercial product. The investigator/BGTC request Agency feedback on the plan for potency testing including any guidance on the use of these assays for commercial release of drug product.

Rationale and Background:

The mechanism of AAV vectors, including rAAV8-hSLC4A11, is to bind to target cells, traffic to the nucleus, uncoat and release the vector DNA. Sanger sequencing of the drug product is included in release testing to ensure that the fidelity of the hSLC4A11 sequence is correctly maintained through manufacture of the drug product. Measurement of transgene mRNA in a cell line susceptible to AAV8 indicates that the transgene has been successfully incorporated in a target cell. Development of a functional cellular potency assay, such as quantification of the downstream protein coded by the transgene, is complex and may be difficult to qualify or validate due to either low or variable expression of the transduced protein. If the vector DNA is released in a reproducible manner matching expectations, the investigator/BGTC's position is that the drug product has functioned correctly. All subsequent steps including translation of the protein and action of the protein are downstream cellular functions stemming from the production of the hSLC4A11 mRNA.

The proposed approach is consistent with the prior CMC informal meeting the BGTC has held with the Agency in 2022 (see [Section 1.3](#)). Feedback provided in this INTERACT meeting will

allow the investigator/BGTC to proceed with development of this assay and additional details on the qualified method will be shared in the future Pre-IND meeting.

8.1.2 Question 3: Stability Plan

Table 1 summarizes the testing plan and time points for the toxicology and clinical GMP batch drug product stability studies. The investigator/BGTC propose to evaluate stability for IND submission on the toxicology drug product and include data on 0 and 6 month timepoints. The investigator/BGTC will commit to submitting data in IND updates, for each subsequent time point from the toxicology stability study (12 months) as well as all time points from the clinical GMP stability study. Judicious use of material is necessary given the small number and size of batches expected for this investigational product. A comparison of differences between the toxicology batch and the clinical GMP batch along with a risk assessment and comparability analysis showing that the toxicology batch is equivalent to or representative of the clinical product will be provided in the IND.

Table 1. Stability tests and time points

QUALITY ATTRIBUTES		TOXICOLOGY LOT			CLINICAL GMP LOT			
		TIME POINT (MONTH)						
TEST	METHOD	0 ¹	6 ²	12	0 ¹	6	12	24
Vector Genome Titer	ddPCR	X	X	X	X	X	X	X
Potency	mRNA expression	X	X	X	X	X	X	X
Purity	CE-SDS	X	X	X	X	X	X	X
Bioburden	USP <61> and Ph.Eur. 2.6.12	X	X	X	N/A	N/A	N/A	N/A
Sterility	USP <71>	N/A	N/A	N/A	X	-	-	X
CCIT	USP <1207>	N/A	N/A	N/A	-	-	X	-
Osmolality	Freezing Point Depression, USP <785>	X	X	X	X	-	-	X
pH	Potentiometric, USP <791>	X	X	X	X	-	-	X
Appearance	Visual inspection, USP<631>, USP<790>	X	X	X	X	X	X	X
Particulate Matter (sub-visible particles)	USP <789>	X	X	X	X	X ³	X ³	X ³
AAV Vector Aggregation	Size Exclusion Chromatography	X	X	X	X	X	X	X

¹Test results from batch release will be recorded for t=0. Note: Sponsor to provide the complete panel of batch release tests at Pre-IND, this table only reflects those release tests appropriate for evaluating stability. ²Corresponds to approximate conclusion of GLP toxicology study. ³Diluent samples will be analyzed for sub-visible particles for this time point only. Diluent will be made on the same production line and filled in the same vials as the DP.

3) Does the Agency agree with the proposed time points and proposed source of stability test samples, with the condition that specifications and methods for these tests will be provided at Pre-IND? The investigator/BGTC wish to obtain agreement with the Agency on these specific points prior to Pre-IND as there is a need to plan for production quantities at this time.

Rationale and Background:

It is likely that only a very limited number of batches (as few as one) will be manufactured to support rAAV8-hSLC4A11 clinical trials and both the yield and scale of clinical production is currently unknown. Given limited availability of GMP batches and the possibility of expanded access if initial clinical results are promising, the investigator aims to be as efficient as possible with the consumption of clinical product. If appropriate and in order to reserve material in clinical trials that would otherwise be consumed for stability testing, the investigator believes samples from toxicology manufacturing runs will be representative of the clinical drug product. Concurrent with the goals of the BGTC, the investigator/BGTC request regulatory flexibility on the typically expansive panel of tests and time points for stability as well as the ability to supplement stability data with comparable investigational product material, as necessary. This topic was discussed briefly at the BGTC CMC informal Agency meeting.

8.2 Nonclinical

8.2.1 Question 4: Design of non-GLP Pilot Minipig Study

4) Does the Agency agree with the design of the non-GLP pilot toxicology and biodistribution studies, including the selection of Gottingen mini pigs as an appropriate large animal model, the dose translation strategy, in vivo safety parameters, biodistribution analysis and study duration?

Rationale and Background:

AAV8-hSLC4A11 safety, toxicology and biodistribution studies in Gottingen minipigs.

Traditionally, rabbits have been a commonly used animal model for research involving the cornea given similar corneal diameter and central corneal thickness to humans.^{5,7} However, given the regenerative capacity of the rabbit corneal endothelium, a phenomenon not seen in humans, they are a less than ideal animal model for investigating the pharmacodynamics, biodistribution, immunogenicity and toxicology of a viral vector targeting the corneal endothelium.¹¹ Gottingen minipigs have become a popular non-rodent animal model to use for ocular research due to the similarity of their ocular anatomy to humans, well-monitored genetics,^{12,13} size and ease of handling.⁶ Gottingen minipigs have been utilized for evaluating the safety and efficacy of ocular therapeutics,¹⁴ as an animal model of glaucoma,¹⁵ for testing novel biomaterials for the management of corneal perforations,¹⁶ and for developing surgical techniques for implanting intraocular devices.¹⁷ Given this, as well as the fact that pigs are the laboratory animal species that has a corneal volume closest to that of humans,⁷ the investigators selected Gottingen minipigs as the large animal model to use for safety, toxicology and biodistribution studies of the therapeutic candidate AAV8-hSLC4A11. Human SLC4A11 and porcine SLC4A11 share high protein conservation as demonstrated by pair-wise sequence alignment ([Figure 6](#)).

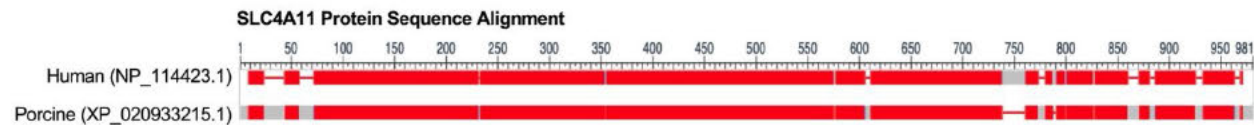


Figure 6. Protein sequence alignment demonstrates high similarity between human SLC4A11 and porcine SLC4A11. A graphical protein alignment shows the highly conserved and less conserved amino acid positions between the human (*Homo sapien*) and porcine (*Sus scrofa*) SLC4A11 protein based on the relative entropy threshold of the residue. Only alignment positions with no gaps are colored. Red indicates highly conserved positions and blue indicates lower conservation. The pair-wise alignment was performed using NCBI Multiple Sequence Alignment Viewer, Version 1.25.2.

The investigators will perform a non-GLP 3-month safety, toxicology, and biodistribution study using the toxicology grade rAAV8-hSLC4A11 vector generated by Andelyn (Tables 2 and 6). Twenty four minipigs (12 male and 12 female) will be used for the study, with each receiving a single administration of one of three doses of the rAAV8-hSLC4A11 vector (1×10^8 vg/ μ L (low), 1×10^9 vg/ μ L (mid), 1×10^{10} vg/ μ L (high)) or control (vehicle only) via intrastromal injection (100 μ L) in one eye. The three dose concentrations selected (1×10^{10} , 1×10^{11} , and 1×10^{12} vg/eye) represent concentrations consistent with those that were utilized in a New Zealand white rabbit model, a canine mucopolysaccharidosis type I (MPS I) model and a feline MPS VI model using the same AAV8 capsid and are one to three log higher than the highest dose that demonstrated therapeutic efficacy in the *Slc4a11*^{-/-} mice.^{1,2} The 100 μ L dosage volume is based on the fact that this volume is consistent with the 50 –100 μ L volume of voriconazole and amphotericin B injected into the cornea when treating fungal keratitis in humans, whose corneas have a volume approximately the same as the pig, and as the posterior corneal surface area in the pig is approximately 25 times that of the *Slc4a11*^{-/-} mice, in which a 3 μ L dose of rAAV8-hSLC4A11 vector preparation was injected into the cornea in one eye. The concentration of the rAAV8-hSLC4A11 vector per μ L will be the same in the 100 μ L dosage volume as in the 3 μ L dosage volume. Slit lamp ophthalmoscopy, tear collection, intraocular pressure (IOP) measurement, AS OCT, endothelial cell imaging with specular or confocal microscopy, hematology, coagulation, and clinical chemistry will be performed on each minipig prior to dosing and at Weeks 2, 4, and 13 (prior to necropsy). Whole blood samples will be also be collected prior to dosing and at weeks 2, 4, and 13 following dosing and processed to serum or plasma for immunogenicity and biodistribution studies. Clinical observations, body weight, and food intake will also be regularly monitored. At Week 13 (terminal endpoint), minipigs will be sacrificed and eyes will be enucleated for ocular tissue and aqueous fluid collection and assessment. Samples of non-ocular tissues will be collected for histopathology and biodistribution studies. The investigators have consulted with BGTC nonclinical experts as well as Agency feedback from the informal nonclinical meeting held on June 27, 2023, for the design of this study.

Table 2. Non-GLP Pilot Study Synopsis: rAAV8-hSLC4A11 Toxicology and Biodistribution Study in Gottingen Minipigs

Objective	The objective of this pilot 3-month study is to: evaluate the appropriateness of the Gottingen minipig as the large animal in which to evaluate the pharmacology, pharmacodynamics, biodistribution, immunogenicity, and toxicity of rAAV8-hSLC4A11 when administered as a single dose via intrastromal injection; and to evaluate a range of doses of the rAAV8-hSLC4A11 vector that will be used to inform the doses selected for a subsequent IND enabling GLP-compliant safety, toxicology and biodistribution study.				
Animals	12 male and 12 female mini-pigs. Two additional animals/sex will be available in case animal replacement is required. Age of animals to be discussed based on pharmacological endpoints (anticipate 3-5 months). Animals will be tested for the presence of anti-AAV8 neutralizing antibody (Nab) status and excluded if positive. Animals will be sacrificed 3 months after dosing.				
Justification for use of the mini-pig	The Gottingen mini-pig has been selected as pigs are the laboratory animal species that has a corneal volume closest to that of humans and the transgene product is pharmacologically active in this species.				
Study Groups	Vector	Dose Conc. (vg/ μ L) (OD)	Fixed Dose Vol (μ L/eye)	Dose (vg/eye) (OD)	No. of Animals
1	Vehicle	0	100	0	3M+3F
2	rAAV8-EF1 α -hSLC4A11	1 x 10 ⁸ (low)	100	1 x 10 ¹⁰	3M+3F
3	rAAV8-EF1 α -hSLC4A11	1 x 10 ⁹ (mid)	100	1 x 10 ¹¹	3M+3F
4	rAAV8-EF1 α -hSLC4A11	1 x 10 ¹⁰ (high)	100	1 x 10 ¹²	3M+3F
Dose Justification	The three dose concentrations of the toxicology (TOX) grade rAAV8-hSLC4A11 vector from Andelyn selected represent concentrations higher than those that demonstrated therapeutic efficacy in the <i>Slc4a11</i> ^{-/-} mice. The amount of the dose to be administered in each eye is a clinically utilized volume for injection into the human cornea, which has a volume that is approximately the same as that of the Gottingen minipigs.				
Immune suppression	Animals will not be administered immunosuppression unless it is required for surgery.				
Dosing Schedule	A single dose on Day 1.				
Dosing Route	The test article (TA) or control articles will be dosed into the cornea of the right eye. The dose volume will be 100 μ L. TA will be administered into the cornea via intrastromal (posterior) injection. Prior to sedation for dosing, flunixin meglumine, a nonsteroidal anti-inflammatory drug, will be administered by intramuscular injection. Upon recovery from anesthesia, buprenorphine, a semi-synthetic opioid, will be administered by intramuscular injection. Additional buprenorphine injection(s)				

	may be administered at the discretion of the technical staff for the management of discomfort.
Dose Preparation	TA or vehicle will be provided in labelled vials at the required dose concentrations for each animal prior to administration for use as supplied. Stability on the TA will be performed as required.
Test article analysis	Not required, as formulations will be provided at concentrations required. Remaining TA will be retained until the completion of the study.
Serial Observations	Clinical observations, body weight and food intake (qualitative). At cageside observations (following dosing and recovery from anesthesia on Day 1, and twice daily thereafter for mortality, abnormalities, signs of pain or distress), animals will be observed within their cage for potential effects of vehicle or test article administration. Detailed observations: at least once during the predose phase, during the dosing, and on the day of scheduled sacrifice. Body Weight: at least once during the predose phase, during the dosing, and on the day of scheduled sacrifice. Food Consumption: Qualitative, by cage; daily.
Ocular Endpoints	Typically these would include the following endpoints at these times:
Slit lamp ophthalmoscopy	Twice prior to dosing, Day 3, Weeks 1, 2, 4, 8 and 13. Animals will be sedated. Both eyes will be examined by board certified veterinary ophthalmologist.
Slit lamp photography	Once prior to dosing, as needed (for documentation of abnormalities) Day 3, Weeks 1, 2, 4, 8 and 13. Animals will be sedated. Both eyes will be imaged by board certified veterinary ophthalmologist.
Tear collection (via STT strips)	Twice prior to dosing, Day 3, Weeks 1, 2, 4, 8 and 13. Animals will be sedated. Samples will be collected by qualified personnel and placed in tubes and stored frozen until shipment/analysis.
IOP (TonoVet or similar)	Twice prior to dosing, Day 3, Weeks 1, 2, 4, 8 and 13. Animals will be sedated. IOP will be measured by qualified personnel.
Indirect ophthalmoscopy	Twice prior to dosing, Weeks 2, 4, 8 and 13. Animals will be sedated. Both eyes will be examined by board certified veterinary ophthalmologist.
Ultrasonic corneal pachymetry	Once prior to dosing, Hour 8, Days 1 and 2 Animals will be sedated. IOP will be measured by qualified personnel.

Anterior segment OCT	Once prior to dosing, Weeks 2, 4, 8 and 13. Imaging will be taken of cross section of cornea and anterior chamber. The OCT images will be evaluated qualitatively and a report will be created. Representative images will be included in the imaging report.	
Specular or Confocal microscopy (with endothelial cell counts)	Once prior to dosing, Weeks 2, 4, 8 and 13. Images will be obtained of the endothelium and any corneal stromal lesions. Images will be evaluated qualitatively, and endothelial cell counts (cells/mm²) will be provided in a report that will also contain representative images.	
Hematology	Once prior to dosing, Weeks 2, 4 and 13 (prior to necropsy).	
Coagulation	Once prior to dosing, Weeks 2, 4 and 13 (prior to necropsy).	
Clinical chemistry	Once prior to dosing, Weeks 2, 4 and 13 (prior to necropsy).	
Urinalysis	Week 13 (a sample can be collected at necropsy).	
Immunogenicity	Nabs: Blood will be collected and processed to plasma or serum (blood sample volume of ~1mL for 2x250 µL serum) for evaluation of Nab upon arrival (within the first five days), prior to dosing, on Day 1, Week 2, 4 and 13 (prior to necropsy). Anti-transgene product antibodies (ATPA): Blood (sample volume of ~1mL) will be collected and processed to plasma or serum for evaluation of ATPA prior to dosing, on Day 1, Weeks 2, 4 and 13 (prior to necropsy). Samples to be shipped to the Test Facility selected for Bioanalysis (sponsor designee). PBMCs will be collected for potential ELISPOT analysis, prior to dosing and prior to necropsy. Blood sampling and volumes to be adjusted based on volume allowances in mini-pigs and Test Facility guidelines.	
Biodistribution	Whole blood will be collected for vector DNA prior to dosing, Day 4, Weeks 2, 4, 8 and 13. For tissues, see terminal procedures.	
Terminal Procedures	Animals will be perfused with PBS (as per Test Facility SOP for perfusion).	
Tissue sampling	Organ weights and tissue retention for all animals. Tissues in accordance with regulatory requirements (to include reporting as tissue:bodyweight and tissue:brain weights). Histopathology – full list (see below). Ensure evaluation of lymphoid organs (spleen, thymus, bone marrow, and lymph nodes [mandibular, axillary, inguinal and parotid]).	
Histopathology Full Tissue List	adrenal (2) aorta	lung with large bronchi

	bone, femur with bone marrow (articular surface of the distal end) bone, sternum with bone marrow brain cecum cervix colon dorsal root ganglion (cervical; 2) dorsal root ganglion (lumbar; 2) dorsal root ganglion (thoracic; 2) duodenum epididymis (2) esophagus eye (OS) with bulbar conjunctiva^{a,b} eyelids, upper with palpebral conjunctiva (2) eyelids, lower with palpebral conjunctiva (2) gall bladder (drained) gut-associated lymphoid tissue(GALT)/ Peyer's patch heart ileum jejunum kidney (2) lacrimal gland (right) larynx liver	lymph node (mandibular, right) lymph node (mesenteric) lymph node (parotid, right) mammary gland (females) muscle, biceps femoris muscle, masseter nasal turbinates^c nasolacrimal duct (right) nasopharynx^c optic chiasm optic nerve^{a,b} oral cavity ovary (right) oviduct (2) pancreas pituitary gland prostate rectum salivary gland (mandibular, right) sciatic nerve seminal vesicle skin/subcutis sinus, frontal spinal cord (cervical, thoracic, and lumbar) spleen stomach testis (right)^d thymus thyroid (2 lobes) with parathyroid tongue trachea urinary bladder uterus vagina
	^a Collected in 4% paraformaldehyde and processed to paraffin block after no more than three overnights in fixative. Prior to placing the eyes in fixative, they will be injected with 4% paraformaldehyde until turgid. ^b The right eyes from all animals in Group 4 will not be collected for microscopic evaluation. ^c Collected and retained in fixative for possible future evaluation. ^d Collected in modified Davidson's fixative and stored in 10% neutral-buffered formalin.	
Pharmacodynamics	Additional samples will be collected to detect the transgene product (SLC4A11 protein) either using bioanalytical methods or histological techniques (e.g. IHC) to demonstrate expression in the target tissue.	

Eye (OD)	At necropsy and immediately following enucleation of each eye, a sample of the aqueous fluid (target volume of 50 µL, if possible) will be collected, and the aqueous fluid will be frozen. For animals in Group 4, the right eye with optic nerve will be designated for fresh tissue collections.
To be confirmed depending on endpoints required	Aqueous humor (target volume of 50 µL) Vitreous humor Choroid – RPE Cornea Conjunctiva Extraocular muscle Lens Iris-ciliary body Optic nerve Retina Sclera Posterior eye cup Eyes will be divided into four quadrants (superior, inferior, nasal, and temporal). From each quadrant, one sample will be taken using an 8 mm biopsy punch. Alternatively, collect one punch for BD only. Filter paper will be used to collect the retina. All remaining posterior eye cup will be collected following collection of the biopsy punches
Biodistribution	In accordance with ICH S12. Tissues collected include: gonads, adrenal gland, right brain (optic nerve, optic chiasm, occipital lobe, frontal cortex, hippocampus, cerebellum, and thalamus), aqueous and vitreous humors, CSF, spinal cord (cervical, thoracic, and lumbar), DRG (cervical, thoracic and lumbar), lymph nodes (mandibular, parotid), salivary gland (mandibular), peripheral nerves, liver, kidney, lung, heart, spleen, thymus and blood., right nasolacrimal apparatus, nasopharynx, larynx, oral cavity, facial muscles, and sinuses. Tissues that are collected will be placed on dry ice immediately after collection, then stored at -80 degrees C.
Scheduled Necropsy Requirements	Necropsy will be supervised by a veterinary pathologist with knowledge of the study protocol. This should be the assigned Study Pathologist, but if he/she is not available the duty pathologist may substitute.
Data Treatment	Individual tables to be provided with mean +/- SD.
Report	The final study report will meet FDA CTD reporting requirements, especially as it relates to TOC, section and figure/table numbering, and PDF requirements of the CTD. The final report will be hyperlinked and contain a CTD table as the final appendix, and be provided as a PDF and Word file.

8.2.2 Question 5: Exemption from SEND

5) The investigator will be submitting a noncommercial IND for rAAV8-SLC4A11 (i.e., the product is not intended for commercial distribution). Does the Agency agree that the preclinical safety studies contained in the IND would be exempt from standardized study data requirements (i.e. SEND) as provided by FDA Guidance for Industry, Providing Regulatory Submissions In Electronic Format – Standardized Study Data and Section 745A(a) of the FD&C Act?

8.3 Clinical

8.3.1 Question 6: Pediatric Subjects

6) Does the Agency agree with the investigator's proposal to include pediatric subjects in the first-in-human trial, in compliance with 21 CFR Part 50, Subpart D, given evidence of a direct benefit for the pediatric population based on the natural history of the disease and efficacy data from the only disease model available?

Rationale and Background:

CHED is a congenital condition present from birth with progressive corneal edema and loss of corneal endothelial cells with age. There is no approved alternative therapy other than corneal transplantation. The median age at the time of the initial corneal transplant for CHED is approximately 9.5 years, indicating individuals with CHED who are older than 18 years old (adult) would most likely have received corneal transplantation in both eyes and no longer need gene therapy treatment. The determination of the age at which corneal transplantation should be performed for CHED takes into account individual patient and family characteristics (the degree of corneal opacification, the amount of family support, etc) as well as a balancing of increased intraoperative and postoperative keratoplasty complications associated with earlier intervention against the increased risk of amblyopia development associated with delayed intervention. Although earlier corneal transplantation in CHED is associated with better visual outcomes,¹⁸ a successful corneal transplant with long-term graft survival in a pediatric patient, especially a young child, requires care across multiple ophthalmic specialties and a long-term commitment by the patient's family.

The potential advantage of rAAV8-SLC4A11 gene therapy in young children with CHED is to offer the benefit of early intervention in preventing amblyopia development without the risk and long-term care associated with corneal transplantation. Additionally, given the progressive loss of corneal endothelial density over time in CHED, and the demonstration that *Slc4a11* gene replacement in *Slc4a11*-deficient mice is most effective at reducing corneal edema and preserving corneal endothelial cell function in younger mice, rAAV8-SLC4A11 gene therapy is most likely to offer maximum visual recovery potential and to avoid risks associated with corneal transplantation in young children with CHED.⁴

FDA guidance document FDA-2022-D-0738, Section III, Subsection A, specifies children should not be enrolled into a clinical investigation unless their participation is necessary to answer an important scientific question directly relevant to the health and welfare of children. The principle of scientific necessity is encompassed in two regulatory requirements: the equitable selection of subjects (21 CFR 56.111(a)(3)) and minimization of risk (21 CFR 56.111(a)(1)). In this case, the safety and effectiveness of rAAV8-SLC4A11 gene therapy in adults with CHED cannot be extrapolated to children with CHED, affirming the scientific necessity to collect safety and effectiveness data in children.

The selection of minimal enrollment age of 3 years old in this study is a result of balancing the following factors:

- 1) The rate of increase in CCT in children with CHED is greatest during first 3 years of life in our natural history data (**Figure 8**), suggesting that the optimal therapeutic window is before 3 years of age.
- 2) The increased risk of anesthesia, including a potential impact on brain development, in children under 3 years of age, as specified in an FDA 2016 warning.
- 3) The ability to perform post-treatment follow-up visits in an outpatient clinic setting, including diagnostic testing (such as corneal thickness and corneal densitometry) in children older than 3 years of age, in contrast to the requirement for frequent eye examinations under anesthesia for children younger than 3 years of age in the operating room, where these diagnostic devices are not available.
- 4) The stage of cognitive development in children older than 3 years old enables the study to assess more safety and functional efficacy outcome measures, e.g. ocular pain, discomfort, visual acuity, contrast sensitivity and quality of life.

FDA regulations in 21 CFR Part 50, Subpart D, specify that a clinical investigation in which more than minimal risk to children is presented by an intervention that holds out the prospect of direct benefit for the individual subject may involve children as subjects only if (a) the risk is justified by the anticipated benefit to the subjects; (b) the relation of the anticipated benefit to the risk is at least as favorable to the subjects as that presented by available alternative approaches; and (c) adequate provisions are made for soliciting the assent of the children and permission of their parents or guardians. Information relevant to these requirements includes the following:

As stated in **Section 8.2**, preclinical studies indicate that intrastromal injection of AAV8-hSLC4A11 offers a reasonable hope for improving vision in affected human subjects and have demonstrated an excellent safety profile. Additionally, FDA approved systemically administered AAV gene therapy for spinal muscular atrophy (Zolgensma (onasemnogene abeparvovec-xioi)) has a reasonable safety profile in infants less than 2 years of age. As stated in **Section 1.1**, corneal transplantation is currently the only proven treatment option for restoration of corneal clarity in infants and children with CHED, but is associated with a relatively high incidence of intraoperative and postoperative complications, including increased rates of donor corneal rejection and failure. Long term topical ocular corticosteroid use to decrease the risk of corneal graft rejection is also associated with an increased risk of secondary cataract and glaucoma. As the mean corneal graft survival for CHED is approximately 10 years, multiple corneal transplants will be needed to maintain vision for individuals with CHED, with a documented decreasing duration of graft survival with each subsequent corneal transplant.

This clinical protocol design requires that the investigational product be tested in 1 participant with severe corneal edema (corneal densitometry score (CDS) ≥ 40 and ≤ 45 grayscale units (GSU)) at each dosage level with 2-month follow-up before enrolling 3 additional participants with mild to moderate corneal edema (CDS < 40 GSU) staggered at least 1 week apart, a strategy designed to ensure safety of the enrolled participants. If the investigational product is found to be unsafe, enrolled participants will be eligible for corneal transplantation as a rescue measure, reverting to the standard of care treatment for CHED. If the investigational product is found to be

ineffective after the Year 1 follow-up visit, enrolled participants will be eligible for corneal transplantation.

8.3.2 Question 7: Dose Translation

7) Does the Agency agree with the dose translation strategy from the planned minipig toxicology study to first-in-human clinical trial?

As porcine and human corneas have a similar volume and posterior surface area, the dosing strategy for the first-in-human (FIH) clinical trial is expected to be similar to that utilized in the Gottingen minipig toxicology and biodistribution studies (**Table 3**). Specifically, we expect the dosage range among low-dose, medium-dose and high-dose groups in the FIH trial to be within the range of 1×10^{10} - 1×10^{12} vg/eye, given previous experience in AAV8 intrastromal delivery in large animals and human donor corneas as listed below:

Administration of rAAV8G9-CMV-IDUA at a dose range of 5×10^{10} - 8×10^{10} vg/eye by intrastromal injection in canine corneas demonstrated therapeutic effect restoring corneal clarity in MPS I canine model.²

Administration of rAAV8-EF1 α -opt-IDUA at a dose of 1×10^9 - 1×10^{10} vg/eye by intrastromal injection in New Zealand White Rabbits produced expression of IDUA protein at therapeutic level in corneal endothelium, stromal keratocytes and corneal epithelium (see Completed Preclinical Studies. Safety and biodistribution studies in large animal models. *rAAV8 intrastromal injection in New Zealand White rabbit model*).¹

Administration of rAAV8-CMV-GFP at a dose of 5×10^{10} vg/eye by intrastromal injection in *ex vivo* human donor corneas demonstrated GFP expression in corneal endothelium. The transduction efficiency of human corneal endothelium is dependent on the injection depth and injection volume, in addition to viral dosage.

Table 3. Corneal biometry comparison in humans and lab animals

	Human	Rabbit	Canine	Cynomolgus	Pig
r (mm) ⁱ	6.5 ¹⁹	7.26 ²⁰	8 ²¹	5.12 ²²	8.95 ²¹
a (mm) ⁱⁱ	11.5 ²³	13.2 ²⁰	14.5 ²⁴	9.8 ²⁵	13.2 ²⁶
A (mm²) ⁱⁱⁱ	141.7	193.2	232.1	116.9	161.8
CCT (mm) ^{iv}	0.553 ²⁷	0.388 ²⁸	0.587 ²⁹	0.457 ³⁰	0.666 ³¹
V (mm³) ^v	78.3	75.0	136.3	53.4	107.8

ⁱ r, the radius of curvature; ⁱⁱ a, corneal diameter; ⁱⁱⁱ A, posterior corneal surface area, $A = 2\pi r(r - \sqrt{r^2 - (a/2)^2})$; ^{iv} CCT, central corneal thickness; ^v V, corneal volume, $V = A \times \text{CCT}$.

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[illegible]

10. SUGGESTED DATES AND TIMES

The investigator is requesting the earliest available meeting date. A meeting time that accommodates a West Coast time zone is appreciated.

11. MEETING FORMAT

Following the written response, the investigator is requesting a teleconference meeting.

12. DETAILED BACKGROUND INFORMATION

12.1 Disease Background

Clinical Manifestations

CHED is an autosomal recessive condition affecting the corneal endothelial cells, which function to maintain corneal clarity. CHED is characterized by bilateral corneal edema and opacification presenting at birth or in the first decade of life (**Figure 7**). Symptoms consist of bilateral visual impairment, which is typically significant, and secondary deprivation amblyopia and nystagmus. The diffuse corneal edema may be associated with increased corneal thickness up to twice normal, although the remainder of the ocular structures typically develop normally. A subset of affected individuals will develop a slowly progressive sensorineural hearing loss (Harboyan syndrome, OMIM# 217400) that is associated with the maintenance of good speech discrimination and may remain undetected until the second to third decade of life. At present, there is no effective pharmaceutical treatment for CHED and thus corneal transplantation is the only treatment option for restoration of vision in infants and children with CHED.



Figure 7. Clinical presentation of Congenital hereditary endothelial dystrophy (CHED). Slit-lamp images of 16-year-old-boy with CHED associated with compound heterozygous *SLC4A11* mutations, demonstrating diffuse corneal stromal edema seen on direct and slit illumination.

Molecular Etiology

CHED is associated with mutations in the *SLC4A11* gene. To date, more than 90 distinct mutations throughout exons 2 to 19 of *SLC4A11* have been identified in individuals with CHED, including missense and nonsense point mutations, insertion or deletion-associated frameshift

mutations, as well as canonical splice-site mutations.³²⁻⁴⁶ Approximately 80% of individuals with CHED screened to date demonstrate biallelic coding region mutations in *SLC4A11*. In CHED cases without *SLC4A11* coding region mutations, an intronic *SLC4A11* mutation was reported to result in aberrant pre-mRNA splicing and functionally null allele, whereas no pathogenic variants have been identified in the *SLC4A11* promoter region thus far.^{39,42,47}

Pathophysiology

SLC4A11 is one of the highly expressed differentiation markers for corneal endothelium and is essential in facilitating energy producing glutaminolysis by maintaining ammonia homeostasis, reducing glutaminolysis-associated oxidative stress, maintaining antioxidant signaling and preventing apoptosis in the corneal endothelium.⁴⁸⁻⁵⁵ The progressive corneal stromal edema observed in CHED is evidence of corneal endothelial dysfunction, either from cell loss or dysfunction of the corneal endothelial “pump-leak” system.⁵⁶⁻⁵⁸ The endothelial pump activity is driven by an ionic electrical-chemical gradient set up by the highly expressed Na⁺/K⁺-ATPase.⁵⁹ As such, corneal endothelial cells have the second highest density of mitochondria among any cell types in the body (second to photoreceptors), necessary to generate sufficient ATP to fuel the Na⁺/K⁺-ATPase driven endothelial pump.⁶⁰ Glucose and glutamine each contribute about half of the energy needed to fuel the corneal endothelial “pump” activity.⁶¹ Multiple lines of evidence support dysfunction of the corneal endothelial “pump” activity secondary to loss of SLC4A11 function as the direct cause of corneal edema in CHED.^{47,60,62-66}

Natural History

To determine the natural history of CHED, we collected demographic and clinical data on 166 individuals (age range: 4 months – 60 years old) with CHED, including 62 published cases (42 male, 20 female) and 104 unpublished cases (57 male, 47 female).^{42,62,67-74} Visual acuity was measured on Snellen visual acuity chart or age-appropriate Cardiff chart for young children. If preoperative VA of both eyes was available at a given age, only the VA of the better-seeing eye was included.

Progressive corneal edema (measured by an increase in CCT) and loss of vision (measured by a decrease in visual acuity) with increasing age were evident in individuals with CHED (**Figure 8A, D**). In 180 eyes with available preoperative corrected visual acuity (VA) at a specified age, the median VA was 20/400 (1.30 logMAR) (95% confidence interval (95% CI) of 20/317 – 20/604 (1.2 – 1.48 logMAR)), with 73.3% eyes reaching the threshold of legal blindness (**Figure 8A**). Preoperative laser interferometer (LI) visual acuity testing performed to assess the visual acuity potential after optical correction and vision therapy demonstrated the potential for visual recovery in 31 of 33 eyes with a mean potential VA improvement of 0.80 logMAR unit (paired t test, $p < 0.0001$) (**Figure 8B**). Similarly, preoperative pin hole visual acuity testing performed to assess visual acuity potential after optical correction revealed the potential for visual recovery in 13 of 14 eyes with a mean potential VA improvement of 0.26 logMAR unit (paired t test, $p < 0.0001$) (**Figure 8C**). These data suggest that individuals with CHED have the potential for significant recovery of vision if corneal edema is corrected with or without subsequent vision therapy for amblyopia.

CCT in individuals with CHED can be fit against age with a one-phase association curve, if it is assumed that corneas in CHED will reach maximum thickness with zero swelling pressure beyond a certain age (Figure 8D, E). Our data of 301 eyes in individuals with CHED with preoperative CCT data demonstrate an increase in CCT between birth and 3 years of age, with a stabilization of CCT after 3 years of age (Figure 8E). In addition to increased CCT, the corneas from children affected with CHED demonstrate decreased endothelial cell density (Figure 8F) and exhibit significantly increased mean corneal densitometry (37.2 ± 1.5 grayscale units (GSU)) compared with controls (15.0 ± 1.9 GSU, $p < 0.0001$) (Figure 8G).

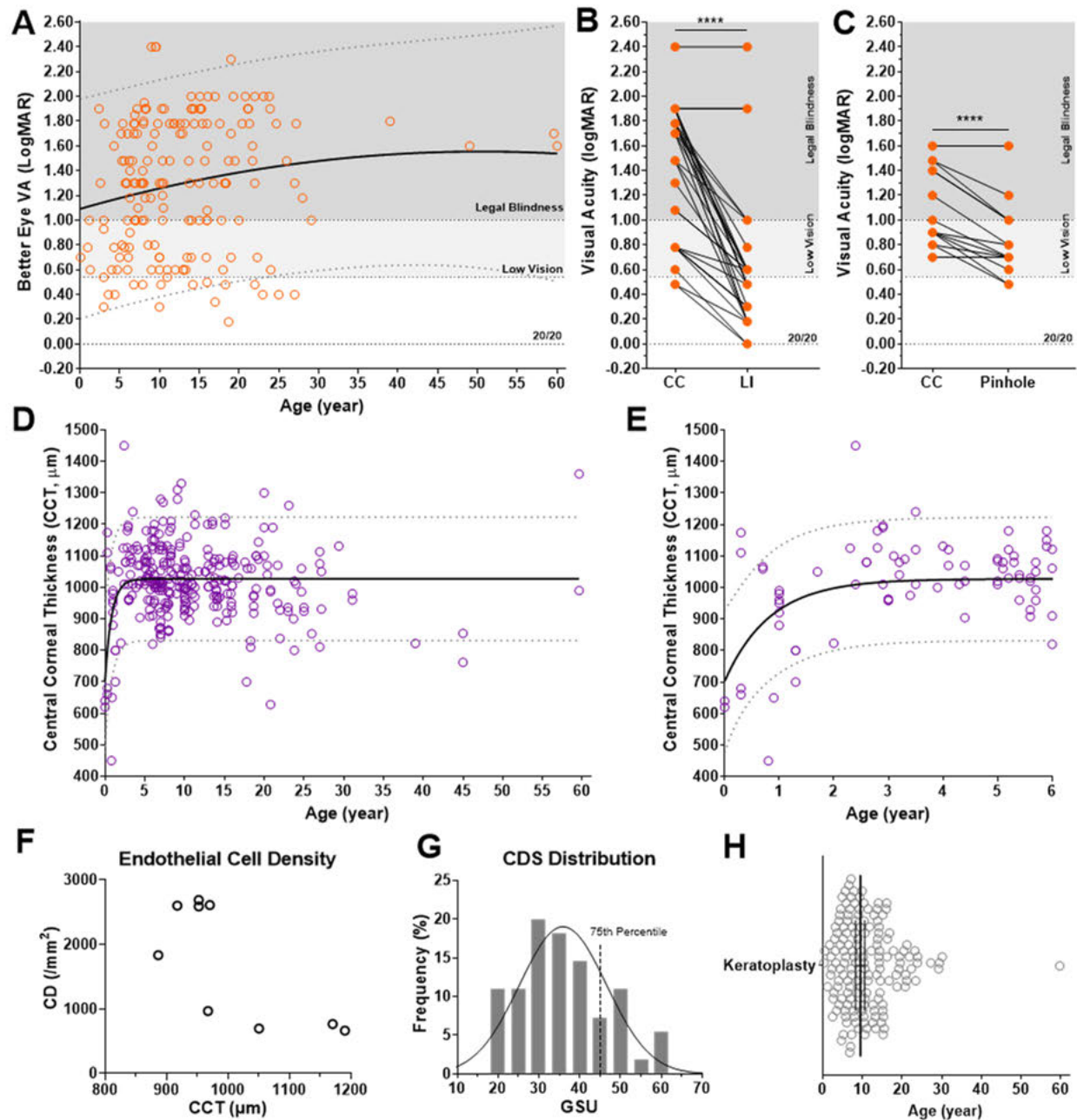


Figure 8. Natural History of CHED. A. Scatter plot of visual acuity (VA) in logMAR unit against age in individuals with CHED. Fitted second order polynomial curve (solid line) with 95% CI (dotted lines) of predicted VA against age were also plotted. Reference lines and shaded areas indicate 20/20 vision, VA range for low vision/moderate visual impairment (light grey) and VA range for legal blindness (dark grey). B. Slope graph of Snellen chart VA in 33 eyes of individuals with CHED measured with correction (CC) and laser interferometry (LI). ****, $p < 0.0001$. C. Slope graph of Snellen chart VA in 14 eyes of individuals with CHED measured with correction (CC) and with pinhole. ****, $p < 0.0001$. D. Scatter plot of CCT against age in individuals with CHED. Fitted one phase association curve (solid line) with 95% CI (dotted lines) of predicted CCT against age were also plotted. E. Zoomed-in figure of scatter plot D, showing CCT plotted against age within first 6 years of life in individuals with CHED. F. Scatter plot showing age at time of initial keratoplasty in individuals with CHED. Median with 95% CI was plotted as line with error bars. G. Scatter plot showing corneal endothelial cell density (CD) plotted against CCT in children 6 – 13 years of age with CHED. H. Histogram of CDS (in grayscale units (GSU)) in 55 eyes of children with CHED. GSU 10 indicates a relatively transparent cornea and 70 indicates a relatively opaque cornea).

In published case series of CHED, the mean age at the time of keratoplasty is reported to be between 8.1 – 13.5 years of age, excluding two case series reporting 42.7 and 3.3 years of age as the mean age at the time of keratoplasty.⁷⁵⁻⁸¹ The first of the two series was published in 1969 and described early experiences of keratoplasty in individuals with CHED, while the second was a case series that specifically described keratoplasty outcomes in young children.^{80,81} Our unpublished data from 157 eyes that underwent corneal transplantation showed a median age at the time of initial keratoplasty of 9.5 years of age (95% CI, 8.3 – 10.6), similar to published series (Figure 8H).

12.2 Completed Preclinical Studies

12.2.1 Vector targeting

Intrastromal injection of AAV8-GFP transduces ex vivo human corneal endothelium

Intrastromal injection of rAAV8-CMV-GFP in donor human corneas at a dosage of 5×10^{10} vg/eye showed the transduction efficiency in corneal endothelium is dependent on the depth and volume of the injection, independent of viral dosage (Figure 9). Viral vectors were diluted in Balanced Salt Solution sterile irrigating solution (BSS™, Alcon) to achieve the desired concentrations; BSS alone was used as the negative control. A single intrastromal injection was performed in each cornea using a 33G needle (Figure 9A). The following injection conditions were tested: 1) central mid-stromal injection of 50 μ L rAAV8-GFP or 50 μ L BSS; 2) central posterior stromal injection of 50 μ L rAAV8-GFP or 50 μ L BSS; 3) central posterior stromal injection of 100 μ L rAAV8-GFP or 100 μ L BSS; and 4) central deep stromal injection of 100 μ L rAAV8-GFP or 100 μ L BSS. Following injection, the corneas were replaced in OptiSol-GS corneal storage medium (Bausch & Lomb) at 37°C with 5% CO₂ until processed for immunostaining. The Descemet membrane was peeled from the corneas immediately before immunostaining, *en face* visualization and quantification of GFP+ cells to estimate the

transduction efficiency (GFP+ / total cells). In the cornea in which a central deep stromal injection of 100 μ L rAAV8-GFP was performed, 37% of corneal endothelial cells were GFP+ (6640 GFP+ cells / 17886 DAPI+ nuclei) on post-injection day 15 (**Figure 9B, C**). The percentage of terminal deoxynucleotidyl transferase (dUTP) nick-end labeling (TUNEL) positive cells, indicative of apoptotic cells, was between 1 -2 % and was not significantly different between the rAAV8-GFP injected and BSS injected corneas.

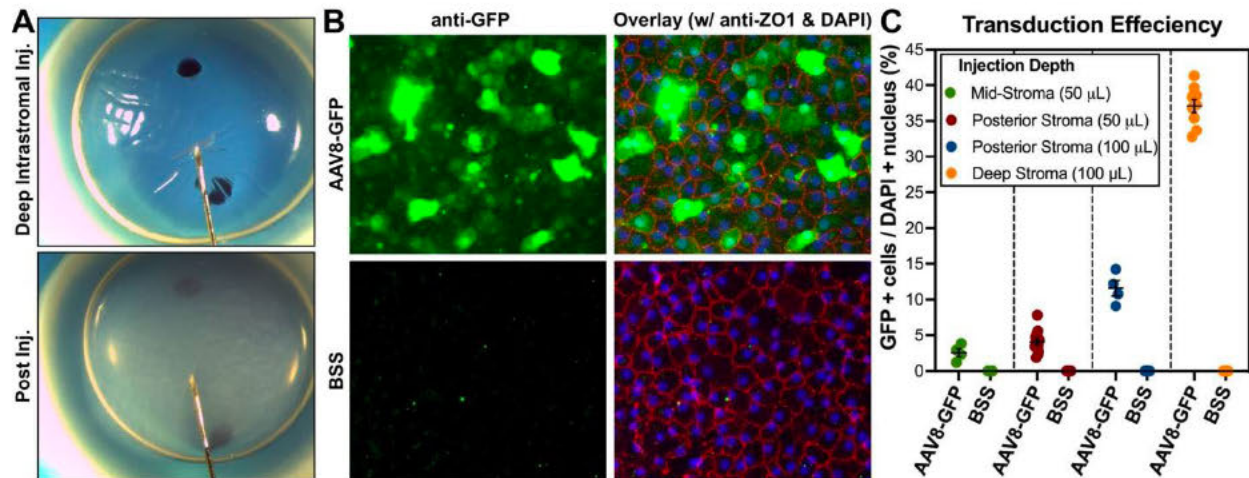


Figure 9. Intrastromal injection of AAV8-GFP in ex vivo donor human corneas transduces the corneal endothelium. A. Photographs of human cornea mounted on artificial anterior chamber prior to and after central deep stromal injection. Air in the anterior chamber facilitates visualization of folds in the Descemet membrane (DM) observed when the needle tip was advanced very close to the DM. B. Representative immunofluorescence staining images of peeled DM from AAV8-GFP or BSS injected human corneas labeled with anti-GFP and anti-ZO1 antibodies with DAPI nuclear staining. C. Scatter plot of the estimated percentage of transduced corneal endothelial cells in AAV8-GFP or BSS injected corneas in four testing conditions with varying injection depth and volume but constant 5e10 vg viral genome dosage.

12.2.2 Efficacy

Therapeutic candidate rAAV8-hSLC4A11 produces mature SLC4A11 protein

The transduction efficiency of rAAV8-GFP in an *SLC4A11*^{-/-} human corneal endothelial cell line was only 0.2% - 1%, consistent with the recognized low transduction efficiency of AAV8 in immortalized cell lines,⁸² and making *SLC4A11*^{-/-} HCEC-21T an unsuitable model for testing the efficacy of our therapeutic candidate rAAV8-hSLC4A11. Therefore, we transduced HEK293 cells with rAAV8-hSLC4A11 following cell transfection with plasmid containing the adenovirus gene E4ORF6, which increases AAV8 transduction in HEK293 cells by ~ 100-fold as shown previously.⁸³

Mature SLC4A11 protein is dimerized and glycosylated when expressed on the plasma membrane.^{66,84} The SLC4A11 monomer is 100 kDa in size and the glycosylated SLC4A11 monomer is ~130 kDa. rAAV8-hSLC4A11 transduction of HEK293 cells resulted in the production of dimerized and glycosylated SLC4A11, as evidenced by Western Blot showing bands at 200 kDa and ~260 kDa when using an anti-SLC4A11 antibody (Invitrogen, PA5-

101889, **Figure 10**). The pAAV-hSLC4A11 plasmid used to produce the AAV8-hSLC4A11 viral vector was transfected in HEK293 cells as a positive control and showed significant over-expression of mature SLC4A11 protein. *SLC4A11*^{-/-} HCEnC-21T cells stably transduced with LV-hSLC4A11vBWT was also included as positive control.

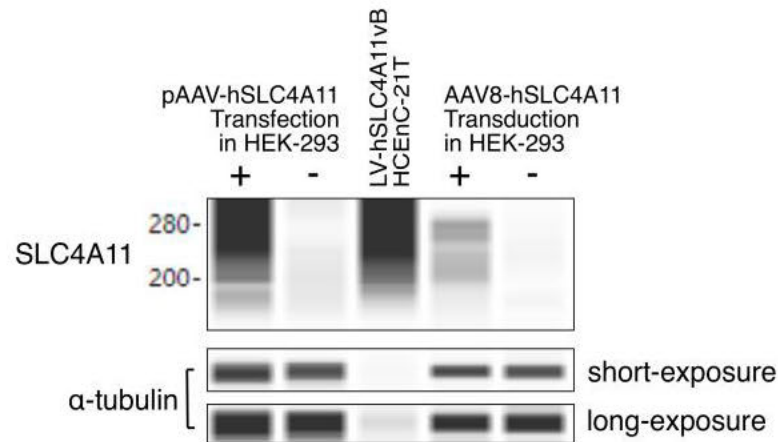


Figure 10. AAV8-hSLC4A11 transduction produces mature SLC4A11 protein. Western Blot results of HEK293 cell lysates after transduction of AAV8-hSLC4A11 blotted for SLC4A11 and α-tubulin proteins.

SLC4A11 replacement restores SLC4A11-mediated NH₃ and H⁺ permeability in *SLC4A11*^{-/-} human endothelial cells.

Central corneal thickness is the functional measure of corneal endothelial “pump” activity *in vivo*. The corneal endothelium *in vivo* expresses SLC4A11 at a high level as evidenced by multiple publications⁸⁵⁻⁸⁸ and our RNAseq data of *ex vivo* human cornea endothelial cells (evHCEnC) and *ex vivo* mouse corneal endothelial cells freshly dissected from *Slc4a11*^{+/+} and *Slc4a11*^{-/-} (exon 9-13 del) mice (*Slc4a11*^{+/+} evMCEnC and *Slc4a11*^{-/-} (exon 9-13 del) evMCEnC) (**Figure 11A**)⁴. For corneal endothelial cells *in vitro*, SLC4A11-mediated plasma membrane NH₃/NH₄⁺ and H⁺ conductance measured by single-cell patch-clamp (voltage-clamp or current-clamp) recordings is a direct, sensitive and physiologically relevant parameter to evaluate mature SLC4A11 protein function. We observed consistent NH₄Cl-induced membrane depolarization with current-clamp in corneal endothelial cell lines expressing wild type human SLC4A11 or murine *Slc4a11*: primary human corneal endothelial cells (pHCEnC), immortalized human corneal endothelial cell lines (HCEnC-21T) and immortalized mouse corneal endothelial cell lines (*Slc4a11*^{+/+} immorto-MCEnC) (**Figure 11B**). In contrast, SLC4A11 knockout resulted in diminished NH₄Cl-induced membrane depolarization in human and mouse corneal endothelial cell-based models of CHED, including CRISPR mediated *SLC4A11* knock-out in HCEnC-21T (CRISPR *SLC4A11*^{-/-} HCEnC-21T) and *Slc4a11*^{-/-} (exon 9-13 del) evMCEnC (**Figure 11B**). Additionally, transducing CRISPR *SLC4A11*^{-/-} HCEnC-21T with lentiviral (LV) vector driving expression of wild-type SLC4A11 variant B or C (LV-SLC4A11varBWT or LV-SLC4A11varCWT) reinstated the SLC4A11-mediated plasma membrane NH₃/NH₄⁺ and H⁺ conductance, while transduction with lentiviral vector driving expression of SLC4A11 mutants showed a variable decrease in the amplitude of depolarization (**Figure 11C**).

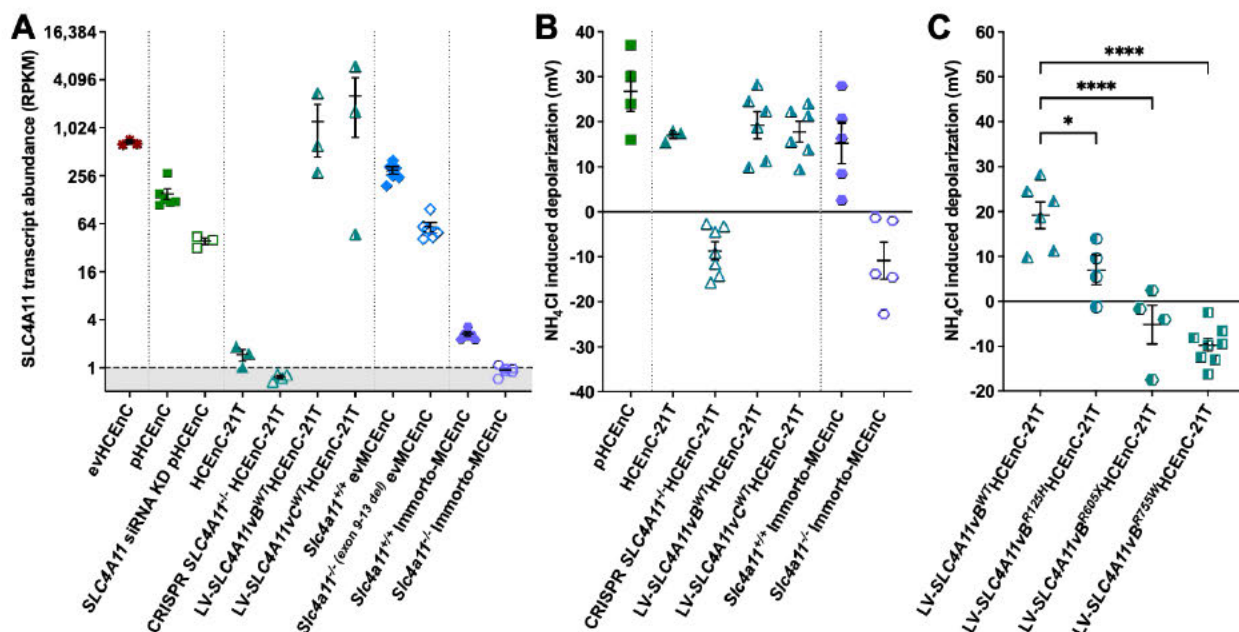


Figure 11. SLC4A11 mediated NH₃/NH₄⁺ and H⁺ conductance is a sensitive measure of mature SLC4A11 function. A. Scatter plot of SLC4A11/Slc4a11 mRNA transcript abundance from RNAseq data of corneal endothelial cells from various sources. RPKM, reads per kilobase of transcript, per million mapped reads; evHCEC, ex vivo human corneal endothelial cells. RPKM less than 1 was considered no expression (region shaded grey). B. Scatter plot of NH₄Cl (10 mM) induced membrane potential changes measured by single-cell current-clamp recordings of corneal endothelial cells from various sources. C. Scatter plot of NH₄Cl (10 mM) induced membrane potential changes of CRISPR *SLC4A11*^{-/-} HCEC-21T transduced with lentiviral (LV) vectors driving expressing of SLC4A11vB wild type, R125H, R605X or R755W mutant, respectively. Mean $\pm$ SEM were plotted; *, $p < 0.05$; ****, $p < 0.0001$.

LV-SLC4A11 rescues NH₃:H⁺ permeability and oxidative stress defense in SLC4A11^{-/-} human endothelial cells

Stable transduction of an immortalized human corneal endothelial cell line in which *SLC4A11* expression was knocked out by CRISPR-Cas9 (CRISPR *SLC4A11*^{-/-} HCEC-21T) using LV vectors with the CMV promoter driving expression of wild type human SLC4A11 variant B (LV-CMV-hSLC4A11vB^{WT}) or variant C (LV-CMV-hSLC4A11vC^{WT}) restored both plasma membrane NH₃/NH₄⁺ and H⁺ conductance and antioxidative stress response with no significant difference observed between cells transduced with LV-hSLC4A11vB^{WT} and LV-hSLC4A11vC^{WT} (Figure 12).

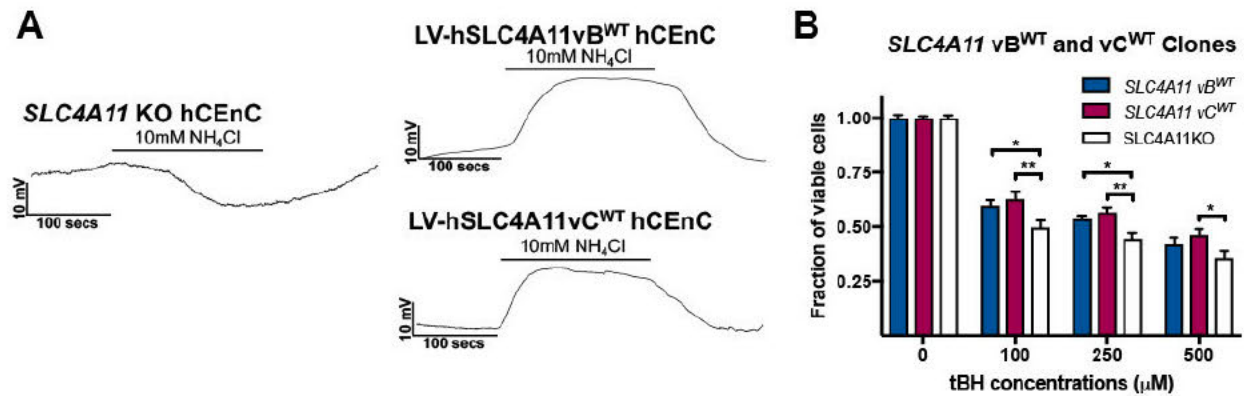


Figure 12. LV-hSLC4A11 transduction reinstated membrane $\text{NH}_3/\text{NH}_4^+$ and H^+ conductance, as well as cellular resistance to oxidative stress stimuli in SLC4A11-deficient HCEnc. A. Representative traces of membrane potential changes induced by 10 mM NH_4Cl in CRISPR *SLC4A11*^{-/-} hCEnc transduced with empty vector, LV-hSLC4A11vB^{WT} or LV-hSLC4A11vC^{WT}. B. Bar graph shows the fraction of viable cells after induction of acute oxidative stress by escalating concentrations of tBH. Stable transductions of CRISPR *SLC4A11*^{-/-} hCEnc-21T with LV-hSLC4A11vB^{WT} or LV-hSLC4A11vC^{WT} exhibit increases in hCEnc viability during tBH-induced oxidative stress compared to empty vector. LV = Lentivirus; hCEnc = human corneal endothelial cells; tBH = tert-butylhydroperoxide; vB = SLC4A11 variant B; vC = SLC4A11 variant C.

Overexpression of human SLC4A11 in an immortalized human corneal endothelial cell line (HCEnc-21T) to ~3000 times its endogenous level, as measured by SLC4A11 mRNA transcript abundance, does not result in evidence of toxicity, and offers a protective effect against an oxidative stress insult (**Figure 12B**). Additionally, overexpression of human SLC4A11 does not over-correct SLC4A11 protein-mediated NH_3/NH_4 and H^+ conductance, as the amplitude of NH_4Cl -induced membrane depolarization following SLC4A11 overexpression in SLC4A11 knock-out HCEnc-21T is similar to that in HCEnc-21T expressing endogenous SLC4A11 (**Figure 12B**), possibly due to the role of ER-mediated SLC4A11 protein turnover,⁶⁶ and the roles of other ion transporters and channels in regulating corneal endothelial membrane potential.⁸⁹

Intracameral AAV9-mSlc4a11 rescues corneal edema in *Slc4a11*^{-/-} mice

Efficacy of rAAV vectors expressing wild type murine *Slc4a11* was demonstrated in *Slc4a11*^{-/-} mice with exon 9-13 deletion.⁴ The estimated corneal endothelial cell transduction efficiency following intracameral injection of rAAV9-CAG-HA-mSlc4A11 in mice was 25% $\pm$ 12%.⁴ A single intracameral injection of rAAV9 with chicken β -actin (CAG) promoter driving expression of murine *Slc4a11* with a N-terminal HA-tag (rAAV9-CAG-HA-mSlc4A11) at the dosage range of 5×10^{11} – 8×10^{11} vg in 5 and 11-week-old *Slc4a11*^{-/-} mice demonstrated reversal or halting the progression of corneal edema. Gene therapy in 5-week-old *Slc4a11*^{-/-} mice was able to reduce corneal edema in the rAAV9-mSlc4a11 injected eye compared to the non-injected contralateral eye (**Figure 13**), whereas in 11-week-old *Slc4a11*^{-/-} mice, rAAV-mSlc4a11 injection prevented further increase in corneal thickness, which was observed in the noninjected eye. rAAV9-

mSlc4a11 injection also resulted in less endothelial cell loss in the treated eye compared with the non-injected contralateral eye in both 5 and 11-week-old *Slc4a11*^{-/-} mice.⁴

No significant change in intraocular pressure and no ocular immune response was observed following intracameral injection of rAAV9-GFP or rAAV9-CAG-null in *Slc4a11*^{+/+} mice and rAAV9-CAG-HA-mSlc4A11 in *Slc4a11*^{-/-} mice.⁴

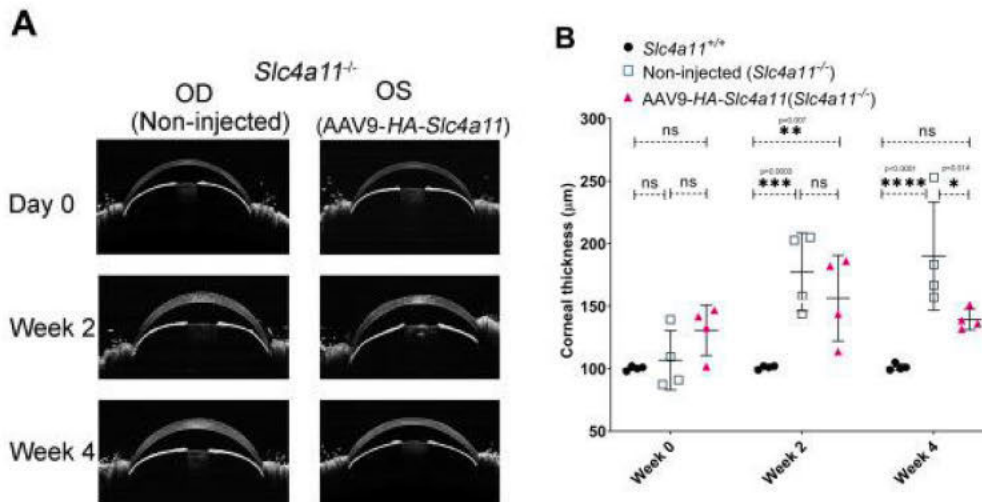


Figure 13. Corneal edema is reduced with AAV9-mSlc4a11. A. Representative OCT images of noninjected and AAV9-HA-*Slc4a11* injected eyes from 5-week-old *Slc4a11*^{-/-} mice. B. Quantification of data from A. Mean ± SD. ns = not significant. OD = right eye; OS = left eye.

Corneal intrastromal AAV8-mediated hSLC4A11 delivery rescues corneal edema in *Slc4a11*^{-/-} mice

We have evaluated the dose-response relationship of the viral vector AAV8-hSLC4A11 delivered by intrastromal injection in *Slc4a11*^{-/-} mice. The intrastromal injections have been performed using an unpurified AAV8-hSLC4A11 viral vector preparation from VectorBuilder. A single corneal intrastromal injection of 3 μL AAV8-hSLC4A11 vector was performed in one eye of *Slc4a11*^{-/-} mice at 4 weeks of age. Five dosages (1 x 10⁹ (n = 14), 3.3 x 10⁸ (n = 10), 1 x 10⁸ (n = 10), 1 x 10⁷ (n = 11), and 1 x 10⁶ (n = 11) vg/eye) were tested. For each mouse, the eye receiving the AAV injection was randomized, and the contralateral eye served as the control. Anterior segment ocular coherence tomography (OCT) was performed in both eyes before injection to measure baseline CCT (mean ± SEM), bi-weekly after injection until post-injection week 14 and every 4 weeks from post-injection week 14 until post-injection week 26. Slit-lamp examination of the anterior ocular segment and retinal OCT were also performed at each time point to monitor for evidence of toxicity and the host immune response. Mixed-effects analysis with post-hoc multiple comparisons was used to compare CCT values between AAV-injected eyes and control eyes at each post-injection time point.

The change in CCT from baseline to each of the post-injection time points was determined in the AAV injected eyes and the contralateral non-injected control eyes. For the 1 x 10⁹ vg/eye group, a significant decrease in CCT from baseline was observed at weeks 4, 6 and 8 in the AAV

injected eyes, with no difference between the baseline CCT and CCT at any of the other time points (**Figure 14A**). In contrast, a significant increase in the CCT from baseline was observed at each time point in the control eyes (**Figure 14A**). For the 3.3×10^8 vg/eye group, a significant increase in the CCT from baseline was not observed in the AAV injected eyes until week 26 (**Figure 14B**). In contrast, the CCT significantly increased from baseline after week 4 in the control eyes (**Figure 14B**). For the 1×10^8 vg/eye group, a significant decrease in CCT from baseline was noted at weeks 2, 6 and 8 and a significant increase in CCT from baseline was noted post-injection at weeks 18 and 26 in the AAV injected eyes (**Figure 14C**). In contrast, a significant increase in CCT from baseline was observed at weeks 10, 14 and 26 in the control eyes (**Figure 14C**). For the 1×10^7 vg/eye group, a significant increase in CCT from baseline was not observed in the AAV injected eyes until week 22, while a significant increase in CCT from baseline was noted after week 4 in the control eyes (**Figure 14D**). For the 1×10^6 vg/eye group, a significant decrease in CCT from baseline was observed at weeks 2 and 4 post-injection and a significant increase in CCT from baseline was observed after week 8 in the AAV injected eyes. In contrast, a significant increase in CCT from baseline was observed at week 8 and after week 10 in the control group.

AAV-injected eyes showed a sustained reduction or maintenance of CCT from baseline, whereas untreated contralateral eyes showed a progressive increase in CCT. For the 1×10^9 vg/eye group, AAV-injected eyes showed a 75 μm reduction in CCT compared to control eyes at week 26 ($p = 0.010$). The CCT (μm) at baseline, week 14 and week 26 in AAV-injected eyes were 161.1 ± 3.3 , 171.5 ± 10.9 ($p = 0.41$), and 161.8 ± 12.8 ($p = 0.99$) and in control eyes were 160.2 ± 2.7 , 237.7 ± 11.1 ($p < 0.0001$), and 236.4 ± 16.2 ($p = 0.0003$) (**Figure 14A, Table 4**).

For the 3.3×10^8 vg/eye group, AAV-injected eyes showed a 46 μm reduction in CCT compared to control eyes at week 26 ($p < 0.0001$). The CCT (μm) at baseline, week 14 and week 26 in AAV-injected eyes were 154 ± 4.0 , 153 ± 4.6 ($p = 0.915$) and 197.4 ± 9.8 ($p < 0.0001$) and in control eyes were 157.2 ± 4.6 , 200.9 ± 9.4 ($p < 0.001$) and 242.9 ± 17.5 ($p < 0.0001$) (**Figure 14B, Table 4**).

For the 1×10^8 vg/eye group, AAV-injected eyes showed a 48 μm reduction in CCT compared to control eyes at week 26 ($p = 0.007$). The CCT (μm) at baseline, week 14 and week 26 in AAV-injected eyes were 162.5 ± 3.2 , 180.1 ± 11.6 ($p = 0.167$), and 207.9 ± 18.6 ($p = 0.020$), and in control eyes were 166.7 ± 5.2 , 208.2 ± 9.9 ($p = 0.004$) and 256.1 ± 22.2 ($p = 0.005$) (**Figure 14C, Table 4**).

For the 1×10^7 vg/eye group, AAV-injected eyes showed an 86 μm reduction in CCT compared to control eyes at week 26 ($p = 0.0005$). The CCT (μm) at baseline, week 14 and week 26 in AAV-injected eyes were 152.8 ± 6.1 , 159.9 ± 4.7 ($p = 0.272$) and 179.2 ± 7.5 ($p = 0.003$), and in control eyes were 154.7 ± 3.4 , 222.6 ± 13.8 ($p = 0.0005$) and 265.3 ± 18.5 ($p = 0.0002$) (**Figure 14D, Table 4**).

For the 1×10^6 vg/eye group, AAV-injected eyes showed a 47 μm reduction in CCT compared to control eyes at week 26 ($p = 0.0007$). The CCT (μm) at baseline, week 14 and week 26 in AAV-injected eyes were 159.6 ± 4.3 , 175.5 ± 5.9 ($p = 0.004$) and 189.0 ± 11.8 ($p = 0.017$), and in

control eyes were 155.1 ± 7.5 , 211.2 ± 12.9 ($p = 0.0001$) and $237.2 \pm 16.2 \mu\text{m}$ ($p < 0.0001$) (Figure 14E, Table 4).

AAV-injected eyes demonstrated a clinically and statistically significant mean decrease in central corneal thickness compared with control eyes at each dosage tested that was maintained up to 26 weeks after injection.

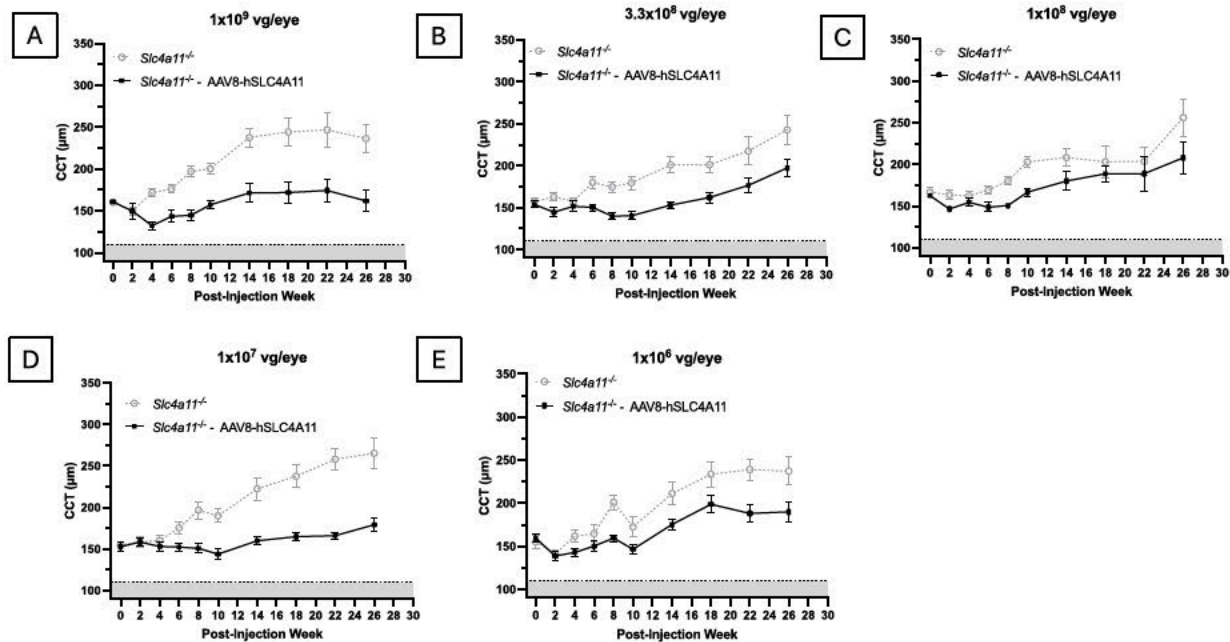


Figure 14. Intrastromal AAV8-hSLC4A11 injection rescues corneal edema in *Slc4a11* knock out mice. Central corneal thickness (CCT) measurements in *Slc4a11*^{-/-} mice following corneal intrastromal injection of five dosages of rAAV8-hSLC4A11 vector in one eye (*Slc4a11*^{-/-} - AAV8-SLC4A11) (1×10^9 ($n = 14$ mice), 3.3×10^8 ($n = 10$ mice), 1×10^8 ($n = 10$ mice), 1×10^7 ($n = 11$ mice), and 1×10^6 ($n = 11$ mice) vg/eye) and in the contralateral untreated eye (*Slc4a11*^{-/-}). vg = viral genomes. Shaded area indicates CCT in *Slc4a11* wild type mice.

Table 4. Intrastromal AAV8-mediated SLC4A11 delivery rescues corneal edema in Slc4a11 knock out mice. Mixed-effect analysis of central corneal thickness measurements Week 2 (W2) - Week 26 (W26) after corneal intrastromal injection of four dosages of AAV8-hSLC4A11 vector in one eye (AAV eye) and in the contralateral untreated eye (CTRL eye).

Vg/eye	AAV eye vs CTRL eye		AAV eye		CTRL eye	
1x10 ⁹	Baseline	p = 0.62	Baseline vs		Baseline vs	
	W2	p = 0.97	W2	p = 0.181	W2	p = 0.016
	W4	p < 0.0001	W4	p < 0.0001	W4	p = 0.011
	W6	p = 0.001	W6	p = 0.048	W6	p = 0.011
	W8	p < 0.0001	W8	p = 0.025	W8	p < 0.0001
	W10	p = 0.0004	W10	p = 0.598	W10	p < 0.0001
	W14	p = 0.002	W14	p = 0.406	W14	p < 0.0001
	W18	p = 0.005	W18	p = 0.448	W18	p < 0.0001
	W22	p = 0.009	W22	p = 0.383	W22	p = 0.0006
	W26	p = 0.010	W26	p = 0.955	W26	p = 0.0003
Overall AAV Treatment Effect (AAV eyes vs CTRL eyes) p = 0.004						
3.3x10 ⁸	Baseline	p = 0.629	Baseline vs		Baseline vs	
	W2	p = 0.009	W2	p = 0.315	W2	p = 0.549
	W4	p = 0.359	W4	p = 0.797	W4	p = 0.957
	W6	p < 0.0001	W6	p = 0.693	W6	p = 0.016
	W8	p < 0.0001	W8	p = 0.133	W8	p = 0.063
	W10	p < 0.0001	W10	p = 0.145	W10	p = 0.017
	W14	p < 0.001	W14	p = 0.915	W14	p < 0.0001
	W18	p < 0.0001	W18	p = 0.387	W18	p < 0.0001
	W22	p < 0.0001	W22	p = 0.017	W22	p < 0.0001
	W26	p < 0.0001	W26	p < 0.0001	W26	p < 0.0001
Overall AAV Treatment Effect (AAV eyes vs CTRL eyes) p = 0.0002						
1x10 ⁸	Baseline	p = 0.327	Baseline vs		Baseline vs	
	W2	p = 0.014	W2	p < 0.0001	W2	p = 0.640
	W4	p = 0.299	W4	p = 0.208	W4	p = 0.550
	W6	p = 0.021	W6	p = 0.034	W6	p = 0.724
	W8	p = 0.001	W8	p = 0.001	W8	p = 0.132
	W10	p < 0.0001	W10	p = 0.511	W10	p = 0.001
	W14	p = 0.088	W14	p = 0.167	W14	p = 0.004
	W18	p = 0.436	W18	p = 0.028	W18	p = 0.116
	W22	p = 0.501	W22	p = 0.154	W22	p = 0.060
	W26	p = 0.007	W26	p = 0.020	W26	p = 0.005
Overall AAV Treatment Effect (AAV eyes vs CTRL eyes) p = 0.023						
1x10 ⁷	Baseline	p = 0.726	Baseline vs		Baseline vs	
	W2	p = 0.975	W2	p = 0.277	W2	p = 0.555
	W4	p = 0.254	W4	p = 0.968	W4	p = 0.371
	W6	p = 0.039	W6	p = 0.933	W6	p = 0.012
	W8	p = 0.001	W8	p = 0.744	W8	p = 0.002
	W10	p = 0.002	W10	p = 0.207	W10	p = 0.001
	W14	p = 0.003	W14	p = 0.272	W14	p = 0.001
	W18	p = 0.001	W18	p = 0.111	W18	p = 0.0001
	W22	p = 0.0001	W22	p = 0.043	W22	p < 0.0001
	W26	p = 0.001	W26	p = 0.003	W26	p = 0.0002
Overall AAV Treatment Effect (AAV eyes vs CTRL eyes) p = 0.0003						
1x10 ⁶	Baseline	p = 0.528	Baseline vs		Baseline vs	
	W2	p = 0.598	W2	p < 0.0001	W2	p = 0.060
	W4	p = 0.023	W4	p = 0.007	W4	p = 0.433
	W6	p = 0.010	W6	p = 0.090	W6	p = 0.204

Vg/eye	AAV eye vs CTRL eye		AAV eye		CTRL eye	
	W8	p = 0.0002	W8	p = 0.967	W8	p < 0.0001
	W10	p = 0.006	W10	p = 0.039	W10	p = 0.121
	W14	p = 0.002	W14	p = 0.004	W14	p = 0.0001
	W18	p = 0.007	W18	p = 0.0004	W18	p < 0.0001
	W22	p < 0.001	W22	p = 0.010	W22	p < 0.0001
	W26	p = 0.001	W26	p = 0.017	W26	p < 0.0001
Overall AAV Treatment Effect (AAV eyes vs CTRL eyes) p = 0.0004						

Light microscopic examination of AAV8-hSLC4A11-treated corneas of *Slc4a11*^{-/-} mice demonstrates compact corneal stroma without evidence of corneal inflammation

Following the week 26 examinations, *Slc4a11*^{-/-} mice treated with 1 x 10⁹ vg/eye of the AAV8-hSLC4A11 vector were sacrificed and histopathologic examination of the injected and the control eyes was performed. The AAV8-hSLC4A11-treated corneas demonstrated a relatively compact stroma compared with the control corneas, which demonstrated separation of the stromal lamellae (**Figure 15**). Interestingly, although significant stromal edema was observed in the control eyes, no epithelial edema was seen. While the density of stromal keratocytes was not quantified, no significant difference was observed between the treated and the control eyes. Neither AAV8-hSLC4A11-treated nor control corneas demonstrated the presence of inflammatory cells or stromal scarring.

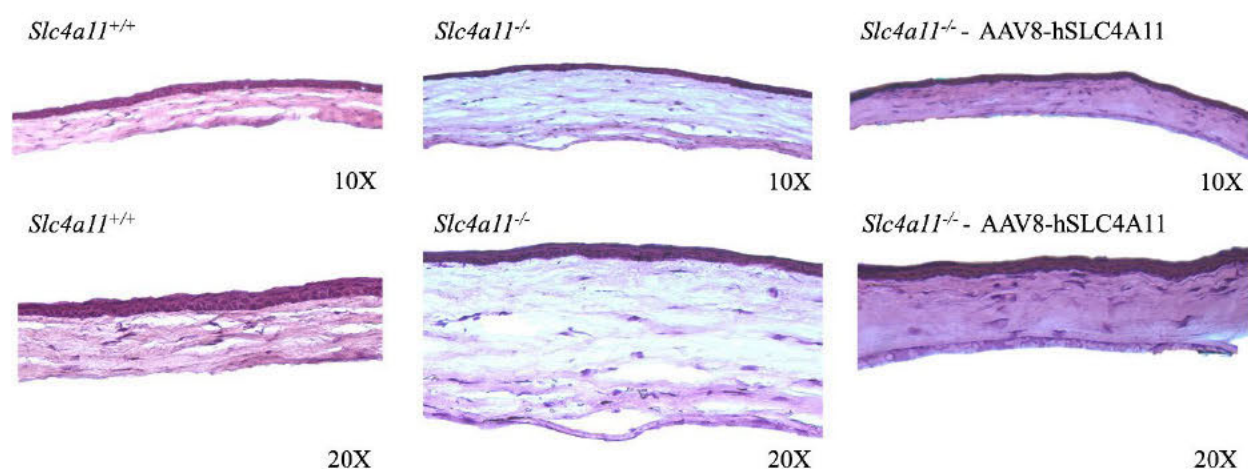


Figure 15. Corneal histologic images from *Slc4a11*^{+/+} mouse and *Slc4a11*^{-/-} mouse following unilateral AAV8-hSLC4A11 intrastromal injection. Representative images of corneal sections from a wild type *Slc4a11*^{+/+} mouse and the AAV8-hSLC4A11-treated (1 x 10⁹ vg/eye) and untreated (control) eyes of a 30 week *Slc4a11*^{-/-} mouse 26 weeks following injection. Images were obtained at 10X magnification (top panel) and 20X magnification (bottom panel) following H&E staining.

Corneal intrastromal AAV8-mediated hSLC4A11 delivery results in SLC4A11 expression in treated corneas of *Slc4a11*^{-/-} mice

Following the week 26 examinations, *Slc4a11*^{-/-} mice treated with 1 x 10⁹ vg/eye of the AAV8-hSLC4A11 vector were sacrificed and immunohistochemical staining of the injected and control eyes with a rabbit anti-SLC4A11 antibody was performed. As expected, no staining of the

corneas in the control eyes was observed, while a few SLC4A11 positive cells were observed in the AAV8-hSLC4A11 treated corneas, suggestive of transgene expression in these corneas (Figure 16).

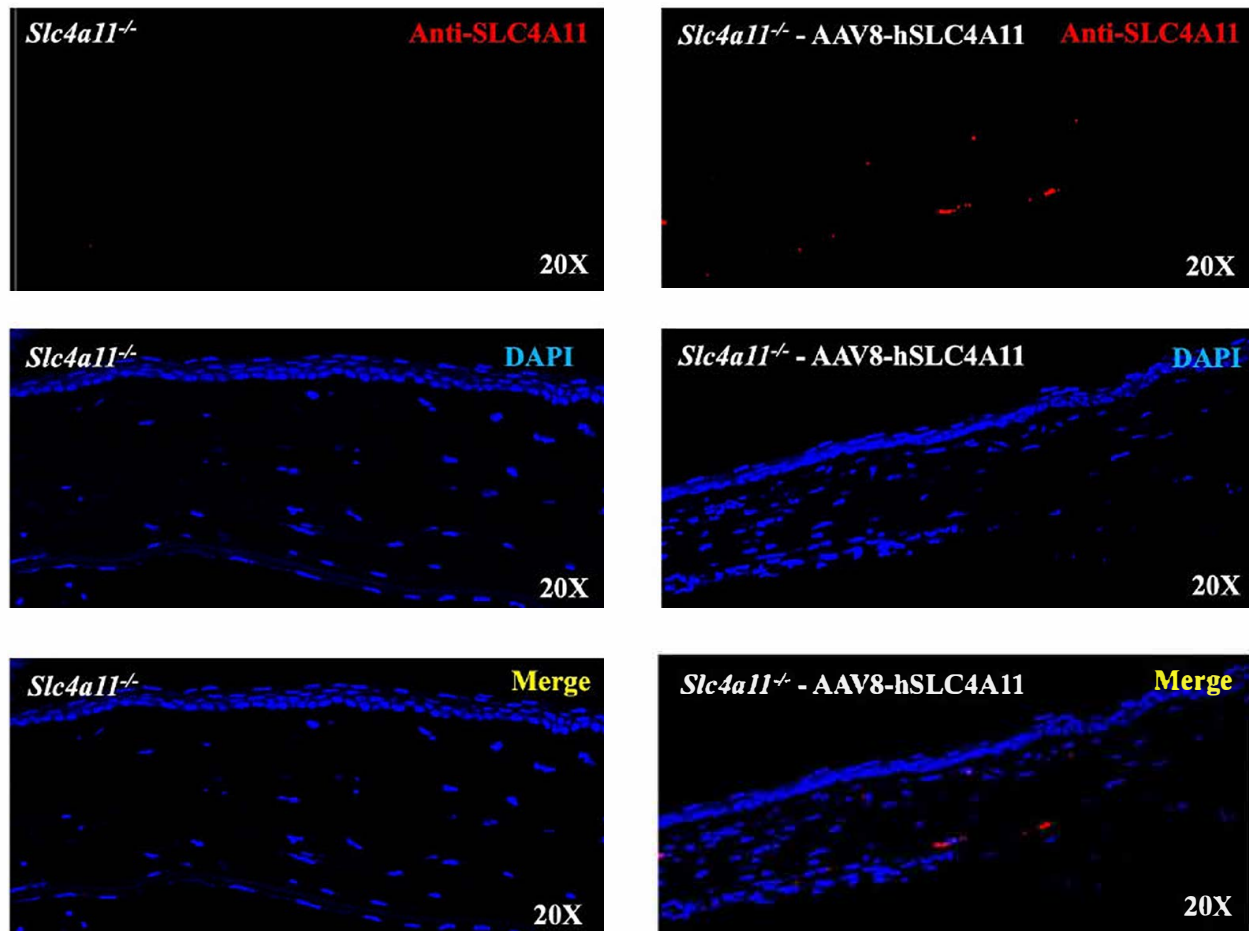


Figure 16. SLC4A11 expression in corneas from *Slc4a11*^{+/+} mouse and *Slc4a11*^{-/-} mouse following unilateral AAV8-hSLC4A11 intrastromal injection. Representative images of corneal sections from a wild type *Slc4a11*^{+/+} mouse and the AAV8-hSLC4A11-treated (1×10^9 vg/eye) and untreated (control) eyes of a 30-week *Slc4a11*^{-/-} mouse 26 weeks following injection. Immunostaining of corneal cryosections was performed with SLC4A11 (red). DAPI staining for nuclei (blue) was also performed. Images were obtained at 20X magnification.

12.2.3 Safety studies in *Slc4a11*^{-/-} mouse model of CHED

Use of purified rAAV8-hSLC4A11 preparation to elucidate cause of corneal subepithelial opacification and vascularization with unpurified rAAV8-hSLC4A11 preparation

The most likely explanations for the cause of the corneal opacification and vascularization observed with the use of the unpurified rAAV8-hSLC4A11 preparation (VectorBuilder) are toxicity of the transgene product, AAV8 capsid, and impurities in the AAV8-hSLC4A11 preparation. To investigate each possibility, a mutant *SLC4A11* plasmid that is missing the transgene product (containing a deletion that removes the ATG start sequence and the following

164 nucleotides, essentially knocking out the *SLC4A11* transgene) (pTR-EF1-SLC4A11^{KO}) was constructed. AAV8 vectors were then produced by the UNC Vector core with a titer of 2.2×10^{12} vg/mL for rAAV8-hSLC4A11^{KO} and 5.8×10^{11} vg/mL for rAAV8-hSLC4A11^{WT}. A single corneal intrastromal injection of 3 μ L AAV8-hSLC4A11 vector was performed in one eye of *Slc4a11*^{-/-} mice at 4 weeks of age. Three dosages (1×10^9 (n = 11), 1×10^8 (n = 10), and 1×10^7 (n = 6) vg/eye) have been tested while one dosage (1×10^6 vg/eye) remains to be tested. For each mouse, the eye receiving the AAV injection was randomized, and the contralateral eye served as the control. Slit-lamp examination of the anterior ocular segment and retinal OCT were performed at each time point to determine the proportion of injected eyes that exhibited corneal opacification and/or vascularization, as well as for other evidence of toxicity and a host immune response. Anterior segment OCT was performed in both eyes before injection to measure baseline CCT (mean $\pm$ SEM) and bi-weekly after injection until post-injection week 14 and every 4 weeks from post-injection week 14 until post-injection week 26. Mixed-effects analysis with post-hoc multiple comparisons was used to compare CCT values between AAV-injected eyes and control eyes at each post-injection time point.

Corneal subepithelial opacification and vascularization infrequently observed with purified AAV8-hSLC4A11 preparation

The subepithelial opacification and vascularization observed with the unpurified AAV8-hSLC4A11 preparation has been observed in a small number of eye injected with the purified AAV8-hSLC4A11 preparation, indicating that these complications are most likely secondary to impurities in the unpurified AAV8-hSLC4A11 vector batch (Table 5). Subepithelial opacification was observed in 9% (1/11) of the treated eyes in the 1×10^9 vg/eye group, in 20% (2/10) of the treated eyes in the 1×10^8 vg/eye group and in none (0/6) of the treated eyes in the 1×10^7 vg/eye group. In both of the eyes with subepithelial opacification, it was observed at 4 weeks after injection, with 10 week post-injection follow-up in all eyes in the 1×10^9 vg/eye group, 6 (n=5), 4 (n=3), and 2 (n=2) week post-injection follow-up in the 1×10^8 vg/eye group and 2 week post-injection follow-up in the 1×10^7 vg/eye group.

Table 5. Corneal complications associated with intrastromal purified AAV8-hSLC4A11 injection in *Slc4a11*^{-/-} mice

Virus Dosage (vg/eye)	Subepithelial opacification	Vascularization	Pigmented Deposits	Vitreous Cells
1×10^9	1/11 (9%)	2/11 (18%)	2/11 (18%)	0/11 (0%)
1×10^8	2/10 (20%)	1/10 (10%)	0/10 (0%)	0/10 (0%)
1×10^7	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)

Corneal vascularization was noted in 18% (2/11) of the 1×10^9 vg/eye treated eyes, 10% (1/10) of the 1×10^8 vg/eye treated eyes, and none (0/6) of the 1×10^7 vg/eye treated eyes. Both of the eyes that developed subepithelial opacification also developed corneal vascularization, indicating that subepithelial opacification and/or vascularization developed in only 3 of the 27 injected eyes (11%), similar to the percentage of uninjected eyes that developed subepithelial opacification and/or vascularization and significantly lower than the percentage of eyes that developed

subepithelial opacification and/or vascularization following injection with the unpurified AAV8-hSLC4A11 preparation.

12.2.4 Safety and biodistribution studies in large animal models

Relevant to our proposed use of rAAV8-hSLC4A11 as a therapeutic intervention for CHED, our group has extensive efficacy and safety data for AAV8 delivery to the cornea using different transgenes in canine and feline corneal disease models and in wild type rabbits. The demonstration of the efficacy of corneal intrastromal gene therapy in the prevention and reversal of corneal opacification in a canine MPS I model and a feline MPS VI model using the same AAV8 capsid, dose range and route of delivery as proposed in this study provides indirect support for the potential effectiveness and safety of ssAAV8-EF1 α -SLC4A11 for CHED. In particular, the extensive AAV8 vector biodistribution and capsid neutralizing antibody data in wild type rabbits is relevant to this study that also employs AAV8 vector administration to the corneal stroma.

Our preclinical results of rAAV8 vector corneal intrastromal administration in large animal models, including rabbit,¹ canine,² and feline (unpublished), showed excellent safety profiles using various transgenes with limited to no ocular and non-ocular immunologic responses. Vector-mediated transgene expression was only detected in the treated cornea and, in general, vector genome biodistribution was restricted to the injected eye. Minimal to no detectable neutralizing antibodies to the rAAV8 capsid were found in the aqueous, vitreous humor or serum.^{1,2}

rAAV8 intrastromal injection in New Zealand White rabbit model

The safety and biodistribution of AAV8-EF1 α -opt-IDUA unilateral corneal intrastromal delivery in New Zealand White rabbits was evaluated. Our published data suggest that there is minimal AAV8 viral genome and transgene-derived transcript present in tissues and organs outside of the injected eye.¹ Twenty-two wild type rabbits received a single intrastromal injection via a 31G needle of either 50 μ L of saline (n = 6), 50 μ L of rAAV8-Ef α 1-opt-IDUA at 1×10^9 vg (n = 8), or 50 μ L of rAAV8-Ef α 1-opt-IDUA at 1×10^{10} vg (n = 8) and were followed for 6 months to assess acute or chronic ocular and extraocular toxicity. Clinical assessments of the eye, including ocular inflammation (measured using the Hackett-McDonald (HM) score), corneal endothelial cell density, CCT, intraocular pressure (IOP), complete blood count (CBC), serum chemistry, histopathology of the eye, ocular and non-ocular transgene expression, ocular and non-ocular viral genome biodistribution, and AAV8 capsid neutralizing antibody titers were evaluated to provide a comprehensive toxicologic safety profile.

Injection-associated adverse events: AAV8-EF1 α -opt-IDUA intrastromal delivery was well tolerated with mild leakage along the needle track, corneal edema in the area of the injection, and mild ocular inflammation immediately following the injection. The corneas of all rabbits regained transparency within 24 hours post-injection and remained clinically unremarkable during post-injection days 4–7. Corneal in vivo confocal microscopy, AS OCT, and histopathologic analyses of the injected corneas demonstrated that all AAV8 injected corneas were indistinguishable from saline injected and non-injected corneas.

Ocular toxicity: HM scores for ocular inflammation were assigned under slit lamp examination and were recorded on days 0 (prior to anesthesia and injection), 1, 4, 7, 10, 14, 18, 21, 24, and 28, and then weekly for 6 months. Mild elevation of HM scores (e.g., mild conjunctival hyperemia and chemosis) was observed within 24 hours of injection (mean cumulative scores for all groups was 2), but HM scores returned to baseline by day 7. No significant ocular pathology was noted throughout the 6-month follow-up period in any group. Mean IOP change from baseline was not significantly different among AAV-injected, saline-injected and non-injected groups. CCT values returned to baseline by post-injection day 4. Six months following the injection, no abnormality was noted on in vivo confocal microscopic imaging in corneas of AAV8-injected, saline-injected and non-injected groups. Corneal endothelial cell density was not different between AAV8-injected, saline-injected and non-injected corneas. AS OCT performed prior to injection (time 0), 24 h post-injection, and day 176 post-injection similarly did not demonstrate a corneal abnormality in any group at any time point.

Systemic toxicity: CBC and serum chemistry profile (basic metabolic panel, BMP) were performed prior to injection and monthly following the injection for all rabbits. All CBC and BMP results were within the normal reference range at all time points, showing no evidence of systemic toxicity following corneal intrastromal AAV8 injection.

Vector shedding: Tear samples collected on days 7 and 14 post-injection were evaluated, and all samples from all rabbits were negative for the transgenic cassette.

Vector genome distribution in ocular tissue: DNA was extracted from the conjunctiva, cornea, iris/ciliary body, and retina/choroid of 4 AAV8-injected eyes and 4 contralateral non-injected eyes to quantify vector genome copy number using qPCR. Vector genomes containing the transgene cassette were detected at significant levels in the cornea and retina in AAV8-injected eyes in a dose-dependent manner. No significant vector genome was detected in other ocular tissues of AAV8-injected eyes. No vector genome was detected in any ocular tissues of the non-injected contralateral eye.

Transgene expression in ocular tissue: RNA recovered from the conjunctiva, cornea, iris/ciliary body, and retina/choroid of 4 AAV8-injected eyes and 4 contralateral non-injected eyes was examined by qRT-PCR. Transgene transcripts were detected at a significant level only in the AAV8-injected corneas.

Biodistribution of persistent AAV8 vector genome in non-ocular tissues: DNA extracted from the liver, kidney, spleen and heart from 6 saline injected rabbits, 8 AAV8 low dose ($1e^9$ vg) injected rabbits, and 8 AAV8 high dose ($1e^{10}$ vg) injected rabbits was examined by qPCR for vector genome copy number. All 22 rabbits were negative for vector genome in the spleen and liver. Only 2 of 16 AAV8-injected rabbits, one from the low dose group and one from the high dose group, had detectable vector genome in the kidney.

AAV8 capsid neutralizing antibody (Nab): Nab titer to AAV8 capsid was measured 6 months post-injection in the serum, aqueous humor and vitreous humor using an in vitro transduction assay. Only 1 of 16 AAV8-injected rabbits had a serum Nab titer (1:2) to AAV8 capsid at 50% neutralization. No AAV8 Nab was detected in the aqueous or vitreous humor of any eye.

Table 6. Completed preclinical studies

Study Identifier or Title	Study Objective(s)	Test Article and Manufacturing Process Used	Model and Number of Animals (samples) per Arm (Treatment Group)	Key Outcome(s)
Vector targeting				
rAAV8 targeting in SLC4A11 CRSIPR KO (<i>SLC4A11</i>^{-/-}) human corneal endothelial cell line (HCEnc-21T)	Assess transduction efficiency of rAAV8 in an <i>in vitro</i> cell based model of CHED	Therapeutic Surrogate (rAAV8-GFP)	1) rAAV8-GFP at 5×10^4 vg / cell (n = 3) 2) rAAV8-GFP at 1×10^6 vg / cell (n = 3) 3) PBS (n = 6)	0.2% of <i>SLC4A11</i> ^{-/-} HCEnc-21T were GFP+ 72 hours after transduction with rAAV8-GFP at 5×10^4 vg/cell. 2.7% of <i>SLC4A11</i> ^{-/-} HCEnc-21T were GFP+ 72 hours after transduction with rAAV8-GFP at 1×10^6 vg/cell.
rAAV8 intrastromal injection in <i>ex vivo</i> human donor corneas	Proof of concept study for rAAV8 intrastromal injection to transduce human corneal endothelium in its native environment and assess corneal endothelial toxicity associated with intrastromal injection of rAAV8 in <i>ex vivo</i> human corneas	Therapeutic Surrogate (rAAV8-CMV-GFP)	Human donor cornea receiving intrastromal injection of either: 1) rAAV8-CMV-GFP in BSS (n = 4) 2) BSS (n = 4)	No significant difference between % of apoptotic cells in rAAV8-injected and BSS-injected corneas.
Efficacy				
AAV8-hSLC4A11 transduction	Assess the capacity of therapeutic candidate in	Therapeutic Candidate (AAV8-hSLC4A11)	HEK293 cells transduced with either: 1) rAAV8-hSLC4A11 (n = 6)	Mature (dimerized and glycosylated) SLC4A11 was produced in HEK293 cells

Study Identifier or Title	Study Objective(s)	Test Article and Manufacturing Process Used	Model and Number of Animals (samples) per Arm (Treatment Group)	Key Outcome(s)
	producing dimerized and glycosylated SLC4A11		2) PBS (n =6)	transduced with therapeutic candidate rAAV8-EF1 α -hSLC4A11
hSLC4A11 gene replacement in SLC4A11 CRSIPR KO (<i>SLC4A11</i>^{-/-}) human corneal endothelial cell line (HCEnC-21T)	Proof of concept study for hSLC4A11 gene replacement therapy in human cell-based model of CHED. Assess cellular toxicity associated with human SLC4A11 over-expression in human corneal endothelial cells	Therapeutic Surrogate (LV-CMV-hSLC4A11)	<i>SLC4A11</i>^{-/-} HCEnC-21T stably transduced with one of the following: 1) LV-CMV-hSLC4A11vB^{WT} 2) LV-CMV-hSLC4A11vC^{WT} 3) LV-CMV-null	Expression of wild type human SLC4A11 variant B or C restored both plasma membrane NH₃/NH₄⁺ and H⁺ conductance, as well as antioxidative stress response in <i>SLC4A11</i>^{-/-} HCEnC-21T. No significant difference observed between cells transduced to express wild type human SLC4A11 variant B and C. Overexpression of human SLC4A11 in <i>SLC4A11</i>^{-/-} HCEnC-21T to ~3000 times its endogenous level, as measured by SLC4A11 mRNA transcript abundance, does not result in evidence of toxicity, and offers a protective effect against an oxidative stress insult
AAV gene replacement in <i>Slc4a11</i>^{-/-} mouse	Proof of concept study for AAV gene therapy in	Therapeutic Analogue (rAAV9-	<i>Slc4a11</i> ^{-/-} mice receiving intracameral	In young (5-week) <i>Slc4a11</i> ^{-/-} mice, AAV9-Slc4a11

Study Identifier or Title	Study Objective(s)	Test Article and Manufacturing Process Used	Model and Number of Animals (samples) per Arm (Treatment Group)	Key Outcome(s)
model of CHED⁴	rodent model of CHED	CAG-HA-mSlc4a11)	injection in one eye ($5 - 8 \times 10^{11}$ vg per eye) of either: 1) rAAV9-mSlc4a11 (n = 71) 2) rAAV9-Null (n = 8) 3) rAAV9-GFP (n = 3) Age-matched <i>Slc4a11</i>^{+/+} mice not injected (n = 53)	reversed corneal edema and endothelial cell loss, and returned mitochondrial oxidative stress, lactate transporter expression and corneal lactate concentration to the levels observed in <i>Slc4a11</i>^{+/+} mice. In the older (11-week) <i>Slc4a11</i>^{-/-} mice, gene replacement did not reverse the phenotype but prevented progression. No significant change was observed in intraocular pressure or ocular immune response following intracameral injection of rAAV9-GFP, rAAV9-CAG-HA-mSlc4A11 or rAAV9-CAG-null in mice.
AAV gene replacement in <i>Slc4a11</i>^{-/-} mouse model of CHED	Preliminary study for AAV8 gene therapy in	Therapeutic Candidate (AAV8-hSLC4A11)	<i>Slc4a11</i> ^{-/-} mice receiving intrastromal injection in one eye of either	rAAV8 injected <i>Slc4a11</i> ^{-/-} mice eyes had a sustained reduction/stabilization of central corneal

Study Identifier or Title	Study Objective(s)	Test Article and Manufacturing Process Used	Model and Number of Animals (samples) per Arm (Treatment Group)	Key Outcome(s)
	rodent model of CHED		1) 1×10^9 vg/eye (n=14) 2) 3.3×10^8 vg/eye (n=10) 3) 1×10^8 vg/eye (n=10) 4) 1×10^7 vg/eye (n=11) 5) 1×10^6 vg/eye (n=11)	thickness from baseline in a dose dependent manner whereas the untreated contralateral eye had a progressive increase in the central corneal thickness. A significant difference was observed in the CCT of rAAV8 treated and control eyes. The AAV8-hSLC4A11-treated corneas demonstrated a relatively compact stroma compared with the control corneas, which demonstrated separation of the stromal lamellae The AAV8-hSLC4A11-treated corneas demonstrated SLC4A11 positive cells, suggestive of transgene expression in these corneas.
Toxicity and Biodistribution				
Corneal intrastromal injection of rAAV8 in rabbits ⁹⁰	Assess the capacity of AAV8 vectors to transduce corneal endothelial cells	Therapeutic Surrogate (rAAV8-EF1 α -IDUA)	Rabbits receiving intrastromal injection of one of the following: 1) rAAV8-IDUA at 1×10^9 vg/eye (n = 8)	Transgene product staining demonstrated IDUA in higher abundance than endogenous level in all layers

Study Identifier or Title	Study Objective(s)	Test Article and Manufacturing Process Used	Model and Number of Animals (samples) per Arm (Treatment Group)	Key Outcome(s)
	Assess ocular and non-ocular toxicity Assess ocular and non-ocular biodistribution of AAV8 vg		2) rAAV8-IDUA at 1×10^{10} vg/eye (n = 8) 3) saline (n=6)	of corneas, including corneal endothelium. AAV8-EFα1-IDUA intrastromal delivery was well tolerated without ocular inflammation requiring topical or systemic corticosteroids and without a significant increase in intraocular pressure. No evidence of systemic toxicity or inflammation was observed on complete blood count or serum basic metabolic panel during the first 6 months after injection. No change in corneal anatomy or histology was observed on <i>in vivo</i> confocal microscopic imaging, AS OCT or histopathologic analyses. No evidence of vector shedding in tear was observed on day 7 or 14 post-

Study Identifier or Title	Study Objective(s)	Test Article and Manufacturing Process Used	Model and Number of Animals (samples) per Arm (Treatment Group)	Key Outcome(s)
				injection in any rabbit. Transgene transcripts were detected at significant levels in AAV8-injected corneas. No transgene transcripts were detected in the conjunctiva, iris/ciliary body or retina of either the AAV8-injected or the saline-injected rabbits. No AAV8 vg was detected in the liver, spleen, heart, or contralateral non-injected cornea in any of the 16 rabbits injected with AAV8. Two of 16 rabbits had detectable AAV8 vg in the kidney. Neutralizing antibody titer to AAV8 capsid was not detectable in the aqueous humor or vitreous humor in the AAV8-injected eye of any of the 16 rabbits but was detected in serum (1:2) in 1

Study Identifier or Title	Study Objective(s)	Test Article and Manufacturing Process Used	Model and Number of Animals (samples) per Arm (Treatment Group)	Key Outcome(s)
				of 16 rabbits injected with AAV8 in one eye.

12.3 Planned Preclinical Studies

12.3.1 AAV8-hSLC4A11 dose optimization in *Slc4a11*^{-/-} mouse model of CHED

12.3.1.1 Determination of the dose-response relationship of AAV8-hSLC4A11 intrastromal delivery in *Slc4a11*^{-/-} mice

Although we have demonstrated a clinically and statistically significant mean decrease in central corneal thickness in eyes injected with an unpurified rAAV8-hSLC4A11 vector preparation compared with control eyes of *Slc4a11*^{-/-} mice at each of five dosages tested, we plan to repeat the study using the research grade rAAV8-hSLC4A11 vector (GMP quality) from Andelyn. We believe that it is important to do so to demonstrate the efficacy of the vector produced by the manufacturer of the rAAV8-hSLC4A11 vector that will be used in the non-GLP pilot toxicology and biodistribution studies and the IND enabling GLP-compliant safety, toxicology and biodistribution studies in the Gottingen minipigs, as well as the first-in-human trial. We will perform a single corneal intrastromal injection in one eye of 10 *Slc4a11*^{-/-} mice (5 male, 5 female) at 4 weeks of age with the research grade rAAV8-hSLC4A11 vector at five dose group (1 x 10⁹ vg/eye, 3.3 x 10⁸ vg/eye, 1 x 10⁸ vg/eye, 1 x 10⁷ vg/eye and 1 x 10⁶ vg/eye). For each mouse, the eye receiving the AAV injection will be randomized, with the contralateral eye serving as the control. Anterior segment OCT will be performed in both eyes before injection to measure baseline CCT (mean ± SEM) and will be performed bi-weekly after injection until post-injection week 26. Slit-lamp examination of the anterior ocular segment and retinal OCT will also be performed at each time point to monitor for evidence of adverse reactions.

The minimal effective dosage (MED) and the maximum tolerated dosage (MTD) in the *Slc4a11*^{-/-} mice will be determined to inform the initial dosages to be used in the non-GLP toxicology and biodistribution studies. MED is the lowest dosage that achieves a statistically significant reduction in corneal edema progression. The progression of corneal edema will be measured by the change in CCT. MTD is the highest dosage that shows no signs of toxicity.

12.3.1.2 Biodistribution studies in *Slc4a11*^{-/-} mice

Biodistribution analysis will be performed after the week 26 time point following the injection in the *Slc4a11*^{-/-} mice treated with each dosage of research grade rAAV8-hSLC4A11 vector from

Andelyn. Treated and untreated eyes will be processed for frozen sections to assess for the ocular distribution of hSLC4A11 protein via immunofluorescence staining using a custom affinity purified anti-hSLC4A11 antibody; serum and aqueous humor will be collected for anti-AAV8 and anti-SLC4A11 antibody measurements; and non-ocular tissues (liver, blood, heart, kidney and gonads) will be collected for digital PCR based vector genome and transgene biodistribution analysis. If no detectable levels of AAV8-hSLC4A11 vector genome are found by digital PCR in the non-ocular tissues in the mice in the highest dose group (1×10^9 vg/eye), then we will consider whether performing biodistribution assays to detect AAV8-hSLC4A11 vector genome in non-ocular tissues within the lower dose groups will likely be informative.

Ocular and non-ocular tissues and aqueous humor and serum samples harvested from male and female wild type *Slc4a11*^{+/+} mice will serve as untreated controls for the biodistribution analyses. *Slc4a11*^{-/-} mice in which a systemic AAV8-hSLC4A11 injection is performed prior to the collection of ocular and non-ocular tissues will serve as positive controls for the biodistribution analyses.

12.3.1.3 Determination if there is a sex-related difference in the therapeutic window of AAV8-hSLC4A11 administration in *Slc4a11*^{-/-} mice

We recently identified a less severe phenotype (decreased corneal edema) both in girls with CHED and in female *Slc4a11*^{-/-} mice compared to age-matched boys and male *Slc4a11*^{-/-} mice, respectively.⁹¹ While the published AAV gene therapy study in *Slc4a11*^{-/-} mice (**Figure 13**) reported an estimated therapeutic window between 5 – 11 weeks of age, the study did not include sufficient numbers of mice of each sex to allow a meaningful post-hoc data analysis based on sex. Therefore, we propose to use the MED identified in **12.3.1.2** to refine the therapeutic window for male and female *Slc4a11*^{-/-} mice. A single intrastromal injection of research grade AAV8-hSLC4A11 viral vector generated by Andelyn at the MED will be administered to one eye of *Slc4a11*^{-/-} mice at the ages listed in **Table 7**, and the contralateral uninjected eye will serve as control. CCT, as an efficacy measure, will be measured before injection and biweekly thereafter until post-injection week 26.

12.3.2 Potency assay and immunoassay development

12.3.2.1 Development of digital PCR based vector potency assays

To ensure consistent dosage and therapeutic efficacy of AAV8-hSLC4A11, we developed an AAV8-hSLC4A11 potency assay based on 2 critical factors, i) vector derived cDNA normalized to internalized vector genomes, and ii) Sanger sequencing of hSLC4A11 cDNA. In short, optimization of this assay has identified a hSLC4A11 ddPCR primer probe set and a cell line permissive to AAV8-hSLC4A11 transduction. Using an AAV8-hSLC4A11 preparation employed in the murine efficacy studies and that serves as our vector standard, a dose of 25,000 vg/cell yielded consistent and reliable detection of hSLC4A11 cDNA that was 3 orders of magnitude above negative controls. When normalized to intracellular AAV8-hSLC4A11 vector genomes this value defines the potency of the vector preparation at the levels of particle entry, trafficking, uncoating, and transgene expression, which are the key steps in defining AAV vector transduction. Sanger sequencing of the transgene-derived hSLC4A11 cDNA complements the

ddPCR data by verifying that the entire therapeutic product precursor is produced as intended to ensure consistent dosing/efficacy. While this assay assumes a direct correlation between translation of hSLC4A11 transcripts and protein production, so do other potency assays that rely on quantitation of transgene product or function, both of which introduce unnecessary variables that decrease assay reliability and consistency.

Table 7. Age of *Slc4a11*^{-/-} mice at the time of AAV8-hSLC4A11 administration.

Age (week)	Male <i>Slc4a11</i> ^{-/-} mice	Female <i>Slc4a11</i> ^{-/-} mice
4	8	8
6	8	8
8	8	8
10	8	8
11	8	8
12	8	8
13	9	6

12.3.2.2 Development of serum anti-AAV8 and anti-SLC4A11 antibody titer assays and T cell response to rAAV8 and SLC4A11 assays

Anti-AAV8 antibody immunoassay. We will detect serum anti-AAV8 antibodies using a commercially available ELISA-based anti-AAV8 antibody assay (Creative Diagnostics) using serum collected from *Slc4a11*^{-/-} mice two weeks following intramuscular injection of rAAV8-GFP.

Anti- SLC4A11 antibody immunoassay. For the detection of serum anti-SLC4A11 antibodies in *Slc4a11*^{-/-} mice following intramuscular injection of AAV8-hSLC4A11, we selected Biosynth, a life sciences reagents, custom synthesis, and manufacturing services company, to synthesize a custom SLC4A11 linear overlapping peptide (OLP) library and map epitopes targeted by anti-*SLC4A11* antibodies. Biosynth has synthesized the customized SLC4A11 linear OLP library peptide array that spans the entire 891 amino acid SLC4A11 protein sequence and consists of over 450 15-mer linear peptides with a 2 amino acid offset, which will be utilized for anti-*SLC4A11* antibody epitope mapping using sera collected from *Slc4a11*^{-/-} mice following intramuscular injections with research grade AAV8-hSLC4A11 viral vector generated by Andelyn and sera from negative controls. Peptide binding on the microarray will be quantified and analyzed to identify epitope candidates. Peptide analyses will be provided in a full technical report that includes plots that visualize the binding of all samples to each peptide.

T cell response to rAAV8 assay. We will utilize a commercially available IFN-γ ELISPOT assay (ImmunoSpot) for detection of IFN-γ secreting white blood cells using murine splenocytes isolated from *Slc4a11*^{-/-} mice four weeks following intramuscular injection of rAAV8-GFP, using an established protocol for murine splenocyte isolation.⁹²

T cell response to SLC4A11 assay. Splenocytes isolated from *Slc4a11*^{-/-} mice 4 weeks following intramuscular injection of research grade AAV8-hSLC4A11 viral vector generated by Andelyn will be used for the IFN-γ ELISPOT-peptide assay.

These immunoassays will be used in the Phase I/II clinical trial to assess hormonal and T cell immune responses to rAAV8 and SLC4A11 after AAV8-hSLC4A11 administration in enrolled participants.

12.3.3 In vitro efficacy assay in the presence of anti-SLC4A11 antibody

12.3.3.1 Generation of mouse monoclonal anti-SLC4A11 antibodies

We will use the custom antibody service provided by Thermo Fisher Scientific to generate three distinct clones of monoclonal anti-SLC4A11 antibodies using B cells isolated from *Slc4a11*^{-/-} mice immunized with intramuscular injection of toxicology grade AAV8-hSLC4A11 viral vector generated by Andelyn. The ELISA-peptide assay for anti-SLC4A11 antibody developed in 12.3.2.2 will be used to screen candidate mice with the strongest hormonal immune response and to screen positive B cell culture supernatants for monoclonal anti-SLC4A11 antibodies.

12.3.3.2 Determination of the effect of extracellular anti-SLC4A11 antibodies on SLC4A11 mediated NH₃:H⁺ conductance

We will perform single cell patch-clamp recordings of NH₄Cl induced depolarization in LV-hSLC4A11vB^{WT} stably transduced *SLC4A11*^{-/-} HCEC-21T (cells shown in Figure 12A) after a 30-min pre-incubation with monoclonal anti-SLC4A11 antibodies generated in 12.3.3.1. This will provide evidence supporting or disputing the hypothesis that circulating antibodies against SLC4A11 protein developed following AAV8-hSLC4A11 administration in participants of the planned Phase I/II trial who previously did not express SLC4A11 protein due to *SLC4A11* mutations will not affect the efficacy of AAV8-hSLC4A11 gene therapy.

Table 8. Planned preclinical studies

Study Objective(s)	Methodology	Outcome Measures
1.1 Determination of the dose-response relationship of AAV8-hSLC4A11 intrastromal delivery in <i>Slc4a11</i>^{-/-} mouse model of CHED 1.2 Biodistribution studies in <i>Slc4a11</i>^{-/-} mice	1. Intrastromal injections of research grade AAV8-hSLC4A11 vector (Andelyn) in one eye and no injection to the contralateral eye 2. AS OCT scans before injection (baseline) and after injection biweekly until post-injection week 26 3. Ocular inflammation quantification using slit lamp biomicroscopy and retinal OCT 4. Collection of frozen sections of treated and untreated eyes post-injection week 26 5. Collection of serum, aqueous humor and vitreous humor for anti-AAV8 and anti-SLC4A11 antibody measurement	1. Central corneal thickness 2. Anterior chamber cell and flare 3. Ocular distribution of hSLC4A11 protein 4. Transgene and biodistribution of AAV8, SLC4A11 in ocular and non-ocular tissues 5. Minimum effective dosage (MED) and maximum tolerated dosage (MTD)

Study Objective(s)	Methodology	Outcome Measures
	6. Collection of non-ocular tissues (liver, blood, heart, kidneys and gonads) for digital PCR based vector genome	
1.3 Determination if there is a sex-related difference in the therapeutic window of AAV8-hSLC4A11 administration in <i>Slc4a11</i>^{-/-} mice	1. Single intrastromal injection of research grade AAV8-hSLC4A11 vector (Andelyn) at the MED identified in 1.1 will be administered to one eye of <i>Slc4a11</i> ^{-/-} mice and the contralateral uninjected eye will serve as control 2- AS OCT scans before injection (baseline) and after injection biweekly until post-injection week 26	Central corneal thickness
2.1 Development of digital PCR based vector potency assays	1. HEK-293 cells will be infected with Andelyn research grade vector at 25,000 vg/cell. 2. Quantitation of SLC4A11 cDNA will be performed using ddPCR and normalized to viral genomes to determine the ratio of vector derived cDNA copy number to the intracellular vector genomes.	1. Internalized vector genomes will be quantified via ddPCR. The number of internalized vector genomes will be compared to the AAV8-SCL4A11 standard
2.2 Development of serum anti-AAV8 and anti-SLC4A11 antibody titer assays and T cell response to rAAV8 and SLC4A11 assays	Anti-AAV8 antibody immunoassay. Serum anti-AAV8 antibodies will be detected using a commercially available ELISA-based anti-AAV8 antibody assay (Creative Diagnostics) using serum collected from <i>Slc4a11</i> ^{-/-} mice two weeks following intramuscular injection of rAAV8-GFP. Anti- SLC4A11 antibody immunoassay. Serum anti-SLC4A11 antibodies in <i>Slc4a11</i> ^{-/-} mice following intramuscular injection of AAV8-hSLC4A11 will be detected using a custom SLC4A11 linear overlapping peptide (OLP) library for anti-SLC4A11 antibody epitope mapping using sera collected from <i>Slc4a11</i> ^{-/-} mice following intramuscular injections with research grade AAV8-hSLC4A11 viral vector generated by Andelyn and sera from negative controls. Peptide binding on the microarray will be quantified and analyzed to identify epitope candidates. T cell response to rAAV8 assay. A commercially available IFN-γ ELISPOT assay (ImmunoSpot) will be utilized for detection of IFN-γ secreting white blood	

Study Objective(s)	Methodology	Outcome Measures
	cells using murine splenocytes isolated from <i>Slc4a11</i>^{-/-} mice four weeks following intramuscular injection of rAAV8-GFP, using an established protocol for murine splenocyte isolation. T cell response to SLC4A11 assay. Splenocytes isolated from <i>Slc4a11</i>^{-/-} mice 4 weeks following intramuscular injection of research grade AAV8-hSLC4A11 viral vector generated by Andelyn will be used for the IFN-γ ELISPOT-peptide assay.	
3.2 Determination of the effect of extracellular anti-SLC4A11 antibodies on SLC4A11 mediated NH3:H+ conductance.	Single cell patch-clamp recordings of NH4Cl induced depolarization in LV-hSLC4A11vBWT stably transduced <i>SLC4A11</i> ^{-/-} HCEC-21T after a 30-min pre-incubation with monoclonal anti-SLC4A11 antibodies.	Membrane NH ₃ /NH ₄ ⁺ and H ⁺ conductance
Non-GLP pilot rAAV8-hSLC4A11 toxicology and biodistribution studies in Gottingen minipigs (see Table 2 for more details)	1. Twenty-four minipigs (12 male and 12 female). Age of animals to be discussed based on pharmacological endpoints. Animals will be tested for the presence of anti-AAV8 neutralizing antibody (Nab) status and excluded if positive. 2. Single administration of one of three doses of toxicology grade AAV8-hSLC4A11 vector (Andelyn) via intrastromal injection (100 μL) in one eye.	Ocular endpoints Slit lamp ophthalmoscopy Slit lamp photography Tear collection IOP Indirect ophthalmoscopy Ultrasound corneal pachymetry Anterior segment OCT Specular or confocal microscopy (with endothelial cell counts) Hematology Coagulation Clinical chemistry Urinalysis Immunogenicity Biodistribution

12.4 Phase I/II Clinical Study Design

This will be a non-randomized, open label, multi-center, Phase I/II dose-escalation and dose-expansion study of the safety and efficacy of rAAV8-hSLC4A11 administered by corneal (deep stromal) injections in one or both (one year apart) eye(s) of individuals with CHED. The primary study endpoint will be safety and the secondary study endpoint will be efficacy.

Each participant will receive rAAV8- hSLC4A11 by a single deep intrastromal injection in the eye that meets enrollment criteria, or the more severely affected eye (based on CDVA and CCT) if both eyes meet enrollment criteria. If both eyes meet enrollment criteria, the less severely affected eye will be considered for a single deep intrastromal injection one year after the injection in the more severely affected eye, as outlined in Table 9.

Table 9. Study Groups

Group		Age	Number of Subjects	Dose Level	
				Volume	vg per eye
Phase I (Dose Escalation)	1	≥ 3 years	1 ^a + 3 ^b	100 µL	Low
	2	≥ 3 years	1 ^a + 3 ^b	100 µL	Medium
	3	≥ 3 years	1 ^a + 3 ^b	100 µL	High
Phase II (Dose Expansion)	4	≥ 3 years	up to 12	100 µL	MTD ^c

^a Corneal densitometry score (in grayscale units (GSU)) ≥ 40 and ≤ 45

^b Corneal densitometry score < 40

^c MTD = maximum tolerated dose determined in Groups 1, 2 and 3

Participants in Groups 1, 2 and 3 will be at least 3 years of age and receive the vector at a low dose (Group 1), medium dose (Group 2) or high dose (Group 3). The first (sentinel) participant in each group of Group 1, 2 and 3 will have a degree of corneal opacification that is worse than the subsequently enrolled 3 participants at that dose level. Participants in Group 4 will be participants enrolled in Group 1, 2 or 3 who are at least one year after receiving the vector in one eye and who have an anti-AAV8 antibody titer less than 1:20. Participants enrolled in Group 4 will receive the vector in the contralateral previously untreated eye at the maximum tolerated dose (MTD) determined in Groups 1, 2 and 3.

Enrollment will begin with one sentinel subject with severe corneal opacity in Group 1, followed by a 2-month follow-up period. After review of safety data by a Data and Safety Monitoring Committee (DSMC), enrollment will proceed to three additional subjects (staggered at least 1 week between each participant) with less severe corneal opacification followed by a 2-month follow-up period. After review of safety data from Group 1, participants will be enrolled in Group 2 with the same 1 + 3 enrollment scheme. After review of safety data from Groups 1 and 2, participants will be enrolled in Group 3 with the same 1 + 3 enrollment scheme. Within Groups 1, 2, and 3, enrollment of the 3 participants with less severe corneal opacification will be staggered by at least 1 week to allow for review of safety information.

After review of safety data from Group 1, 2 and 3, participants who are already enrolled in Group 1, 2 or 3 who are at least 1 year after the initial dose administration, with an anti-AAV8 antibody titer < 1:20, will be recruited into Group 4. Within Group 4, enrollment of first 3 participants will be staggered by at least 2 weeks to allow adequate time for review of safety information by the investigators and sponsor.

Safety will be monitored by evaluation of ocular and non-ocular AEs, hematology and clinical chemistry parameters and immune responses to SLC4A11. Efficacy will be measured by evaluation of visual acuity, contrast sensitivity, central and peripheral corneal thickness, corneal densitometry and quality of life questionnaires. Other data that will be collected includes corneal photography, immune responses to AAV and SLC4A11 and the presence of vector DNA in blood and tears.

Twelve participants will be enrolled in Phase I of this study and up to 12 participants will be enrolled in Phase II of this study. Enrollment is anticipated to take approximately 12 months for Phase I and 12 months for Phase II. Enrolled participants will have frequent follow-up visits during the first year after investigational product administration.

To monitor for delayed AEs and assess the duration of any changes in visual function or structure that occur, participants enrolled into both Phase I and Phase II will be followed annually for an additional 3 years after the Year 2 visit.

Study Population

Inclusion Criteria. In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. Congenital corneal opacification consistent with a diagnosis of CHED
2. Confirmation of the diagnosis of CHED by the identification of homozygous or compound heterozygous presumed pathogenic mutations in the *SLC4A11* gene
3. At least 3 years of age
4. Willing and able to travel to Study Site for treatment and follow-up visits
5. Good general health without significant physical examination findings or clinically significant abnormal laboratory results
6. Able to undergo AS OCT, corneal ultrasonography, Scheimpflug-based optical densitometry and age-appropriate visual acuity testing.
7. Visual acuity not better than 0.60 logMAR unit and not worse than hand motion in both eyes
8. Stable hematology, clinical chemistry and urinalysis parameters
9. For female participants of childbearing potential, a negative pregnancy test at screening, and agreement to use effective contraception for 12 months after administration of rAAV8-hSLC4A11
10. For male participants of reproductive potential, agreement to use effective contraception for 12 months after administration of rAAV8-hSLC4A11

Exclusion Criteria. An individual who meets any of the following criteria will be excluded from participation in this study:

1. Prior keratoplasty in either eye
2. Corneal endothelial density < 500 cells/mm² on specular microscopy in both eyes
3. Bowman layer scarring or splitting observed on AS OCT in both eyes
4. CDS > 45 GSU on Scheimpflug-based optical densitometry using the Pentacam HR (Oculus) in both eyes
5. Pre-existing eye conditions that would preclude the planned surgery or interfere with interpretation of study endpoints or complications of surgery (e.g. glaucoma, retinal diseases, uveitis, cataract, or history of retinal detachment) in both eyes
6. History of immunodeficiency or other medical conditions that might increase the risk of rAAV8-hSLC4A11 administration
7. History of allergy or sensitivity to medications planned for use in the peri-operative period

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8. For females of childbearing potential, a positive pregnancy test at screening or baseline (within 2 days before rAAV8-hSLC4A11 administration)
 9. Females who are breast feeding
 10. Use of any investigational agent, systemic corticosteroid or other immunosuppressive drug within 3 months prior to enrollment
 11. Prior receipt of any AAV gene therapy product
 12. Serum anti-AAV neutralizing antibody titer against AAV8 >1:20
 13. Any condition that leads the investigator to believe that the participant cannot comply with the protocol requirements or that may place the participant at an unacceptable risk for participation

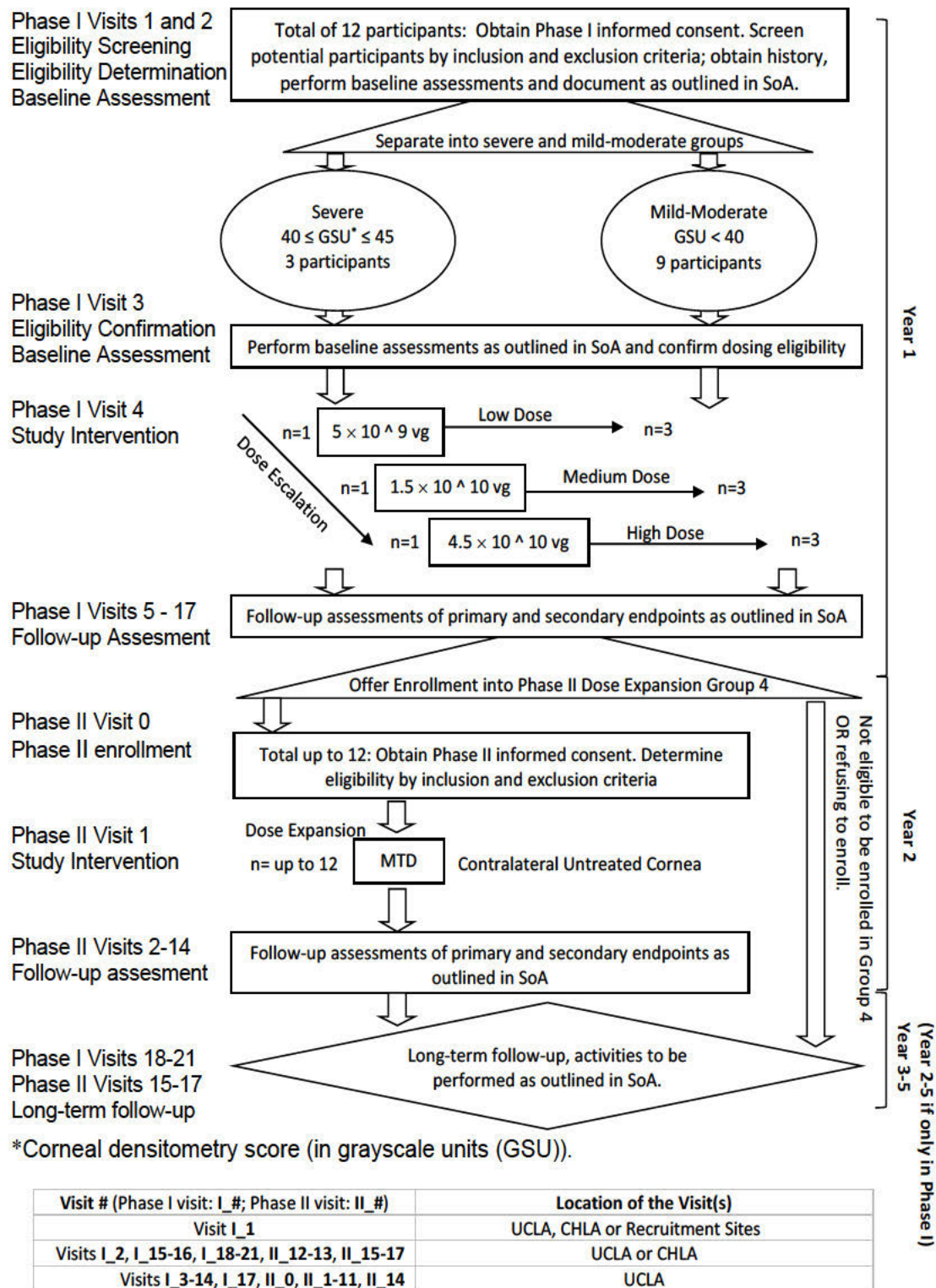


Figure 17. Schematic of Phase I/II clinical Study Design

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