Chapter 6

AAV-Mediated Gene Therapy for *CRB1*-Hereditary Retinopathies

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Additional information is available at the end of the chapter

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Abstract

Variations in the Crumbs homolog-1 (*CRB1*) gene lead to autosomal recessive retinal dystrophies such as early-onset retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA). No treatment is yet available for these patients. Adeno-associated virus (AAV) mediated gene therapy for hereditary retinal diseases holds great promise proven by the large number of active clinical trials. We here summarized the knowledge about the localization and function of CRB1 in the retina and the main pathological features resulting from loss of CRB1 function in humans and in rodents. This know-how is being applied to design and develop AAV gene therapy vectors for the treatment of *CRB1*-Hereditary retinopathies. Knowing which cell types express the CRB proteins, the possible redundancy of function between CRB1 and CRB2, and the AAV tropism in the human retina, will allow us to rationalize about the AAV capsid, promoter and route of administration that should be used in the AAV vector in order to efficiently and specifically deliver *CRB1* or *CRB2* into the human retina.

Keywords: crumbs homolog-1 (CRB1), retinitis pigmentosa, Leber congenital amaurosis, gene therapy, adeno-associated virus (AAV)

1. Introduction

A new generation of medicines emerged in 2012 with the first ever European market authorization of Glybera (alipogene tiparvovec), an adeno-associated virus (AAV) gene therapy medicine for the treatment of a rare inherited autosomal recessive lipid disorder, lipoprotein lipase deficiency. Five years later the company did not seek for renewal of the marketing authorization for Glybera due to patient's lack of demand [1]. Despite the marketing failure

Targeted disease	AAV serotype	Promoter	Gene	Delivery route	Volume injected	Dosage	ClinicalTrials.gov Identifier	Ref.
LCA	AAV4	RPE65	*hRPE65*	Subretinal	400 or 800 μL	1.22×10^{10} vg 4.8×10^{10} vg	NCT01496040	[17]
	AAV2	CBA	*hRPE65*	Subretinal	450 μL	1.8×10^{11} vg 6×10^{11} vg	NCT00749957	[18, 19]
	AAV2	hRPE65	*hRPE65*	Subretinal	up to 1 mL	up to 3×10^{12} vg	NCT00643747	[4, 20]
	AAV2	CB^{SB}	*hRPE65*	Subretinal	150–300 μL	8.94×10^{9} 3.58×10^{10} vg	NCT00481546	[21]
	AAV2	CBA	*hRPE65v2*	Subretinal	150 μL	1.5×10^{10} vg 4.8×10^{10} vg 1.5×10^{11} vg	NCT00999609	[22]
Choroideremia	AAV2	CBA	*REP1*	Subretinal	60–100 μL	10^{10}–10^{11} vg	NCT01461213 NCT02407678 NCT02077361	[5]
	AAV2	NR	*hCHM*	Subretinal	NR	NR	NCT02341807	NR
RP (*RLBP1*)	AAV8	sRLBP1 CPK850	*hRLBP1*	Subretinal	NR	NR	NCT03374657	[23]
RP (*PDE6B*)	AAV5	RK	*hPDE6B*	Subretinal	NR	NR	NCT03328130	[24]
RP (*MERTK*)	AAV2	VMD2	*hMERTK*	Subretinal	NR	NR	NCT01482195	[25]
X-linked RP	NR	NR	*RPGR*	Subretinal	NR	NR	NCT03116113	NR
	AAV2tYF	GRK1	*RPGR*	Subretinal	NR	NR	NCT03316560	[26, 27]
Achromatopsia	AAV8	NRx	*hCNGA3*	Subretinal	NR	1×10^{10} vg 5×10^{10} vg 1×10^{11} vg	NCT02610582	NR
	AAV8	hCAR	*CNGB3*	Subretinal	NR	NR	NCT03001310	NR
	AAV2tYF	PR1.7	*CNGA3*	Subretinal	NR	NR	NCT02935517	[28]
	AAV2tYF	PR1.7	*CNGB3*	Subretinal	NR	NR	NCT02599922	[29]
X-linked retinoschisis	AAV8	scRS/IRBP	*shRS*	Intravitreal	NR	NR	NCT02317887	[30, 31]
	AAV2tYF	CB	*hRS1*	Intravitreal	NR	NR	NCT02416622	[32]
Leber hereditary optic neuropathy (LHON)	scAAV2 (Y444,500,730F)	CMV/CBA	*P1ND4v2*	Intravitreal	200 μL	5.00×10^{9} vg 2.46×10^{10} vg 1.0×10^{11} vg	NCT02161380	[8, 33]
	AAV2	CMV	*ND4*	Intravitreal	90 μL	3×10^{10} vg $9 \times x10^{10}$ vg 1.8×10^{11} vg	NCT02064569 NCT02652767 NCT02652780 NCT03293524	[34, 35]

Targeted disease	AAV serotype	Promoter	Gene	Delivery route	Volume injected	Dosage	ClinicalTrials.gov Identifier	Ref.
Age-Related Macular Degeneration (AMD)	AAV2	CMV	*sFLT01*	Intravitreal	100 μL	2×10^8 vg 2×10^9 vg 6×10^9 vg 2×10^{10} vg	NCT01024998	[36]
	AAV8	NR	*soluble anti-VEGF*	Subretinal	NR	3×10^9 vg 1×10^{10} vg 6×10^{10} vg	NCT03066258	NR

CBA: chicken β-actin promoter (CBA); CB^{SB}: Hybrid modified short cytomegalovirus (CMV) enhancer and chicken β-actin promoter (CBA); GRK1: G protein-coupled receptor kinase; hCAR: human cone arrestin; NR: not reported; PR1.7: 1.7-kb L-opsin promoter; REF: References; RK: Rhodopsin kinase; scRS/IRBP: Retinoschisin/interphotoreceptor retinoid binding protein; VMD2: Vitelliform macular dystrophy-2.

Table 1. Summary of the clinical trials for retinopathies using AAV as delivery system registered on ClinicalTrials.gov database.

of Glybera, the use of AAV gene therapy in the eye is very attractive since the marketing prospects look better for the small amounts of AAV medicine to be transferred into the retinal tissue or retinal pigment epithelium. The eye is well accessible for surgery and allows direct observation, *in vivo*, of the retinal tissue in microscopic detail. Moreover, the eye is considered an immune-privileged tissue. Therefore, the risks of an immune response against the virus and/or the transgene itself are reduced. The local application in the "compartmentalized" eye of low amounts of AAV drug will minimize side effects expected if systemically applied at high doses [2]. But most importantly, potential drug efficacy for retinal orphan diseases can be efficiently proven thanks to a plethora of non-invasive retinal investigation techniques.

At the end of 2017, Luxturna (voretigene neparvovec-rzyl) became the first FDA-approved AAV gene therapy medicine for patients with hereditary retinal disease caused by biallelic *RPE65* gene mutations [3, 4]. The market approvals of the first gene therapy medicines in Europa and in the USA paved the road to similar programs, reflected on the large number of clinical trials registered on the ClinicalTrials.gov website using AAVs as a delivery strategy to treat hereditary retinal diseases such as choroideremia (*CHM* or *REP-1*) [5], achromatopsia (*CNGA3*) [6], wet age-related macular degeneration (AMD) (*VEGFR1*/*FLT* and a gene encoding soluble anti-VEGF protein) [7], Leber hereditary optic neuropathy (LHON) (*ND4*) [8], autosomal recessive retinitis pigmentosa (arRP) (*MERTK*) [9], X-linked RP (*RPGR*) [10], RP (*PDE6B*) [11] and (*RLBP1*) [12] and X-linked Retinoschisis (*RS1*) [13, 14] (**Table 1**). Developing an AAV gene therapy to treat patients with mutations in the Crumbs homolog-1 (*CRB1*) gene was particularly challenging due to its large cDNA (4.2 kb) which approached the packaging limit of the AAV genome (~4.7–4.9 kb). Thus, to build an AAV vector that allowed efficient packaging of the human *CRB1* cDNA, the use of a short promoter (<350 bp) and a short synthetic polyadenylation sequence was required to efficiently express the CRB1 protein *in vivo*. Codon optimization of the *CRB1* cDNA was used to achieve sufficient levels of expression [15]. A second strategy that implied the replacement of CRB1 by its structural and functional family member CRB2 was used to overcome the size limitation and potential toxicity due to expression of CRB1. *CRB2* cDNA was only

3.85 kb in size and gave more flexibility to design the AAV gene therapy vector in terms of promoter sequence size, polyadenylation sequence and other optimized sequences that stabilized the transcript [16].

2. *CRB1*-Hereditary retinopathies

More than 240 different mutations in the *CRB1* gene have been described so far (http://www.LOVD.nl/CRB1). These gene variations are associated with a wide variety of retinal dystrophies, including autosomal recessive retinitis pigmentosa (RP), Leber congenital amaurosis (LCA), cone-rod dystrophy, isolated macular dystrophy and foveal retinoschisis [37]. Furthermore, mutations in *CRB1* are responsible for 7–17% of all the LCA cases and for approximately 3–9% of all cases of RP [38, 39]. Retinitis pigmentosa type 12 (RP12) due to mutations in the *CRB1* gene was initially characterized by RP with preservation of para-arteriolar retinal pigment epithelium (PPRPE), progressive visual field loss starting from the first decades of life, and early macular involvement. Later on it became clear that RP12 commonly presents early-onset retinitis pigmentosa, hyperopia and optic disc drusen, with or without PPRPE [37, 40, 41]. Leber congenital amaurosis type 8, due to mutation in the *CRB1* gene (LCA8), is a severe form of retinal dystrophy characterized by roving eye movements or nystagmus, nonrecordable or severely reduced cone and rod electroretinography amplitudes and severe loss of vision within the first years of life. Retinas of LCA8 patients with *CRB1* mutations are about 1.5 times thicker than normal retinas, while retinas of patients with LCA due to mutations in other genes such as *RPE65* or *GUCY2D* are thinner [42]. In addition, LCA8 retinas showed abnormal retinal architecture suggesting that loss of CRB1 function might interrupt the naturally occurring process of proliferation, apoptosis and cell migration during retinal development [42–44].

No treatment is yet available for *CRB1*-associated retinal dystrophies. We achieved proof-of-concept for retinal *CRB1* gene therapy, using an AAV9-CMV-*hCRB2* vector in two mouse models. A first model lacked CRB1 and had reduced levels of CRB2 in Müller glial cells and photoreceptors, and a second model lacked CRB2 from Müller glial cells and photoreceptors [16]. These two pre-clinical studies opened the perspective for therapeutic trials for human *CRB1*-associated dystrophies.

Intriguingly, there is no clear genotype–phenotype correlation for *CRB1* mutations [45]. This fact associated with the large spectrum of retinal dystrophies observed in patients with mutations in the *CRB1* gene [37], reinforced the need to study in detail the clinical features and natural disease progression of *CRB1*-associated retinal dystrophies before moving towards a clinical trial. This knowledge is required to establish patient eligibility criteria and clinical outcomes for the forthcoming clinical trial.

2.1. The *CRB1*-complex in the retina

In the developing mouse retina, the retinal neuroepithelium is composed of multipotent retinal progenitor cells that differentiate in a time-dependent manner, giving rise to six major types of neuronal and one type of glial cells. The first cell type to be generated from the

progenitors are the ganglion cells, followed in overlapping sequential phases by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and the Müller glial cells. The seven retinal cell types organize or "laminate" in three orderly distinct nuclear layers divided by two plexiform layers [46]. The CRB complex plays a crucial role during retinogenesis by the establishment of polarity, adhesion, retinal lamination and restricting proliferation and apoptosis of progenitors and the number of late born cells such as rod photoreceptors, bipolar cells, late-born amacrine cells and Müller glial cells [47–52].

The CRB family in mammals consists of three members CRB1, CRB2 and CRB3. Both the CRB1 and CRB2 have a large extracellular domain with epidermal growth factor-like and laminin-A globular domains, a single transmembrane domain and a short intracellular C-terminal domain. The C-terminal domain of 37 amino acids has a single FERM-protein-binding motif juxtaposed to the transmembrane domain and a single C-terminal PDZ protein-binding motif [53–55]. While CRB3, the third family member, contains the transmembrane and C-terminal domain but is very short in length since it lacks the large extracellular domain. The C-terminal PDZ motifs of CRB proteins bind to the PDZ domain of PALS1 (also called MPP5). PALS1 binds via its N-terminal L27 domain to the L27 domain of the multiple PDZ proteins PATJ and MUPP1 [56]. The multi-adapter protein PALS1 recruits MPP3 and MPP4 to the subapical protein complex at the so called subapical region adjacent to adherens junctions at the outer limiting membrane [57, 58]. Loss of the CRB1, CRB2, PALS1, or MPP3 but not MPP4 resulted in disruption of adhesion between photoreceptors and Müller glial cells. In summary, the core of the retinal CRB-complex is composed of CRB1, CRB2, PALS1, PATJ, MUPP1, and MPP3 [52, 59].

In the embryonic mouse retina, CRB1, CRB2, PALS1, PATJ and MUPP1 are expressed at the subapical region adjacent to the adherens junctions of the retinal progenitor cells [49]. In the adult mouse retina, CRB2 is present at the subapical region in photoreceptors and Müller glial cells. The mouse *Crb1* gene transcript is expressed in photoreceptors and Müller glial cells but expression of the CRB1 protein is limited to the subapical region of Müller glial cells [60, 61]. CRB3 has a broader expression pattern being located at the subapical region in both photoreceptors and Müller glial cells [52, 60], at the photoreceptor inner segments and photoreceptor synaptic terminals and at sub-populations of amacrine and bipolar cells in the inner plexiform layer [62]. The expression patterns of CRB1 and CRB2 observed in the mouse retina do in part match with the ones observed in the human retina. In the first trimester human fetal retina, CRB2 but not CRB1 is expressed at the subapical region. While in the second trimester CRB1, CRB2 and PALS1 localize at the subapical region. A similar expression pattern is observed in early (differentiation day 28) versus late (differentiation day 160) human induced pluripotent stem cells (iPSCs)-derived retinas [63]. Immunoelectron microscopic protein localization studies performed on adult human retinas, collected at two to 3 days post-mortem, showed CRB1 and CRB2 localization at the subapical region of Müller glial cells as found in the mouse retina. Human CRB1 localized also at the subapical region in photoreceptor cells, whereas human CRB2 localized at vesicles in the photoreceptor inner segments some distance away from the subapical region [52, 60] (**Figure 1**).

Interestingly, the overexpression of human CRB2 protein specifically in mouse photoreceptors that lacked endogenous mouse CRB2 in photoreceptors and Müller glial cells, caused aberrant localization of human CRB2 predominantly at vesicles in photoreceptor inner segments

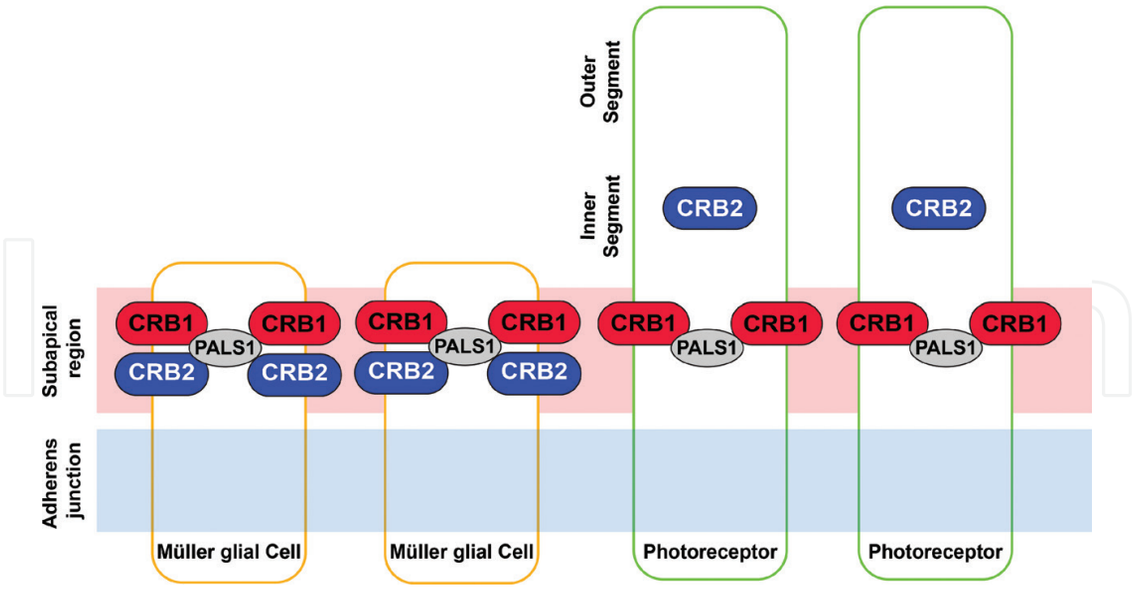

Figure 1. Model depicting the localization of CRB1 and CRB2 proteins in the human retina at 2 days post-mortem. CRB proteins are present at the subapical region above the adherens junctions between Müller glial cells, between photoreceptor and Müller glial cells and between photoreceptor cells. CRB1 is located in both Müller glial cells and cone and rod photoreceptor cells at the subapical region. CRB2 is located in Müller glial cells at the subapical region, and in photoreceptors at vesicles in the inner segments at a distance from the subapical region.

at a distance from the subapical region. However, when expressed in both photoreceptors and Müller glial cells, human CRB2 localization was restricted to the subapical region, which suggested that expression of CRB2 in both cells types might be required for proper protein localization and function [16].

2.2. Animal models for *CRB1*-retinopathies

Animal models able to recapitulate features of the *CRB1*-retinopathies are of value to understand the molecular mechanism behind retinopathies and to test new AAV gene therapy vectors. Over the recent years several rodent models were described in the literature. The retinal phenotypes observed in these animals mimic the wide spectrum of clinical features as described in *CRB1*-patients, including early and late onset RP, LCA and telangiectasia [44, 49, 50, 52, 64–67]. The onset and severity of the phenotype observed in these animal models seem closely associated with the total levels of the CRB proteins in the different cell compartments. The available models can be grouped into three major categories:

a. late onset-RP: homozygous knockout *Crb1* [52], hemizygous knockin $Crb1^{C249W/-}$ [67] and homozygous naturally occurring mutant $Crb1^{rd8}$ [66] mice showed, at foci, loss of integrity of the outer limiting membrane, with protrusions of rows of photoreceptor nuclei into the inner- and outer segments layer and ingression of photoreceptor nuclei into the photoreceptor synaptic layer. Microglial cell infiltration and upregulation of glial fibrillary acidic protein (GFAP) were observed at the foci of photoreceptor dysplasia. Conditional ablation of *Crb2* specifically in Müller glial cells resulted in disruptions at the outer limiting

membrane and ectopic photoreceptor nuclei in the inner- and outer segment layer [50]. The morphological abnormalities observed in all these models do not lead to a decrease in electrical retinal function.

b. early onset-RP: ablation of *Crb2* from retinal progenitor cells, and consequent loss of CRB2 in cone and rod photoreceptors and Müller glial cells [47, 49] or ablation of *Crb2* specifically in immature photoreceptors [50] leads to disruptions at the outer limiting membrane during late-stage embryonic development resulting in abnormalities in retinal lamination, severe retinal degeneration and early loss of retinal function. More recently, a naturally occurring substrain of Brown Norway rats (BN-J) was described as a model for retinal telangiectasia due to homozygous variations in the *Crb1* gene. Interestingly the retinal phenotype observed in this *Crb1* rat strain differs from the phenotype observed in the *Crb1* knockout mice. The *Crb1* rat displays retinal dysplasia at early postnatal days, leading to early-onset disruption of photoreceptor synapses and subsequent loss of retinal function at 1 month of age and near to complete photoreceptor cell death at 6 months of age [64].

c. LCA: mouse retinas with loss of CRB1 and CRB2 proteins from retinal progenitor cells showed lack of a proper retinal lamination with loss of a photoreceptor synaptic layer, intermingling of photoreceptor nuclei with the nuclei of inner nuclear layer cells, and early loss of retinal function [44].

The lack of a genotype–phenotype correlation in humans might correlate with the different retinal phenotypes as observed in mice with lowered levels of CRB1 and/or CRB2 in retinal progenitors, photoreceptors and Müller glial cells. Cumulative data suggest that not only the levels of CRB1 are important for the pathogenesis observed in humans but also the total levels of CRB1 and CRB2 proteins. Or that the levels of functional CRB2 variants in retinal progenitors, photoreceptors or Müller glial cells might play a role in determining the severity of the retinal dystrophy caused by mutations in the *CRB1* gene.

3. Adeno-associated virus (AAV) biology

Adeno-associated virus belongs to the parvovirus family, but is placed in the genus Dependovirus since it is dependent on co-infection with other viruses, mainly adenoviruses, in order to replicate. AAV is a small, non-enveloped single-stranded DNA virus. The genome of the AAV is approximately 4.7 kb and has three open reading frames to express the *rep* (Replication), *cap* (Capsid) and assembly activating protein (*aap*) (Assembly) genes, flanked by two 145 nucleotide-long inverted terminal repeats (ITRs). The ITRs self-assemble into hairpin structures required for genome replication, integration and encapsidation. The *rep* gene encodes four proteins (Rep78, Rep68, Rep52 and Rep40), which are required for viral genome replication and packaging. While *cap* gene transcripts gives rise to the viral capsid proteins, virion protein 1 (VP1), VP2 and VP3, with molecular weights of 87, 72 and 62 kDa, respectively. These capsid proteins assemble into an icosahedral symmetry protein shell of 60 subunits, in

a molar ratio of 1:1:10 (VP1:VP2:VP3). The *aap* gene encodes the assembly-activating protein (AAP) that is thought to have a scaffolding function for capsid assembly [68]. Wild-type AAV integrates into the human host genome at a specific site, AAVS1 on chromosome 19.

In gene therapy a recombinant AAV (rAAV) and not the wild-type AAV are used. In rAAV the viral genome required for viral replication, the rep and *cap* genes, and the element required for site-specific integration are deleted. A sequence containing a promoter, a 5′-untranslated region, the cDNA of a transgene of interest, and a 3′-untranslated region containing a polyadenylation site are then inserted in between the AAV vector containing the two ITRs. To produce AAV particles from the AAV gene therapy plasmid in a human cell line, the *rep* and *cap* genes are supplied in trans on a helper plasmid along with helper genes from adenovirus (*E4*, *E2a* and *VA*) necessary for replication.

The major advantages of the use of rAAVs are the safety profile, low immunogenicity, lack of toxicity and the property that the rAAV genomes do not integrate into the host genome. The rAAV capsid enters the cells by receptor-mediated endocytosis, the rAAV genomes are processed into nuclear episomal structures and are maintained extrachromosomally. Dependent on the gene therapy vector used, and the life span of the targeted cell, the rAAV genomes can express a transgene for more than 10 years. AAV vectors also have the ability to transduce non-dividing cells, including non-dividing retinal neurons like photoreceptors. One major disadvantage of the rAAV technology is the size limitation of the total DNA that can be efficiently packaged in the AAV vector (4.7–4.9 kb) which makes it difficult to design AAV-mediated gene therapy for larger genes (≥ 4 kb). The development of dual and triple AAV vectors with a maximum transfer capacity of around 9 and 14 kb, respectively, might in the future overcome in part this limitation [69].

The generation of a gene therapy vector able to deliver CRB1 is particularly challenging due to its large size of cDNA (4.2 kb). To assemble the gene therapy vector, the 4.2 kb *CRB1 cDNA* and the two ITR sequences (0.29 kb) need to be added which make up to 4.49 kb. Therefore, only 0.2–0.41 kb space is left for the promoter and polyadenylation sequences. Although challenging it was shown that it is possible to efficiently package human *CRB1 cDNA* in AAV vectors and to express CRB1 protein *in vivo* [15, 16]. Another strategy to overcome the size limitation is to use the 3.85 kb *CRB2 cDNA* as replacement [16].

3.1. Gene delivery in the retina using AAVs

The eye offers a set of unique features for the application of gene therapy vectors. The eye is a small, compartmentalized, immunoprivileged, paired organ and easily accessible using minimally invasive techniques. There are also high resolution functional and structural diagnostics, such as, optical coherence tomography, scanning laser ophthalmoscopy and electroretinography, as well as psychophysical tests such as microperimetry, kinetic perimetry, visual acuity testing, and multi-luminance mobility test (MLMT) in the ophthalmology field that allow to examine the eye/retina structure and to test as well retinal function and vision. Gene therapy vectors for retinal disease can be delivered mainly by two routes: subretinally into the "subretinal space" between the neural retina and the RPE, or intravitreally, into the vitreous body, both approaches are described below. The administration route is an

important parameter to take into consideration in the testing of gene therapy vectors, together with the selection of the AAV capsid and promoter since all these parameters have effects on the tropism of the vectors.

3.2. Route of delivery

3.2.1. Subretinal injection

In pre-clinical studies performed in rodents *ab externo* subretinal injections are commonly performed [16, 70]. This method uses a small needle (34 gauge) to penetrate (*ab externo*) the sclera at the limbus and under direct observation the needle can be guided through the retina to create a subretinal space between the retinal pigment epithelium and the outer limiting membrane. Normally, a volume of 1 μL is injected to form an injection fluid bleb that transiently detaches a large portion of neural retina from the RPE in one single injection. Incorrect surgery might cause cataract due to damage to the lens. It is also common to have a large volume of backflow of injected AAV particles when the injection needle is retracted.

Subretinal injections in human can be performed using the "single-step" or the "two-step" approach [71]. With the "single-step" approach the fluid, containing the gene therapy vector, is directly delivered into the subretinal space without previous retinal detachment [22].

The "two-step" approach consists of first the generation of a bleb in the subretinal space by injection of a balanced salt solution (BSS), followed by injection of the therapeutic agent using a controlled flow rate [4, 5, 72]. The second approach offers several advantages like the possibility to better assess the direction of bleb spread as well as to minimize vector loss by misguided injection [71]. The subretinal surgery and injection is a specialized technique and can in principle be executed by surgeons operating an ophthalmic surgery robot to obtain most reproducible results. According to information collected from the different clinical trials registered in the *Clinicaltrials.gov* database, a volume ranging from 60 to 1000 μL can be injected via this route (**Table 1**).

Subretinal injections seem the logical choice when RPE or photoreceptors are the target cells, since these cells will be in direct contact with the fluid containing the AAV particles. However, degenerating retinas at an advanced stage are often quite thin, with disruptions at the outer limiting membrane, loss of inner/outer segments and/or photoreceptor cells, neovascularization and infiltration of microglial cells. All these features might lead to a reduction in the potential subretinal space between the neural retina and the RPE, or to leaking of the AAV vector to the choroid vasculature system and influence the AAV tropism. The retinal detachment caused during the subretinal injection might potentially also either aggravate or alleviate the processes of retinal degeneration.

3.2.2. Intravitreal injection

Intravitreal injection implies direct delivery into the space in the back of the eye called the vitreous cavity, which is filled with a jelly-like fluid called the vitreous humor gel. Intravitreal injections are generally limited to volumes of up to 2 μL in mice [15, 16, 73], while in rats the

volumes are limited to 3–5 μL [74]. The main surgical complications observed are cataract formation due to lens-induced damage and retinal perforation [75].

In humans intravitreal injections are generally performed under local anesthesia [71], by inserting a 30 gauge needle through the sclera at the pars plana region, 3.5–4 mm posterior to the limbus between vertical and horizontal muscles with limited reflux [71, 73]. In clinical trials volumes between 90 and 200 μL have been injected via this route (**Table 1**).

Intravitreal administration of AAV gene therapy might look tempting since it is an easier procedure with less potential surgical complications compared to the subretinal injection, especially when treating thinned degenerative retinas. However, administration of AAV intravitreally has its own caveats namely the difficulty of AAV capsids to cross the thick inner limiting membrane in the human retina and the current lack of AAV serotypes capable of transducing efficiently the human photoreceptors or RPE cells. Another obstacle is the potential AAV transduction and subsequent expression in other eye tissues, as for example, the ciliary body especially when using a ubiquitous promoter.

Pre-clinical studies in mice and rats showed that Müller glial cells can efficiently be infected after intravitreal administration of AAV2/6 or AAV2/shH10^{Y445F} [15, 76], therefore these AAV capsids might be used to deliver CRB1 or CRB2 into Müller cells. AAV serotype shH10^{Y445F} is however known to transduce efficiently the ciliary body epithelium when applied intravitreally [16].

3.3. AAV capsids and cell type specific promoters

The existence of 11 natural AAV serotypes and derivatives that differ in their tropism, and the different types of cells they infect, makes AAV a very useful system to infect the various cell types of the retina. The cell specificity of the AAV vector can be further increased by using cell type specific promoters, for example RPE65 or VMD2 to drive expression in retinal pigment epithelium. Or by using e.g. the rhodopsin (RH), G protein-coupled receptor kinase 1 (GRK1), 1.7-kb L-opsin promoter (PR1.7) or cone arrestin (hCAR) promoter to drive expression in rod and/or cone photoreceptors. Or using e.g. the RLBP1, GFAP or NR2E1 promoter to drive expression in Müller cells [17, 23, 24, 28].

Several pre-clinical studies showed the tropism and/or potency of the different capsids and promoter (cell specific or ubiquitous) in infecting retinal cell types such as RPE, photoreceptors and Müller glial cells. However, AAV tropism might differ *in vivo* between rodent species, dogs, non-human primates and human. AAV tropism is dependent of the route of administration, the stage of retinal development and severity of retinal dystrophy. Therefore, is quite difficult to extrapolate the data from pre-clinical studies performed in rodents directly to the human *in vivo* setting. To obtain evidence-based data for clinical gene therapy studies, researchers optimize culture protocols for human retinal organotypic cultures [77–79] or human iPSC-derived retinas to study the AAV tropism [80]. Recently, the capacity of different AAV serotypes to infect and express in human retinal cells was studied in organotypic

cultures. This study suggested that serotypes AAV4, AAV5 and AAV6 were particularly efficient at transducing photoreceptor cells, whereas serotype AAV8 displayed consistently low transduction of these cells [79, 81]. Actually several AAV serotypes and ubiquitous promoters or cell specific promoters are being used in clinical trials (**Table 1**), the results from these studies will provide us with important clues about the best promoters and capsids to use in the human retina.

In order to deliver *CRB1* or *CRB2* into rod and cone photoreceptor and Müller glial cells in the human retina an AAV capsid able to infect all the three cell types needs to be used in combination with a promoter active in the same cells. Studies performed in mice suggested that a combination of AAV9 and a CMV promoter might be a possibility but further studies are required to test its suitability for human retinal cells [16]. Subretinal injection of expression vectors packaged into serotypes AAV5 or AAV9 infect photoreceptors *in vivo* in macaques [82, 83]. Tropism studies in human retinal explants reported that AAV5 would be more efficacious than AAV9 [84]. Another strategy would be the use of one vector to deliver *CRB1* or *CRB2* specifically in Müller glial cells and a second vector to deliver specifically in photoreceptors. Besides regulatory and financial issues, the main technical issues here resides with the lack of a short promoter (≤ 300 bp) specific for Müller cells, and the lack of an AAV serotype that in human retina efficiently infects Müller glial cells upon intravitreal or subretinal injection.

4. Conclusion

In recent years the scientific progress in the field of gene therapy for inherited retinal dystrophies culminated in the first ever approved AAV gene therapy medicine to treat LCA patients carrying mutations in the *RPE65* gene. The number of engineered AAV capsid variants and new promoters to drive expression in the different retinal cell types is raising at great speed allowing the design of more specific and more efficient viral vectors. Likewise, the number of clinical trials using AAV gene therapy is increasing at a similar rhythm, the data collected from these studies will be very useful for the development of similar therapies. Pre-clinical studies performed in mice demonstrated that AAV-mediated *CRB2* gene augmentation therapy might be a promising medicine to prevent progression of retinitis pigmentosa in patients with mutations in the *CRB1* gene. In mice at mid-stage retinal disease *CRB2* gene augmentation therapy successfully improved retinal morphology with preservation of photoreceptor cells and retinal function, therefore providing good perspectives for the forthcoming clinical trial in patients with RP due to mutations in *CRB1*.

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Conflict of interest

The LUMC is the holder of patent application PCT/NL2014/050549, which describes the potential clinical use of CRB2; JW is listed as inventor on this patent, and JW is an employee of the LUMC.

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