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Gene therapy on inherited retinal dystrophies: an update

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ABBREVIATION INDEX

AAV: Adeno-associated virus
ABCA4: ATP Binding Cassette 4
Ad: Autosomal dominant
AdV: Adenovirus
ADA-SCID: Adenosine deaminase-Severe combined immunodeficiency
AMD: Age-related macular degeneration
Ar: Autosomal recessive
Cas9: CRISPR associated protein-9
CMV: Cytomegalovirus
CNTF: Ciliary Neurotrophic Factor
CRISPR: Clustered regularly interspaced short palindromic repeats
DNA: Deoxyribonucleic acid
DSB: Double Stranded Break
EIAV: Equine infectious anemia virus
EMA: European Medicines Agency
ERG: Electroretinogram
FDA: Food and Drug Administration
FGF2: Fibroblast growth factor 2
fMRI: Functional magnetic resonance imaging
GT: Gene therapy
GHR: Genetics home reference
Gp: Genome particle
HDR: Homology Directed Repair
hESCs: Human embryonic stem cells
hiPSCs: Human induced pluripotent stem cells
IRD: Inherited retinal dystrophies
ITR: Inverted terminal repeat sequence
kDa: Kilo-dalton
LCA: Leber Congenital Amaurosis
LHON: Leber Hereditary Optic Neuropathy
MLMT: Multi-luminance mobility test
NGS: Next Generation Sequencing
NHEJ: Non Homologous End Joining
OCT: Optical coherence tomography
ORF: Open reading frame
OTC: Ornithine transcarbamylase
PAM: Proto adjacent motif
RP: Retinitis pigmentosa
RPE: Retinal pigment epithelium
RNA: Ribonucleic acid
sgRNA: Single guide ribonucleic acid
TALENs: Transcription activator-like effector nucleases
VA: Visual acuity
VF: Visual field
XL: X linked
WT: Wild type
ZFNs: Zinc finger nucleases

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1. INTRODUCTION

Inherited retinal dystrophies (IRDs) are a heterogeneous group of disorders characterized by mutations in one of more than 290 identified genes and loci (https://sph.uth.edu/retnet/disease.htm), resulting in the dysfunction or death of photoreceptors and the alteration of the retinal pigment epithelium (RPE), the choroid or the visual cycle pathways, ultimately leading to visual loss.[1,2] IRDs may appear as syndromic traits, or be presented in a non-syndromic manner and it is estimated that 1 in 2300 individuals is affected by an IRD.[3] Remarkably, a recent study by Irigoyen found that Retinitis Pigmentosa (a particular form of IRD) affects 1 in 4244 people in Gipuzkoa.[3]

1.1. CLASSIFICATION OF INHERITED RETINAL DYSTROPHIES

Classifications of IRDs may be based on clinical features, age of onset, progression and/or affected retinal cells or areas. However, these are being replaced by disease groups based on molecular diagnosis. In order to simplify such classification as much as possible, IRDs will be divided according to affected retinal areas and to the progressive or stationary nature of each disease.[4] Most commonly mutated genes will be exposed too.[1]

1.1.1. Diffuse photoreceptor dystrophies

1.1.1.1. Retinitis pigmentosa (RP)

Retinitis Pigmentosa gives name to a group of diseases that diffusely affect the RPE and the photoreceptors and show altered electroretinograms (ERGs). Rod photoreceptor function is initially lost and in most cases cones are affected in advanced stages, leading to a complete loss of vision. The ability to seeing under dim light and peripheral vision are affected, driving patients to suffering from nyctalopia and tunnel vision. Funduscopic examination **(Figure 1)** shows three classic alterations: arteriolar attenuation, optic disc pallor and pigment accumulation causing bone spicule formation.[3,4]

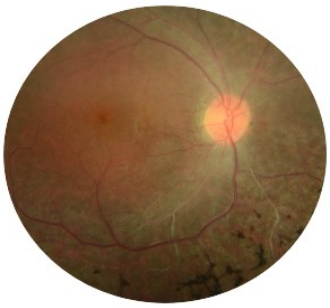


Figure 1. Funduscopy of an eye affected by retinitis pigmentosa. This figure shows the fundus examination of a patient affected by retinitis pigmentosa. Characteristic optic disc pallor and bone spicule formation can be seen. Courtesy of Irigoyen C.[3]

Despite RP is a disease usually confined to the eye, 20 to 30% of patients suffer from non-ocular diseases, and those cases are part of more than 30 different syndromes.[5] Usher's syndrome is the most frequent form, in which hearing impairment is associated with RP. Another major form is the recessively inherited Bardet-Biedl syndrome, which encompasses RP, obesity, polydactily, hypogenitalism, renal abnormalities and delayed psychomotor development. Mutations in more than 70 genes are associated to RP; mutated RHO, USH2A and RPGR genes accounting for 30% of cases.[5]

Leber Congenital Amaurosis (LCA) gives name to a group of conditions within the RP group which associate early onset visual loss, nystagmus, slow pupillary reactions and altered rod and cone ERG responses.[6,7] It usually derives in complete blindness by the third decade of life.[8] Mutated genes that cause LCA phenotype include CEP290, RPE65, GUCY2D and CRB1.[1]

1.1.1.2. Cone and cone-rod dystrophies

Cone dystrophies lead to loss of visual acuity (VA) and colour perception starting at youth or adolescence. Peripheral visual field (VF) usually remains unaltered. Cone-rod dystrophies feature progressive rod alterations as well.

1.1.2. Macular dystrophies

1.1.2.1. Stargardt's disease (STGD)

In this case, VA decreases and colour vision abnormalities occur bilaterally with onset in the first two decades of life. Slow disease progression is common and yellow lipofuscin flecks and foveal atrophy are usually present in the fundus examination.[4] ABCA4, PROM1 and STGD2 would be among the affected genes.[1]

1.1.2.2. Vitelliform degenerations

Best disease features bestrophin alterations that cause damage to RPE cells and lipofuscin accumulation in the macula, in the form of egg like (vitelliform) clusters.[4] Eventually, the macular area becomes atrophic and VA decreases.

Other described macular dystrophies include dominant familial drusen, pattern dystrophies and Sorsby's macular dystrophy.[4]

1.1.3. Choroidal dystrophies

Choroideremia (CHM) involves diffuse choroid, RPE, retinal vessel and photoreceptor degeneration. Pigmentation clusters accumulate in the macular area and annular scotomas progressively disturb the VF. Nyctalopia is also present. On the other hand, atrophia gyrata encompasses serum ornithine level increases with RPE and choroid alterations. Panretinal hyperpigmentation is usually helpful for differentiating atrophia gyrata and choroideremia as it is absent in the latter (**Figure 2**).[4] The affected gene related to choroideremia is CHM gene.[1]

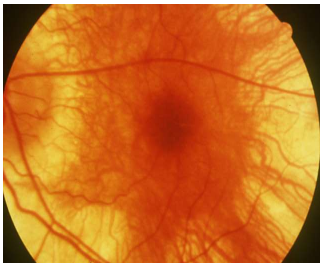


Figure 2. Fundus examination of an eye affected by choroideremia. This figure shows a hypopigmented fundus showing choroidal vessels due to chorioretinal atrophy. Courtesy of Irigoyen C.[3]

1.1.4. Internal retinal and vitreoretinal dystrophies

1.1.4.1. X linked juvenile retinoschisis (XLJR)

Retinal layers split and cystic spaces and vitreous veils form in XLJR. Besides newly formed cavities, the first synapse between photoreceptors and bipolar cells is usually altered too. Visual acuity usually deteriorates in latest stages.[4]

1.1.4.2. Goldmann-Favre syndrome

Night blindness, increased blue light sensitivity and VF and ERG alterations characterize it. Nr2e3 gene alterations are present and rods can only be stimulated under bright light. Tissular studies have shown significant rod reduction and doubled cone quantity with up to 90% of short wave length stimulated cones (S cones).[4]

1.1.5. Congenital stationary retinopathies

1.1.5.1. Congenital achromatopsia

It is characterized by very poor cone responses with normal rod ERG resulting in colour perception and VA impairment. Rod monochromatism (completely malfunctioning cones that only permit sight in a range of grey shades) and blue colour cone monochromatism are the two forms in which it is usually presented.[4] Mostly, it affects CNGA3, CNGB3 and GNAT2 genes.[1,9]

1.1.5.2. Congenital stationary night blindness (CSNB)

It is characterized by nyctalopia, nystagmus, strabismus and/or myopia from a very early onset. Eye fundus may be normal.[4]

Other described IRDs include: familial exudative vitreoretinopathy, ocular-retinal developmental diseases and optic atrophy.

1.2. CURRENT THERAPIES

Some available therapeutic options **(Figure 3)** have tried to reverse visual loss although none of them has proven to be definitive. Patients suffer from difficulties for performing daily activities and vision rehabilitation is usually offered with the hope to maintain an acceptable quality of life.[10] Patient associations form invaluable supportive nets for patients.[11]

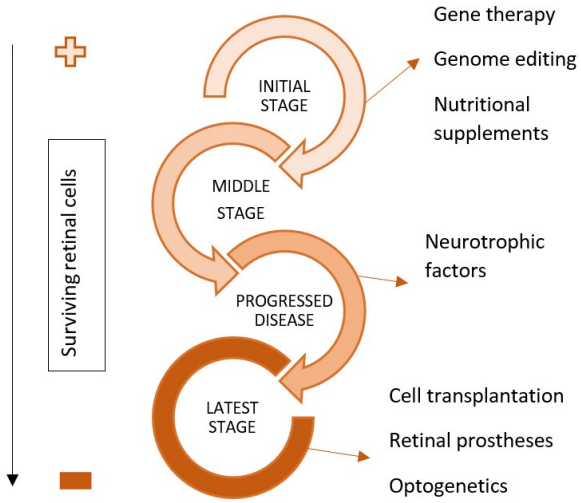


Figure 3. Disease phases and associated therapeutic options for each stage. Earlier detection may provide wider unaffected retinal areas, with functioning cells that may be benefited from treatments such as gene therapy. Latest stages involve massive affection and require rescue treatments.

1.2.1. Retinas with intact photoreceptors: first approaches

1.2.1.1. Nutritional supplementation

Nutritional supplements aim to improve visual function by giving patients certain substances such as vitamins or fatty acids. Vitamin A and β-carotene and lutein carotenoids have proven discrete ability to halt RP progression. Two early phase studies to evaluate the safety of oral QLT091001 (9-cis-retinyl acetate) in RP subjects with mutations in RPE65 have already finished (NCT01543906, NCT01014052) and show positive safety and VA/VF results.[1,12] However, some research groups have solely reported positive ERG records, but neither VA nor VF

improvements and a detrimental effect seems to be associated to vitamin A supplements in patients with ABCA4 mutations.[3,10]

Docosahexanoic acid (DHA) may be involved in rhodopsin regeneration. Nonetheless, no further improvement was achieved from DHA in patients already taking vitamin A in randomized trials, and just a temporary effect in those patients that had not been previously treated with vitamin A.[13,14] Vitamin E has proven to be harmful and should be avoided.[10,13]

Some particular conditions such as abetalipoproteinemia, alpha-tocopherol transport protein deficiency and Refsum's disease benefit from nutritional supplements. This should be taken into account when treating newly diagnosed patients.[3]

1.2.1.2. Gene therapy (GT)

The objective of GT is to manipulate or provide wild type genes (**Figure 4**) to treat genetic diseases.[15] Recessive diseases often benefit from gene supplementation as a means to forming functional proteins, whereas dominant diseases could require gene silencing and reduction of toxic protein influence.[16] A difference is to be made between gene specific approaches in patients with well described mutations and gene independent strategies where more general approaches can be a solution for a wide range of diseases. Correct genotyping of patients is essential in order to conduct accurate therapeutic pathways.[3]

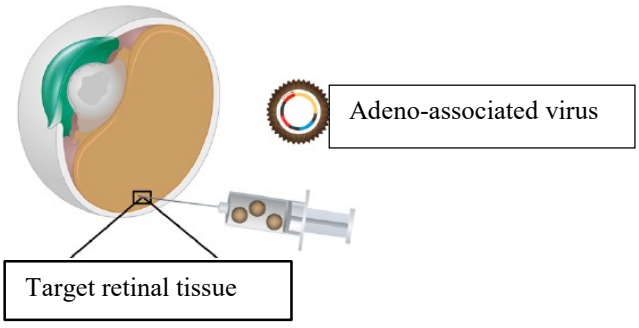


Figure 4. In vivo gene therapy approach. An adeno-associated virus vector is being used for gene delivery. This vector contains the wild type or recombinant gene that intends to modify the patient's phenotype. Modified from Ovando-Roche P. et al.[17]

1.2.1.3. Genome editing (CRISPR-Cas9)

Novel mutations or gene insertions and deletions can be created via genomic modification. CRISPR associated nuclease (Cas9) and its attached guide RNA create double-strand breaks (DSB) in the host DNA.[18] These are corrected by non-homologous end joining (NHEJ) or via homology directed repair (HDR). NHEJ involves insertions and deletions leading to premature STOP codons and HDR results in specific genomic changes in the host DNA.[17]

1.2.2. Preventing photoreceptor degeneration: damage prevention approaches

Halting photoreceptor death by creating protective environments is intended when neuroprotective or neurotrophic therapy is used. Nutritional supplements and pharmacological therapies could also contribute to ameliorating this stage.

1.2.2.1. Neurotrophic factors

Several neurotrophic factors such as fibroblast growth factor 2 (FGF2) or ciliary neurotrophic factor (CNTF), the latter alone or in combination with adeno-associated viruses (AAVs), seem to halt retinal deterioration in preclinical studies.[2,19] Rod derived cone viability factor (RdCVF) also induces cone survival in mice.[16]

Of particular interest is the NT-501 implant for CNTF delivery using an encapsulated intraocular device. It has been tried in patients with RP and appears to be safe for up to 2 years. In terms of efficacy (VA and VF sensitivity), however, its beneficial effects remain to be seen.[2] CNTF has also been tested for achromatopsia.[20] Further studies or the consideration of alternative and clinically relevant parameters could prove more encouraging results.

1.2.3. Photoreceptor and visual pathway signal substitutions: latest stages

Latest stages usually involve extensive photoreceptor degeneration and selected devices should maintain visual function on behalf of non-working cells.

1.2.3.1. Retinal cell transplantation

Cell transplants would be suitable for patients in latest stages as substitutes of photoreceptor functions or for patients with unknown genetic alterations (where no clue of potential gene targets is available).[11,17] The implantation of precursor cells at the correct stage and with a correct photoreceptor-like development, could allow their integration into the host retina and substitution of retinal cell functions.[2] In fact, it has been achieved in mouse models.[21] Hypotheses claim that trophic stimuli originated from donor cells could enhance host photoreceptor survival with subsequent visual improvement too.[11,17] Neural stem cells, somatic cells, human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs, **Figure 5**) and RPE cells are being tested for transplantation.[17] Two phase I trials for the use of retinal transplants in humans have reported promising results, particularly for Stargardt's disease and for age-related macular degeneration (AMD). Visual acuity gains were not as good as expected, but a good safety profile was discovered for these ESC-derived transplants.[21] Interestingly, degenerated retinas could lose blood-retinal-barrier efficacy, and hiPSC cells could be a valid alternative with no requirements of immunosupresion, as they are originated from the patient's cells.[21]

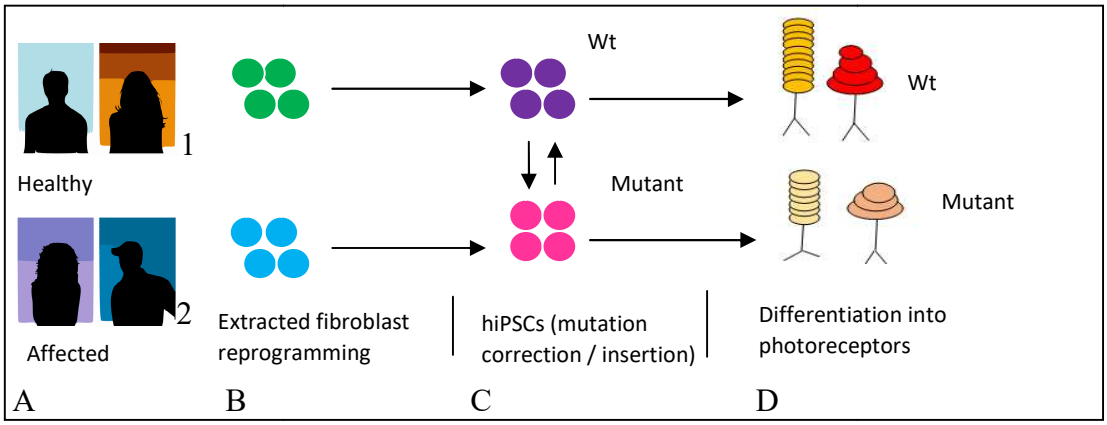


Figure 5: Image representation of *in vitro* fibroblast reprogramming into human iPSC. Fibroblasts (B) are taken from healthy (A1) and affected (A2) individuals. This can be associated to CRISPR/Cas9 strategy to correct disease causing mutations in affected individuals' cells or to insert mutations in healthy individuals' cells for investigational purposes (C). Disease models and potential therapeutic sources can be formed when differentiated into healthy (D1) or affected (D2) photoreceptors. (WT: wild type, hiPSCs: human induced pluripotent stem cells). Modified from Ovando-Roche P. et al.[17]

1.2.3.2. Retinal prostheses and sensory substitution devices

Retinal prostheses **(Figure 6)** aim to transduce light into electrical impulses that travel directly to the inner retina, the optic nerve or even the visual cortex. Currently on the market, the Argus II, the Alpha AMS and the Iris implants are good examples. Camera recorded videos are converted into electrical signals, then transmitted into electrodes located in the patient's retina.[10] Sensory substitution devices can even convert tactile or auditory stimuli into visual sensory-like information allowing motion and object location. These techniques might well be used as complementary of other approaches.

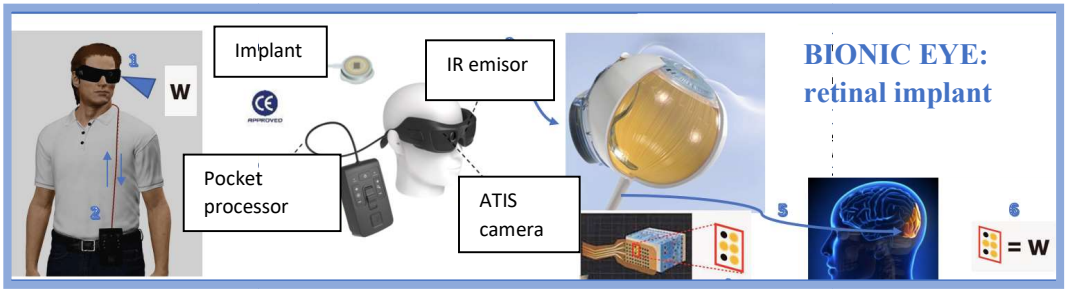


Figure 6. Graphic representation of the Bionic Eye. 1. A processor receives an image sent by a video camera located in the goggles. 2. The processor transforms the image into electrical information that is sent back to the goggle receptor. 3. The electrical signal is sent to the retinal implant located in the eye. An electrode net located in the retina is stimulated by the electrical pulses sent by the retinal implant. 5. The optic nerve transduces the retinal stimuli into the occipital cortex. 6. The brain perceives different light and shadow patterns dependent upon the different electrodes stimulated in the retinal implant. Courtesy of Irigoyen C.[3]

1.2.3.3. Optogenetics

The transformation of retinal cells into *`artificial photoreceptors'* or the creation of electrical signals that mimic those produced by photoreceptors is part of optogenetics.[2] Remaining cells in late stages of retinal diseases could be photo-sensitized and depolarized using light activated proteins or channels (via gene delivery) such as archaebacterial halorhodopsin or channelrhodopsin, enabling visual pathways and visually stimulated behaviours.[2,11,22]

Table 1 exposes advantages and limitations of the different treatment options. Limited proof of efficacy is available, and results do not totally support any treatment. However, advances towards treating these severe ocular conditions are being made.

Disease stage	Treatment	Vision improvement	Limitations	Additional considerations
Early stages	Nutritional supplements	Vitamin A, beneficial in RP	Limited improvement Vitamin E: harmful Limited evidence	Liver toxicity, lung cancer risk in high risk patients, teratogenic
	GT	Encouraging in animals and humans Different visual areas improved	Genotyping needed Gene size Retinal degeneration despite visual gains	Luxturna (Approved by FDA: 12-19-17)[23]
	CRISPR/Cas9	Preclinical promising results (mice: Usher syndrome, rats: RP)[24,25]	Ethical concerns	Could be corrective in a single treatment session
Halting degeneration	Neurotrophic factors	CNTF implants are safe	No RP correction	Could enhance GT
Latest stages	Cell transplants	Early phase trials	Ethical concerns (stem cells)	Could combine with genome editing
	Retinal prostheses	Motion sense Large object location	Suboptimal visual gains	Already commercialized
	Optogenetics	Encouraging in mice and postmortem human retinas	Possible phototoxicity	Potentially useful for advanced stages

Table 1. Summary of available treatments for IRDs. Several aspects concerning these treatments have been summarized. (CNTF: Ciliary Neurotrophic Factor, FDA: Food and Drug Administration, RP: retinitis pigmentosa, GT: gene therapy, CRISPR: clustered regularly interspaced short palindromic repeats).

Inherited retinal dystrophies (IRDs) are rare diseases that profoundly compromise patients' quality of life due to associated visual impairment. No cure is available and in most cases, treatments focus on preventing complications or ensuring an acceptable quality of life for these patients. It is claimed that gene therapy could radically change this scenario and improve visual function in patients with IRDs.

1.3. HISTORY OF GENE THERAPY AND CURRENT APPLICATIONS

Rogers et al performed the first direct human GT trial in 1973 using the wild-type Shope papilloma virus for treating two patients with hyperargininemia based on gene deliveries by viruses already seen in nature.[26] In 1990, two adenosine deaminase deficiency (ADA-SCID) patients were treated by Blaese and colleagues using *ex vivo* modified autologous T cells. A normal copy of adenosine deaminase was inserted into retroviral vectors and it was successfully integrated into the cellular genome.[27] Subsequent trials paved the way for success until 1999, when a teenager participant of an ornithine transcarbamylase (OTC) deficiency trial conducted by Wilson and colleagues died of a brutal immune response due to adenovirus administration. In 2002, researchers reported 5 new acute T cell lymphoblastic leukaemia cases in participants of two Severe Combined Immunodeficiency trials (SCID-X1) as a result of insertional transactivation of LMO2 proto-oncogene and insertions/deletions of other genes.[28] Insertional mutagenesis supposedly responsible for these cases is related to retroviral capacity of allowing the expression of endogenous oncogenes.[28] Several ethical concerns arose regarding patients' safety and research was restricted.

In 2003, the State Food and Drug Administration of China approved Gendicine, the first GT drug for commercial use (for head and neck squamous cell carcinoma) and in 2005, Oncorine was also approved in China to treat refractory nasopharyngeal cancer. In 2012, the European Commission approved Glybera (alipogene tiparvovec), the first GT product to be commercialized in Europe, which featured an Adeno-Associated vector and aimed at lipoprotein lipase deficiency (LPLD). The EMA also approved Strimvelis for the treatment of ADA-SCID. The FDA has recently validated Kymriah (tisagenlecleucel), Yescarta (axicabtagene ciloleucel) and Luxturna (voretigene neparvovec). This last one was approved in December 2017 to treat Leber Congenital Amaurosis type 2 (LCA2).[23]

Despite all the drawbacks GT has suffered since it was first developed, positive results have arisen that encourage the idea that it could proximately be a useful treatment option for several diseases such as IRDs.[26]

2. OBJECTIVE

Based on previously introduced premises, the main purpose of this project is to conduct a systematic review of the scientific literature to evaluate the safety and efficacy of gene therapy for the treatment of inherited retinal dystrophies. As a means to assessing this, the following objectives will have to be completed:

1. Assessment of safety and efficacy of different gene therapy approaches (such as gene delivery and genome editing).
   - Review of gene therapy performance under clinical trial conditions.
   - Best gene therapy performance strategies: evaluation of different vectors and surgical techniques.
2. Identification of current challenges faced by gene therapy.
3. Evaluation of the most common mutations causing retinitis pigmentosa in Gipuzkoa.
4. Quantification of the amount of potential candidates to be benefited from these approaches. Study of the applicability of worldwide advances to patients in the Donostia University Hospital.
5. Assessment of future prospects in the treatment of IRDs.

3. METHODOLOGY

For ensuring a complete and organized review, the main research question has been built in terms of the PICO system and would be as follows:

Patient: patients suffering from inherited retinal dystrophies. No narrower patient group has been selected. The reasons for this include the difficulties of rare diseases to generate sufficient data for a narrower review and the interest of evaluating solutions for patients with many different IRDs. Many do not have a certain molecular diagnosis yet, all of this making it tough to select a single disease pathway to explore.

Intervention: gene therapy. Genome editing has also been selected as part of gene therapy and a brief comment about optogenetics will be available too.

(Comparison, if any): Due to heterogeneous and scarce information regarding each treatment, other therapies have been assessed but will not be strictly compared to GT.

The **primary outcome** would be visual acuity gains. Secondary outcomes include: visual field gains, night vision changes, light sensitivity, brain responses to optical stimulation, nystagmus amelioration, functional vision improvements (the ability of sight to allow individuals to complete usual tasks) and self reported quality of vision.

3.1. INFORMATION SOURCES AND SEARCH STRATEGY

As it is a question related to an intervention, the best research studies for answering it would be randomised clinical trials. In addition, systematic reviews of the literature and if available, meta-analysis studies would be of the best quality. Non randomised clinical trials, observational studies and other types of studies could also be included, but quality aspects should be taken into account.

For data gathering, Pubmed database, Cochrane Library and TRIP database were searched from inception to March 2018 **(Figure 7)**. To complete a systematic search, reference lists from retrieved articles were examined for additional citations. No language restriction was initially applied. Articles in foreign languages were tried to be translated and if such translation was not possible, the article was excluded.

The following searching terms were used: `gene therapy´, `inherited retinal dystrophies´, `adeno associated virus´, `adenovirus´, `CNTF´, `lentivirus´, `nonviral´, `vector´, `subretinal´, `intravitreal´, `CRISPR-Cas9´, `treatment´, `history´, `Leber congenital amaurosis´, `retinitis pigmentosa´, `achromatopsia´, `choroideremia´, `X linked retinoschisis´, `stargardt's disease´, `Usher syndrome´, `cideciyan´, `maclaren´, `maguire´, `hauswirth´, `jacobson´, `Leber hereditary optic neuropathy´, `optogenetics´, `RPE65´, `CEP290´, `ABCA4´, `RPGR´, `AMD´, `MYO7A´, `USH2A´, `briard dog´, `ZFN´, `TALEN´ and combinations thereof.

Most articles were from the last 9 years (when the first clinical trials of gene therapy in IRDs were performed) but commonly referenced and highly regarded older publications were not excluded.

Specific databases were also searched. Clinicaltrials.gov was interrogated for all the gene therapy related and retinally directed clinical trials developed in the past or in the present (https://clinicaltrials.gov/).[29]

Retinal Network (RetNet) database was searched for updated information related to inherited retinal dystrophies (https://sph.uth.edu/retnet/disease.htm).[1] Genetics home reference (https://ghr.nlm.nih.gov/) and Online Mendelian Inheritance in Man (https://www.omim.org/) databases were also searched for a complete understanding of inherited retinal diseases.[7,30]

3.2. SELECTION CRITERIA

Articles followed inclusion and exclusion criteria prior to being selected for this project. Inclusion criteria include research articles covering the different vectors for GT delivery in the human retina, the surgical techniques developed for human patients, current treatments for IRDs and genome editing in human ocular diseases. Research papers covering the results of the clinical trials made to assess GT in ocular diseases, particularly, in IRDs were also included. As additional information, articles including the history of GT in humans and institutional regulation papers regarding rare disease trials were read.

Moreover, the clinical trials studying gene therapy for IRDs and for other ocular conditions were listed **(Appendix 2)** based on Clinical Trials database (https://clinicaltrials.gov/).

Exclusion criteria include preclinical studies not relevant to any of the clinical studies commented, GT for other medical purposes (other than studying its history), information related to the natural history of the diseases with no updated information about potential treatments and opinion essays or letters to newspapers (due to being merely hypothetical or too brief to be considered). Old articles containing inaccurate statements in the light of the available data were also discarded.

This project has been developed as a review of the literature. As part of my academic pathway, I completed a three month internship at the Biodonostia Institute, and some of the lessons learned there have been added to this project too **(Appendix 1)**.

Therefore, the added value of this review includes: updated, exhaustively searched and comprehensively reviewed information of studies and available data from worldwide trusted sources. Medical concerns have been combined with research interests in order to provide high quality information for all kinds of professionals. Relevant topics in the GT area have been precisely addressed and conclusions summarize all the evidence and future approaches.

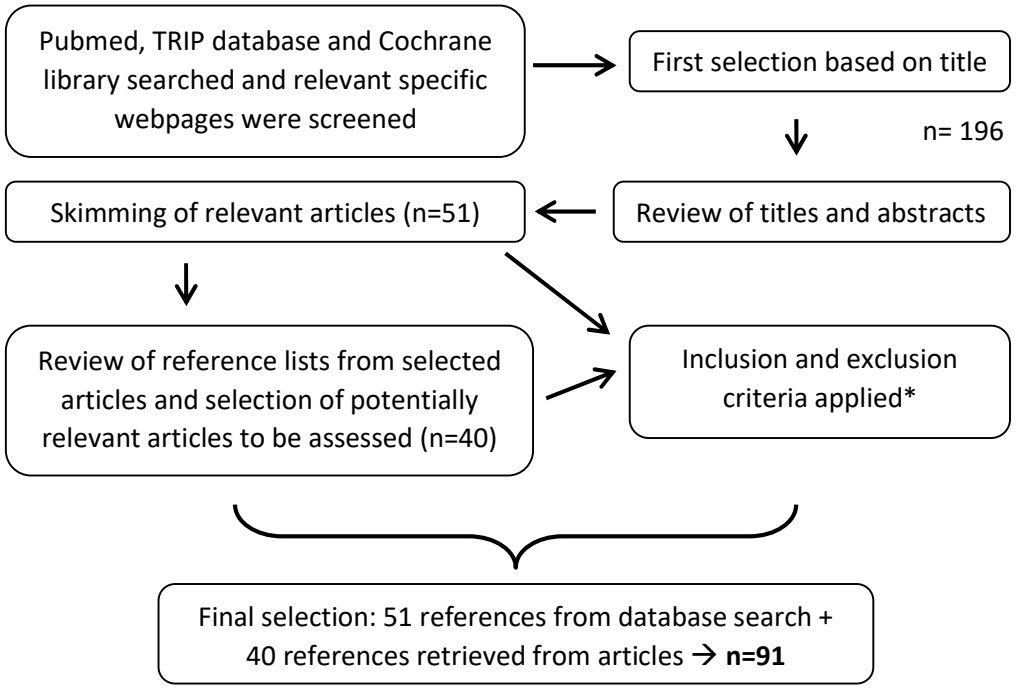


Figure 7. Article selection process. This diagram shows the summary of the search and selection process. A search was performed in information databases and it was completed reading the reference lists from selected articles. *Discarded articles were related to: preclinical studies in animals (59 articles), cellular models (8 articles), old fashioned or partial reviews (8 articles), newspaper letters (7 articles), gene therapy for other diseases (6 articles), topics related to IRDs, beyond the scope of this review (114 articles).

3.3. METHODOLOGICAL QUALITY ASSESSMENT AND SYNTHESIS

CASP (Critical Appraisal Skills Programme) tools were initially selected to assess the quality of the evidence and the risk of bias (www.redcaspe.org).[31] Particularly, tools for assessing clinical trials and systematic reviews were selected. The specific tool used for clinical trials comprises 11 items that study if the results are valid, of significant importance and applicable to our local population. For systematic reviews, 10 items assess those same aspects. A preliminary review of the clinical trials showed some differences with regards to methodology compared to traditional clinical trials for more prevalent diseases, and those differences can be explained if regulations and conditions for rare diseases are understood.

Usual standards of methodological quality are not met by clinical trials evaluating therapies for rare diseases, and thus, updated regulatory statements by FDA and EMA were studied for methodological support of the gathered evidence.

An orphan drug intends to treat a rare disease (defined by the FDA as a disease affecting less than 200,000 Americans) and faces challenges such as high patient heterogeneity, lack of reliable preclinical models or usually devastating diseases to be investigated in few patients.[23] In the light of these problems, the international drug regulating agencies have developed programmes to support orphan drug development and offer scientific, technical and economic aids to those promoting research on this area. As a result of this, trials assessing orphan drug safety and efficacy do not meet all the criteria requested to other drugs and often benefit from accelerated marketing procedures. CASP tools and FDA and EMA regulatory aspects have therefore been combined when understanding and evaluating gathered data from studies. Having said that, advice from regulatory agencies and the unmet need of thousands of patients make this an exception. Undeniably, future research will be needed for extended recommendations to patients. Before prescribing these drugs or selecting patients for clinical trials, clinicians will have to explain all the light and shadows of the evidence and make individualized recommendations based on risk-benefit and patient needs and concerns for each disease. **Appendix 2** features a more developed assessment of the individual clinical trials.

Because of the high heterogeneity in methodological quality, interventions and outcome measures in the studies, it was not feasible combining data in the means of a meta-analysis.

4. RESULTS

4.1. GENE THERAPY

Recombinant nucleic acids are used in gene therapy (GT) for repairing, regulating or replacing a disease causing genetic sequence.[26] The modification of the genetic material can be performed *in vivo*, or be administered to cells *ex vivo* before patient delivery. Moreover, somatic cells or germ line cells can be targeted, the latter being capable of transmitting changes to offspring.[26] Recessive monogenic diseases are perfect candidates for GT as they usually comprise a functional protein absence and can be treated with gene replacement strategies, sometimes with partial replacement levels being enough for phenotype correction.[16,32] Gain of function mutations (in autosomal dominant diseases) include toxic products that must be eliminated and therefore pose additional therapeutic challenges.[16] Additional advantages when treating the eye include the easily reachable and immune privileged retina, which allows the prevention of severe immune responses to administered compounds. Additionally, the blood-retina barrier (formed by tight junctions between retinal capillary endothelial cells and RPE cells) permits the retention of the treatments within the eye, with reduced risks of affecting other tissues.[16] Moreover, the eye can be explored by non-invasive methods such as optical coherence tomography (OCT) and autofluorescence (techniques that can also be used to assess clinical trial results), and if only one eye is treated, the contralateral eye works as a control.[33]

4.1.1. Most widely used vectors

Naked DNA is a large, hydrophilic and negatively charged molecule that is rarely able to enter cells on its own. As a result, vectors have been developed for gene transfer. Ideal vectors should be very specific to the targeted region, avoid the immune system so as not to generate any dangerous immune responses and have enough loading capacity for the required gene to be transported.[34,35] Vectors can be viral or non-viral, and researchers usually prefer viral gene carriers, as broader experience in clinical trial and preclinical study conditions is available and more efficient transduction with viral vectors has been reported.[16]

4.1.1.1. Viral vectors

Viruses possess special infectious features that enable viral DNA to be introduced into host cells.[34,36] They can be enriched with phenotype correcting human genes that can be delivered to desired cells. Some concerning viral complications such as immunogenic responses, cytotoxicity and insertional mutagenesis are currently being addressed. The following viruses are commonly used for gene delivery **(Table 2)**.

4.1.1.1.1 Adeno-associated viruses (AAVs)

AAVs belong to the *Parvoviridae* family and require coinfection with other viruses because they lack replicative functions.[16] They hold small storing capacity (4.5-5kb), and the material transferred by them remains predominantly episomal (not contained within chromosomes) and on that account, insertional mutagenesis risk is reduced by leaving native DNA information unaltered in host cells.[34] They can be eliminated by host humoral responses in previously exposed patients, but the retina remains almost unaffected due to aforementioned immune privilege status, so their immunogenicity rates are very low.[16,37] Stable transgene expression has also been reported.[32] These features have put AAVs in the spotlight.

AAV genome takes form of a linear single stranded DNA. Two open reading frames (ORF) named cap and rep genes encode proteins required for replication and viral structure formation. Two palindromic inverted terminal repeat sequences (ITRs) enable DNA replication.[38] During vector design, cap and rep genes are removed and the genetic sequence of interest is introduced instead.[38] Moreover, capsid variants confer these viruses different cell tropisms. AAV serotypes 1, 2, 4, 6 and genetically engineered AAV2-7m8, rAAV2/1, rAAV2/4 and rAAV2/5 have been successfully used to target RPE cells.[36,38] These cells have a phagocytic profile, and some studies claim that almost any AAV serotype could potentially reach them.[32,36] AAV serotypes 2, 5-9 and recombinant AAV2-7m8 and AAV8BP2, allow gene transfer to the photoreceptors.[39] More versatile AAV2/2 and rAAV2/5 can reach both RPE and photoreceptors.[38] Speed and efficacy of transgene expression are improved using capsids of AAV5, AAV8 and AAV9.[38-40] Retinal ganglion cells are reached via intravitreal AAV delivery with significantly less success than via subretinal

pathway.[36] No easy approach for bipolar, horizontal and amacrine cells is available, as they are anatomically located between dense layers of cells. Müller cells can be approached intravitreally or subretinally.[36]

Also interesting is the fact that answers to cargo capacity difficulties are being tested. Dual AAV mediated strategies combine genetic information contained in two AAVs. Subsequently, homologous recombination permits overlapping sequences, transplicing allows concatemerization of both sequences and hybrid dual vectors mix both strategies.[36,38,41-43] Oversized AAV vectors and `gutless´ viruses have also been reported.[43] These could be useful approaches for gene delivery of larger genetic materials and particularly, mouse models of Stargardt's disease (mutated ABCA4 gene) and Usher1B (mutated MYO7A gene) have been studied. Although therapeutic levels of the gene of interest could be obtained, high heterogeneity between these approaches requests alternative solutions or further investigations.

4.1.1.1.2. Adenoviruses (AdVs)

Adenoviruses are lytic, non-enveloped double stranded DNA viruses from the *Adenoviridae* family. Most cells express AdV receptors and they can infect both dividing and non-dividing cells.[38] In addition, their genes remain episomal, reducing undesirable mutation possibilities. Group C serotype 5 and group B serotype 35 have been particularly useful for gene transfers. Human infection by AdVs usually triggers mild febrile episodes, albeit immunocompromised patients may suffer severe immune reactions.[44] Most people have faced community based contacts with AdVs and have created antibodies against them. People receiving AdV injections also develop antibodies that reduce subsequent gene transfer successes, hence making AdVs suitable for transient transgene expression.[38,44] In order to reduce immune recognition, different AdV serotypes are being considered.[44]

4.1.1.1.3. Lentiviruses (LV)

Lentiviruses are enveloped, single-stranded RNA viruses of the *Retroviridae* family. They use reverse transcriptase to convert RNA into double stranded DNA. Their genetic information is integrated into the host cell genome (providing longer gene expression) and behave as mostly non-immunogenic in humans.[45,46] Replicating and

non-replicating cells can be targeted and offspring cells can also be reached. Although these are remarkable advantages, two main drawbacks concerning LVs must be cautiously taken into account. The first would be the insertional mutagenesis that could arise from gene integration into cells and the second, the unlikely but possible option of these HIV-1 derived viruses of becoming actively replicative and disease causing. Genome editing strategies are being assessed for solving mutational problems that could arise and LVs are being improved by ensuring that potential integration remains away from oncogenes that could be activated.[32]

4.1.1.1.4. Herpes simplex virus (HSV)

Herpes simplex virus has also been used and has the advantage of its large size (50Kb).[45] Although most humans present previous contact with HSV, it has successfully proven to avoid immune destruction. However, it is not frequently used for GT purposes.

	Immunogenicity	Cargo capacity	Gene delivery
AAV	Mild	4.5-5Kb	Episomal
Adenovirus	Moderate	26-45Kb[45]	Episomal
Lentivirus	Potentially severe	8-10Kb[45,47]	Integrating
HSV	Mild	Up to 50Kb[45]	Episomal

Table 2. Most frequent viral vectors and special features. This table summarizes remarkable features of each viral vector. AAVs have been widely used for gene therapy due to their interesting and helpful delivery characteristics. However, limited cargo capacity can be solved by novel strategies such as dual AAV vectors or by using different viral vector such as lentiviruses. (AAV: Adeno associated virus, HSV: Herpes simplex virus, Kb: Kilobase).

4.1.1.2. Non-viral vectors

The creation of non-viral strategies intends to pose a valid alternative to viruses and their potential risks.

4.1.1.2.1. Physical techniques

Electroporation uses electric pulses through local electrodes to create pores in the cell membrane that disappear after gene delivery.[48] However, target cells may end damaged and membrane trespassing is not always achieved. On the other hand, `gene gun´ technique uses DNA coated gold or silver particles to penetrate target cells using electricity. Low and transient gene expression have been reported.[48]

Sonoporation uses ultrasounds and magnetofection uses magnetic forces to facilitate DNA transference.[48]

4.1.1.2.2. Chemical techniques

Nanoparticles have been studied for their large capacity and ease of production. Natural polymers such as chitosan show high biocompatibility and biodegradability (they are derived from exoskeletons). Several compounds such as calcium phosphate (CaP) have also shown the ability to improve DNA uptake by treated cells.[37]

Lipid-based approaches (lipoplexes or lipid/DNA complexes) are also useful for transfections of DNA and they have been studied in X linked retinoschisis mice models.[36] However, their high instability and possible retinal toxicity on administration pose some difficulties.[48]

Both natural and syntethic polymers have also been tried for GT. Polyethylene glycol in particular, has shown ability to treat mice affected from Stargardt´s disease.[36]

Non-viral strategies are capable of handling larger genes, but still do not compete against viral approaches due to lower transfection rates and dependency upon associated promoters. Strategies should focus on combining advantages from viral and non-viral tools.

4.1.2. Administration routes and surgical technique

There are two main options for the surgeon to deliver the vector to the retina **(Figure 8)**: it can be injected intravitreally (vectors are diluted within the vitreous) or in the subretinal space (between the photoreceptors and the RPE).[49,50] Intracameral, subconjunctival and suprachoroidal injections have also been described.[37,38] Generally, the injection route is selected according to the targeted cells' location for the best gene expression in the selected area **(Figure 9)**. Thus, if the target is the inner retina, intravitreal route would be of choice, and if outer retina is our objective, subretinal route should be chosen. However, there is no standardized protocol that guides this decision.

Intravitreal delivery has two main advantages: its simplicity of performance and its capability of direct drug delivery into the eye.[51] Negative adverse events that could appear after intravitreal treatments include: ocular inflammation and/or haemorrhage, endophthalmitis, retinal detachment and cataract.[37,49] It has also been related to more immune responses than subretinal delivery.[37] Also interesting is to notice that intravitreal approach is widely used for treating age-related macular degeneration (AMD) with anti-VEGF.[51] Therefore, it is an efficient route for some uses, albeit it is not sufficient for outer retinal treatment in some cases (not enough for targeting RPE and photoreceptors). Intravitreal route injections have proven to obtain inner retinal gene expression after 2-4 weeks after injection. Outer retinal expression of intravitreally delivered genes requires about 6-8 weeks.[36]

Subretinal route injections usually include pars plana vitrectomy (PPV) with a subsequent retinotomy and viral vector injection into the subretinal space (the space between the photoreceptors and the RPE).[37] This process is followed by a temporary retinal detachment but has the advantage of reaching RPE and photoreceptors in a direct manner (which are frequently affected in IRDs). If using a viral vector, it infects the host cell and makes it express new genetic material providing tools for correcting the treated disease.

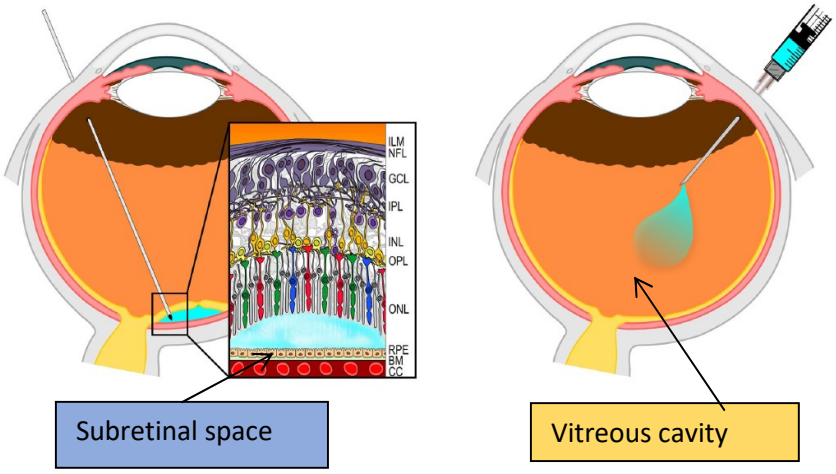
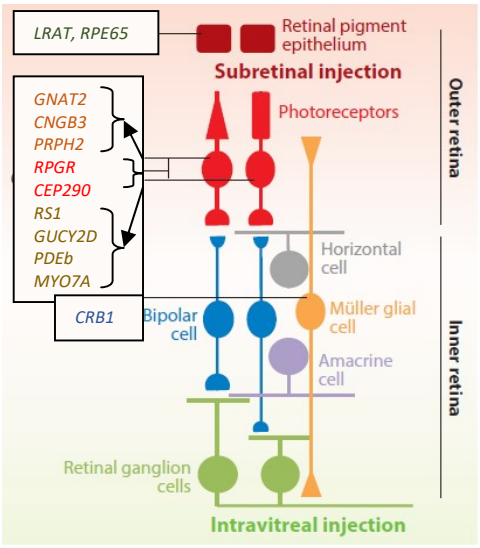


Figure 8. Intravitreal VS subretinal delivery of gene therapy to the retina. This image shows the difference between the two main injection types of retinal gene delivery: subretinal and intravitreal. Left image shows the subretinal approach in which a needle is inserted through the pars plana and the area between the photoreceptors and the RPE is reached after a vitrectomy, and the vector is injected. The intravitreal path is shown on right image and it can be seen how a needle passes through the pars plana and then delivers the vector in the vitreous cavity for a theoretically widespread distribution of the vector. Modified from Ochakovski GA.[52]

The surgeon uses a surgical microscope to complete the procedure. Nowadays, there is the possibility to use an OCT during surgery that allows to locate the injection site in real-time.[37,53] The formation of a subretinal bleb is usually expected and is used to confirm the adecuacy of the delivery (as the injected vector can spread across the subretinal space).[51] Viral transmission should be guaranteed in the surgical subretinal procedure with the least possible damage. Vector reflux to the vitreous is one of the described drawbacks of this technique and should be taken into account if the risk of vitritis is to be avoided. A proposed change of this technique could be doing the injection under the internal limiting membrane.[54]

Successful targeting of the retinal pigment epithelium (RPE) and photoreceptors involves an optimal gene therapy vector delivery into the subretinal space. An optimal surgery is critical for clinical efficacy and safety. Described possible complications associated to these surgeries are: vector reflux from the subretinal space to the vitreous and subsequent vitritis, excessive retinal stretch, macular holes, retinal detachment or air bubble formation in the injection system.[37]



Intravitreal approach	Subretinal approach
AAV2-7m8	AAV1-9
AAV88P2	AAV2-7m8
AAV2.3YF	AAV88P2
AAV2.4YF	AAV2.3YF/ AAV2.4YF
AAV2	
AAV2-7m8	AAV2-7m8
ShH10	ShH19
AAV88P2	
AAV2.3YF/AAV2.4YF	

Figure 9. Genes amenable to retinal gene therapy and candidate viral vectors. In this image gene delivery routes and retinal cell types, location and interactions are shown. Indicated genes are candidates for gene therapy and affect related cells as marked in the picture. Some vectors used with this purpose are on the right side of the image. Modified from: Planul A.[36]

A study by Parikh and colleagues demonstrated a successful transcleral posterior approach that can be used for retinal gene therapy studies in mice as a form of facilitating retinal reattachment after the procedure and preserving eye integrity by minimizing complications.[55]

As stated before, subretinal approach has proven to be better for reaching the RPE and the photoreceptors, and as these cells are usually affected in IRDs, subretinal delivery has been most widely tried in human clinical trials.

4.1.3. Genome editing via CRISPR/Cas9 strategy

A broad range of diseases in a limited resource environment, require treatments that cover the highest possible number of cases. CRISPR/Cas9 strategy has some interesting characteristics that could make it suitable for this to be achieved. CRISPR/Cas9 is a RNA-guided DNA editing tool which can be used to modify a DNA sequence in vitro or in vivo. It is based on a series of clustered repeat sequences present in achaea and some bacteria.[56] Such sequences are formed by about 29 homologous nucleotides interspaced by nonrepetitive sequences of 32

nucleotides. A series of genes located adjacent to these sequences were named Cas genes. CRISPR/Cas system is described to confer bacteriae resistance against viral infection.[56]

A single guide RNA (sgRNA) guides a Cas9 nuclease to create a double-strand break (DSB) in the target DNA.[57] DNA is binded to the Cas9 by matching PAM (protospacer adjacent motif) sequences.[58] After this break is formed, two ways of repairing DNA have been described **(Figure 10)**. Homology Directed Repair (HDR), wherein DNA repair is obtained by exchanging homologous DNA sequences (externally provided oligonucleotides, for instance) and Non-Homologous End Joining (NHEJ) where targeted regions are directly matched. The latter can cause random insertions or deletions which is a serious caveat to this approach.[56,58] More complex approaches include the use of a transcription inhibitor or activator associated to Cas9.

This system has shown greater efficiency and ability for editing genes in a multiplexed manner (that is, targeting various sites with various guide RNAs at the same time) than previous approaches such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). This is an interesting advantage in complex diseases where multiple genes contribute to the formation of an altered phenotype or where the cleavage and elimination of large DNA fragments is required.[56] Cas9 and sgRNA complex have the capacity of integrating into the host genome and of passing on to offspring cells permiting stable knockout of selected genes.[58]

In vivo gene therapy in RP and LCA animal models has also been tried.[58,59] A remarkable approach has intended to transform a mutation sensitive cell type into a mutation resistant cell type by celular reprogramming, to eliminate mutation influence and tissular loss of function. Inactivating Nrl or Nr2e3 has demonstrated rod reprogramming into cone-like photoreceptors, making the rod specific mutation irrelevant and thus stimulating both rod and cone preservation in RP mice.[49]

Cas 9 nucleases are limited by potential mutations associated with off-target DNA cleavages. Several strategies have been described to detect potential off-target

cleavage sites such as Next Generation Sequencing (NGS) strategies.[56] They could be amenable to correction via further genome editing strategies.

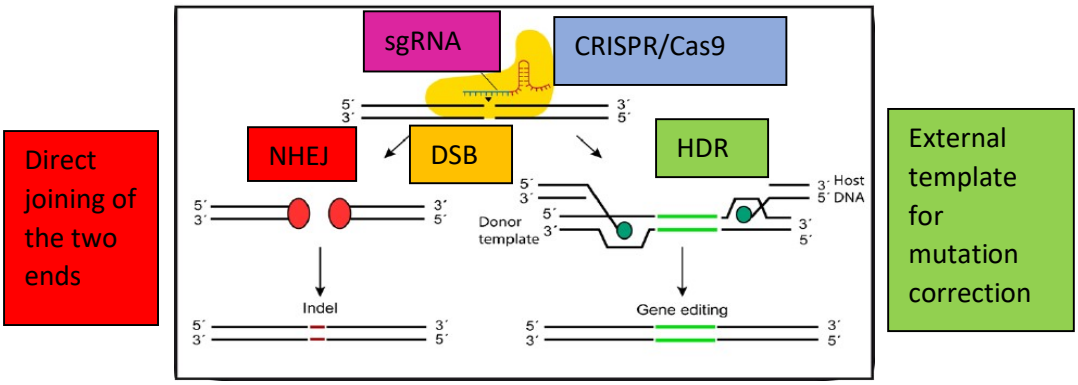


Figure 10. CRISPR/Cas9 system possibilities for gene editing. A single guide RNA searches for a desired sequence that is cut off by the cas 9 nuclease. That double strand break (DSB) can be corrected by Non Homologous End Joining (NHEJ) or via Homology Directed Repair (HDR). NHEJ derives in insertions and deletions, as the replacing sequence is not intended to be exactly matching. HDR, leads to gene engineering with more precision. Modified from Ovando-Roche et al.[17]

Vectors used in CRISPR/Cas system include integrating viruses and non-viral vectors which could be of help in this strategy as they are in gene replacement approaches.[56, 60] AAVs and lentiviruses are the most commonly used viral vectors in CRISPR/Cas9. AAVs repeat to face the important drawback of small cargo capacity, in fact, *Streptococcus pyogenes* derived Cas9 protein and sgRNA form a sequence of about 4.2kb and the maximum capacity of AAV is about 4.5kb.

Lentiviruses have recently been used for treating patients suffering from Burkitt's lymphoma (eliminating Epstein-Barr virus genome from cells). This study showed lymphoma cell proliferation reduction and virus-infected cell apoptosis. Alas, non-infected cells did not end affected by cytotoxicity systems.[61] It is also being tested for eliminating HIV-1 genomic information from $CD4^+$ T cells with promising results.[60]

Multiple CRISPR/Cas9 associated clinical trials have been approved and started and some complicated diseases, such as cancer or HIV, may benefit from genome engineering in the foreseeable future.[57]

This CRISPR/Cas9 revolutionary approach by Emmanuelle Charpentier and Jennifer Doudna has gained public recognition and was awarded with the Princesa de Asturias prize in 2015.[62] The creation of various start-ups such as Editas Medicine, Intellia Therapeutics and Crispr therapeutics has also hit the market.

4.1.4. Optogenetics

Optogenetics is another therapeutic approach, potentially more suitable for retinas with advanced degeneration, as it could be capable of stimulating non functioning cells, and passing light derived stimuli to the brain. This can prove invaluable, as many patients enter clinical trials when advanced disease is already present. No definitive data on humans is yet available, but deeper research and increasing interest in this approach and in gene therapy, could bring important changes in the near future. In fact, two phase I/IIa open label studies of safety and tolerability of intravitreal RST-001 (channelrhodopsin-2) and GS030 (channelrhodopsin) in patients with advanced RP are being investigated (NCT02556736), (NCT03326336).[1]

Possible phototoxicity associated to the high light irradiance required is a drawback that should be kept in mind.[22]

4.2. GENE THERAPY CLINICAL TRIALS REVIEW

Multiple clinical trials have been already run to assess the safety and efficacy of gene therapy in humans **(Figure 11)**. A precise review of the available evidence has been performed and the observed results will be analysed.

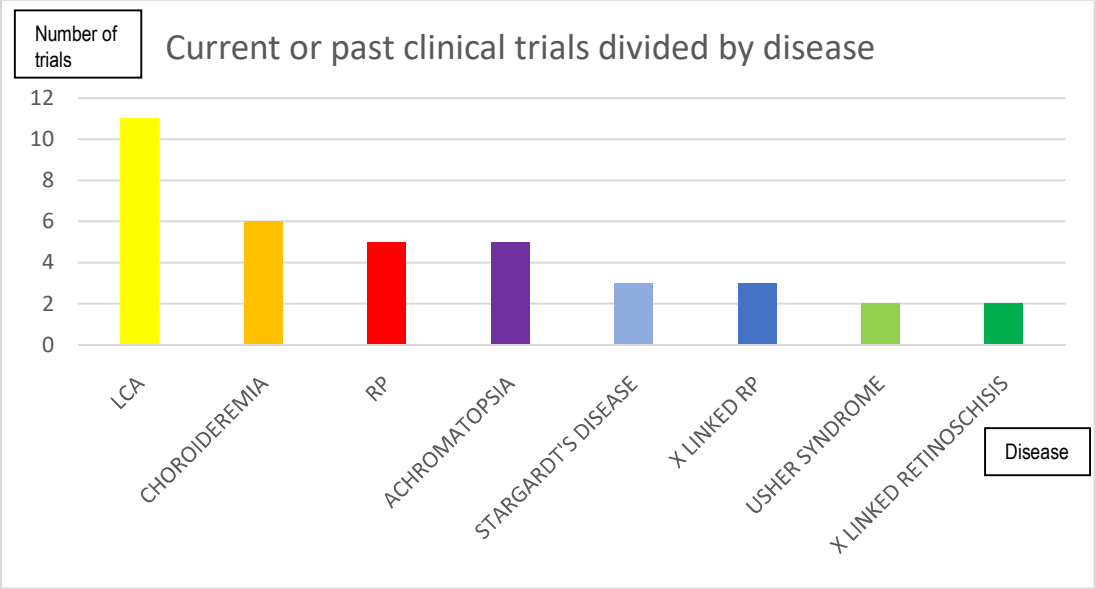


Figure 11. Quantitative representation of gene therapy clinical trials developed for IRDs' treatment. This graphic shows the number of studies performed per particular retinal disease. Exact NCT numbers can be found throughout this chapter. Pubmed and www.clinicaltrials.gov where searched for exact numbers.

4.2.1. Leber Congenital Amaurosis

The RPE65 gene encodes a RPE specific 65kDa isomerase that performs a key role in the regeneration of visual pigments after exposure to external light.[8] About 5-10% of LCA cases (LCA2, in particular) are caused by RPE65 protein defficiency and suffer from profound vision loss with relatively well preserved outer retinal structures until late stages.[33,63] Enzyme substitution by gene augmentation started with promising studies in mice and Swedish Briard dogs (a natural animal model of the disease).[32] Encouraged by these successes, three phase I clinical trials were simultaneously performed by Maguire et al. (NCT00516477), Bainbridge et al.

(NCT00643747) and Jacobson et al. (NCT00481546) to assess the safety of subretinally injected RPE65.[32,64-67]

Maguire et al. used an AAV2 to subretinally deliver RPE65 DNA to 12 patients in their worst eye.[64] The macula was targeted and the dose ceiling was established in $1.5x10^{11}$ vector genomes. No serious adverse events or immune reactions were observed, even though transient post surgical vector exposure occurred in some patients from the highest dose cohort as reported from blood tests. Visual acuity gains were observed in 7 out of 12 patients, and 1 experimented VA loss. Gains in visual field (VF) were observed in all patients as well as self reported improved vision in low lit spaces. Scotomas were reduced if covered by injection sites. Adults with higher retinal deterioration improved to a lesser extent than children with better preserved retinas. The team reported sustained changes even after 3 years.[68] Up to 11 out of the 12 original patients were subsequently enrolled for contralateral eye treatment (NCT01208389).[40] This approach involved further gains in visual function even in patients with preformed antibodies against AAV. Only mild adverse events were observed and all patients reported subjective vision changes, but no VA change was seen in nine patients' objective tests. Brain plasticity adaptations in the form of newly activated cortical areas were seen in functional magnetic resonance imaging (fMRIs) performed to three patients under high and medium contrast stimuli.[69]

Bainbridge et al. intended to analyse the effects of subretinal AAV2/2 expressing RPE65 cDNA in three young adults (as first results from a cohort of 12 patients reported).[65] No serious adverse responses were reported, but mild post surgical inflammation occurred. No significant improvements were seen in VA, VF or electroretinogram (ERG). One of the patients improved in the microperimetry ($p<0.05$) and dark adapted perimetry tests. Despite macular inclusion, no improvement in central vision was reported. Possible explanations include the necessity of higher dosages or the congenital amblyopic effects related to these patients. In a three year period, improvement in retinal sensitivity was observed in 6 out of 12 patients which showed decreasing trends after the first year of follow up. ERG responses remained non significant. Two patients experimented significant VA loss (of more than 15 letters on the ETDRS chart).[63,70]

Jacobson et al. also conducted a phase I trial to assess the safety of subretinal delivery of rAAV2-CB^{SB}-hRPE65 in patients with mutated RPE65 gene (NCT00481546).[67] Three young patients aged 21-24 were included and all of them self reported increased visual sensitivity in dim light environments in treated eyes. No systemic toxicity or vector associated serious immune responses occurred. One patient showed foveal thinning in the OCT examination. Posterior controls showed significant increases in light sensitivity in treated eyes ($p<0.001$) compared to control eyes ($p>0.99$) and same results were maintained by the end of the first year.[71] Furthermore, maintained visual improvement was seen after three years[72] with similar photoreceptor loss in the treated and untreated retinal areas. Follow up proved visual deterioration by the 5th to 6th year for all patients.[41,63]

Maguire et al. went a step further, following 3 subjects until 1.5 years after subretinal AAV2-hRPE65v2.[73] Retinal function improvement seen in the short term trial was maintained through the extended follow up period and despite antibody formation against the vector in two patients, those did not lead to dangerous reactions and disappeared by the first year. Weleber and colleagues ran a phase I/II clinical trial including 12 patients to assess the safety and efficacy of GT 2 years after subretinal injection of rAAV2-CB-hRPE65 (NCT00749957). Surgery related common adverse events were ocular irritation and subconjunctival haemorrhage and solved within a month. As a whole, 9 out of 12 patients benefited from improvements in one or more visual function aspects. Further gains were seen in children.[74]

LeMeur and colleagues attempted to use a rAAV2/4-RPE65 (NCT01496040). Nystagmus improved and a general trend towards vision function amelioration was observed, with sustained cortical responses.[75] Cideciyan et al. evaluated 15 patients who had received GT and observed the formation of pseudofoveas in four of them which served for letter fixation in different light conditions. Light sensitivity was preserved as seen in cortical fMRI for the areas correlated to those pseudofoveas, exclusively.[76]

In the light of these discoveries, a phase III clinical trial involving a subretinal injection of AAV2 and associated RPE65 started (NCT00999609).[77] After

randomization, 20 patients received treatment, and 9 ended up as controls. Treated patients showed improvements of mean 1.8 lux (SD 1.1) in standardized multi-luminance mobility test (MLMT) at one year vs 0.2 lux (SD 1.0) in the control group (difference of 1.6; 95% CI 0.72-2.41, p=0.0013). One third of treated patients improved 15 letters in VA and 13 of the 20 were able to complete the MLMT at 1 lux one year after treatment (the lowest luminance assessed). These compared to no improvements in the control group. Full-field light sensitivity threshold for white light also got significantly better in treated patients. Furthermore, no treatment related serious adverse events occurred. These encouraging results led to the approval of voretigene neparvovec (Luxturna) by the FDA in December 2017.

More genes can cause LCA and particularly, CEP290 mutations are most commonly reported. CEP290 is crucial in ciliogenesis and a study performed by Burnight and colleagues showed that an LV vector transporting CEP290 could transduce photoreceptor precursors derived from patients' iPSCs in vitro and achieve deficiency corrections.[78] Additionally, over-expression of genes could produce cell toxicity and should be carefully assessed when treating patients. A phase I/II clinical trial has recenly started to evaluate intravitreal delivery of CEP290 gene in 12 patients (NCT03140969).[29]

Important advances usually carry challenges too. Cideciyan et al. reported continued retinal degeneration and slow rod function despite GT.[79,80] Incomplete transduction of retinal cells may prove inefficient for disease correction too.[33] Moreover, long term protection against retinal degeneration in canine models has been proven both in early and more advanced stages.[33] It is suggested though, that treatment within an early free of degeneration phase holds greater chances of being successful than later treatment to already altered photoreceptors. Probably, treatment should focus on combination strategies where retinal cell degeneration is also halted.

4.2.2. Choroideremia

CHM gene mutations associated to Rab escort protein 1 (REP1) alterations, are known causes of choroideremia. MacLaren et al. performed a phase I/II trial to assess the effects of subfoveal injection of AAV.REP1 in six patients between 35-63 years

(NCT01461213).[53] Despite retinal detachment (usually related to visual loss) two patients with low baseline VA improved 21 and 11 letters. The rest of the participants with better baseline VA only gained between 1 to 3 letters. Dark adapted microperimetry increased from 23dB (SE1.1) to 25.3dB (1.3) after treatment (2.3 dB increase (95% CI 0.8-3.8)). A positive effect of treatment was also seen at 6 months (increased sensitivity in the treated eye of mean 1.7dB (SE1.0), p=0.04). Three and a half years after treatment, treated eyes preserved gained visual function (and non treated eyes gradually lost visual function).[32,33] This research path is expected to continue in the form of a phase III clinical trial soon.[32]

A significant number of phase I and II studies have been started for treating patients with confirmed CHM mutations: NCT02341807 (using AAV2-hCHM), NCT02077361 (rAAV2.REP1), NCT02553135 (AAV2-REP1), NCT02671539 (rAAV2.REP1 in 6 patients) and NCT02407678 (REGENERATE study AAV2-REP1 estimated to be in 30 participants).[1,37] Results from these studies will guide future decisions.

The search for an appropriate animal model has been fruitless and so iPSC based and fibroblast derived celular models have been generated. Particularly, proof of concept studies showed phenotype correction using AAV2/5 and AAV8 in preclinical conditions and could be of interest for future human trials.[32,81]

4.2.3. Achromatopsia

Clinical trials have been initiated to assess the safety of post-vitrectomy, subretinally injected and AAV mediated CNGA3 and CNGB3 (NCT02610582, NCT02599922, NCT02935517, NCT03001310).[29] Used AAV serotypes (AAV5, rAAV8 and AAV2tYF) have preclinically rescued achromatopsia phenotypes.[9] Secondarily, the trials focus on VA, color vision improvements and ERG changes. With potential future inclusions of younger patients, they will try to discover potential differences between the degree of benefit obtained by adults and children (as seen in younger dogs).[9,33] Phase I/II trial on safety of CNTF implants for CNGB3 achromatopsia has been conducted (NCT01648452) and no gains in cone function have been observed despite preclinical positive results in dogs.[20]

4.2.4. Retinitis pigmentosa

A MERTK (Mer tyrosine kinase) associated, RP related phase I clinical trial developed with the administration of subretinally (submacularly) injected MERTK in 6 patients. Two of them showed peak gains of more than three lines of vision.[82] One of both maintained visual improvement and the other lost it at 2-year control. Studies showed correct central macular thickness and though disease progression cannot be excluded, loss of visual gains may be associated to a posterior subcapsular cataract secondary to vitrectomy. This trial (NCT01482195) showed encouraging results for the safety outcome but MERTK expression levels may have been insufficient for stable positive results.[33] GT in patients with mutated PDE6B gene and mutated RLBP1 gene are also being assessed (NCT03328130), (NCT03374652).[29]

As for X linked RP, RPGR gene is also being tested in several clinical trials of recent start (NCT03116113), (NCT03252847), (NCT03316560).[29]

4.2.5. Usher syndrome

MYO7A gene absence leads to missing apical RPE melanosomes. It is involved in the Usher syndrome phenotype and forms a complementary DNA of about 7kb, which complicates the use of certain viruses due to exceeding their cargo capacities.[47] First studies of MYO7A GT used lentiviruses and demonstrated irregular integration of the virus into host cells and non generalized cell rescue (although photoreceptor loss was reduced).[32] The problem of protein overexpression was also seen as high expression levels of MYO7A were associated with cell toxicity and death.[47] NCT01505062 and NCT02065011 trials use UshStat, which features equine infectious anemia virus (EIAV) vector, cytomegalovirus (CMV) promoter and MYO7A gene needed for disease rescue. No results are available yet but they should help clarify relevant aspects on safety and efficacy.[29,33]

DNA-compacted nanoparticles, featuring compacted polyethyleneglycol-substituted polylysine 30-mers (CK30PEG) are also being investigated for Usher syndrome and although they provide lower transduction rates, an interesting 20kb cargo capacity will surely direct research into further efforts for carrying these to the market.[32]

4.2.6. Stargardt's disease

ABCA4 (ATP binding cassette transporter gene) participates in the transport of vitamin A derivatives in the visual cycle and its mutations have been associated to several retinal degeneration phenotypes, particularly, to Stargardt's disease. Promising results after EIAV lentivirus carrying ABCA4 gene with a cytomegalovirus (CMV) promoter, resulted in the start of a phase I/II clinical trial by Oxford BioMedica for patients affected by STGD1 (NCT01367444).[83] The lentivirus was selected due to larger cargo capacity necessary for ABCA4 delivery. It features subretinally delivered SAR422459 (a lentivirus and associated ABCA4 gene) at 3 different concentrations.[37] Data on long term safety is being collected and a follow up study has also been registered (NCT01736592).[29] A phase IIb double blind, sham controlled trial using Zimura (avacincaptad pegol, intravitreally delivered complement C5 inhibitor) for STGD1 is also underway (NCT03364153).[29,82] Furthermore, an interesting clinical trial has been conducted using human embryonic stem cell derived RPE cells (MA09-hRPE) delivered to patients with different visual acuities in a phase I/II trial (NCT01345006) and results suggest a good safety profile and improvements in VA, VF and visual function in some patients.[40] This data poses it as a potential future contribution to the treatment of Stargardt´s disease.[37]

4.2.7. X linked retinoschisis

Retinoschisin (RS1) protein is supposed to participate in cell adhesion and photoreceptor-bipolar cell synapse.[33] Mutations in RS1 gene have been described as causing juvenile X linked retinoschisis. Two phase I/II clinical trials of intravitreally delivered RS1 are currently underway. NCT02416622 study comprises the assessment of dose escalation of rAAV2tYF-CBA-hRS1.[1,33] Patients in the highest dose group, will also be randomized for complementary carbonic anhydrase inhibitor use.[37] The schisis cavity sizes and the changes in visual parameters are going to be investigated. NCT02317887 study intends to cover patients with mild or moderate vision loss with intravitreal delivery of AAV8-scRS/IRBPhRS.[40]

4.2.8. Gene therapy for other ocular conditions

Gene therapy has started to be tested for additional ocular conditions such as AMD. Several phase I studies by Racokzy et al, Heier et al proved that rAAV.sFLT-1 (a natural VEGF inhibitor) was safe for wet AMD both subretinally and intravitreally.[84,85] Using this technique, the production of antiangiogenic factors could be produced in the exact location where is required.[86-88] Moreover, NCT01678872 trial evaluated the LV driven and subretinally injected RetinoStat (featuring angiostatin and endostatin, against angiogenesis). A good safety profile and a sustained transgene expression were noticed, but no clear clinical beneficial effects were seen. This may be due to advanced patient disease, and should be evaluated in future trials.[46]

After preclinical studies, human studies for the treatment of Leber Hereditary Optic Neuropathy (LHON) started.[89] Wan and colleagues performed a study to assess the efficacy and safety of rAAV2-ND4 in patients affected by LHON. Intravitreal delivery of ND4 was used in nine patients with point mutations in mitochondrial DNA (NCT01267422).[82,90] Nine month follow-up showed VA amelioration in six patients. These patients also ended with broader VFs. No serious adverse events were reported. A closely related study included fourteen patients to test the safety and efficacy of intravitreal injection of AAV2-P1ND4v2 (NCT02161380).[91] Some phase III clinical trials of recent start could provide further evidence on the use of gene therapy for the treatment of LHON.[29]

5. INTERNSHIP AT BIODONOSTIA RESEARCH INSTITUTE

In September 2017, I started an internship at the Biodonostia Research Institute with the `Lehen Aukera´ scholarship programme. I have had the opportunity to learn from the Sensorial Neurodegeneration research group. In combination with Cristina Irigoyen's guidance, I have learned about all the aspects related to inherited retinal disease, genotyping and gene therapy that are currently being explored at Biodonostia.

5.1. GIPUZKOA'S MOST PREVALENT MUTATIONS

In a study conducted at the Donostia University Hospital in San Sebastian, as part of the PhD dissertation of Cristina Irigoyen, a recount of the prevalence of disease causing genes in retinitis pigmentosa was performed **(Table 3)**.

Disease	Affected genes	Number of families affected	Families of Gipuzkoan origin
adRP	RHO	9	1
	SNRNP200	8	-
	PRPF8	3	2
arRP	CERKL	5	4
XLRP	RP2	1	-
Bardet-Biedl	BBS1	2	1
	BBS12	1	1
Usher syndrome	USH2A	4	2

Table 3. Gipuzkoa's most prevalent retinitis pigmentosa mutations. This table shows the number of families affected by the most prevalent genes. The first column was not associated with a geographical area, and the second column, refers to patients from Gipuzkoan origin. Data was obtained from the Biodonostia Institute database. (ad: autosomal dominant, ar: autosomal recessive, XLRP: X linked retinitis pigmentosa).

5.2. POTENTIAL GENE THERAPY DERIVED BENEFITS IN THE DONOSTIA HOSPITAL PATIENT COHORT

Having discussed gene therapy in depth, a measurement of the benefits that would potentially affect local patients from the Ophthalmology service in the Donostia University Hospital was done. Data were obtained from the Biodonostia Research Institute database. Appropriate informed consents for the storage and use of information derived from the samples had already been obtained by the Sensorial Neurodegeneration group at the Biodonostia Research Institute.

The database includes 606 DNA samples, 214 affected index cases and a total number of 260 affected individuals (including indexes' families). 73 families have been specifically diagnosed and associated to disease causing mutations. www.clinicaltrials.gov database was searched on the 5th of March, 2018, looking for all the gene therapy clinical trials that were finished or were being conducted. Biodonostia Research Institute database was then compared to obtain the number of patients that could benefit if these trials led to approved products **(Table 4)**.

Genes under GT clinical trials	Affected families	Total number of affected patients
CNGB3	1	1
RLBP1	1	1
CHM/REP1	1	2
RPGR	1	3
MYO7A	2	3
CNGA3	2	3
ABCA4	17	19
TOTAL	**25**	**32**

Table 4. Potential benefits of gene therapy in the Donostia University Hospital cohort. This table shows the number of patients from the Ophthalmology service in the Donostia University Hospital that could benefit from gene therapy that is currently being tested in human clinical trials. Data was obtained from the Biodonostia Institute database.

As stated, up to 32 patients could be benefited from the promising new therapies that could reach the market in the next few years. With new trends towards patient genotyping and exploration of potentially affected family members, the whole diagnostic and therapeutic process is rapidly changing. A huge effort has been already made, but research continues, and will hopefully answer to patient's needs.

Preclinical studies in animals also hold promising hopes for these patients. Gene therapy in RHO gene has been tested in animals and if successful, it could pass on to human trials which would pose hope for 9 more families.[82]

A remarkable number of patients in the group are affected by mutations in USH2A (4 families), EYS (4 index cases), CERKL (5 families) and SNRP200 (8 families). Encouraging results always pave the way for future research and vector improvements and therapy advances could lead to solutions for them.

6. FUTURE

Gene therapy is expected to form a solid response to IRDs. Although GT influence is still modest, it must be considered that new sequencing technologies and preclinical studies, will progressively give more answers to current concerns. After the approval of Luxturna last year, the way is being paved for future approvals of GT products that will produce information about long term effects and safety issues.

6.1. LOCAL COMPANIES AT THE FOREFRONT

Viralgen Vector Core, Asklepios Biopharmaceutical and Vive Biotech form part of gene therapy product producers in Gipuzkoa. Invaluable benefits to local and European communities in terms of gene therapy product availability are supposed to be guaranteed thanks to local work completed by these companies. Given that the future holds great promise on the field, Gipuzkoa may be a special protagonist in the whole developmental process of gene therapy for retinal dystrophies.

6.2. FUTURE APPROACHES

The future of gene therapy for IRDs will unite patients' concerns and new patient profiles (in terms of genotyping and knowledge about more disease causing genes) with increasing strategies to offer safe and effective treatments. Creating multi-centre collaborative studies and international registries will offer richer databases and wiser conclusions that will create a path towards better and more homogenized treatments. Rare diseases will only be defeated if all efforts are put towards the same direction. Futhermore, different research areas combined will produce innovative tools for reversing or ameliorating every patient's illness.

7. DISCUSSION

Gene therapy for IRDs has proven to be a promising tool for massive changes in the approach of previously devastated patients. Visual improvements have been reported in several clinical trials assessing different vectors and targeting distinct diseases, thus posing hopes for thousands of people. Even patients with advanced degeneration might be benefited from therapies such as optogenetics. Furthermore, safety of treating both eyes has been reported, and no serious adverse events have happened so far that could compromise therapeutic success. However, although several clinical trials regarding GT have been performed and knowledge regarding IRDs is constantly growing, results have to be carefully taken into account.

Few clinical trials have been conducted to date and they involved small cohorts of few individuals since retinal gene therapy targets diseases that affect a small number of patients. This does not meet usual trial criteria in terms of the number of patients that usually need to be tested before market approval. Besides, follow up studies cover short time periods with no safety reports from side effects associated to long time uses neither rare side effect reports only seen when applied to massive patient cohorts. Masking techniques do not always apply either. All of this increases the challenge of taking these drugs to human use. International drug agencies are currently working on supporting these drugs' development, but longer follow ups and treatment to broader patient populations will justify vector choices and surgical procedure standardization towards stable and long term treatments of these diseases.

In addition to this, high prices may prevent patients with no financial support from affording potentially life changing treatments. Luxturna, for example, is said to have a market pricing of 425000USD for each treated eye. International regulatory agencies have tried to technically and financially protect drug sponsors, but international health systems and patient organizations will have a tough path to protect patients' interests and to preserve medical interventions for all the community.

As previously said, safety and mutational problems also need to be considered. Gene expression must remain stable in some illnesses for patients to notice a real

improvement, but multiple GT sessions could compromise patient adherence to treatment or result in toxic outcomes for retinal cells. These negative or challenging aspects are currently being changed by scientific advances and it is expected that they will not pose a seriouscaveat to GT development.

Uncertainties regarding standardized protocols and correct dosages for optimal phenotype correction still threaten the field. For perfect approaches, questions about whether treated retinal areas are enough will have to be answered. Presumably, GT will be more profitable in younger patients in earlier stages.[64] Retinas with higher numbers of intact photoreceptors and structures may be more suitable for gene therapy approaches as those structures may make good use of the delivered proteins. However, no consistent information has been extracted on this topic, and further studies will be needed to correctly classify patients to the treatments that will benefit them the most.

It is also capital to establish priorities in consensus with patients, determining the most important clinical outcomes that make patients' lives easier. If patients' quality of life is to be improved, we have to focus on relevant outcomes on which to focus future clinical trials. Devastating diseases, often request drastic solutions that intend to ameliorate patients' daily life. Limited results are nowadays available and patient counseling must be careful, always explaining realistic outcomes and potential risks, before recommending emerging therapies.

Research will be very important to overcome difficulties and to advance towards definitive solutions for IRDs. The need for reliable animal and cellular models is known, but preclinical discoveries merely orientate human studies. The scientific community should carefully assess those discoveries and put efforts for translating them into clinical practice. Novel strategies such as iPSC derived disease models will certainly be helpful for this purpose.

However, there are solid reasons to be excited about what is coming. To begin with, aforementioned diseases are expected to have a treatment option soon owing to GT development. Diseases with existing therapies could also benefit from GT as a suitable approach for some candidates. If reported current low toxicity and low

immunogenicity are maintained, GT could be less altering than many other pharmacological products. A great amount of studies and clinical trials are being developed generating supportive data that answer previously commented questions and concerns. A local search of candidates for GT has also shown near benefits for Donostia University Hospital cohort. Moreover, GT also provides new ways for science development and for a deeper knowledge on human genetics and disease mechanisms.

This discussion about GT has been reignited after successful proof of concept studies but consensus should be slowly reached in order to provide unified alternatives for vulnerable patients.

8. CONCLUSIONS

1. Gene therapy has proven to be safe for bilateral ocular treatment in patients with inherited retinal dystrophies. No serious adverse events have compromised clinical trial development.

2. Adeno-associated viral vectors (AAVs) are the most widely used vectors for gene therapy in IRDs due to benefitial safety and transduction efficacy results.

3. Subretinal delivery of genes or genomic tools is preferred for the treatment of frequently affected photoreceptors and RPE. Intravitreal delivery is also being studied for inner retinal cell treatment.

4. Results on efficacy of GT show positive trends, although no sustained improvement in all the outcomes of interest has been recorded.

5. Recovered visual function appears to be of slow kinetics for some cells and processes; no perfect wild type phenotype recovery has been possible.

6. Patients in earlier stages seem to have higher improvements in visual function.

7. Genome editing strategy CRISPR/Cas9 has proven to be benefitial in preclinical models and future research in humans could support this therapeutic pathway.

8. Most commonly mutated genes in patients with retinitis pigmentosa include: RHO gene in autosomal dominant RP, CERKL gene in autosomal recessive RP and USH2A in autosomal recessive RP and Usher syndrome patients.

9. A considerable number of affected patients from the Biodonostia Institute cohort could be benefited from treatments currently under clinical trials if participated as study subjects or if those compounds were commercialized.

10. Future objectives include international collaboration for accurate diagnosis of patients, supportive scientific databases for better understanding of diseases and for preclinical model creation and projects that cover wider populations for treatment purposes.

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APPENDIX 1: SUBRETINAL INJECTIONS IN MICE

The purpose of this appendix is for the reader to learn about one possible technique of preclinical gene delivery to the retina that could be performed for investigational purposes **(Figure 12)**. Local research groups have focused interests on this topic and gene therapy will surely continue to be at the forefront of research with interesting results for patients. I learned about one possible procedure during my internship at the Biodonostia Institute and solely intend to make an explanatory description of such technique. No analysis or comparison has been made and no statistical data have been included. Technique explanation and images on this appendix were courtesy of Anasagasti A. as part of his PhD collection.

Rd10 mice can be used for subretinal delivery technique. General anesthesia is usually delivered subcutaneously before the procedure. Visual control of heart beats can be used as a safety procedure. Eyelid opening is expected to happen at day 13 (P13). If the mice used in the process are younger, the eyelids should be opened using a thin needle and tweezers. A surgical microscope is then used during the process. Tropicamide intends to cause mydriasis due to its anticholinergic properties and topical ocular anesthesia must be applied to minimise suffering.

The eyeball must be extruded from the orbit maintaining its unions to the eye cavity (with no total enucleation). Being the eye held by tweezers, a beveled needle is introduced posterior to the limbus (which should be appreciated as a white-to-grey stained area involving the eye posterior to the cornea). A blunt needle is then inserted in the small hole made by the first needle (the blunt needle reduces the risk of eye perforation and associated complications and subsequent deficient gene delivery). Once the vitreous cavity is crossed a vector is subretinally delivered. Fluorophores such as mCherry compound can be associated to such vector for posterior analysis under a fluorescence microscope. The injected fluid is also stained with a blue dye to externally prove that the injection has been successfully performed. A blue-to-greenish colour seen through the pupil indicates that the injection is supposed to have occurred. Once the vector is injected, a bleb originates that allows the injected substance to spread over the widest possible subretinal area. The bleb is supposed to vanish after some time.

Complications that could arise from this procedure include holes in the posterior pole and vector reflux into the vitreous cavity. After the procedure, the eye must be replaced into the eye cavity and Methocel is usually applied for eye recovery.

Figure 12. Training session of subretinal AAV8 and fluorophore delivery in mice. The left image shows the initial procedure of eye extrusion from the treated mouse. The whole procedure is performed with a surgical microscope. The right image shows part of the injection procedure where the researcher inserts the viral vector and the fluorophore in the subretinal space of the mouse. Images were courtesy of Anasagasti A.

Two weeks later mice are sacrified after sedation with a mixture of isoflurane and oxygen. Such sacrifice can include CO_2 and posterior cervical dislocation or only the latter procedure.

Sample containers are used to hold the retinas. After this, eyes are enucleated and the limbus is cut with a scalpel and linearly separated with small scissors. The eye is moved until orientation allows a further cut and a `T´ form incision directed towards the optic nerve is performed. The choroid and sclera are removed with tweezers and once the lens and the retina are the only remaining structures, the eye is placed upside down, the lens remaining on top and it is removed. Small scalpel cuts are made in the remaining retina in order to flatten the retinal tissue for posterior processing (the lasting image ought to resemble a four leaf clover and flattening is essential for correct counting of stained areas). During this procedure, the RPE should also be removed in order to avoid autofluorescence signals.

The flattened retinas are treated with paraformaldehyde and the final samples can be preserved at 4ºC. Half an hour later, Fluoromount reactant is applied to the samples after paraformaldehyde aspiration and samples are covered with special slice covers.

Retinal flattened distribution is called whole mount. In the fluorescence microscope, the Texas red filter is red and is able to read mCherry covered samples, in the event of having applied it to studied samples (**Figure 13**, **right**). A simple bright light filter allows seeing whole mount pictures in their natural shape (**Figure 13**, **left**).

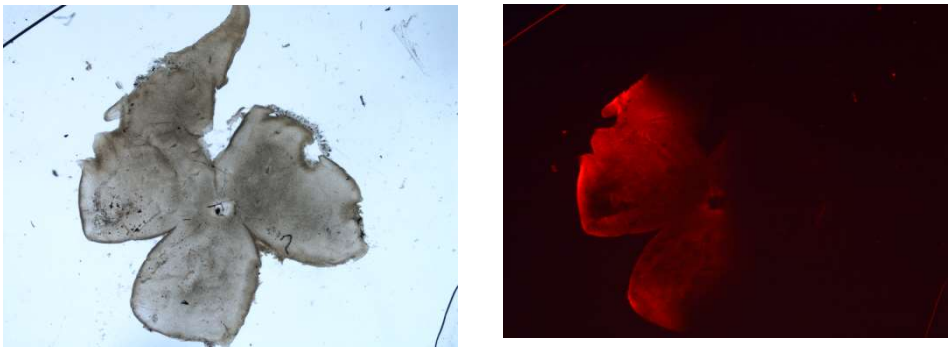

Figure 13. Retinal whole mount images. The first image shows the flatttened retina with no associated fluorescence emission under a bright light filter. A clear image of a three and a half leaf clover can be observed. The second image shows different fluoresce emission in each half due to differences in vector transduced areas. The percentage of covered areas is measured after comparisons of the results obtained with different filters. In this particular case, an approximate area of 50-60% of the retina had been transfected with the vector. A Texas Red filter permits to read this mCherry^{+} sample. Images were courtesy of Anasagasti A.

APPENDIX 2: METHODOLOGICAL INFORMATION ABOUT ASSESSED CLINICAL TRIALS OF GENE THERAPY ON IRD

A summary of several methodology related aspects have been summarized in **Table 5**.

Phase	Target disease	NCT number	Masking	Allocation	Estimated enrollment (number of patients)
I	LCA	NCT00516477	OL	NR	12
I/II	LCA	NCT00643747	OL	NR	12
I	LCA	NCT00481546	OL	NR	3
I/II	LCA	NCT00749957	OL	NR	12
I/II	LCA	NCT01496040	OL	NR	9
I/II	LCA	NCT01208389	OL	NR	12
I/II	LCA	NCT02781480	OL	NR	27
I	LCA	NCT00821340	OL	NR	10
I/II	LCA	NCT02946879	-	OBS	27
I/II	LCA	NCT03140969	OL	SEQ	12
III	LCA	NCT00999609	OL	R	31
II	CHM	NCT02553135	OL	NR	6
I/II	CHM	NCT01461213	OL	NR	14
I/II	CHM	NCT02077361	OL	NR	6
II	CHM	NCT02671539	OL	NR	6
II	CHM	NCT02407678	OL	R	30
I/II	CHM	NCT02341807	OL	NR	15
I/II	STGD	NCT01367444	OL	NR	46
I/II	STGD	NCT01736592	OL	NR	46
IIb	STGD	NCT03364153	DB	R	120
I/II	ACHM	NCT02610582	OL	NR	9
I/II	ACHM	NCT02599922	OL	NR	24
I/II	ACHM	NCT02935517	OL	NR	24
I/II	ACHM	NCT03001310	OL	NR	18

I/II	ACHM	NCT03278873	OL	NR	18
I	RP	NCT01482195	OL	NR	6
I/II	RP	NCT03328130	OL	NR	12
I/II	RP	NCT02556736	OL	NR	21
I/II	RP	NCT03374657	PM	NR	15
I/II	RP	NCT03326336	OL	SEQ	18
I/II	XLRP	NCT03116113	OL	NR	24
I/II	XLRP	NCT03252847	OL	NR	36
I/II	XLRP	NCT03316560	OL	NR	15
I/II	Usher S.	NCT01505062	OL	NR	18
I/II	Usher S.	NCT02065011	OL	NR	18
I/II	XLRS	NCT02416622	OL	NR	27
I/II	XLRS	NCT02317887	OL	NR	24

Table 5. Clinical trials' methodological quality assessment. This table shows selected aspects related to methodological quality assessment performed to reviewed gene therapy clinical trials. As stated before, CASP tools have guided this assessment, and therefore, this table reflects some aspects that are evaluated by those tools, such as masking and allocation. Most trials are open label (OL) and non-randomized (NR). These aspects have to be taken into account as they reduce the quality of the study (randomized and blinded trials are preferred as combine reductions in the risks of bias and generally reduce the quality of the conclusions). Phase I trials are usually performed in healthy individuals, and rare diseases often select affected patients, and often combine phase I and II trials in a row (safety and efficacy are subsequently addressed). Even for early phase trials, few patients constitute each cohort. All these aspects have been considered for this project, and results that arise from these trials have been carefully considered.

(LCA: Leber congenital amaurosis, RP: Retinitis Pigmentosa, XLRP: X linked Retinitis Pigmentosa, CHM: Choroideremia, ACHM: Achromatopsia, STGD: Stargardt's disease, Usher S: Usher syndrome, XLRS: X linked retinoschisis, OL: open label, NR: non-randomized, R: randomized, OBS: observational study, PM: partially masked (mixture ofmasked and unmasked staff), SEQ: sequential assignment, p: patients).[29]