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Utilizing CRISPR/cas9-mediated technology to treat Inherited Retinal Diseases: A systematic review

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Abstract

Inherited retinal diseases are considered as a leading cause of vision loss in a young population. Neither a permanent cure nor long-term treatment has yet been discovered. However, treating congenital visual impairment by utilizing a sequence-specific nuclease gene editing tool, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-associated protein 9), has appeared to have a positive impact in restoring eyesight. The following systematic review was implemented to gain more knowledge about the safety and efficiency features of CRISPR/Cas9 technology when used in clinical trials to treat inherited retinal diseases (IRDs). The review focuses on trials with Leber Congenital Amaurosis and Autosomal Dominant Retinitis Pigmentosa, common childhood IRDs. The studies were synthesized in a proportional meta-analysis using MedCalc software, and supplemented with a narrative literature review, considering both qualitative and quantitative data. The review covers different aspects related to the use of CRISPR/Cas9s and provides an overview of IRDs and future treatment methods. In conclusion, CRISPR/Cas9, indeed, is seen as a potential technique to treat IRDs. However, different complications do arise, and researchers need to be reminded of the side effects and downsides of CRISPR/Cas9. So, they can further enhance this innovative gene-editing technique in exchange for ultimately achieving long-term treatments for blindness and other inherited diseases.

List of abbreviations

adRP	Autosomal dominant Retinitis Pigmentosa
ad	Autosomal dominant
ar	Autosomal recessive
AVV	Adeno-associated viral
BRB	Blood-retina barrier
CC	Connecting cilium
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRi	CRISPR interference
CRX	Cone-rod homeobox transcription factor
crRNA	CRISPR RNA
dCas9	endonuclease deficient Cas9
DSBs	Double-stranded breaks
ERG	Electroretinography
ERGs	Electroretinograms
GC1	Guanylate cyclase-1
gRNA	Guide ribonucleic acid
HDR	Homology-Directed Repair
IRDs	Inherited Retinal Diseases
IS	Inner segment
LCA	Leber Congenital Amaurosis
NHEJ	Non-Homologous End Joining
OCT	Optical coherence tomography
OGT	Ocular gene therapy
ONL	Outer nuclear layer
OS	Outer segment
PAM	Protospacer adjacent motif

PICO	Population, Intervention, Comparison and Outcome
pegRNA	Prime editing guide RNA
PR	Photoreceptor
RPE	Retinal pigment epithelium
SE	Standard error
sgRNA	Single guide RNA
SR/MA	systematic review/meta-analysis
TALENs	Transcription activator-like effector nucleases
TSS	Transcription site
xl	X-linked
ZFNs	Zinc finger nucleases
QA	Quality Assessment

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1. INTRODUCTION

The eye is a fundamental organ for human life, which helps to observe and gather information from the surroundings (Jin et al., 2019). The importance of vision for humans and the easy accessibility of the eye for examination have driven researchers to characterize more than one thousand genetic eye conditions (Liu et al., 2019). The fundus of the eye was first examined in detail in the mid-19th century when Charles Schepens invented new and astounding ophthalmoscopy in 1947 (Francis, 2006; Pearce, 2009). Schepens's instrument incorporated liable illumination of the fundus and more comprehensive experiments could be implemented for future retinal research (Pearce, 2009). An enormous amount of articles have been dedicated to the description of the countless patterns that are found in hereditary retinal diseases, also known as retinal dystrophies. Improvements over the last two decades in gene identification and cell biology techniques have encouraged researchers to provide more realistic insights into genetic defects and to understand the molecular pathophysiological mechanisms that underlie these conditions (Francis, 2006).

In this systematic literature review, I provide background information about ophthalmology and inherited retinal diseases (IRDs) by concentrating on two of the most common congenital recessive dystrophies, Leber Congenital Amaurosis (LCA) and autosomal dominant Retinitis pigmentosa (adRP). Advances, in understanding the use of CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas9 technology to treat inherited blindness, are described by focusing on previously conducted gene-editing trials that include experiments performed on the following genes: *Rho*, *Nrl/Nr2e3, Mertk, Pde6b, Rpe65, Cep290, and Gucy2d*. These genes are strongly associated with adRP and LCA diagnosis. A systematic review including a meta-analysis was conducted to combine the findings from the independent studies and to provide a more precise estimate of the treatment effect while answering the research objective itself, about the efficiency and safety of CRISPR/Cas9 application to treat inherited retinal disease. Finally, I summarize the future aspects regarding the use of CRISPR/Cas9 as a gene therapy tool to treat IRDs and appraise other possible treatment strategies.

1.1 Eye as in research focus

The eye has received a lot of attention in the field of gene-based technology due to its several unique anatomical and physiological properties (Sengillo et al., 2017). The beneficial properties of the eye include the eye's limited regenerative capacity, meaning the eye can go to exceptional lengths to reduce immune-mediated inflammation by tolerating the introduction of foreign antigens (like viral vectors) (Niederkorn, 2019; Peddle & MacLaren, 2017; Vázquez-Domínguez et al., 2019). This phenomenon is known as "immune privilege" (Niederkorn, 2019; Peddle & MacLaren, 2017). In other words, the ocular immune privilege enables the researchers to apply different gene therapies into the eye and follow the repairing of the damaged tissues without major concern about the inflammatory response (Niederkorn, 2019). This significantly weaker immune response is due to a lack of lymphatics, immunomodulatory factors in the vitreous humor, and antigen-presenting cells (Sengillo et al., 2017). Another important and useful characteristic of the eye is the presence of the blood-retina barrier (BRB), which reduces the risks of viral vectors from potentially migrating to any other areas, eliminating the off-target effects of

the treatment (Peddle & MacLaren, 2017). In addition, the target site of the eye for gene therapy is easily accessible via modern ophthalmic techniques and the treatment can be directly applied to the target under local anesthesia (Peddle & MacLaren, 2017; Peddle et al., 2020). Furthermore, the untreated eye can serve as a control during the clinical trials if the condition is expected to progress symmetrically, and the optical transparency of the eye greatly enables in vivo, longitudinal and non-invasive supervision of a disease progression and a treatment response (Sengillo et al., 2017). A wide variety of eye phenotypes can be directly visualized as well as documented photographically and their effects on the organ can be quantified by psychophysical measures like acuity, field, and color contrast. Retinal electrophysiology enables the function of each retinal cell type to be dissected individually. Since retinal dystrophies are slowly progressive and may not become visually detrimental until adult life, families with these conditions often become large enough for genetic linkage studies (Francis, 2006).

1.2 Inherited retinal diseases (IRDs)

Inherited retinal diseases are a group of heterogeneous and neurodegenerative disorders that cause visual impairment, and are estimated to affect around 2 to 5.5 million people worldwide (Chen et al., 2021; Cideciyan & Jacobson, 2019; Hanany et al., 2020; Vázquez-Domínguez et al., 2019). IRDs are considered a leading cause of vision loss in a younger population, those between 15 and 45 years old (Hernández-Juárez et al., 2021). The propagation of IRDs includes all modes of inheritance - autosomal recessive (ar), autosomal dominant (ad), X-linked (xl), and mitochondrial (Peddle et al., 2020; Vázquez-Domínguez et al., 2019). Over 250 individual genes have been associated with clinical subtypes of retinal dystrophies that can cause irreversible progressive retinal thinning and degeneration, resulting in vision loss or total blindness (Chiu et al., 2021; Motta et al., 2018). Penetrance and the disease severity is varying widely among IRD patients but drastic and early visual loss can negatively affect the quality of life and other areas of child development, behavior, and social skills in particular (Chen et al., 2021; Kumaran et al., 2017).

The retina is a tissue that lines the inner back of the eye, composed of a single-layered retinal pigment epithelium (RPE) and multi-layered neuroretina (Diakatou et al., 2019). In neuroretina, the light-sensitive neuronal retina cells called photoreceptors detect the entering light, capture the light photons by chromophore molecules, transform them into electrical signals, and transmit the signals to the brain for image formation (Diakatou et al., 2019; Hanany et al., 2020). Depending on the light sensitivity, photoreceptors can be divided into two types of photoreceptors; rods that are responsible for night and peripheral vision, and cones that allow day and night vision to occur. RPE closely contributes to photoreceptors' overall functioning by for example providing nutrients and growth factors, removing water and ions when needed, as well as by absorbing excess light. Therefore, dysfunction in RPE or in photoreceptors, or both can result in serious visual defects (Diakatou et al., 2019). The mutations causing inherited retinal diseases can cause maldevelopment of cells and directly lead to a defect in the neighboring RPE cells, where usually a visual cycle produces a continuous supply of 11-cis-retinal chromophore that is fundamental for generating vision. The mutations can also interrupt a variety of other pathophysiological mechanisms that cause progressive loss of sight over the years or decades (Cideciyan & Jacobson, 2019; Hanany et al., 2020; Peddle et al., 2020).

1.3 Retinitis pigmentosa/adRP

Retinitis pigmentosa is a major cause of visual disability and blindness, characterized as a vastly diverse disease of inherited retinal dystrophies. Retinitis pigmentosa is mainly causing progressive degeneration of retinal pigment epithelium and degeneration of rod and cone

photoreceptors (Ramsden et al., 2013; Sengillo et al., 2017; Verbakel et al., 2018). RP is genetically heterogeneous and is associated with a large number of genetic defects affecting one in 3000-5000 people worldwide (Gumerson et al., 2021; Verbakel et al., 2018). Until today, mutations in more than 200 genes have been associated with Retinitis pigmentosa, and more than 150 missense/nonsense *RHO* mutations are linked with an adRP phenotype (Athanasiou et al., 2018; Gumerson et al., 2021). adRP requires protein reduction to mitigate toxicity, meaning that gene replacement therapy is an ineffective method for treating adRP whereas, recessive RP is characterized by protein deficiency (Shahin et al., 2022). The age of onset for RP varies among patients; some patients develop symptomatic visual loss in early childhood, while others can remain moderately asymptomatic until mid-adulthood. Diagnosis with Retinitis pigmentosa can be challenging, especially during childhood, as children have the ability to compensate for peripheral vision loss. RP typically starts with the degeneration at the mid-periphery and progresses towards the macula and fovea (Ferrari et al., 2011; Verbakel et al., 2018). The clinical course can vary extensively from person to person due to the number of genes involved but most RP patients report night blindness (nyctalopia) as one of the first symptoms to occur. Nyctalopia is then followed by progressive peripheral visual field loss resulting in tunnel vision and gradual reduction of central vision, ultimately leading to legal blindness or complete blindness (Ferrari et al., 2011; Ramsden et al., 2013; Sengillo et al., 2017; Verbakel et al., 2018). The classical clinical findings during examination include bone-spicule formations (the pigment deposition in the peripheral retina), retinal arteriolar narrowing, optic nerve pallor that indicates optic nerve damage, epiretinal thickening, reduced visual fields, and subjective changes in visual functioning (Fishman et al., 2005; Daiger et al., 2015; Musarella & MacDonald, 2010; Ramsden et al., 2013). Important findings can also be obtained from reduced/abnormal electroretinograms (ERGs) recordings (primarily among rod photoreceptors in the early stages of the disease), and from changes in structure image by optical coherence tomography (OCT) (Fishman et al., 2005; Musarella & MacDonald, 2010; Verbakel et al., 2018).

1.3.1 RHO

Rhodopsin (*RHO*) gene was the first gene discovered to be linked to Retinitis pigmentosa (Meng et al., 2020). The researchers have found more than 150 different mutations in the *RHO* gene that are associated with 25 to 40% of all autosomal dominantly inherited RP cases (Anthanasiou et al., 2019; Ferrari et al., 2011; Meng et al., 2020). Rhodopsin initiates the visual transduction pathway in the light-sensitive rod photoreceptor cells. The rod and cone photoreceptors are polarized cells that consist of a synaptic region, a cell body, an inner segment (IS), and an outer segment (OS). Over 90% of the total protein in the OS consists of rhodopsin (Ferrari et al., 2011; Meng et al., 2020). The diverse mutations in *RHO* genes have been found to cause abnormalities in protein folding and irregularities in 11-*cis* retinal chromophore binding, as well as affect the G-protein coupling/activation and the trafficking of rhodopsin (Ferrari et al., 2011). The majority of pathogenic mutations in *RHO* are gain-of-function mutations straightforwardly leading to adRP (Meng et al., 2020).

1.3.2 NRL/Nr2e3

NRL and *Nr2e3* are predominantly expressed in photoreceptor cells in the retina. *Nr2e3* mutations account for approximately 1.4% of adRP whereas *NRL* mutations contribute to approximately 1% of ADRP (Yang et al., 2010). *NRL* gene encodes for neural retina leucine zipper (NRL) protein, and its downstream repressor *Nr2e3* codes for a nuclear receptor that is specific to the photoreceptor transcription cascade (Milam et al., 2002; Pachydaki et al., 2009; Yang et al., 2010). They both act interactively with the cone-rod homeobox transcription factor (*CRX*, MIM 602225) to initiate the rod photoreceptor development, and maintain cell differentiation and homeostasis (Bessant et al., 2003; Hao et al., 2011; Moore et al., 2020; Yang et al., 2010). Subsequently, the transcriptional regulators function collectively to regulate photoreceptor-

specific genes in the mature retina, suppressing cone-specific gene expression and activating numerous rod-specific genes like *Rhodopsin* in mature rods. (Hao et al., 2011; Montana et al., 2011; Yang et al., 2010). Therefore, germline mutations in *NRL* and *Nr2e3* may lead to dysfunction of the photoreceptors. Rods in the adult retina will be reduced due to defective initiation of the rod gene expression, causing cells to specialize into cones. (Montana et al., 2011; Moore et al., 2020; Yang et al., 2010). *NRL* expression is the earliest known biomarker used to identify complications in the rod photoreceptor activity, and mutations in human *NRL* are mostly due to heritable retinal diseases, most commonly autosomal dominant retinitis pigmentosa (Montana et al., 2011).

1.3.3 MERTK

Within the retina, mer tyrosine kinase receptor (*MERTK*) is an important factor in recycling photoreceptor outer segments. Retinal pigment epithelium manages to phagocytize the shed disks by photoreceptors that repeatedly require the light-sensitive disks to be renewed in their outer segments (Al-khersan et al., 2017). To complete this process, *MERTK* protein is necessary, though the mechanism by which *MERTK* acts during phagocytosis remains unclear (Al-khersan et al., 2017; Tang et al., 2021). Ksantini et al. concluded that "*MERTK* mutations lead to severe retinitis pigmentosa with discrete dot-like autofluorescent deposits at early stages, which are a hallmark of this MERTK-specific dystrophy."

1.3.4 PDE6B

The phosphodiesterase 6 beta (*PDE6B*) gene is part of a protein complex that consists of three other subunits. *PDE6B* plays a central role in vertebrate phototransduction (Kuehlewein et al., 2021; Li et al., 2022). Mutations in *PDE6B* are known to cause adRP (Li et al., 2022).

1.4 Leber Congenital Amaurosis (LCA)

Leber Congenital Amaurosis is the most common congenital recessive dystrophy, which causes severe childhood blindness with an estimated prevalence of 2–3 per 100,000 births (Haider et al., 2014; Koenekoop, 2004; Miyamichi, et al., 2019; Sengillo et al., 2017). LCA occurs within months following birth and results in profound vision loss, primarily due to maldevelopment and neurodegeneration of cells, as well as a fault in the retinal pigment epithelium (RPE) cells (Cideciyan & Jacobson, 2019; Haider et al., 2014). This inherited retinal disease was first described by Theodor Leber in 1869, and ever since, up to 25 genes have been identified to contribute to the cases of LCA (Haider et al., 2014; Miyamichi et al., 2019). Some common characteristics when diagnosing LCA include involuntary eye movement (nystagmus), slow pupillary movement, extinguished or reduced rod and cone responses in the course of electroretinography (ERG), and repetitive pressing, poking and rubbing of the eye (Oculodigital-sign of Franceschetti). Moreover, LCA diagnosis can be confirmed by genetic testing (Miyamichi et al., 2019). In order to understand the molecular events that determine normal and abnormal retinal development, Leber Congenital Amaurosis is considered a truly important research topic (Koenekoop, 2004). The most common genes that participate in different retinal pathways in Leber Congenital Amaurosis include *GUCY2D*, *CEP290*, *CRB1*, *RDH12,* and *RPE65* (Kumaran et al., 2017). In particular, gene therapy trials for *RPE65*-associated LCA are undeniably among the major accomplishments in modern medicine (Sengillo et al., 2017).

1.4.1 *RPE65*

RPE cells are incapable of regeneration after birth, therefore, death or dysfunction of these cells can cause permanent vision loss (Jin et al., 2019). The *RPE65* (retinal pigment epithelium-specific 65 kDa protein) gene, also called retinoid isomerase, is an essential component of the retinoid cycle pathway to provide visual function in vertebrates (Hanany et al., 2020; Kiser et al., 2015). The *RPE65* gene encodes for the retinal isomerase enzyme, which is an essential catalyst in isomerization to recycle all-*trans* retinyl esters back into the chromophore 11-*cis*-retinylidene, and for rod and cone opsins to initiate light perception. The continuous regeneration of visual chromophore by retinoid cycle empowers the activity of sustainable vision, therefore reduction of this non-heme Fe-dependent monotopic membrane protein limits the response of photoreceptors, and 11-cis-retinal cannot be regenerated, resulting in opsin being left without its chromophore (Chrenek et L., 2016; Kiser et al., 2015; Pierce & Bennett, 2015; Sengillo et al., 2017). The disturbed enzyme activity leads to the degeneration of the retinal pigment epithelial cells (Pierce & Bennett, 2015). Loss-of-function mutations in *RPE65* can lead to Leber Congenital Amaurosis, but by providing the exogenous wildtype *RPE65* for the patients, vision can be restored (Chrenek et al.., 2016; Kiser et al., 2015). RPE65-LCA has been declared to be the first successfully treated inherited dystrophy using gene augmentation therapy with prominent improvement in vision (Hanany et al., 2020). Regardless of the substantial improvement in vision, patients that were treated with adeno-associated viral (AAV) vectors with the functional *RPE65* gene have not maintained vision over longer periods, but the improved vision was discovered to endure at least three years (Chrenek et al., 2016; Hanany et al., 2020).

1.4.2 *CEP290*

Mutations in the centrosomal protein 290 kDa (CEP290, MIM610142) gene vastly contribute to LCA. In the retina, CEP290 protein is mainly expressed in the connecting cilium (CC) of photoreceptors, where it plays an essential role in both cilium assembly and ciliary protein trafficking (Hernández-Juárez et al., 2021; Ruan et al., 2016) The most common cause (up to 30% of the cases) of LCA is the biallelic loss-of-function mutations in CEP290 that are identified as LCA10 (Leroy et al., 2021). LCA10 patients usually preserve some cone photoreceptors but these photoreceptors have abnormal inner and outer segments that prevent the proteins and lipids from trafficking, therefore typically leading to significant vision loss (Hernández-Juárez et al., 2021; Leroy et al., 2021).

1.4.3 *GUCY2D*

Guanylate cyclase-1 (*GUCY2D)* was the first gene found to be associated with Leber congenital amaurosis (Perrault et al. 1996). Mutations in the *GUCY2D* gene are a major cause of LCA, approximately affecting 10-20% of all cases. (Boye, 2015; Jacobson et al., 2021). *GUCY2D* encodes for retinal guanylate cyclase-1 (GC1), a protein that is predominantly expressed in cones in the outer segments of photoreceptors (Dizhoor et al., 1994; Lowe et al., 1995). Due to the mutations in *GUCY2D*, no normal photocurrent can be maintained leading to rod and cone dysfunction but surprisingly photoreceptor integrity is moderately preserved (Jacobson et al., 2021).

1.5 Gene therapy treatments for IRDs

Prior to new gene therapy treatments, inherited retinal diseases were medicated with nutrient supplements so as to slow the disease (Cideciyan & Jacobson, 2019). On the contrary, new, safe and effective gene augmentation therapies based on protein-directed DNA editing techniques followed. (Hung et al., 2016; Peddle & MacLaren, 2017). Gene therapy is premised on making site-specific modifications to the human genome by replacing an abnormal, disease-causing gene with a normal gene (Gonçalves & Paiva, 2017). Gene therapy strategies have required a careful and

precise re-evaluation of ethical and safety considerations. More advanced inspection with vector choices and delivery methods have been particularly important, as well as additional testing with animal models for preclinical safety measures (Sengillo et al., 2017).

Depending on the genetic defect and its pattern of inheritance, gene therapy can involve two approaches to treating the defect: delivering the genetic material to the target organ to silence a pathogenic mutation, or in cases like recessive and null genetic diseases, supplementing patients with a wild type copy of a mutated gene (Chrenek et al., 2016; Sengillo et al., 2017). This includes the use of subretinal injection of AAV vectors to deliver the wild-type function of the gene, for example, the human *RPE65* cDNA to the RPE cells of the treated eyes (Pierce & Bennett, 2015). Alternative gene delivery techniques targeting the eye/retina/RPE include the use of plasmids that can fuse with the target cell membrane, and lentiviral delivery systems that deliver synthetic non-viral DNA into cells (Chrenek et al., 2016). To date, researchers have used ocular gene therapy (OGT), especially in mice, dogs, and humans with positive results but despite the improvements in the vision for patients who undergo gene augmentation therapies to treat loss-of-function mutations, degeneration of vision over time, could still be observed (Musarella & MacDonald, 2010; Peddle & MacLaren, 2017; Wojno et al. 2013). To no extent, have gene augmentation therapies been able to treat dominant gain-of-function mutations (Peddle & MacLaren 2017). As most inherited retinal diseases are monogenic disorders, gene therapy based on the use of gene-editing tools can be seen as a feasible treatment for them, and more hope exists that these blinding conditions can be permanently cured (Francis, 2006; Peddle et al., 2020).

1.6 Application of CRISPR/Cas9 to treat IRDs

CRISPR/Cas9 system was first discovered in bacterial and archaeal genomes in 1987, as an adaptive immune mechanism for the cells to defend themselves against foreign organisms (Ishino, 1987; Liu et al., 2017; Sengillo et al., 2017). In 2012, Doudna and Charpentier adapted the CRISPR/Cas9 system to be used in Molecular Biology for gene editing and curiosity around this new invention arose (Chrenek et al., 2016). Before the discovery of utilizing CRISPR/Cas9 as gene therapy treatment, two predominant tools have been extensively used in gene-editing: zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), but the disadvantages of these gene-editing tools have limited the use of them *in vivo* (Peddle & MacLaren 2017; Xu et al., 2018). It has been recorded that ZFNs have low on-target efficiency, as well as difficulty to locate a potential target site, whereas TALENs are large in size and the delivery to the cell is challenging (Peddle & MacLaren 2017). As a molecular tool CRISPR/Cas9 has a significant potential for future medicine as it takes advantage of the RNA-based defense system to cut DNA at a very precise location dependent on the guide RNA (gRNA) sequence, and enables the removing, adding, and editing of the genome (Chrenek et al., 2016; Mali et al., 2013). Therefore, CRISPR/Cas9 genome surgery used for future ocular gene therapies could eliminate the need for patients with retinal diseases from undergoing repetitive procedures such as drug injections and provide long-term treatment for monogenic retinal diseases like Leber Congenital Amaurosis and Retinitis Pigmentosa (Xu et al., 2018).

1.6.1 The phases of CRISPR/Cas9 adaptive immune system

The CRISPR-Cas9 immune mechanism functions through three phases: adaptation of CRISPRs to invaders, crRNA (CRISPR RNA) biogenesis, and invader silencing (Terns & Terns, 2011). The phases of CRISPR-Cas9 Adaptive immune system are shortly presented in Figure 1. The adaptation of CRISPRs to invaders is initiated by microorganisms recognizing the invading genetic material and processing it into short fragments called ‘protospacers’ with the help of Cas nuclease. The viral DNA spacers that preserve memories of prior infections are then integrated into CRISPR loci as new spacers in the sequence and separated by repeat sequences (Behler &

Hess, 2020; Carter & Wiedenheft, 2015; Chrenek et al., 2016; Sengillo et al., 2017; Terns & Terns, 2011; Wiedenheft et al., 2012). The second phase, CRISPR RNA biogenesis, is initiated by the expression of the CRISPR array from a transcription site (TSS) (Behler & Hess, 2020). Transcription of the locus yields long precursor crRNAs (pre-crRNA) that are later processed into mature crRNAs by hybridizing with trans-activating CRISPR RNA (tracrRNA) to form a double-stranded RNA complex, termed gRNA (Behler & Hess, 2020; Deltcheva et al., 2011; Sengillo et al., 2017). RNase III cleaves the complex and produces smaller targeting crRNA complexes that are able to bind to Cas9 proteins to form the CRISPR/Cas9 complex (Deltcheva et al., 2011). The third phase includes invader silencing also referred to as target interference. The short cRNAs guide Cas proteins to recognize and bind to the invading nucleic acids and together with cellular nucleases degrade the foreign genome and, moreover, provide immunity (Behler & Hess, 2020; Peddle et al., 2020).

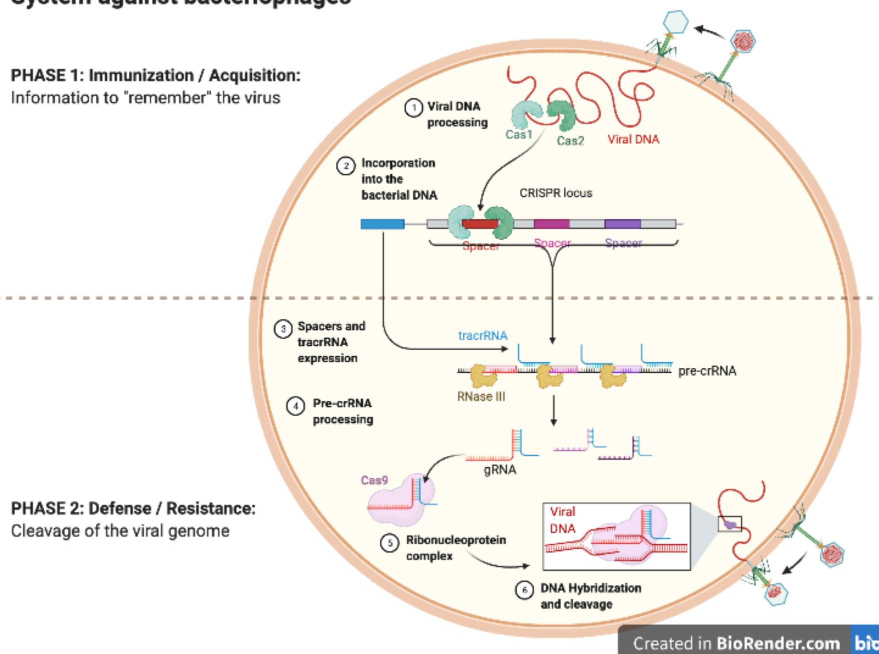

Figure 1. The phases of CRISPR-Cas9 Adaptive immune system. (**1**) Viral DNA processing; foreign DNA is detected in the cell and processed into protospacers by Cas nuclease. (**2**) Incorporation into the bacterial DNA; viral DNA fragments are incorporated into the CRISPR locus as spacers leaving repeated CRISPR sequences between them. (**3**) Spacers and tracrRNA expression; tracrRNAs recognize CRISPR RNA repeat sequences on the pre-crRNA and base pairs with them. (**4**) Pre-crRNA processing; RNase III and other CRISPR-associated proteins modify the pre-crRNA/tracrRNA duplex to form a guide RNA and inactive Cas9 protein can bind to the gRNA to activate. (**5**) Ribonucleoprotein complex; the activated guide RNA/Cas9 complex binds with the invading DNA. (**6**) DNA Hybridization and cleavage; the Cas9 protein together with cellular nucleases cleave the target DNA and inactivates it. Figure 1. was created by using a professional online application for science figures at (https://biorender.com/).

1.6.2 Engineering CRISPR/Cas9 mechanisms

Gene editing with CRISPR/Cas9 requires the presence of three components: a protospacer adjacent motif (PAM) binding site (around 2-8 bp in length), the endonuclease protein Cas9, and an artificially engineered piece of single guide RNA (sgRNA) (Peddle & MacLaren, 2017; Jiang & Doudna, 2017). Bioinformatics programs are used to screen DNA sequences for PAM sites, and

design single guide RNAs that contain 18 to 25 bp long sequences corresponding to the sequence immediately upstream of the PAM (Peddle & MacLaren, 2017). Synthesized sgRNAs mimic the natural tracrRNA-crRNA interaction and are capable of coordinating Cas9 to the target site for DNA cleavage (Peddle & MacLaren, 2017; Jiang & Doudna, 2017). Cas9 proteins cleave double-stranded DNA and cut at a specific site of interest to disable the viral DNA by preventing DNA transcription (Ishino, 2018; Peddle et al., 2020). When Cas9 binds to the PAM site it cleaves approximately 3 bp upstream of PAMs' location at the 3' end (Corsi et al., 2022; Jinek et al., 2012; Hernández-Juárez et al., 2021). The PAM is a necessary targeting component, which discriminates between 'self' and 'non-self' to prevent the CRISPR/Cas9 complex from cleaving its own locus and to avoid autoimmunity (Geditzsch et al., 2018; Mali et al., 2013). Therefore, PAM motifs are not located near the spacers of the CRISPR locus (Gleditzsch et al., 2018). After Cas9 cleaves double-stranded breaks (DSBs), it initiates homeostatic DNA repair in the host cell (Hung et al., 2016). Cells can employ two distinct repair systems in response to DSBs in the DNA to prevent cell death: Non-Homologous End Joining (NHEJ) or Homology-Directed Repair (HDR) (Chrenek et al., 2016; Xu et al., 2018).

In NHEJ, two broken ends of double-stranded DNA are directly constrained together, resulting in inaccurate ligation due to the absence of a homologous complementary sequence (Hung et al., 2016; Peddle & MacLaren, 2017). This process is prone to errors due to shortened or extended ends of DNA before the ligation (Chrenek et al., 2016). Small insertions and deletions called indels can affect the cleavage site and create frame-shift mutations, or exon-skipping mutations in protein-coding sequences. This would disrupt the normal gene activity, yielding a dysfunctional protein (Chrenek et al., 2016; Hernández-Juárez et al., 2021; Hung et al., 2016; Peddle & MacLaren, 2017; Sengillo et al., 2017; Xu et al., 2018). This method would be beneficial when desiring to create knockout mutations to disrupt the function of a dominant-negative allele, but uncertain outcomes could be detrimental and this should be taken into consideration (Chrenek et al., 2016). Controversially, the HDR pathway follows the homologous chromosome template to ensure that the double-stranded break is repaired according to the correct sequence before ligation (Sengillo et al., 2017). Considering the gene editing context, a custom-made template with a short fragment of single- or double-stranded exogenous DNA sequence together with HDR, allow the replacement of a sequence at the cut site with our desired gene (Chrenek et al., 2016; Hernández-Juárez et al., 2021; Hung et al., 2016; Peddle & MacLaren, 2017; Xu et al., 2018) Loss-of-function and gain-of-function alleles can be replaced with HDR-based strategies by performing knock-in genetic modifications at the definite locus. In such a manner, gene function can be retained, and pathogenic effects can be eliminated (Cox et al., 2015).

1.6.3 Aim

Treating Leber Congenital Amaurosis and Autosomal Dominant Retinitis Pigmentosa that cause inherited blindness are particularly suitable for the application of CRISPR/Cas9 technology. Finding a long-term cure for these inherited retinal diseases could be achieved by utilizing CRISPR/Cas9 technology, and more research on the topic could lead to better future treatments for IRDs in general. Not many studies have yet been conducted to examine this topic, and to be able to revolutionize IRD patients' lives conclusively, still awaits to be accomplished.

Research question: To what extent is CRISPR/Cas9 seen as a safe and efficient gene-editing tool for treating IRD patients?

Research aim:
1) To conduct a literature review based on the available literature found on the chosen topic, and more closely to focus on the two most common IRDs; Leber Congenital Amaurosis and Autosomal Dominant Retinitis Pigmentosa.

2) To perform a meta-analysis by combining the findings from independent studies and to provide a more precise estimate of the overall treatment effect.

Expected outcome: This study is designed to assess the hypothesis that using CRISPR/Cas9-mediated technology to restore vision loss is safe and efficient.

2. METHODS

1.7 Flow Diagram

The workflow for this systematic review/meta-analysis (SR/MA) can be seen in Figure 2. The purpose of visualizing the workflow was to help to follow the steps required to perform SR/MA, moreover, to aid to achieve the overall aim of the study.

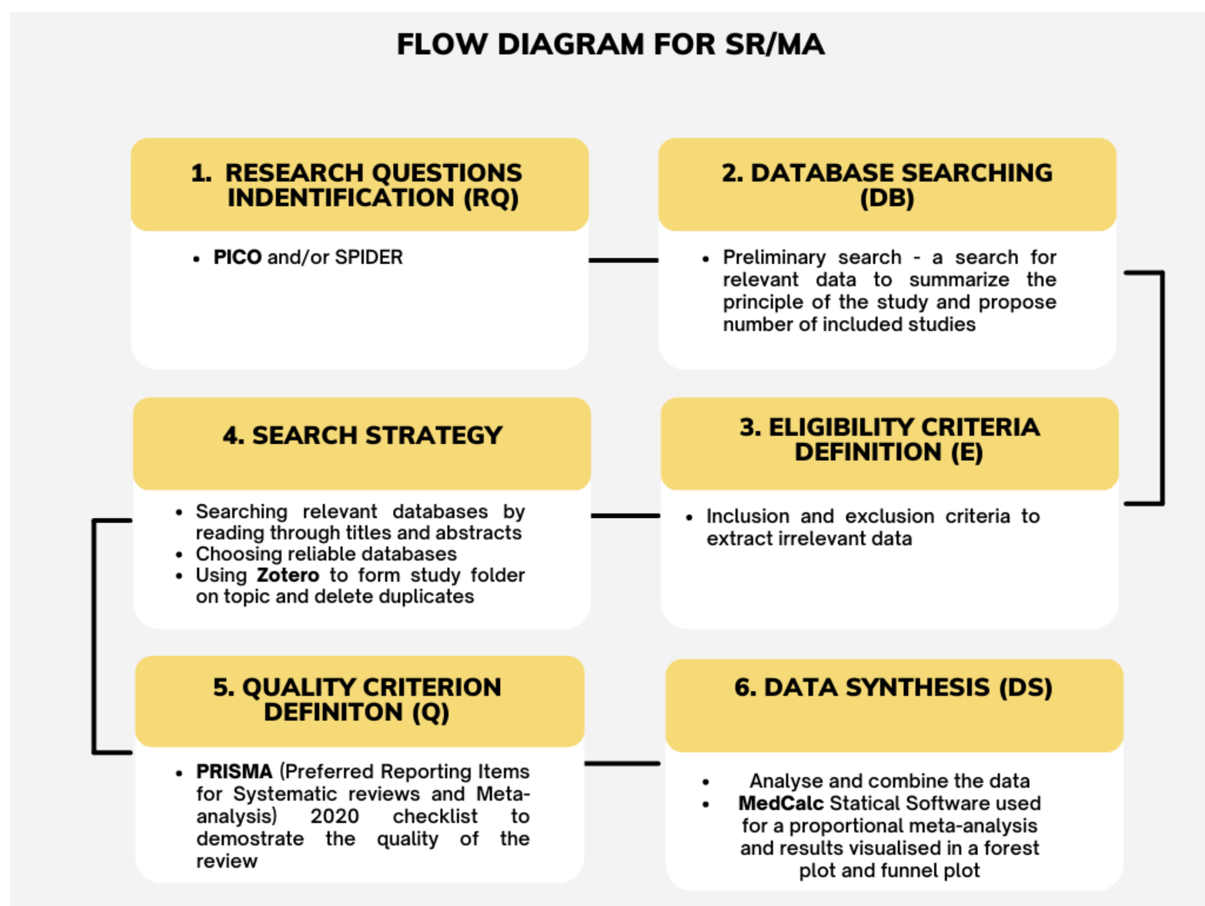

Figure 2. Detailed flow diagram guideline to follow systematic review and meta-analysis steps. Created with Canva software.

1.8 PICO

PICO stands for Population, Intervention, Comparison, and Outcome, and it is commonly used for systematic review and meta-analysis of clinical trial studies (Tawfik et al., 2019). PICO was used as a guidance tool to formulate an interesting, relevant, and executable research question. This study focuses on clinical trials conducted to treat the following Inherited Retinal diseases: Leber Congenital Amaurosis and autosomal dominant Retinitis Pigmentosa with the help of

CRISPR/Cas9 technology. The study aimed to find out if using this revolutionary gene-editing technology is a safe and efficient way of treating IRD patients, which led to the creation of the following research question: To what extent is CRISPR/Cas9 seen as a safe and efficient gene-editing tool treating IRDs patients? **P:** LCA and adRP male/female subjects, **I:** CRISPR/Cas9 gene-editing tool used to help to treat LCA and adRP, **C:** indel percentage, **O:** safety or adverse effects.

1.9 Inclusion and exclusion criteria

The eligibility criteria (Table 1.) was constructed to have borderlines for the research topic and to enable focus on the specific inherited retinal diseases, adRP, and LCA. The time period was chosen according to the use of CRISPR/Cas9 in the latest research, and the overall time limit of the project was considered when limiting the extent of the research.

Table 1. Eligibility criteria presented

Criteria	**Inclusion**	**Exclusion**
Time period	2012 to 2022	Any study out of the stated dates
Language	English	Non-English
Type of article	Original research articles or editorials published in peer-reviewed journals	Publications that were not original research or were unpublished research and Ph.D. theses and reports
Study focus	Articles about clinical trials that focused on using CRISPR/Cas9 to treat LCA or adRP	Any studies concluded that did not include the study focus
Participants	Any mammals	Other than mammals
Geographical location of the study	Worldwide	None
Full-text articles	Free	Chargeable
Reported outcomes	Stated as edited sequences and total sequences (Indel percentage)	If no outcomes were stated as indel percentages including edited sequences and total sequences

1.10 Search strategy

The literature search consisted of searching relevant literature through the following databases: ScienceOpen, AJHG, American Academy of Ophthalmology, ARVO Journals, BMC, BMJ Journals, ClinicalTrials.gov, CSH Perspectives in Medicine, Frontiers, Indian Journal of Ophthalmology, Google Scholar, JAMA Ophthalmology, Mary Ann Liebert, 2Inc., MDPI, Molecular Therapy Nucleic Acids, National Center for Biotechnology Information (NCBI), Nature Biotechnology, New England Journal of Medicine (NEJM), Oxford Academic, PNAS, PubMed, ResearchGate, Science, Science Advances, ScienceDirect, Taylor & Francis Online, The American Journal of Pathology, The Company of Biologists, Trends in Molecular Medicine, Wiley Online Library. The following terms were used while conducting the research: "Utilizing CRISPR/Cas9 to treat LCA", *RPE65* treated with CRISPR/Cas9", "Clinical trials to treat LCA AND CRISPR/Cas9", "*RPE65*", "*CEP290*", "CRISPR/Cas9 AND *CEP290*", "Inherited retinal diseases", "Treating IRDs with CRISPR/Cas9", "LCA2 AND CRISPR/Cas9", "Gene therapy for retinal diseases", "Genome-editing AND Inherited retinal diseases", "Curing blindness", "Leber Congenital Amaurosis", "Research on the eye", "Future aspect of CRISPR/Cas9", "Application of CRISPR/Cas9 to treat IRDs", "Gene mutations in IRDs", "Clinical trials on gene-editing CEP290 AND CRISPR/Cas9", "CRISPR/cas9 AND retinal diseases", "Retinitis pigmentosa rhodopsin", "CRISPR/Cas9 AND LCA", "adRP AND CRISPR/Cas9" and "treating autosomal dominant Retinitis pigmentosa with CRISPR/Cas9".

Zotero connector

Zotero connector was used as a tool to collect and organize research articles, as well as to easily delete and tag unrelated, duplicated, and unavailable full texts. Zotero allowed an easy way to create citations in the text and bibliographies on the reference list.

1.11 PRISMA 2020 checklist

PRISMA 2020 checklist was used as a helping tool to understand the necessary criteria that are needed when publishing a systematic review and to improve the transparency of the report. It was not essential to accurately follow the PRISMA checklist for the Bachelor thesis project, but the checklist provided important knowledge that could be respectively exploited in this literature review.

1.12 Quality assessment (QA)

Systematic reviews and meta-analyses are truly important methods to summarize and evaluate medical literature as well as to help improve previously conducted research (Tran et al., 2021). Including quality assessment in the reviews enables the addressing of unethical and misconducted research studies, but according to Tran et al., only 51.6% out of 244 *vitro* SRs/MAs had performed the QA on their reports. Similarly, research by Minelli et al. (2009) affirmed that meta-analyses are often missing the quality assessment, and this is extremely worrying as meta-analyses provide strong research evidence and should be conducted as methodologically rigorously as possible. In this paper, the methodological quality of the studies was assessed with the help of the "Checklist for systematic reviews and research syntheses", provided by the University of Adelaide, retrieved from https://jbi.global/critical-appraisal-tools.

1.13 Data synthesis

Proportional meta-analyses are used to summarize the impact of treatments as well as to identify evidence regarding the effectiveness of the treatments, although this type of single-group estimate is seen as less informative compared to a comparative meta-analysis (Barker, T.H. et al., 2021). Part of the collected data to answer the research objective on the efficiency and safety of CRISPR/Cas9 application for treating inherited retinal diseases was synthesized in a proportional meta-analysis, and another part was summarized as a narrative literature review, including qualitative and quantitative data. Two software were used to conduct the meta-analysis: MedCalc and JBI SUMARI. MedCalc uses the Freeman-Tukey transformation (arcsine square root transformation; Freeman and Tukey, 1950) to estimate the weighted summary proportion and JBI SUMARI was used for a better visual appearance of the results (DerSimonian & Laird, 1986). Data was entered to MedCalc as three variables (difference between means and continuous data) to compare Indel Percentages between studies: 1. Study: containing an identification of the different studies, 2. Edited_sequences; containing the number of edited sequences from the different studies and 3. Total_sequences: containing the total number of sequences from the different studies. A Forest plot and Funnel plot were generated to present the results from the meta-analysis, whereas a summary table was created to aid with the data presentation of narrative description.

3. RESULTS

1.14 Data extraction

The PRISMA 2020 flow diagram template was used (Figure 3) to show the flow chart of the selection process of relevant studies. This flow chart was constructed to give an overview of the total number of articles that were screened and to point out articles that were ultimately included in the meta-analysis.

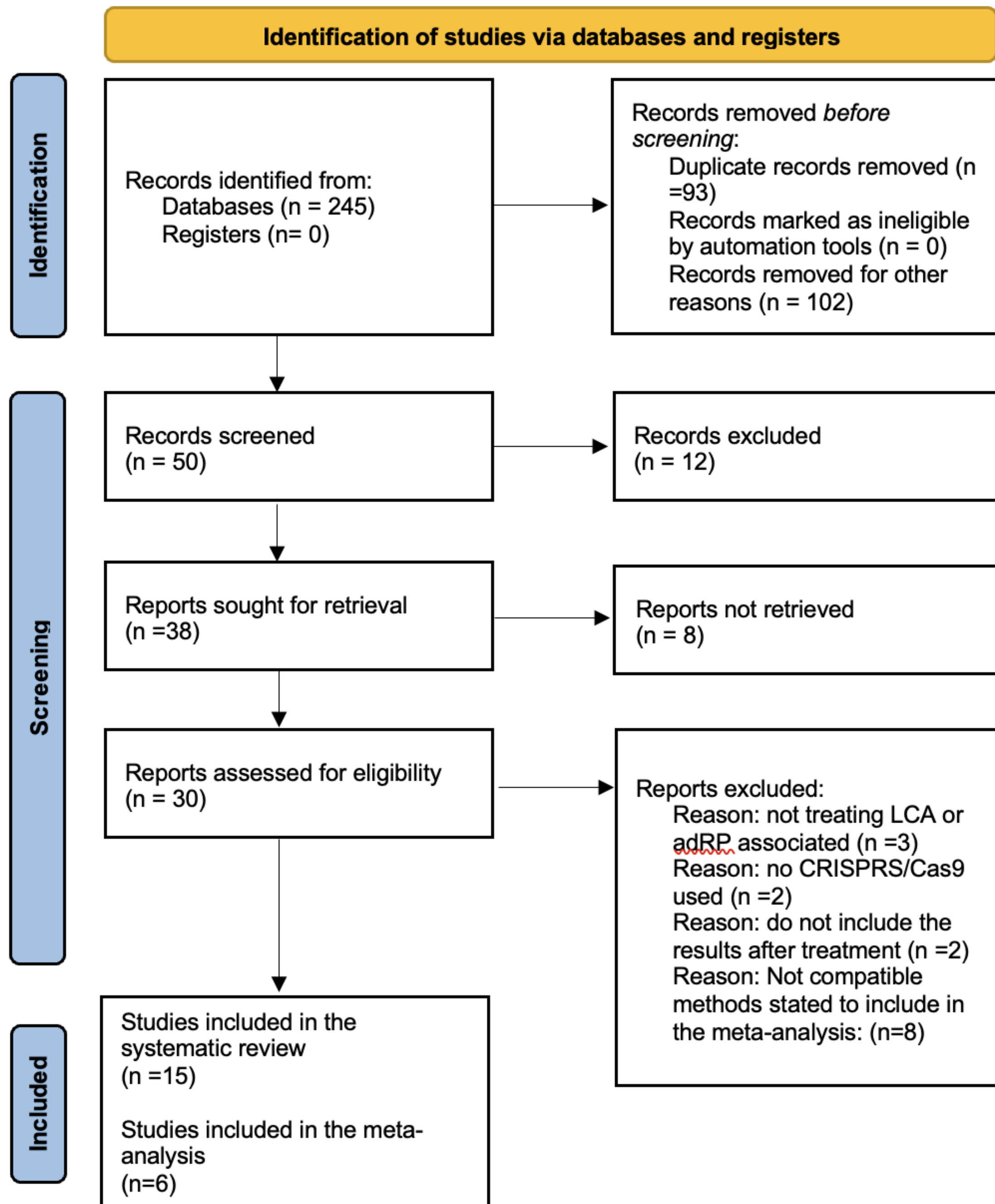

Figure 3. PRISMA 2020 flow diagram represents studies identified, included, and excluded. 245 publications were identified from the literature research. The following records were removed: 93 duplicates, and 102 records for other reasons like irrelevant topics and reports with no full-text availability. Other reports were excluded for the following reasons: 2 were not associated with IRDs, 2 did not include the use of CRISPR/Cas9, 2 were without proper results, and the final 8 articles did not include compatible methods to be selected. In total 16 articles were used more closely in this systematic review and only 6 articles with comparable results were used in the meta-analysis.

1.15 Data analysis

1.15.1 Meta-analysis

All six studies that were included in the meta-analysis had stated the indel percentage in the papers, referring to the gene editing efficiency. In addition, chosen studies specifically quantified all detected sequences that were different from the wild type in their research papers. Merging the indel percentages from different clinical IRD studies that utilized CRISPR/Cas9 technology, enabled the formation of a more accurate estimate of the overall gene editing efficiency of CRISPR/Cas9. Sample sizes of the individual studies somewhat varied from each other, therefore naturally affecting the sampling error. As Figure 4 illustrates, MedCalc calculated the proportions (expressed as indel percentage) with a 95% confidence interval (CI) for each individual study included in the meta-analysis. The random effects model was chosen as a default for the data synthesis as more than five studies were included in the meta-analysis, and heterogeneity was inspected among the effect sizes (Tufanaru et al., 2015). Heterogeneity was tested using Cochran's Q test, resulting in a p-value less than 0.0001, indicating that the proportion of success is not equal across 3 or more groups (Figure 4). $I^2 = 100$ also indicated considerable heterogeneity (Deeks et al., 2022). Each black square in Figure 4 represents the proportion of indel percentage rate conducted from individual studies. The pooled prevalence shows 0.443, or 44.3% (95% CI:0.246-0.649), indicating an average of 44.3% positive gene editing rate of CRISPR/Cass9 within the studies. Egger's test (Egger et al., 1997) was used to detect the presence of potential publication bias and to visualize the outcome with the funnel plot (Figure 5). Severe asymmetry seen in Figure 5 indicates the presence of publication bias, but subcontrary Egger's test resulted in a P-value of 0.5575 indicating, no significant difference. Although Eggers's test is known to have low power to detect heterogeneity and it is recommended to use a value of 0.10 as a cut-off for significance, the following P-value designates that no publication bias was presented (Higgins et al., 2003). The output using MedCalc software for proportional meta-analysis can be found in Appendix 2.

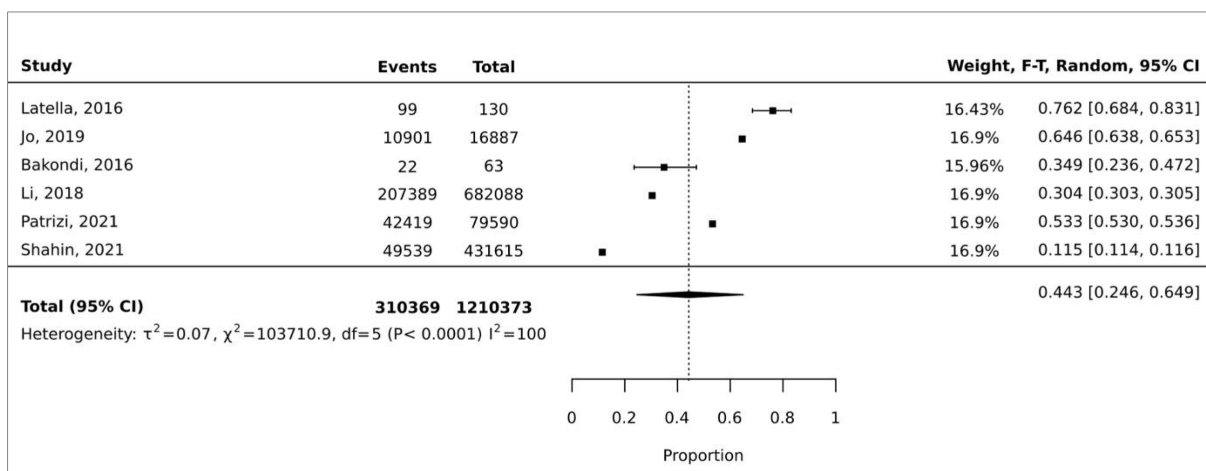

Figure 4. Forest plot presenting a proportional meta-analysis. This forest plot has been recreated in JBI SUMARI using data from a systematic review investigating the differences between indel percentages indicating the gene editing efficiency rate when treating LCA and adRP patients with the help of CRISPR/Cas9 technology. This synthesis included data from six different studies.

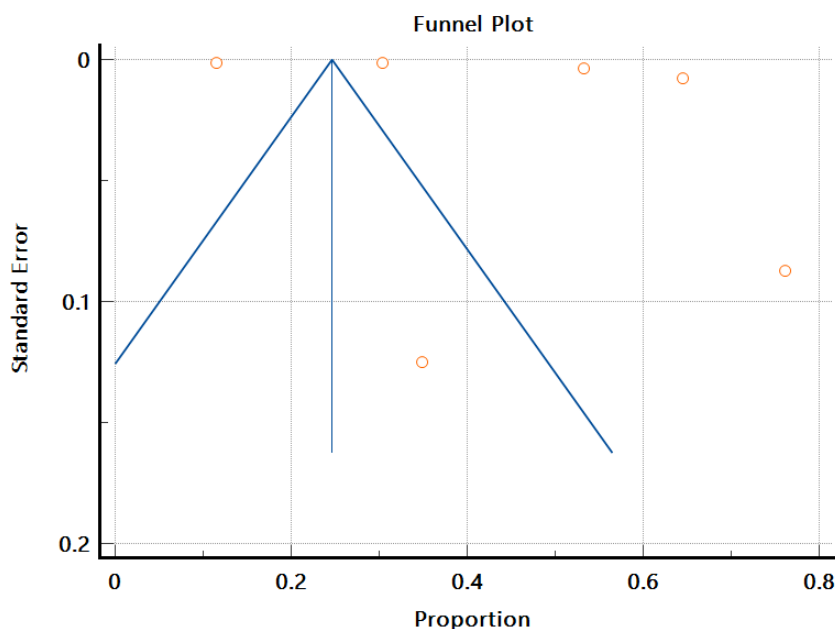

Figure 5. Funnel plot visualizing possible publication bias. Dots represent individual studies that took part in the data synthesis. The plot shows the standard error (SE) of the difference in proportion (Y-axis) versus the reported percent of Indel Percentage (X-axis) of gene editing using a random effects model. The vertical line indicates the pooled effect estimate.

1.15.2 Data Narrative

Nine research studies were chosen to be included in the systematic review but not in the meta-analysis due to their diverse treatment durations and data measurements, as well as differences in treatment approaches including methods and tools applied during the clinical trials. These studies together with the studies that were included in the meta-analysis, specified extremely important evidence and knowledge to answer the research question. Therefore, a table of 'Summary of Findings' was created to adduce some of the main results. Table 2. presents all the important findings from the 15 articles that enclose the safety and efficiency sides of CRISPR/Cas9 during clinical trials of treating Leber congenital amaurosis or autosomal dominant Retinitis pigmentosa.

Table 2. Summary of findings. The table states the main results of the studies related to the research question and the summary section at the bottom divides the results according to the outcomes. Asterix (*) indicates results not stated in the paper.

Study	Indel percentage %	Off-target %	Photoreceptor (PR) degeneration	Retinal pigment epithelium (RPE) degeneration	Outer nuclear layer (ONL)	Electroretinograms (ERGs) – a & b waves	Outer segment layer (OS)	Light sensitivity & visual acuity	Time frame
Bakondi, B. et al., 2016 (adRP)	35%	Not detected	9-fold recovery	*	Thicker	*	*	Up to 53% higher	*

Li, P. et al., 2018 (adRP)	45%	3.12 +- 2.28%	Reduced	*	Delayed degeneration	*	*	*	Active, 5 weeks post
Tsai, Y.T. et al., 2018 (adRP)	91%	*	Reduced	*	Thicker	Higher (130 % for b-wave)	~142 %	*	Active, 2 weeks post
Shahin, S. et al., 2022 (adRP)	12%	Not detected	Reduced	Slowed down	Thicker	Higher	Damage partly rescued	2-fold higher	Active, post 15 months post
Giannelli, G.S., et al., 2018 (adRP)	65 %	Not detected	Reduced	Slowed down	No difference	Higher	Damage partly rescued	Higher	Active 3 months post
Latella, M.C. et al., 2016 (adRP)	76%	Not detected	Possible recovery	*	*	*	*	*	*
Patrizi, C. et al., 2021 (adRP)	53%	Not detected	Reduced	Slowed down	No difference	b-waves higher	Rho more evident	Higher	Active, 1 month post
Zhu, J. et al., 2017 (adRP)	39%	*	Recovered (25 % cones)	*	Thicker	b-waves higher (60%)	No difference	*	*
Yu, W. et al., 2017 (adRP)	98 %	Not significant	Significantly reduced	No change	Thicker	Higher	Damage partly rescued	No Change	Active, 4 months post
Suzuki, K., et al., 2016 (adRP)	~93 %	Not significant	*	*	Thicker	Higher (a-waves, 4-fold)	*	Higher	Active, 4 weeks post
Cai, Y., et al., 2019 (adRP)	*	Not significant	Possible recovery	*	*	Slightly higher	*	Slightly higher	*
Vagni, P., et al., 2019 (adRP)	22 %	*	Slowed down	Slowed down	*	Slightly higher	*	Higher (50 %)	Active, 3 months post
Jo, D.H., et al., 2019 (LCA)	65 %	Not significant	*	*	Delayed degeneration	Higher a (21 %) b (40 %)	*	*	Active, 7 months post
Maeder, M.L., et al., 2015 (LCA)	90%	Less than 0,9%	*	*	*	*	*	*	*
Ruan, G., et al., 2017 (LCA)	64 %	Not detected	*	*	*	*	*	*	*

Summary	61%	6/15, 6/15 *3/15	9/15 2/15 *4/15	4/15 1/15 *10/15	6/15 2/15 2/15 *5/15	10/15 *5/15	5/15 1/15 *9/15	7/15 1/15 *7/15	2 weeks- 15 months

An average of 61 % gene editing efficiency was found among the studies indicating a great indel percentage. No off-targets or significant off-targets were detected from 12/15 articles from the analysis, showing prominent evidence for the safety of CRISPR/Cas9. Three articles had not performed the off-target analysis at all. 13/15 articles revealed either recovery of photoreceptors, reduced degeneration of RPE, or thicker outer nuclear layer formation. 10/15 studies measured the electrical activity of the retina in response to light stimuli with ERG, and each of those studies resulted in higher visual activity compared to the controls. These findings present incredible reliability for the CRISP/Cas9 efficiency. Supplementary information about the 15 studies that took part in this systematic review can be found in 'Supplementary materials', attached to this thesis work.

1.16 Assessment of methodological quality

The "Checklist for systematic reviews and research syntheses" was used to assess the methodological quality of a study, and to determine the extent to which a study has addressed the possibility of bias in its design, conduct, and analysis (Aromataris et al., 2015). Authors and consumers need to consider the resulting bias as they conduct this systematic review and possibly incorporate the findings into clinical practice and policy-making (Drucker et al., 2016).

- Language bias, only articles in English included.
- Grey literature (unpublished studies) not included.
- Critical appraisal for the chosen studies was not conducted by at least two reviewers working independently from each other.

4. DISCUSSION

This systematic review was implemented to understand the efficiency and safety of CRISPR/Cas9 gene-editing technology through trials to treat inherited retinal diseases. The studies included in the review focus on two of the most common inherited retinal diseases, Leber congenital and autosomal dominant retinitis pigmentosa. In the studies, CRISPR/Cas9 was used to examine future treatments for LCA and adRP, to permanently change the genome of the target organisms, and eventually provide long-term treatment for blindness. The Summary of findings (Table 2.), showed 61% gene editing efficiency in all 15 studies but results from the meta-analysis with MedCalc were distinct. It can be comprehended why CRISPR/Cas9 tool is the most widely used genome editing system with 61% gene editing efficiency, but considering the results from the forest plot with 44.3% gene editing efficiency, one can argue; if these studies are testing the same intervention, why are the results varying so much from each other? (Chang et al., 2022; Jiang & Doudna, 2107). Are the differences caused by chance or by something more significant? Therefore, another statical test called I^2 was used to calculate the consistency of the analyzed papers. The result, $I^2 = 100$ indicated a considerable heterogeneity, thus representing variation between studies that might relate to population, intervention, comparator, outcome measure, risk of bias, study methods, and other factors that took part in the individual studies (Chang et al., 2022). MedClac was recommended to be used by Barker and his team as appropriate software to be exploited with proportional meta-analysis, thus it can be excluded that the wrong software would have been applied. Additionally, excluding one of the studies as an "outlier" was tested to understand such a high heterogeneity but no significant effect was discovered. Therefore, the

pooled estimate presented by the Forest plot needs to be considered with caution regarding the high heterogeneity and cannot be straightforwardly trusted. A better study design with a larger sample size in the meta-analysis could provide a more precise estimate of gene editing efficiency. Turner et al. (2013) proved how big a difference it can make when comparing small studies to larger studies since small studies tend to express more extreme treatment effects. For comparison, an example study showed a combined analysis of 13 meta-analyses that were evaluating effects on pain in osteoarthritis patients, versus comparing trials with fewer than 100 patients per arm with larger trials resulting in the average difference of −0.21 (95% CI −0.34 to −0.08) (Nüesch et al., 2010).

Potential publication bias in the meta-analysis was tested with Egger's test and presented through a funnel plot as a graphical presentation of sample sizes plotted against the effect size they report (Lee & Hotopf, 2012). A P-value of 0.5575 suggests that no publication bias was presented in the meta-analysis, but conversably the funnel plot asymmetry indicated the opposite. Interestingly, small SE within the studies can be interpreted as the sample mean being a more accurate reflection of the actual population mean, which is very abnormal with such a small sample size as six. One might ponder the applicability of a Funnel plot as an unsuitable method to express publication bias. According to Cheema et al., the use of funnel plots and Egger's test to access publication bias in pooled proportions gives erroneous outcomes and can easily lead to biased results from the meta-analysis. Instead, a supplementary method called the Doi plot and LFK index should be used when evaluating publication bias in meta-analysis of prevalence and when less than 10 studies are being synthesized (Shahid & Cheema, 2022). Therefore, publication bias in the meta-analysis were furthermore tested with MetaXL tool for meta-analysis in Microsoft Excel. Unfortunately, MetaXL was not able to run the available data. This could be due to MetaXL using unique algorithms that are not yet designed for studies that use Indel percentages to calculate gene editing efficiency. Meta-analyses that are centered around gene-editing with CRISPR/Cas9 are still a diminutive and new area of investigation.

Examining the safety features of CRISPR/Cas9 used during eye research was primarily determined from the off-target analysis. Off-target analysis is an important method to detect unintended cleavage and mutations further away from the target site (Modrzejewski et al., 2019). The results obtained from the analysis indicated high safety standards for CRISPR/Cas9 with no significant off-targets found. However, it is important to consider that the off-target analysis often results in ambiguous or no detection of off-target mutations, especially when the sequencing error rate is lower than 0.5%, meaning there is always a slight chance for some sort of off-targets (Kang et al., 2020). Kang et al. developed a new prediction-based highly sensitive CRISPR off-target validation using target-specific DNA enrichment to detect small amounts of mutated DNA. Using Kang et al.'s method with CRISPR/Cas9 when applied to treat inherited retinal diseases could allow the detection of genome editor-induced off-target mutations with higher sensitivity and in a non-biased manner, rising the safety standards for CRISP/Cas9 in clinical trials (Kang et al., 2020).

Each stage of this systematic review was predefined to the last detail. This precise planning included all the parts that are needed in a well-conducted systematic review and to perform an accurate methodology. The research question itself has a high value in the research of ophthalmology and is the first one of its kind for this specific topic. There is no need for concern about the mass production of this type of systematic review. Furthermore, it is essential to investigate the efficacy and safety of CRISPR/Cas9 technology due to its possible side effects, in order to expand the research to human trials and to create long-lasting treatments. The results were presented both, qualitatively and quantitatively due to a variation between study procedures and to gain more reliability and accuracy. Additionally, an extensive search of all the available data was conducted by following the preferred search criteria and using the main keywords. The systematic review could be easily repeated. On contrary, weaknesses of the review

were similarly considered and unfortunately, bias could not be entirely avoided. The grey literature, referring to primary sources like unpublished research directly from researchers or government reports should be included to attain more truthful results. However, the time limit and other limitations of the Bachelor thesis do not allow for the construction of a precise systematic review but an adequate one. Another important concept to be considered is the peer reviewers. This project does not require researchers to peer-review the studies that were synthesized, which naturally increases the possibility of bias. Yet at least two researchers should have reviewed the included studies before analyzing them in the meta-analysis (Charrois, 2015). Regrettably, the research topic chosen was very new in medical research and not many studies have yet been implemented, which created a challenge to gather suitable data. More comparable studies should be designed. Additionally, certain tools and software that are usually used within literature reviews and meta-analyses, could not be used with proportions and with a small sample size. Moreover, working with gene editing and testing gene-editing efficiency includes a huge diversity of techniques and trials. The studies tended to differ immensely each time compared to other studies. This made it hard to find comparable studies and ultimately a different bias emerged. Fortunately, most possible biases were thoroughly discussed to avoid misrepresentation of the study results.

This systematic review deliberates whether CRISPR/Cas9 is an efficient and safe tool to treat inherited retinal diseases. Treating inherited retinal diseases has been a prevalent topic in ophthalmology research. Currently, effective treatment procedures exist to treat recessive or X-linked loss-of-function mutations through gene augmentation, but to treat dominant gain-of-function mutations the pathogenic gene must be silenced (Peddle et al., 2020). The aim is to sustain a permanent cure for blindness, therefore, CRISPR/Cas9 system has been prominently applied in the clinical trials of retinal research. However, the permanent changes that CRISPR/Cas9 causes in the genome have still been the focus of debates, due to possible off-targets and side effects that may follow the treatment (Peddle et al., 2020; Tasan & Zhao, 2017). Moreover, even a low frequency of unintended mutations might be damaging (Tasan & Zhao, 2017). Hence, there remains a dilemma to ensure that treating IRD patients with CRISP/Cas9 is truly safe and efficient, thus supplementary techniques to treat IRD patients should be considered further. Based on the previous studies and this systematic review, it can be agreed to a certain extent that it is safe and efficient to use CRISPR/Cas9 to treat IRDs, but more extensive evaluation needs to be executed. The hypothesis itself was rejected because it cannot with high enough certainty promise safe and effective CRISPR/Cas9 to treat IRDs, and more detailed research on the topic is required.

1.17 Future research

To date, no permanent cure for IRDs has yet been discovered (Diakatou et al., 2019). CRISPR/Cas9 technology has modernized retinal gene therapy to a new era, but due to the genetic heterogeneity of inherited retinal diseases, developing IRD gene therapy further can be challenging (Chiu et al., 2021). CRISPR requires highly allele-specific designs for delivery and construction that specifically consider the context of the patient's disease (Chrenek et al., 2016). All mutations contributing to a certain IRD, need to go through additional clinical trials in to find out the underlying genetic defects of each disorder and the associated disease phenotype, as well as to test the age of onset, speed, and severity of progression, and which cell type is primarily affected (Chiu et al., 2021; Chrenek et al., 2016; Diakatou et al., 2019). Moreover, IRDs presenting with early onset degeneration or causing irregular retinal development could be treated with early gene augmentation by gene editing in human zygotes and embryos, but ethical and practical encounters would strictly arise (Chiu et al., 2021; Chrenek et al., 2016). Application of gene therapy on human adult eyes in another hand requires more demanding technical solutions as development and cell differentiation in the retina have already occurred. Before directly focusing on curing IRDs it is necessary to understand that considerable improvements to the application of CRISPR/Cas9 are needed, even in experimental clinical trials (Chrenek et al., 2016).

Researchers are continuously modifying CRISPR/Cas9 tools with improved designs to reach higher potential to treat IRDs. They aim to improve the safety and efficiency features of CRISPR/Cas9 to execute gene editing in vivo. CRISPR interference (CRISPRi) is one alternative method to silence pathogenic genes. This technique is a modified version of Cas9 protein, where the Cas9 is catalytically inactivated referred to as endonuclease deficient Cas9 (dCas9), though can still bind to DNA but no longer can cleave it. Instead, CRISPRi causes transcriptional repression of the target gene while dCas9 and gRNA are present. CRISPRi could be seen as a more promising tool to treat IRDs with a higher safety profile due to rare off-target repression (Peddle et al., 2020). Currently, there are fascinating development strategies for *in vivo* gene editing that have been discussed. One approach is using the base editing method that allows direct editing of individual bases. By changing bases with an impaired Cas9 that only induces a single-strand break together with cytidine deaminase, it is possible to precisely convert the mutated nucleotide to the wildtype one. Correction of the mutant allele with low efficiency of HDR in the post-mitotic retinal cells could be innovative for gene editing (Chrenek et al., 2016). Another approach uses primer editing that focuses on using an engineered reverse transcriptase fused to Cas9 nickase and a prime editing guide RNA (pegRNA) that adds to the complementary sequence and has a sequence reading of the desired sequence changes. mRNA editing is yet another future gene editing method that has the potential due to possibly circumvent the creation of off-target events (Quinn et al., 2021). A fascinating approach to optimize the use of CRISPR/Cas9 with IRDs could include the use of induced pluripotent stem cells (iPSCs) to repair inactive cells. iPSCs could be produced from patients' own fibroblasts and transplanted cells back into patients' retinas after being modified with CRISP/Cas9 (Bassuk et al., 2016; Chrenek et al., 2016). Bassuk et al. reported success with using CRISPR/Cas9 to correct a pathogenic mutation in iPSCs derived from a patient with photoreceptor degeneration. The trials to treat inherited retinal diseases with the help of CRISPR/Cas9 remain, and more safer and efficient gene editing techniques as well as data presentation tools are constantly generated for future research.

5. CONCLUSION

In conclusion, it is important to understand that even though CRISPR/Cas9 has proven to target specific mutations with great accuracy, we still need new developments to improve this accuracy to its maximum. With such a powerful gene editing tool, naturally, complications appear, and the focus should be shifted to creating more accurate, safe, and efficient editing techniques. Several articles with clinical trials testing to find different treatment methods for IRDs had excitingly tried CRISPR/Cas9 tool based on other trials, possibly with slight altering but with great confidence to change the genome. Sometimes no off-target analysis was even performed to confirm safety. The overwhelming idea of CRISPR/Cas9 as an editing tool needs to be tempered and more dedication set on the safety and efficiency of CRISPR/Cas9 to reach the overall objectives to cure IRDs and other inherited diseases. The meta-analysis confirmed great heterogeneity between studies, which explains the desire of constructing study designs according to needs instead of safety and accuracy. One of the major challenges to gene editing *in vivo,* is efficiency, thus indel percentage should always be mentioned in the studies as it indicates the efficiency of the gene-editing tool used. This systematic review provides great insight into the use of CRISPR/Cas9 in the field of medical research to treat IRDs, emphasizes the importance of this unique gene-editing tool, as well as speculates the negative side effects of CRISPR/Cas9. The study also discusses alternative genome editing approaches with improved CRISPR/Cas9 techniques to ultimately create better safety and efficiency features. This is mandatory if in the future we want to apply CRISPR/Cas9 to treat inherited diseases in humans without greater concerns.

6. ETHICS AND IMPACTS OF THE STUDY ON SOCIETY

The literature review/m eta-analysis study is exempt from ethical approval. This study collected and summarized data from previous clinical trials in which informed consent has already been obtained by the trial researchers. Generally, while conducting this literature review, I as an author, have presented the content honestly and any information stated in the report has not been distorted solely to support or validate the study's aim. This literature review directly impacts society by providing a comprehensive summary of the use of CRISPR/Cas9 technology to treat IRD patients, thus encouraging more researchers to participate in discovering a permanent cure for inherited blinding diseases and giving hope for people struggling with an impaired vision since birth.

7. ACKNOWLEDGEMENTS

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9. APPENDICES

Appendix 1: Input of Indels

Study	Positive	Total	Disease
Latella, 2016	99	130	adRP
Jo, 2019	10901	16887	LCA(2)
Bakondi, 2016	22	63	adRP
Li, 2018	207389	682088	adRP
Patrizi, 2021	42419	79590	adRP
Shahin, 2021	49539	431615	adRP

Figure 1. Input used in meta-analysis, including detected sentences and total sentences

Appendix 2: Output using MedCalc software for proportional meta-analysis

Study	Sample size	Proportion (%)	95% CI	Weight (%)	
				Fixed	Random
Latella, 2016 (adRP)	130	76.154	67.892 to 83.187	0.011	16.26
Jo, 2019 (LCA)	16887	64.553	63.826 to 65.274	1.40	17.05
Bakondi, 2016 (adRP)	63	34.921	23.337 to 47.973	0.0053	15.50
Li, 2018 (adRP)	682088	30.405	30.296 to 30.514	56.35	17.06
Patrizi, 2021 (adRP)	79590	53.297	52.950 to 53.644	6.58	17.06
Shahin, 2021 (adRP)	431615	11.478	11.383 to 11.573	35.66	17.06
Total (fixed effects)	1210373	24.634	24.557 to 24.711	100.00	100.00
Total (random effects)	1210373	44.257	28.956 to 60.139	100.00	100.00

Test for heterogeneity

Q	103712.0927
DF	5
Significance level	P < 0.0001
I^2 (inconsistency)	100.00%
95% CI for I^2	99.99 to 100.00

Publication bias

Egger's test	
Intercept	56.8596
95% CI	-190.1569 to 303.8761
Significance level	P = 0.5575
Begg's test	
Kendall's Tau	-0.3333
Significance level	P = 0.3476

Figure 2. All the included studies taking part of the meta-analysis. Sample size indicates the total reads of indels and proportion (%) states the indel percentage, which includes all detected sequences that are different from the wild type (insertions/deletions). Heterogeneity was tested using Cochran's Q test and Egger's test.