EGAS MONIZ SCHOOL of HEALTH & SCIENCE | INSTITUTO UNIVERSITÁRIO EGAS MONIZ

INSTITUTO UNIVERSITÁRIO EGAS MONIZ

MESTRADO INTEGRADO EM CIÊNCIAS FARMACÊUTICAS

PRECISION GENOME EDITING: UNLEASHING THE THERAPEUTIC POTENCIAL OF CRISPR/CAS9 IN GENETIC DISEASE TREATMENT

Trabalho submetido por
Martim Maria Pedro e Dinis
para a obtenção do grau de Mestre em Ciências Farmacêuticas

novembro de 2024

EGAS MONIZ SCHOOL of HEALTH & SCIENCE | INSTITUTO UNIVERSITÁRIO EGAS MONIZ

INSTITUTO UNIVERSITÁRIO EGAS MONIZ

MESTRADO INTEGRADO EM CIÊNCIAS FARMACÊUTICAS

PRECISION GENOME EDITING: UNLEASHING THE THERAPEUTIC POTENCIAL OF CRISPR/CAS9 IN GENETIC DISEASE TREATMENT

Trabalho submetido por
Martim Maria Pedro e Dinis
para a obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho orientado por
Professor Doutor Nuno Taveira

novembro de 2024

Dedicated to

To those who demonstrated faith in me.

Thanks to

My family, especially my parents Luis and Carla, grandparents Lourdes, Isilda and Jose and sister Matilde, for all their support and belief in me, which would not have been possible without them.
My girlfriend Rita, for all her patience, for being there every step of the way to help me and also to look at things and situations differently.
My friends, for all the help they gave me to finish this course, and for all the good times.
To Professor Nuno for agreeing to help me build this work, for all his knowledge and for all of his time.

Abstract

O principal objetivo deste estudo foi investigar as potenciais aplicações terapêuticas do CRISPR-Cas9 no tratamento de doenças genéticas. Em particular, o estudo examina as aplicações atuais do CRISPR-Cas9, delineia os desafios associados e oferece insights sobre melhorias prospectivas nas tecnologias de edição de genes.

Esta pesquisa emprega uma extensa revisão da literatura, abrangendo artigos de revisão e artigos de pesquisa originais publicados entre 2019 e 2024, aumentados por trabalhos fundamentais. Os artigos selecionados abordam uma série de tópicos, incluindo edição de genes, as aplicações terapêuticas do CRISPR-Cas9 e considerações éticas. As bases de dados pesquisadas incluem PubMed, Google Scholar, Web of Science, ScienceDirect e IEEE Xplore. Foram utilizadas as seguintes palavras-chave para a pesquisa: "CRISPR-Cas9," 'edição de genes,' e "doenças genéticas."

O CRISPR-Cas9 demonstrou a sua eficácia na correção in vitro de mutações genéticas associadas a doenças como a fibrose quística e a doença de Huntington. Apesar da eficácia demonstrada pelos sistemas de entrega, como os vectores virais, as nanopartículas lipídicas e os vectores adenovirais, estes ainda enfrentam limitações relacionadas com as respostas imunitárias e a entrega a tecidos específicos. O potencial para efeitos fora do alvo representa uma preocupação significativa, levantando importantes questões éticas e de segurança, particularmente no contexto de aplicações clínicas e terapêuticas. O advento dos sistemas CRISPR de próxima geração, como a edição de bases e a edição de primes, oferece soluções potenciais, minimizando as quebras de cadeia dupla e melhorando a precisão.

A aplicação clínica do CRISPR-Cas9 para o tratamento de doenças genéticas representa um avanço significativo, mas a tecnologia ainda enfrenta uma série de desafios técnicos e éticos. À medida que a tecnologia se desenvolve, as melhorias nas metodologias de entrega, a redução das consequências fora do alvo e o estabelecimento de diretrizes éticas serão vitais para garantir a incorporação segura e eficaz do CRISPR-Cas9 em terapias médicas.

Palavras chave: CRISPR-Case, Edição de genes, Doenças genéticas, Efeitos fora do alvo, Sistemas CRISPR de próxima geração.

Abstract

The principal aim of this study was to investigate the potential therapeutic applications of CRISPR-Cas9 in the treatment of genetic disorders. In particular, the study examines the present applications of CRISPR-Cas9, delineates the associated challenges, and offers insights into prospective enhancements in gene editing technologies.

This research employs an extensive literature review, encompassing both review articles and original research papers published between 2019 and 2024, augmented by key foundational works. The selected articles address a range of topics, including gene editing, the therapeutic applications of CRISPR-Cas9, and ethical considerations. The databases searched include PubMed, Google Scholar, Web of Science, ScienceDirect, and IEEE Xplore. The following keywords were used for the search: "CRISPR-Cas9," "gene editing," and "genetic diseases."

CRISPR-Cas9 has demonstrated efficacy in the in vitro correction of genetic mutations associated with diseases such as cystic fibrosis and Huntington's disease. Despite the demonstrated efficacy of delivery systems such as viral vectors, lipid nanoparticles, and adenoviral vectors, they still face limitations related to immune responses and targeted tissue delivery. The potential for off-target effects represents a significant concern, raising important safety and ethical issues, particularly in the context of clinical and therapeutic applications. The advent of next-generation CRISPR systems, such as base editing and prime editing, offers potential solutions by minimising double-strand breaks and improving precision.

The clinical application of CRISPR-Cas9 for the treatment of genetic diseases represents a significant breakthrough, yet the technology still faces a number of technical and ethical challenges. As the technology develops, enhancements in delivery methodologies, reductions in off-target consequences, and the establishment of ethical guidelines will be vital for ensuring the secure and effective incorporation of CRISPR-Cas9 into medical therapies.

Keywords: CRISPR-Case, Gene editing, Genetic diseases, Off-target effects, Next generation CRISPR systems.

Índice

Abreviations list

- AAV: Adeno-Associated Virus
- Cas9: CRISPR-associated protein 9
- CF: Cystic Fibrosis
- CFTR: Cystic Fibrosis Transmembrane Conductance Regulator
- CFUs: Colony-Forming Units
- CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
- DDR: DNA Damage Response
- DSBs: Double-Strand Breaks
- EVs: Extracellular Vesicles
- HD: Huntington's Disease
- HSPCs: Hematopoietic Stem and Progenitor Cells
- HTT: Huntingtin Gene
- IDLV: Integrase-Deficient Lentiviral Vectors
- LNPs: Lipid Nanoparticles
- p53: Tumor Protein 53
- sgRNAs: Single-Guide RNAs
- SNPs: Single Nucleotide Polymorphisms
- TALENs: Transcription Activator-Like Effector Nucleases
- TMA: Thrombotic Microangiopathy
- ZFNs: Zinc Finger Nucleases

1 Introduction

Genetic diseases are a major health challenge worldwide, affecting approximately 1 in 17 people [1]. The diversity of these conditions, including rare diseases, requires innovative and effective strategies for the development of genetic therapies.

CRISPR-Cas9 technology has emerged as a revolutionary tool for the therapy of genetic diseases [1]. The genetic changes made possible by CRISPR-Cas9 enable the repair of disease-causing genetic mutations through precise targeting of the genome potentially ushering in a new era of personalised treatments. However, the implementation of CRISPR-Cas9 or any other method of gene editing in the therapy of human diseases faces several challenges [1]. Issues such as the personalised nature of editing, the efficiency of correcting mutations, of-target effects, delivery mechanisms and the potential for recognition of the donor component as a foreign protein, are barriers that need to be overcome. In addition, the ethical considerations of gene editing in humans, including germline modifications and potential long-term effects on the genetic pool, require careful consideration and regulatory oversight. Ensuring equitable access to these potentially life-saving therapies is also a major challenge. As the technology advances, responsible management of these issues, with a focus on maximising benefits while minimising risks, will be crucial. [5]

CRISPR-Cas9 has been used in the correction of mutations associated with Cystic fibrosis (CF) and Huntington disease and has the potential to treat a wide range of other genetic diseases such as, Duchenne muscular dystrophy and sickle cell anaemia. [4].

The future of CRISPR-Cas9 therapeutic applications is bright, with ongoing research expanding its potential and addressing existing limitations. In addition, the development of next-generation CRISPR systems with improved capabilities indicates that genome editing will continue to be a critical tool in the fight against genetic diseases. In the future, the integration of CRISPR-Cas9 into clinical practice is likely to revolutionise genetic medicine, offering hope to patients with genetic disorders and opening up new avenues for treatment strategies. [5]

1.1 Objectives of the work

The aim of this thesis is to explore the therapeutic potential of CRISPR-Cas9 technology in the treatment of genetic diseases. Specifically, this study aims to review the current applications and advances of CRISPR-Cas9 in genetic disease therapy, identify and analyse the challenges and limitations associated with the technology, and

discuss future perspectives and potential improvements in CRISPR-Cas9-based therapies.

The paper is divided into several sections. The Introduction provides an overview of genetic diseases and the role of CRISPR-Cas9 in genetic therapy. The theoretical background discusses the basics of genetics and genetic diseases, including the mechanisms of CRISPR-Cas9. The Mechanism of CRISPR-Cas9 section explains the discovery, development and molecular mechanisms of CRISPR-Cas9. The Applications of CRISPR-Cas9 in Genetic Diseases section reviews specific applications of CRISPR-Cas9 in the treatment of monogenic and complex genetic diseases. The Challenges and Considerations section examines challenges such as off-target effects, delivery methods and ethical concerns. Finally, the Discussion and Future Directions section analyses current research findings and suggests potential improvements for CRISPR-Cas9 therapies.

1.2 Structure of the work

The research included the analysis of both review articles and original research articles. Review articles provided a broad overview of the field, while original research articles provided detailed insights into specific studies and experimental results. The articles selected for this study were published within the last five years to ensure that the information was current and relevant to the rapidly evolving field of CRISPR-Cas9 technology. However, important seminal papers were included regardless of publication date if they provided important background or foundational knowledge.

Keywords used to search for relevant articles included 'CRISPR-Cas9', 'gene editing', 'genetic diseases', 'therapeutic applications', 'off-target effects' and 'gene therapy'. Articles were retrieved from several scientific and academic databases, including PubMed, Google Scholar, Web of Science, ScienceDirect and IEEE Xplore. These methods ensured a comprehensive and up-to-date literature review, covering the most significant advances and ongoing research in CRISPR-Cas9 techn

1.3 Theoretical background

1.3.1 Genetics and genetic diseases

Genetics is concerned with the mechanism of inheritance, which involves passing genetic information from parents to offspring. Humans have 46 chromosomes, organized into 23 pairs, which contain genes that guide physiological and biochemical processes. The inheritance of these chromosomes, determines an individual's sex and influences a wide range of genetic traits.

Genetic disorders arise from mutations in the genome and manifest in diverse forms. These disorders are classified based on the affected chromosome (autosomal or X-linked) and the mode of inheritance (dominant or recessive), exemplifying the variability and complexity of genetic diseases Dominant disorders require only a single mutated gene for manifestation, while recessive disorders require mutations in both gene copies. This classification helps to understand the hereditary patterns and potential risks associated with genetic diseases. [4][5]

Single Gene Disorders are caused by mutations in a single gene, which directly affect health. Examples of these disorders include sickle cell disease, cystic fibrosis, Huntington's disease, and Fragile X syndrome, which demonstrate autosomal and X-linked inheritance patterns, respectively.

Chromosomal Abnormalities, such as trisomies and deletions, result from deviations in chromosome number or structure and underpin a range of disorders. Down syndrome is a condition caused by an extra chromosome 21, which affects developmental and physiological pathways. Many diseases are the result of genetic predispositions and environmental factors, known as multifactorial etiology. This is evident in chronic diseases such as heart disease and diabetes. [5]

1.3.1.1 Cystic fibrosis

Cystic fibrosis (CF) represents a significant challenge in the field of genetic disorders, affecting over 70,000 people in the world and holding the status of the most common inherited genetic disease in the Caucasian population [7]. Over the past 20 years, the comprehension of CF has evolved from a pediatric digestive and lung condition to a complex multisystem disorder persisting into adulthood. This shift is underpinned by an enhanced genetic understanding, where over 2000 mutations of the CFTR gene have

been identified, illuminating the pathophysiology of CF and paving the way for targeted treatments. Concurrently, research efforts have intensified, focusing on gene therapy to introduce normal CFTR copies into lung epithelial cells and developing drugs to optimize ion transport and manage inflammation.

The most prevalent CF mutation, ΔF508, results in the premature degradation of the CFTR protein by the cell, preventing it from reaching the membrane. Other mutations may result in the production of a functional protein that is nevertheless unable to perform its intended function at the membrane. The G551D mutation results in a CFTR protein that reaches the cell membrane but exhibits markedly diminished chloride channel activity [121]. The channel fails to open correctly, resulting in impaired ion transport. The G542X mutation is a nonsense mutation that results in the production of a truncated, non-functional CFTR protein. The premature stop codon results in the production of a protein that is too short to function correctly. The N1303K mutation affects the ATP-binding domain of the CFTR protein, which is essential for its function as a chloride channel. The resulting protein is misfolded and therefore unable to function effectively at the cell membrane. The W1282X mutation is analogous to the G542X mutation, resulting in the production of a truncated CFTR protein. The truncated protein is non-functional and rapidly degraded. The R117H mutation causes a mild defect in the CFTR protein's function. The protein reaches the cell membrane but has reduced efficiency as a chloride channel, often associated with milder forms of cystic fibrosis.

A major achievement in CF care has been the dramatic improvement in survival. The predicted median survival for babies born with CF in the 21st century is now over 50 years in the world, a testament to advances in treatment modalities, early diagnosis through newborn screening and a holistic approach to patient management [107]. Early diagnosis facilitates immediate nutritional support and specialized care, significantly improving quality of life and survival rates. However, the approach also encounters challenges, such as the potential misidentification of carriers and the risk of overlooking classic CF cases, underscoring the need for a nuanced understanding of screening outcomes [107].

The landscape of CF research is rich with potential, notably in gene therapy and the development of novel pharmacological agents aimed at correcting the underlying

CFTR dysfunction. These endeavors represent the frontier of CF treatment, potentially shifting the paradigm from symptom management to disease modification [107].

The pursuit of a cure for cystic fibrosis presents a significant challenge, primarily due to the fact that it is a genetic disorder resulting from mutations in the CFTR gene. As mentioned above, these mutations result in the production of a CFTR protein that is unable to fulfil its normal function, thereby disrupting the process of ion transport across epithelial cells. The CFTR gene is substantial in size and intricate in structure, with in excess of 2,000 identified mutations that can result in a spectrum of protein dysfunction. This complexity presents a significant challenge in the design of a universal therapeutic approach.

The current challenge in developing a cure for cystic fibrosis is the difficulty of correcting the underlying genetic defect in all affected cells. The implementation of gene therapy, which aims to replace or repair the defective CFTR gene, is confronted with considerable challenges, including the effective delivery of the therapy to the relevant tissues (in particular, the lungs) and the assurance that the corrected gene is expressed at functional levels. Furthermore, even if gene therapy were to prove successful, it would still need to address the irreversible damage already caused by the disease in tissues such as the lungs and pancreas.

The heterogeneity of CFTR mutations further complicates the development of a single, universal cure. Different mutations affect the CFTR protein in different ways, necessitating the use of tailored treatments. While advances in gene editing technologies, such as CRISPR, offer promise, these approaches are still in the early stages of development and face numerous technical and ethical challenges before they can be considered as viable cures.

1.3.1.2 Huntington's disease

Huntington's disease (HD) stands as a paradigmatic example of how a single genetic mutation can lead to a complex array of neurological and psychiatric symptoms.

The genetic foundation of HD is attributed to an autosomal dominant mutation involving the expansion of CAG trinucleotide repeats within the HTT gene on chromosome 4. This mutation results in an elongated polyglutamine (polyQ) tract in the huntingtin protein, leading to its dysfunctional and toxic gain of function. Normal alleles typically contain fewer than 36 repeats, whereas alleles with more than 39 repeats invariably cause HD. This expansion is not static across generations; a phenomenon

known as anticipation, where the number of repeats can increase in successive generations, leading to earlier onset and more severe manifestation of the disease[11][12].

The mutant huntingtin protein (mHTT) exerts a myriad of detrimental effects at the cellular level, disrupting normal cellular functions and leading to neuronal death, particularly in the striatum and cortex. The pathogenic mechanisms include impairment of proteostasis, leading to the accumulation of misfolded proteins; interference with transcriptional regulation, affecting gene expression; disruption of mitochondrial function and energy metabolism; and impairment of axonal transport, affecting neuronal communication. The accumulation of mHTT also leads to the formation of neuronal inclusion bodies, further contributing to cellular dysfunction and death[10][11].

The elevated number of glutamines in the huntingtin protein, resulting from the expansion of the CAG trinucleotide repeat in the HTT gene, directly exacerbates these pathogenic mechanisms. The extended polyglutamine (polyQ) tract in mHTT. The expanded polyQ tract increases the propensity of the huntingtin protein to misfold, which in turn leads to its aggregation. This aggregation has a deleterious effect on cellular proteostasis mechanisms, such as the ubiquitin-proteasome system and autophagy, resulting in the accumulation of toxic protein aggregates that the cell is unable to effectively clear. The mutant huntingtin protein with an expanded polyQ region exhibits aberrant interactions with a range of transcription factors and co-regulators. This aberrant interaction impairs the normal regulation of gene expression, resulting in the dysregulation of essential genes, particularly those involved in neuronal survival and function. The expanded polyQ tract in mHTT has been demonstrated to disrupt mitochondrial function, leading to impaired energy metabolism. Neurons, which are highly dependent on mitochondrial function, experience increased oxidative stress and energy deficits, which contribute to neuronal damage and death. The presence of the expanded polyQ tract in mHTT disrupts the normal transport of organelles and proteins along axons, which are essential for maintaining neuronal communication and function. This disruption contributes to the progressive degeneration of neurons. The accumulation of mHTT with an expanded polyQ tract leads to the formation of neuronal inclusion bodies, which are aggregates of misfolded proteins that further disrupt cellular functions and contribute to neurodegeneration.Notably, the striatum, a critical component of the basal ganglia involved in motor control and cognitive functions, is one of the earliest and most severely affected brain regions. This selective vulnerability contributes to the

characteristic motor symptoms of HD, including chorea, dystonia, and bradykinesia, as well as cognitive decline and behavioral changes[7].

The management of HD is currently symptomatic, focusing on alleviating the diverse manifestations of the disease. No cure exists, and treatments that can modify the disease course are the subject of intensive research. Among the promising strategies is the use of antisense oligonucleotides (ASOs) to target and reduce the levels of mHTT, potentially slowing the progression of the disease. Clinical trials are underway to assess the efficacy and safety of these and other novel therapeutic approaches, including small molecule inhibitors, gene therapy, and stem cell therapy[8].

Despite these advancements, several challenges complicate the management of HD. The broad spectrum of symptoms necessitates a multidisciplinary approach to care, involving neurologists, psychiatrists, physical therapists, and genetic counselors, among others. Psychiatric symptoms, including depression, anxiety, and apathy, are particularly challenging to manage and significantly impact the quality of life of individuals with HD and their families. Furthermore, the genetic nature of HD raises complex ethical and psychological issues related to genetic testing and counseling, especially for at-risk individuals[12].

Huntington's disease exemplifies the challenges and opportunities inherent in the fight against neurodegenerative disorders. Through a combination of genetic research, innovative therapeutic strategies, and comprehensive care approaches, there is hope for improving the lives of those affected by HD. Huntington's disease is regarded as a promising candidate for gene therapy, primarily due to the fact that it is caused by a specific mutation in a single gene, the HTT (huntingtin) gene. This mutation results in the production of an abnormal huntingtin protein, which is responsible for the progressive neurodegeneration observed in the disease. The fact that Huntington's is a monogenic disorder, where a single gene mutation is the clear and sole cause, renders it a more straightforward target for gene therapy interventions (Nance, 2017).

In addition, Huntington's disease follows an autosomal dominant inheritance pattern, whereby the presence of a single mutated gene is sufficient to cause the disease. This simplifies the therapeutic objective, which can be defined as the reduction of mutant HTT gene expression or the correction of the mutation, with the potential for a significant impact on disease progression. As the target is unambiguous and specific, gene therapy approaches such as CRISPR-Cas9 can be employed to directly modify or silence the

mutant gene, which may potentially result in the cessation or deceleration of the disease's progression (Kay, 2020).

The nervous system, which is primarily affected in Huntington's disease, is also relatively accessible for gene therapy. Advances in viral vector technology have enhanced the capacity to deliver therapeutic genes directly to the brain, where they can target the affected neurons. These factors collectively render Huntington's disease an appealing target for gene therapy research, as addressing the root genetic cause has the potential to provide significant therapeutic benefits (Tabrizi et al., 2019).

1.3.2 Gene editing systems.

The CRISPR-Cas9 system, derived from a bacterial immune defense mechanism, has become the most widely used gene editing tool due to its simplicity, efficiency, and versatility. The system is comprised of two principal components: the Cas9 endonuclease and a single-guide RNA (sgRNA). The sgRNA is designed to match the target DNA sequence, thereby guiding Cas9 to the specific location in the genome where it introduces a double-strand break (DSB). The break is then repaired by the cell's natural repair mechanisms, which may include non-homologous end joining (NHEJ) or homology-directed repair (HDR). This process results in targeted genetic modifications [13,14].

Transcription activator-like effector nucleases (TALENs) are another important tool, similar to ZFNs in that they use engineered nucleases to target specific DNA sequences. However, TALENs use transcription activator-like effectors (TALEs) to bind DNA, making them easier to design than ZFNs. Despite their ease of design, TALENs can be large and difficult to deliver into cells, limiting their practical application in some cases (Bogdanove & Voytas, 2011).

The CRISPR-Cas systems, particularly CRISPR-Cas9, have attracted the most attention due to their simplicity, versatility and ease of use. CRISPR-Cas9 uses a guide RNA to direct the Cas9 nuclease to a specific DNA sequence, where it induces a double-strand break. This system has been adapted for a wide range of applications, including gene knockouts, insertions and base editing. Other variants, such as CRISPR-Cas12 and CRISPR-Cas13, have been developed, with Cas12 capable of cutting both single- and double-stranded DNA and Cas13 targeting RNA, further expanding the potential applications of CRISPR-based technologies (Jinek et al., 2012; Abudayyeh et al., 2017

Base editors are a more recent technology in gene editing, allowing the direct conversion of one DNA base to another without causing a double-strand break. This method is particularly useful for correcting point mutations and typically involves a modified version of Cas9 fused to a deaminase enzyme. For example, cytosine base editors (CBEs) convert C to T and adenine base editors (ABEs) convert A to G, providing a precise tool for specific genetic modifications (Gaudelli et al., 2017).

CRISPR-Cas9 technology has been employed to correct mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene in vitro and in vivo. By designing sgRNAs specific to the CFTR gene mutations, researchers have successfully introduced precise edits to restore normal function of the CFTR protein. One notable approach involved the use of CRISPR-Cas9 to correct the F508del mutation, the most common CFTR mutation associated with CF. This correction was achieved in patient-derived stem cells, which were then differentiated into lung epithelial cells displaying restored chloride channel function [15].

In addition to in vitro studies, in vivo applications have demonstrated the potential of CRISPR-Cas9 for CF therapy. For example, adenoviral vectors and lipid nanoparticles have been employed to deliver CRISPR components to the lungs of animal models, resulting in notable improvements in lung function and a reduction in mucus viscosity [16].

CRISPR-Cas9 represents a promising approach to target and disrupt the mutant HTT gene. Researchers have designed single-guide RNAs (sgRNAs) to specifically target the expanded CAG repeats, allowing the Cas9 protein to introduce double-strand breaks (DSBs) in the mutant allele while sparing the normal allele. This selective targeting is of great importance in order to minimise the potential for off-target effects and to preserve the normal function of the HTT protein [17].

In vivo studies utilising mouse models of HD have demonstrated that CRISPR-Cas9 is an effective means of reducing the levels of mutant huntingtin protein in the brain, resulting in improvements in motor function and survival. The delivery of CRISPR components to affected brain regions has been achieved using adeno-associated viruses (AAVs), demonstrating the feasibility of this approach for future clinical applications [17].

Genetic diseases are a major health challenge worldwide, affecting approximately 1 in 17 people [1]. The diversity of these conditions, including rare diseases, requires innovative and effective strategies for the development of genetic therapies.

CRISPR-Cas9 technology has emerged as a revolutionary tool for the therapy of genetic diseases [1]. The genetic changes made possible by CRISPR-Cas9 enable the repair of disease-causing genetic mutations through precise targeting of the genome potentially ushering in a new era of personalised treatments. However, the implementation of CRISPR-Cas9 or any other method of gene editing in the therapy of human diseases faces several challenges [1]. Issues such as the personalised nature of editing, the efficiency of correcting mutations, of-target effects, delivery mechanisms and the potential for recognition of the donor component as a foreign protein, are barriers that need to be overcome. In addition, the ethical considerations of gene editing in humans, including germline modifications and potential long-term effects on the genetic pool, require careful consideration and regulatory oversight. Ensuring equitable access to these potentially life-saving therapies is also a major challenge. As the technology advances, responsible management of these issues, with a focus on maximising benefits while minimising risks, will be crucial. [5]

CRISPR-Cas9 has been used in the correction of mutations associated with Cystic fibrosis (CF) and Huntington disease and has the potential to treat a wide range of other genetic diseases such as, Duchenne muscular dystrophy and sickle cell anaemia. [4].

The future of CRISPR-Cas9 therapeutic applications is bright, with ongoing research expanding its potential and addressing existing limitations. In addition, the development of next-generation CRISPR systems with improved capabilities indicates that genome editing will continue to be a critical tool in the fight against genetic diseases. In the future, the integration of CRISPR-Cas9 into clinical practice is likely to revolutionise genetic medicine, offering hope to patients with genetic disorders and opening up new avenues for treatment strategies. [5]

Transcription activator-like effector nucleases (TALENs) are another important tool, similar to ZFNs in that they use engineered nucleases to target specific DNA sequences. However, TALENs use transcription activator-like effectors (TALEs) to bind DNA, making them easier to design than ZFNs. Despite their ease of design, TALENs can be large and difficult to deliver into cells, limiting their practical application in some cases (Bogdanove & Voytas, 2011).

The CRISPR-Cas systems, particularly CRISPR-Cas9, have attracted the most attention due to their simplicity, versatility and ease of use. CRISPR-Cas9 uses a guide RNA to direct the Cas9 nuclease to a specific DNA sequence, where it induces a double-strand break. This system has been adapted for a wide range of applications, including gene

knockouts, insertions and base editing. Other variants, such as CRISPR-Cas12 and CRISPR-Cas13, have been developed, with Cas12 capable of cutting both single- and double-stranded DNA and Cas13 targeting RNA, further expanding the potential applications of CRISPR-based technologies (Jinek et al., 2012; Abudayyeh et al., 2017).

Base editors are a more recent technology in gene editing, allowing the direct conversion of one DNA base to another without causing a double-strand break. This method is particularly useful for correcting point mutations and typically involves a modified version of Cas9 fused to a deaminase enzyme. For example, cytosine base editors (CBEs) convert C to T and adenine base editors (ABEs) convert A to G, providing a precise tool for specific genetic modifications (Gaudelli et al., 2017).

CRISPR-Cas9 technology has been employed to correct mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene in vitro and in vivo. By designing sgRNAs specific to the CFTR gene mutations, researchers have successfully introduced precise edits to restore normal function of the CFTR protein. One notable approach involved the use of CRISPR-Cas9 to correct the F508del mutation, the most common CFTR mutation associated with CF. This correction was achieved in patient-derived stem cells, which were then differentiated into lung epithelial cells displaying restored chloride channel function [15].

In addition to in vitro studies, in vivo applications have demonstrated the potential of CRISPR-Cas9 for CF therapy. For example, adenoviral vectors and lipid nanoparticles have been employed to deliver CRISPR components to the lungs of animal models, resulting in notable improvements in lung function and a reduction in mucus viscosity [16].

CRISPR-Cas9 represents a promising approach to target and disrupt the mutant HTT gene. Researchers have designed single-guide RNAs (sgRNAs) to specifically target the expanded CAG repeats, allowing the Cas9 protein to introduce double-strand breaks (DSBs) in the mutant allele while sparing the normal allele. This selective targeting is of great importance in order to minimise the potential for off-target effects and to preserve the normal function of the HTT protein [17].

In vivo studies utilising mouse models of HD have demonstrated that CRISPR-Cas9 is an effective means of reducing the levels of mutant huntingtin protein in the brain, resulting in improvements in motor function and survival. The delivery of CRISPR components to affected brain regions has been achieved using adeno-associated viruses AAVs), demonstrating the feasibility of this approach for future clinical applications [17].

2 Mechanism of CRISPR-Cas9

2.1 Discovery and Evolution

The history of the CRISPR-Cas9 system begins in 1987 with the discovery of unusual DNA sequences in Escherichia coli by Ishino[18]. These sequences were later identified as clustered regularly interspaced short palindromic repeats (CRISPR). However, the significance of these sequences remained unclear until the early 2000s. In 2002, Jansen et al. identified CRISPR-associated (Cas) genes, which indicated the system's role in adaptive immunity in bacteria and archaea. [18]

Francisco Mojica and colleagues made a pivotal contribution to the field by suggesting that CRISPR sequences were part of an adaptive immune system, protecting bacteria and archaea from viral infections [123]. Subsequent research demonstrated that CRISPR-Cas systems could acquire new spacers from viral DNA, thereby providing immunity against future infections [18].

2.2 Molecular Mechanism

The Cas-9 enzyme is responsible for cleaving the DNA at a specific locus. This enzyme is able to recognise and bind to the DNA, guided by the single guide RNA (sgRNA). Once bound, it introduces a double-strand break at the targeted site, which is a crucial step in the subsequent DNA repair processes that enable genetic editing. The Cas-9 enzyme can be derived from different bacterial species, with Streptococcus pyogenes (SpCas-9) being the most commonly used variant [20].

The single guide RNA (sgRNA) is a synthetic RNA molecule designed to direct the Cas-9 enzyme to the precise location in the genome where editing is desired. The system comprises two components: a CRISPR RNA (crRNA) that is complementary to the target DNA sequence, and a trans-activating crRNA (tracrRNA) that binds to the Cas-9 enzyme. In practice, these two RNAs are often fused into a single guide RNA (sgRNA) in order to simplify the system [20].

The recognition process is initiated by the sgRNA, which directs the Cas-9 enzyme to the target DNA sequence through complementary base pairing [20]. The sgRNA binds to the target DNA sequence, thus ensuring that the Cas-9 enzyme is positioned correctly for cleavage. Once bound, the Cas-9 enzyme creates a double-strand break in the DNA at a specific site, typically three base pairs upstream of the Protospacer

Adjacent Motif (PAM) sequence. This is a short DNA sequence that is required for the Cas-9 enzyme to recognise and bind to the target DNA. In the case of SpCas-9, the PAM sequence is 5'-NGG-3' where the letter 'N' can be any nucleotide and is followed by two guanine (G) nucleotides [20]. The presence of this PAM sequence near the target DNA site is of critical importance for the Cas-9 enzyme to initiate the binding process. Upon binding to the complementary DNA sequence, the sgRNA prompts the Cas-9 enzyme to scan for the PAM sequence. The recognition of the PAM sequence by the Cas-9 enzyme is a prerequisite for the enzyme to undergo a conformational change that activates its nuclease activity, thereby enabling it to create a double-strand break at the targeted site. This mechanism should ensure that the CRISPR/Cas-9 system only cuts at specific locations in the genome, guided by the presence of the PAM sequence and the sgRNA [109]. The Cas-9 enzyme employs two nuclease domains, HNH and RuvC, to create a double-strand break in the DNA. The HNH domain cleaves the DNA strand complementary to the sgRNA, while the RuvC domain cleaves the non-complementary strand, resulting in a precise double-strand break at the target site. Subsequently, the cell's DNA repair machinery is activated to repair the double-strand break. The break can be repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR), depending on the availability of a homologous DNA template and the phase of the cell cycle. Non-homologous end joining (NHEJ) is a more prevalent and expedient repair mechanism, yet it can result in insertions or deletions (indels) in the DNA sequence. In contrast, homology-directed repair (HDR) enables precise genetic modifications through the use of a donor template [110]

The utilisation of knockout (KO) and knock-in (KI) techniques is of paramount importance and constitutes a fundamental aspect of gene function study and the development of disease models [111]. In constitutive knockout models, genes are permanently disrupted or "knocked out" using methods such as non-homologous end joining (NHEJ), a DNA repair pathway that often introduces small insertions or deletions (indels) at the site of double-strand breaks. Such insertions and deletions can result in a shift in the gene's reading frame, which effectively leads to a loss of function of the gene, or 'knockout'. This approach enables researchers to gain insight into the function of the targeted gene by observing the phenotypic effects that arise in its absence. Conversely, knock-in models entail the insertion of particular genetic sequences into a specific locus within the genome. This enables the introduction of mutations, gene tags, or other modifications, thus facilitating the study of specific genetic variants or the expression of

reporter genes. Knock-in models are particularly useful for mimicking human genetic diseases or studying gene function in a more controlled manner, by replacing or introducing alleles of interest [112].

3 Applications of CRISPR-Cas9 in genetic diseases

3.1 Monogenic Disorders

Recent research on cystic fibrosis (CF) has focused on using CRISPR gene editing to correct the ΔF508 mutation in the CFTR gene, aiming to restore functional chloride channel proteins and improve ion transport. Approaches like CRISPR-Cas9, prime editing, and base editing have shown promise in laboratory and animal models, with some success in restoring CFTR function in patient-derived cells. Despite these advances, challenges such as efficient delivery to lung tissue, minimizing off-target effects, and ensuring long-term safety remain. Continued research aims to refine these techniques, bringing gene-editing therapies closer to a potential cure for CF [105].

Huntington's disease (HD) is another well-known example of a monogenic disorder, but in contrast to CF, it follows an autosomal dominant inheritance pattern. Huntington's disease is caused by an expansion of a CAG trinucleotide repeat within the HTT (Huntingtin) gene, which is located on chromosome 4. This expansion results in the production of a mutant huntingtin protein with an elongated polyglutamine tract, which has a toxic effect on neurons, particularly in the striatum and cortex of the brain. The disease is characterised by a progressive decline in motor control, cognitive abilities, and psychiatric health, with symptoms typically manifesting in mid-adulthood. The length of the CAG repeat expansion correlates with the age of onset and severity of the disease; longer repeats are associated with earlier onset and more rapid progression. Huntington's disease is ultimately fatal, with patients typically succumbing to complications such as pneumonia, heart failure, or other infections [9].

Monogenic disorders such as cystic fibrosis (CF) and Huntington's disease (HD) serve to illustrate the considerable impact that single-gene mutations can have on human health. Notwithstanding the evident genetic aetiology of these conditions, their clinical management remains a significant challenge. However, recent advances in molecular genetics have led to the development of targeted therapies that aim to address the underlying genetic defects. For example, CFTR modulators have been developed to enhance the function of the defective CFTR protein, thereby improving outcomes for patients with specific mutations [106]. Similarly, research into gene silencing techniques, such as RNA interference and antisense oligonucleotides, offers the potential for the treatment of Huntington's disease by reducing the expression of the mutant huntingtin protein [9].

3.2 Complex Genetic Diseases

The convergence of Chimeric Antigen Receptor (CAR) T-cell therapy and CRISPR/Cas9 genome-editing technology has opened new avenues in cancer immunotherapy. The efficacy of CAR T-cell therapy in treating refractory or relapsed malignancies, particularly haematological cancers such as acute lymphoblastic leukaemia (ALL) and multiple myeloma, is remarkable, with response rates as high as 80-100%. Despite these successes, several challenges persist. These include the insufficient quantity and poor quality of autologous T cells, CAR T-cell exhaustion, suppressive tumour microenvironments, and potential self-killing or uncontrollable proliferation of CAR T cells [21].

CRISPR/Cas9 technology is being employed to address these challenges and advance CAR T-cell therapy. The principal applications of CRISPR/Cas9 in this field are the production of allogeneic universal CAR T cells. One of the most significant advancements is the development of "off-the-shelf" universal CAR T cells derived from healthy donors, which can overcome the limitations of autologous CAR T cells. By knocking out genes responsible for T-cell receptors (TCRs) and human leukocyte antigens (HLAs), researchers can prevent graft-versus-host disease (GVHD) and rejection of infused allogeneic T cells. Studies have demonstrated the feasibility of generating universal CAR T cells through the simultaneous knockout of TRAC (TCRα constant) and B2M (beta-2 microglobulin) loci using CRISPR/Cas9, achieving high editing efficiency and potent antitumor activity [21].

Another significant advancement is the disruption of inhibitory signalling molecules. To enhance the potency of CAR T cells, CRISPR/Cas9 has been employed to disrupt inhibitory receptors such as PD-1 and CTLA-4, which are upregulated in exhausted T cells within the tumour microenvironment. For instance, CAR T cells with triple knockouts of TRAC/TRBC (TCRβ constant), B2M, and PD-1 have demonstrated enhanced antitumor functionality compared to dual knockout cells [22].

The use of CRISPR/Cas9 enables the exploration of safer and more controllable CAR T cells. This encompasses the incorporation of safety switches and suicide genes into CAR T cells to mitigate life-threatening toxicities such as cytokine release syndrome (CRS) and on-target/off-tumor effects [113]. The insertion of an inducible caspase-9 (iC9) suicide gene allows for the selective elimination of CAR T cells in the event of severe adverse effects, thereby enhancing the safety and controllability of the therapy.

Furthermore, CRISPR/Cas9 is being investigated to generate antigen-specific CAR T cells for the treatment of acute myeloid leukemia (AML) and T-cell malignancies. For example, the use of CRISPR-mediated knockout of the CD33 gene in hematopoietic stem cells enables the delivery of effective CAR T-cell therapy against AML without the risk of destroying normal myeloid cells [21,22].

3.2.1 CRISPR-Based Genome Editing for Drug Target Prioritization and Disease Model Development

CRISPR-based genome editing tools are of significant value in the prioritization of candidate drug targets, as they enable precise and efficient genetic modifications. These tools permit the systematic knockout of genes, thereby enabling the study of their functions and the identification of potential drug targets. By observing the phenotypic effects of gene disruptions, researchers can identify genes that play critical roles in disease pathways and prioritize them for drug development [115]. CRISPR/Cas9 can also be employed in high-throughput genetic screens to identify genes that, when altered, affect cell viability, proliferation, or drug sensitivity. This approach facilitates the discovery of novel drug targets and the elucidation of the genetic dependencies of various diseases [116]. Furthermore, CRISPR/Cas9 enables pathway analysis by targeting multiple genes simultaneously, thus allowing researchers to elucidate the contributions of different genes to disease processes and identify key regulatory nodes that could serve as therapeutic targets. Furthermore, CRISPR/Cas9 enables the creation of precise disease models by introducing specific mutations or gene edits that mimic human diseases. These models are invaluable for evaluating the efficacy of potential drug targets in a controlled environment [21,22].

3.2.2 Challenges and Considerations in the Therapeutic Application of CRISPR/Cas9 Technology

Despite the considerable promise of CRISPR/Cas9 technology in the field of therapy development, a number of challenges must be addressed. One of the most significant concerns associated with CRISPR/Cas9 is the potential for off-target effects [22]. These occur when technology introduces unintended mutations at sites other than the intended target, which can lead to several undesirable consequences. The introduction of double-strand breaks (DSBs) by CRISPR/Cas9 can lead to large deletions and complex genomic rearrangements, which have an impact on genomic stability and may promote

the induction of off-target mutations [22]. This includes the activation of oncogenes or the disruption of tumour suppressor genes. Strategies to minimise these off-target effects include optimising sgRNA design and Cas9 activity, as well as using high-fidelity Cas9 variants[53]. Approaches such as base editing and prime editing, which do not rely on double-strand breaks, are also being developed. In the treatment of genetic diseases, researchers have used base editing to correct single point mutations responsible for genetic disorders. For example, base editing has been used in the laboratory to correct the mutation responsible for sickle cell anaemia by changing an A-T base pair to a G-C pair, effectively restoring normal gene function.

In preclinical studies, Prime Editing has been used to correct mutations associated with Tay-Sachs disease. The technique precisely edits the HEXA gene mutation responsible for the disease in human cells, offering a potential therapeutic approach for treating this devastating neurodegenerative disease[122].

The efficient and safe delivery of CRISPR/Cas9 components into target cells remains also a significant challenge [22]. A variety of delivery methods, including viral vectors, electroporation, and lipid or protein nanoparticles, are currently under investigation with the aim of enhancing the efficiency of delivery while minimizing toxicity and immune responses.

4 Delivery Methods for CRISPR-Cas9

4.1 Viral Vector Systems

One of the most significant challenges in the field of gene editing is the development of delivery systems that provide stable and long-term expression while minimising the risks associated with genomic integration[28,29].

Adeno-associated virus (AAV) has emerged as a popular viral vector for gene therapy due to its low immunogenicity and effective therapeutic outcomes in both animal models and clinical trials. AAV can integrate specifically into the AAVS1 site on human chromosome 19, a safe genomic location for stable gene expression. The limited packaging capacity of AAV (up to 4.7 kb) restricts the delivery of full-length genes, often necessitating the use of truncated versions or multiple vectors. Despite these limitations, AAV vectors have demonstrated efficacy in preclinical models and clinical trials for the treatment of various genetic disorders [25]. The current limitations of Adeno-Associated Virus (AAV) vectors in gene therapy are predominantly associated with immunogenicity and toxicity. One of the most significant challenges is the immune response that some patients develop against the AAV capsid or the transgene product. This immune response, particularly from T cells, can result in the inflammation and rejection of transduced cells, thereby reducing the efficacy of the therapeutic intervention, particularly in the liver [96]. Furthermore, B cells have the capacity to produce neutralising antibodies against the AAV capsid. This not only impedes the successful reapplication of the vectors but can also neutralise the transgene product itself, thereby complicating the therapy [38]. Another significant challenge is the activation of the complement system, particularly at high doses of AAV vectors [96]. This can result in the development of thrombotic microangiopathy (TMA), a severe condition characterised by the presence of haemolytic anaemia, low platelet counts and kidney damage. Thrombotic microangiopathy (TMA) has been observed in clinical settings and, in some cases, has resulted in fatalities despite the implementation of intervention strategies.

Additionally, hepatotoxicity represents a significant concern, particularly in patients with pre-existing liver conditions. The administration of high doses of AAV vectors has resulted in severe liver damage, which in some cases has been fatal [97]. In some cases, this hepatotoxicity is unresponsive to immunosuppressive treatments, which highlights the difficulty in managing these adverse effects [38]. Another observed complication is dorsal root ganglia (DRG) toxicity, which has been documented in non-

human primates and in some human trials [38] This condition involves neuronal damage and inflammation, which can result in significant neurological side effects. Immunosuppressive drugs have been employed to dampen some of these immune responses, yet they are not universally efficacious, and it remains challenging to predict which patients will benefit from such interventions [38]. Developing more efficient AAV vectors that achieve therapeutic effects at lower doses could potentially reduce these risks, though this remains an ongoing area of research [38].

Adenovirus and lentivirus vectors are also frequently employed for the delivery of CRISPR/Cas systems. Adenoviral vectors have the capacity to accommodate up to 8 kb and are highly efficient at delivering genes into a variety of cell types. Nevertheless, their utilisation is frequently constrained by robust immune responses, which can diminish the duration of gene expression and precipitate inflammation [25].

Lentiviral vectors, which integrate into the host genome, can deliver 8-10 kilobases (kb) or more. This allows them to deliver larger genes that AAVs cannot accommodate, or to carry multiple genes within a single vector [25]. They are particularly useful for stable gene editing in both dividing and non-dividing cells. Nevertheless, the potential for insertional mutagenesis and oncogenesis remains a significant concern with lentiviral vectors. Lentiviral vectors integrate their genetic material directly into the host genome, which carries the risk of insertional mutagenesis [96]. This implies that the insertion may occur randomly within the host's DNA, with the potential to disrupt essential genes. In the event of the lentiviral vector inserting in close proximity to or within an oncogene or tumour suppressor gene, it has the potential to disrupt the normal functioning of cells, leading to uncontrolled cell division and the subsequent development of cancerous cells [39]. Recent developments have included the introduction of integrase-deficient lentiviral vectors, which aim to mitigate the aforementioned risks by preventing genomic integration [25].

4.2 Non-Viral Vector Systems

Electroporation is a process whereby an electric field is applied to cells to increase membrane permeability, thereby allowing components of the CRISPR system to enter the cells [26]. This method is effective for ex vivo applications but can cause cell damage and is less suitable for in vivo applications due to its invasiveness and limited tissue specificity.

Lipid nanoparticles (LNPs) have gained attention for their ability to encapsulate and deliver CRISPR components efficiently. LNPs protect CRISPR components from degradation and can be modified for targeted delivery to specific cell types. The development of improved LNP formulations has enhanced their stability, biocompatibility, and delivery efficiency, rendering them a promising non-viral vector for gene editing [26].

Hydrodynamic injection is a method of introducing CRISPR components into cells by rapidly injecting a large volume of solution into the bloodstream. This creates transient pores in cell membranes, allowing the components to enter. This method has been demonstrated to facilitate the delivery of CRISPR components to the liver with efficacy; however, its applicability to other tissues and the potential for inducing tissue damage remain significant limitations [27].

4.3 Challenges in Delivery

Challenges in the clinical delivery of CRISPR/Cas components for gene therapy include the need for vector optimization, purification challenges, transient expression, enhancement of packaging capacity, regulation of Cas9 expression (on and off systems), minimisation of insertional mutagenesis, and the utilisation of personalised medicine approaches.

Vector optimization entails the engineering of viral vectors with heightened specificity and diminished immunogenicity, with the objective of enhancing delivery efficiency and reducing immune responses. This encompasses the modification of viral capsids to circumvent the immune system and enhance tissue targeting [28]. Temporary immunosuppression can be employed during the delivery of viral vectors to prevent immune responses. However, this approach carries inherent risks and must be carefully managed to avoid compromising the patient's overall immune function. The repeated administration of viral vectors is often necessary for the sustained expression of genes. Strategies to mitigate immune responses to repeated doses include the use of different serotypes of the same virus or the implementation of alternative delivery methods for subsequent treatments [28,29].

The enhancement of the packaging capacity of viral vectors will permit the delivery of larger or multiple CRISPR components, which is of paramount importance for the execution of complex gene editing tasks [28].

The controlled and transient expression of Cas9 minimises off-target effects and reduces the risk of unintended genetic modifications. This can be achieved by inducible promoters or self-limiting gene circuits [124].

The development of non-integrating viral vectors or the utilisation of targeted integration techniques serves to reduce the risk of insertional mutagenesis associated with viral delivery.

Personalized medicine approaches implies the tailoring of viral vectors to the genetic backgrounds of individual patients and can enhance the precision and effectiveness of CRISPR/Cas9 therapies [29]. The process of tailoring viral vectors to the genetic backgrounds of individual patients involves the customisation of the vectors to better align with the specific genetic characteristics of each patient. Such customisation can enhance the precision and effectiveness of CRISPR/Cas9 therapies by ensuring that the delivery system is optimised for the patient's unique genomic landscape [98]. To achieve this, it is first necessary to perform a detailed analysis of the patient's genetic background. This entails the identification of any specific mutations, variations, or polymorphisms that may influence the interaction of the CRISPR/Cas9 system with the patient's DNA. By gaining an understanding of these genetic nuances, it is possible to select or engineer viral vectors that are more efficient at targeting the desired DNA sequences in the patient's genome [99]. For instance, some patients may have distinctive single nucleotide polymorphisms (SNPs) that could potentially influence the binding efficacy of the CRISPR/Cas9 components. In such cases, the guide RNA (gRNA) sequences within the viral vector can be designed or modified to ensure precise targeting and to avoid off-target effects that could lead to unintended genetic modifications. Furthermore, the selection of a viral vector (such as an AAV, lentivirus, or adenovirus) may be influenced by the patient's antibody profile, with the objective of ensuring that the vector is less likely to be neutralised by antibodies present in the patient and can deliver the CRISPR/Cas9 components effectively [100]. It is of paramount importance to ascertain the pre-existing antibodies present in the patient, as these may neutralise the viral vector before it can deliver the CRISPR/Cas9 components to the target cells. This is particularly pertinent in the context of vectors such as AAV (adeno-associated virus), adenovirus and lentivirus, which are frequently employed in gene therapy.

It is possible that many people may have been previously exposed to these viral vectors or their relatives (in particular, adenoviruses, which are the viruses responsible for the common cold), leading to the development of neutralising antibodies. The

presence of high levels of antibodies in the patient's immune system could result in the rapid destruction of the viral vector, rendering it ineffective for gene delivery. Consequently, immune profiling in this context entails the assessment of the patient's antibody response to the viral vectors under consideration for therapy. This approach facilitates the selection of a vector that is less susceptible to neutralisation by the immune system and ensures the effective delivery of the CRISPR/Cas9 machinery to the target cells.

As mentioned before, the integration of viral DNA into the host genome has the potential to disrupt endogenous genes and regulatory elements, which could result in the development of cancer or other genetic disorders. Some viral vectors, such as adenoviruses, do not integrate into the host genome, resulting in transient expression of CRISPR components and requiring repeated administrations for sustained effect. The large-scale production and purification of these viral vectors are complex and costly, which limits their widespread use in clinical settings [29].

Extracellular vesicles (EVs) are emerging as a promising alternative for the delivery of CRISPR/Cas9 components. Extracellular vesicles (EVs) are small, naturally occurring membrane-bound particles released by cells that transfer proteins, lipids, and nucleic acids between cells. These advantages include safety, cost-effectiveness, targeting capability, non-integrating nature, and versatility [28,29].Extracellular vesicles (EVs) are biocompatible and less immunogenic than viral vectors, reducing the risk of adverse immune responses. EVs can be produced from readily available cell sources, obviating the need for the complex manufacturing processes associated with viral vectors. EVs can be engineered to display targeting ligands on their surface, thereby enhancing their ability to deliver CRISPR/Cas9 components to specific cell types or tissues. EVs do not integrate into the host genome, thereby reducing the risk of insertional mutagenesis. EVs can transport a diverse range of cargo, including proteins, mRNA, and ribonucleoprotein complexes, thereby offering versatility in the delivery of various CRISPR/Cas9 components [28,29].

Despite the potential of EVs and viral vectors, achieving efficient and safe in vivo delivery of CRISPR/Cas9 still presents several challenges. These include ensuring that the delivery is targeted with sufficient specificity, that the delivery is efficient, that off-target effects are minimised, that the expression is maintained over an extended period, that the regulatory and ethical considerations are addressed, that the delivery is scalable and that manufacturing is feasible [123]. Minimizing off-target effects requires

the implementation of precise targeting mechanisms and the rigorous validation of delivery systems. High levels of delivery efficiency are required in order to ensure effective gene editing. This process necessitates the optimisation of the delivery vehicle and administration routes in order to achieve the greatest possible uptake by target cells [28,29].

The utilisation of CRISPR/Cas9 in humans has the potential to give rise to significant regulatory and ethical concerns especially considering recent data showing that off-target effects are frequent and serious [124]. The assurance of the safety, efficacy, and ethical utilisation of gene editing technologies necessitates the establishment of robust regulatory frameworks and the engagement of the public [28,29].

Isogenic control iPSCs are generated using the CRISPR/Cas9 system to correct the mutations responsible for Huntington's Disease. These genetically corrected isogenic lines are invaluable for studying disease pathogenesis and for the development of therapeutic strategies. By providing a genetic background identical to the diseased cells, except for the mutation, these isogenic controls enable precise comparison and better understanding of the disease [30].

In addition to CRISPR/Cas9, other genome editing approaches, including Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Peptide Nucleic Acid (PNA), are employed. These methods offer different mechanisms for DNA binding and gene regulation, thereby expanding the range of tools available for genomic modifications. Each approach has distinctive advantages and applications, thereby enabling researchers to select the most suitable method for their specific requirements [30].

CFTR overexpressing cell models are frequently employed in the study of CFTR mutants. These heterologous cell models, where a CFTR cDNA or minigene is overexpressed, permit the functional study of CFTR mutants in the absence of endogenously expressed wild-type CFTR. Immortalised cell lines, such as Human Embryonic Kidney (HEK)293T cells or Fischer Rat Thyroid (FRT) cells, are frequently employed in CF research. These cell lines can be differentiated into polarized epithelium for electrophysiological read-outs, thereby providing a robust system for the analysis of CFTR function and potential therapeutic interventions [31].

The combination of different cell and animal models allows for a more comprehensive evaluation of novel gene editing strategies in CF research. Each model offers distinctive advantages, and their integration can facilitate a more comprehensive

understanding of disease mechanisms and therapeutic efficacy. This combination approach is of particular importance in the translation of proof-of-concept studies into viable clinical therapies [30,31].

4.4 Utilizing CRISPR Screens for High-Throughput Analysis of Genes in Hereditary Diseases

CRISPR screens operate by introducing a library of single guide RNAs (sgRNAs) into a population of cells. Each sgRNA targets a specific gene, thereby creating a knock-out or knock-down effect. By examining the phenotypic consequences of these genetic perturbations, researchers can infer the function of the targeted genes. In the context of hereditary diseases, CRISPR screens can be employed to identify novel genes and pathways that contribute to disease development and progression.[23]

One of the principal advantages of CRISPR screens is their capacity for high-throughput analysis. This is particularly useful for identifying genes involved in complex hereditary diseases where multiple genetic factors may be at play. For instance, in instances such as cystic fibrosis or muscular dystrophy, CRISPR screens can identify additional genetic modifiers that influence the severity of the disease and the efficacy of treatment [23].

The process of conducting a CRISPR screen is comprised of a series of critical steps [24]. The initial step is the design and production of the library. A comprehensive sgRNA library, comprising a wide array of genes, is designed, synthesised and packaged into viral vectors for efficient delivery into cells. Subsequently, the sgRNA library is introduced into the target cell population in the cell transduction step. Efficient transduction ensures that each cell receives at least one sgRNA, thereby generating a diverse pool of mutants. Subsequently, the cells are subjected to selection and screening in accordance with the experimental design. For example, in a screen for drug resistance genes, the cells are treated with the drug of interest. Only those cells with relevant genetic alterations will survive, indicating resistance. Subsequently, genomic DNA is extracted from the surviving cells, and the sgRNA sequences are amplified and sequenced in order to identify which genes were targeted in these cells [24].

4.5 Discussion of crispr problems CAS 9

4.5.1 Chromossome loss.

Studies indicate that chromosome loss is a widespread phenomenon across various genomic regions in cells targeted by Cas9 [32]. This loss can persist over weeks in cultured T cells, demonstrating its broad impact on different genomic areas. Tsuchida et al. (2023) conducted systematic analyses revealing that chromosome loss occurs not just at specific sites but generally across the genome, resulting in partial or whole chromosome loss. This finding highlights that chromosome loss is a universal outcome of CRISPR-Cas9 genome editing, affecting diverse target sites throughout the genome [32].

The frequency of chromosome loss in CRISPR-Cas9-edited cells is dependent on the specific guide RNA (gRNA) utilised, with estimates ranging from 5% to 20% of targeted cells. Tsuchida et al. observed that different gRNAs targeting the TRAC locus resulted in varying frequencies of chromosome loss, emphasising the importance of gRNA selection in genome editing experiments. The observed variability in chromosome loss frequency highlights the necessity for meticulous monitoring and validation in CRISPR-Cas9 applications [32].

This persistence raises concerns about the long-term effects of chromosome loss on the functionality and stability of genome-edited cells [32].

The clinical implications of chromosome loss are significant, particularly in therapeutic applications involving CRISPR-Cas9-edited T cells. Tsuchida et al. [32] have highlighted that chromosome loss may interfere with the intended therapeutic effects of engineered T cells, potentially leading to adverse outcomes in patients. This finding emphasises the need for comprehensive studies to assess the influence of chromosome loss in clinical trials and to develop strategies to mitigate its effects.

One approach to mitigating chromosome loss in CRISPR-Cas9-engineered T cells is to modify T cell preparation protocols prior to clinical application. Tsuchida et al. [32] developed a modified protocol that significantly reduced chromosome loss while preserving genome editing efficacy. The protocol involved the introduction of Cas9 RNPs prior to T cell activation, resulting in elevated expression of p53, a key protein involved in DNA damage response. The elevated p53 levels were found to correlate with reduced chromosome loss, thus suggesting a potential mechanism for mitigating this genotoxicity in clinical applications.

The principal mechanism by which chromosome loss occurs in cells that have been edited using the CRISPR-Cas9 system is the formation of double-strand breaks (DSBs) at the site of Cas9 action. In the absence of appropriate repair, these DSBs can give rise to extensive deletions or chromosome fragmentation. The study by Tsuchida et al. [32] employed single-cell RNA sequencing (scRNA-seq) to identify reduced gene dosage, a marker of chromosome loss, in cells treated with TRAC-targeting gRNAs. The study revealed the occurrence of both partial and whole chromosome loss, indicating that DSBs at specific loci could result in varying degrees of chromosomal damage.

The research presented in the document identifies the potential for chromosome-arm truncations to result from Cas9-induced double-strand breaks (DSBs), particularly in proximity to centromeres. This can result in chromosome loss and other forms of genomic instability, including aneuploidy and chromosome mis-segregation. In the event that the DSBs are not correctly repaired, these truncations and large-scale chromosomal rearrangements have the potential to compromise the viability of edited cells and may present risks in therapeutic applications [117].

The persistence of chromosome loss over time, particularly in long-term culture or in vivo settings, underscores the need for rigorous monitoring of edited cells. This persistence raises concerns about the long-term safety and stability of CRISPR-Cas9-engineered cells, especially when used in clinical therapy. Understanding the mechanisms that drive chromosome loss is critical to developing strategies that can mitigate these risks.

4.5.2 P53 Expression

The activation of p53 following DNA double-strand breaks (DSBs) is a well-documented phenomenon [34]. Upon the recognition of double-strand breaks (DSBs), p53 induces the transcription of genes involved in DNA repair and cell cycle arrest, including p21. This arrest primarily occurs in the G1 phase, allowing cells time to repair DNA damage before proceeding with division. In the context of gene editing, this response can be disadvantageous. Prolonged cell cycle arrest can significantly reduce the proliferation of edited cells, thereby limiting the overall efficiency of the gene editing process [35]. Furthermore, in the event of irreparable damage, p53 can induce apoptosis, resulting in the programmed death of edited cells and a further reduction in the pool of viable, corrected HSPCs [35].

One of the unanticipated consequences of p53 activation during gene editing is the potential enrichment of p53-deficient cells. These cells, which have mutations in the TP53 gene which codes for p53, may survive and proliferate preferentially under the selective pressure of a high DNA damage response environment. This outcome is of particular concern given that TP53 is one of the most frequently mutated genes in cancer. The enrichment of p53-deficient cells may predispose patients to oncogenesis post-transplantation, thereby raising significant safety concerns for the clinical applications of gene editing technologies [35].

The activation of p53 and the associated DNA damage response (DDR) can significantly impair the repopulation capacity of genetically edited hematopoietic stem and progenitor cells (HSPCs) [36]. The capacity of haematopoietic stem and progenitor cells (HSPCs) to self-renew and repopulate the haematopoietic system is of paramount importance for the long-term success of gene therapies targeting blood disorders. Nevertheless, p53-induced cell cycle arrest and apoptosis result in a reduction in the number of functional HSPCs available for engraftment. Studies have demonstrated that the extent of the DNA damage response (DDR) is correlated with a reduction in colony-forming units (CFUs) and overall engraftment efficiency [36]. For example, electroporation of HSPCs with low-specificity sgRNA, resulting in multiple DSBs (double-strand breaks) leads to activation of p53 and this leads to a significant upregulation of p21 and a corresponding reduction in CFU output and cell number [37]. High levels of p21 result in RB-E2F complex formation and downregulation of a large number of cell cycle genes. The reduced expression of the many regulators leads to cell cycle arrest [42].

One strategy for mitigating the deleterious effects of p53 activation is the use of highly specific single guide RNAs (sgRNAs) that minimize off-target double-stranded breaks (DSBs) [37]. It has been demonstrated in studies that high-specificity sgRNAs can reduce p53 activation, thereby improving the clonogenic output and survival of edited HSPCs. This strategy is based on the precise targeting capabilities of CRISPR-Cas9, which reduces the overall burden of DNA damage and subsequent DNA damage-responsive cellular processes. This can be achieved through the co-delivery of dominant-negative p53 mutants, such as GSE56, which can suppress p53 transcriptional activity. Research has demonstrated that transient p53 inhibition can reverse the proliferation delay, augment the number of colony-forming units, and enhance the efficiency of

engraftment in immunodeficient mice [37]. This approach does not compromise chromosomal stability or significantly increase mutations in cancer-associated genes.

The selection of the donor template and the chosen delivery method can also influence the extent of p53 activation. For example, rAAV6 vectors are frequently employed for the delivery of HDR templates, yet they can exacerbate DDR and p53 activation [37]. The use of integration-defective lentiviral vectors (IDLVs) or single- and double-stranded DNA templates can result in a reduction in p53 activation and an improvement in the outcomes of gene editing. Studies have demonstrated that the combination of IDLVs and GSE56 results in a higher clonogenic capacity and more stable HDR-edited populations compared to rAAV6 [37]. The advent of novel gene editing technologies, such as base editing and prime editing, has provided alternative avenues that circumvent the necessity of inducing Double Strand DNA Breaks. These techniques employ Cas9 nickase fused to deaminase enzymes or reverse transcriptase to introduce precise genetic modifications without triggering a full DNA damage response (DDR) [42]. For instance, base editing can achieve high editing efficiencies with minimal p53 activation, thus preserving the functionality of haematopoietic stem and progenitor cells (HSPCs) [43]. Prime editing, which also avoids double-strand breaks, has demonstrated promising results in achieving substantial editing efficiencies with reduced DNA damage response in preclinical models [44].

Nevertheless, the challenges presented by p53 activation require meticulous preclinical assessment and refinement of the editing protocols. The results of recent clinical trials for conditions such as sickle cell disease (SCD) and β-thalassaemia have demonstrated a range of outcomes, including varying degrees of success and complications [46]. For instance, the Graphite Bio trial, which employed rAAV6 for HDR, was terminated due to the occurrence of severe adverse events, thereby underscoring the necessity for the development of safer and more efficacious strategies [47].In order to fully realize the potential of HSPC gene editing therapies, future research must focus on refining the balance between HDR and NHEJ, minimizing p53 activation, and enhancing the overall efficiency and safety of the editing process [48]. The development of novel delivery platforms, improved sgRNA design, and innovative gene editing techniques will be crucial for the advancement of these therapies. Furthermore, it is of paramount importance to gain a deeper understanding of the long-term implications of gene editing on HSPC function and to ensure the genomic stability of edited cells in order to advance these therapies to the clinic.

5 Off-Target Effects and Strategies for Mitigation

5.1 Understanding Off-Target Effects

The probability of off-target effects in CRISPR-Cas9 technology is contingent upon a number of variables. One critical factor is the specificity of the guide RNA (gRNA) sequence. The gRNA is responsible for directing the Cas9 enzyme to the specific DNA sequence that requires editing. Consequently, the sequence specificity of the gRNA is crucial for minimising off-target cuts. A well-designed gRNA sequence that exhibits minimal similarity to other genomic sequences markedly reduces the probability of unintended interactions, thereby decreasing the likelihood of off-target effects [49]. Another significant factor is the degree of mismatch tolerance exhibited by the Cas9 protein. The Cas9 protein is capable of tolerating certain mismatches between the gRNA and the target DNA, which can result in unintended cleavage events at sites that are similar, but not identical, to the intended target. The degree of tolerance varies among different Cas9 variants, with some engineered versions exhibiting reduced off-target activity due to enhanced specificity [49]. Furthermore, the genomic context, including chromatin accessibility, is of great consequence. DNA regions that are tightly packed in chromatin may be less accessible to Cas9, thereby reducing the probability of both on-target and off-target cuts. Conversely, regions of open chromatin, where DNA is more exposed, may be more susceptible to off-target effects. Thus, the genomic context, including chromatin structure and epigenetic modifications, is a critical determinant of where off-target effects might occur [50].

The potential for off-target effects to have serious implications is particularly pronounced in instances where unintended mutations result in the disruption of important genes. Such disruptions have the potential to contribute to the development or exacerbation of diseases such as cancer. To illustrate, an off-target cut in a tumour suppressor gene may result in its inactivation, thereby potentially promoting uncontrolled cell growth [51]. Furthermore, multiple off-target cuts can result in larger genomic alterations, including deletions, inversions, or translocations of DNA segments. Such alterations contribute to genomic instability, which is a hallmark of numerous cancers and other genetic disorders. The accumulation of such genomic alterations can have a deleterious impact on cellular integrity and function. In the context of therapeutic applications, the potential risks associated with off-target effects are a significant concern. The occurrence of unintended edits has the potential to elicit an immune response or even

contribute to tumour development, thereby raising concerns regarding the safety of CRISPR-based therapies. The potential for adverse effects underscores the necessity for comprehensive validation and safety assessments in clinical settings. Furthermore, the unpredictability of off-target effects represents a significant concern in the context of germline editing, where changes are inheritable[51]. The potential for off-target mutations to be introduced into germline cells and subsequently passed on to future generations gives rise to significant ethical concerns regarding the long-term implications of such modifications. These concerns underscore the necessity for rigorous ethical guidelines and oversight in the utilisation of CRISPR technology, particularly in the context of human germline editing.

5.2 Strategies for Reducing Off-Target Activity

5.2.1 Advances in gRNA Design

The efficacy and precision of CRISPR-Cas9 gene editing are largely contingent upon the design of guide RNA (gRNA) sequences, which direct the Cas9 enzyme to specific sites within the genome. A significant challenge in this technology is the minimisation of off-target effects, which occur when the gRNA binds to non-target regions, leading to unintended genetic modifications. To address this issue, considerable effort has been invested in optimising gRNA sequences, reducing their similarity to non-target genomic regions and thereby enhancing the specificity of the CRISPR-Cas9 system [52].

Computational algorithms have been developed to facilitate the design of optimised gRNAs. These algorithms analyse the entire genome in order to identify potential off-target sites, employing a range of criteria including sequence homology, thermodynamic properties and chromatin accessibility. By incorporating factors such as mismatch tolerance and binding energy, these algorithms can generate gRNA sequences with a reduced likelihood of off-target binding, thereby increasing the precision of gene editing [52].

A noteworthy advancement in gRNA design is the introduction of truncated gRNAs (tru-gRNAs). In contrast to the typical 20-nucleotide length of standard gRNAs, tru-gRNAs are shorter by a few nucleotides. This truncation results in a reduction in the binding affinity of the gRNA to the target site, particularly in the presence of mismatches, which in turn decreases the likelihood of off-target effects. Tru-gRNAs have been

demonstrated to retain high on-target efficacy while markedly enhancing specificity, rendering them a valuable instrument in contexts necessitating precise genome modifications [53].

In addition, chemical modifications to gRNAs represent an emerging strategy for enhancing their stability and reducing off-target activity. For instance, the incorporation of 2'-O-methyl and phosphorothioate linkages at the 5' and 3' ends of the gRNA can safeguard it from degradation by nucleases, thereby extending its activity within the cell. Furthermore, these modifications have the additional effect of reducing the binding of gRNAs to mismatched DNA sequences, thereby further minimising off-target cleavage. This approach has been particularly beneficial in the context of therapeutic applications, where the prolonged activity and high specificity of gRNAs are of paramount importance[54].

5.2.2 Modified Cas9 Proteins

Research has been conducted into the engineering of Cas9 variants with enhanced specificity and diminished off-target effects. One of the most significant developments in this field is the creation of high-fidelity Cas9 variants, such as SpCas9-HF18 [48]. This variant was created through the introduction of specific point mutations within the Cas9 protein, which serve to reduce its affinity for non-target DNA sequences. By reducing non-specific interactions, SpCas9-HF1 achieves higher fidelity, thereby reducing the likelihood of off-target cleavage and improving the safety of CRISPR-based interventions[55].

Another engineered Cas9 variant is the enhanced specificity Cas9 (eSpCas9), which incorporates structural changes that weaken the interactions between the Cas9 protein and the non-target DNA strand. This modification serves to reduce the likelihood of off-target cleavage, whereby the enzyme is rendered more dependent on perfect base-pairing with the gRNA. The eSpCas9 variant offers superior specificity in comparison to wild-type Cas9, [55].

As mentioned previously, the use of nickase Cas9 is another innovative approach which has been designed to enhance the precision of genome editing. In contrast to the wild-type Cas9, which induces double-strand breaks (DSBs) by cleaving both DNA strands, Nickase Cas9 has been engineered to cut only one strand of the DNA.

5.2.3 Alternative Strategies

Besides the modifications of gRNAs and Cas9 proteins, alternative strategies have been developed with the objective of further mitigating off-target effects in CRISPR-Cas9 systems. One such strategy employs catalytically inactive Cas9 (dCas9), a variant of Cas9 that lacks nuclease activity and therefore does not induce double-stranded breaks (DSBs). dCas9 can be fused with other effector proteins to modulate gene expression or epigenetic states without resulting in a permanent alteration of the DNA sequence. This approach is particularly advantageous in contexts where gene regulation is the primary objective, as it circumvents the potential issue of introducing off-target mutations[55].

Another CRISPR-associated nuclease that has gained attention as an alternative to Cas9 is Cas12a, also known as Cpf1. There are several notable differences between Cas9 and Cas12a, including their recognition of distinct protospacer adjacent motifs (PAMs) and their capacity to process their own guide RNA (gRNA) from a precursor transcript. Furthermore, Cas12a produces staggered DSBs with 5' overhangs, which may be more conducive to certain types of genetic modifications. The distinctive DNA recognition mechanism and PAM sequence of Cas12a can mitigate off-target effects in genomic regions where Cas9 may exhibit reduced specificity, thereby offering a complementary tool for genome editing [118].

Finally, the dual gRNA approach, whereby two gRNAs are designed to target adjacent sequences, represents an additional strategy for enhancing the specificity of CRISPR-Cas9. In this approach, Cas9 is engineered to cleave only when both gRNAs are bound to their respective target sites, thereby ensuring that off-target effects are minimised [125]. The dual approach, which necessitates the binding of two gRNAs, markedly diminishes the likelihood of off-target cleavage, thereby enhancing the overall accuracy of the CRISPR-Cas9 system [54]. This approach may be particularly important in complex genomes such as the human genome where a single gRNA may have multiple potential off-target sites.

5.3 Assessment and Validation

Whole Genome Sequencing (WGS) is a comprehensive method that involves sequencing the entire genome of an organism to detect any genetic mutations, including those that may arise as off-target effects from CRISPR-Cas9 editing. This technique offers an unbiased approach to identifying potential off-target sites, obviating the necessity for prior knowledge of where these effects might occur. However, WGS is

costly and resource-intensive, and may fail to identify low-frequency mutations, particularly in instances where off-target effects manifest in a limited subpopulation of cells. Furthermore, the differentiation between natural genetic variations and CRISPR-induced mutations can prove to be a challenging undertaking, particularly in the context of complex genomes characterised by high genetic diversity [54].

GUIDE-seq (Genome-wide, Unbiased Identification of DSBs Enabled by Sequencing) is a specialised method for the detection of double-strand breaks (DSBs) induced by CRISPR-Cas9 [120]. The method entails the integration of a synthetic double-stranded oligodeoxynucleotide (dsODN) into the genome at double-strand break (DSB) sites, which are subsequently identified and sequenced.

In order to facilitate the incorporation of synthetic double-stranded oligodeoxynucleotides (dsODNs) at double-strand break (DSB) sites prior to the intervention of cellular repair mechanisms, researchers have sought to increase dsODN concentrations and to precisely time their delivery in conjunction with DSB induction. This synchronisation ensures that the template is immediately available at the break site. It should be noted that the temporary inhibition of the cell's default repair pathway, non-homologous end joining (NHEJ), favours homology-directed repair (HDR), which is more likely to incorporate the dsODN. Furthermore, conducting these edits during the S or G2 phase, when HDR activity peaks, increases the chance of successful template integration before cellular repair mechanisms close the break[120].

In GUIDE-seq, the integration of synthetic double-stranded oligodeoxynucleotides (dsODNs) at sites of CRISPR-Cas9-induced double-strand breaks (DSBs) is achieved by harnessing the cell's natural repair mechanisms, specifically the non-homologous end joining (NHEJ) pathway. When CRISPR-Cas9 induces a DSB, the cell's repair machinery quickly attempts to repair the break. By introducing a high concentration of dsODNs immediately after DSB formation, the dsODNs are incorporated into the DSB before the cell can fully repair the site. Because NHEJ does not require sequence homology, it can efficiently integrate the dsODNs as part of the repair process. This approach relies on the rapid and error-prone nature of NHEJ, which is biased towards incorporating the dsODNs, allowing researchers to mark and subsequently sequence the DSB sites across the genome. The success of this method depends on the abundance of dsODNs, which outcompete the cell's intrinsic repair mechanisms, leading to their effective integration into the breaks before repair is complete.

GUIDE-seq is highly sensitive and can detect off-target DSBs at very low frequencies, thereby providing a precise map of off-target sites at the nucleotide level. However, it requires the use of living cells, which restricts its applicability to in vitro experiments and specific cell types. Furthermore, GUIDE-seq may exhibit a propensity to detect off-target effects in genomic regions that are actively transcribed or accessible in the specific cell type under consideration [54,55].

CIRCLE-seq (Circularization for In Vitro Reporting of Cleavage Effects by Sequencing) is an in vitro method for the detection of off-target cleavage sites. It involves the circularization of DNA fragments, which are then subjected to amplification and high-throughput sequencing. This technique does not necessitate the use of living cells, thereby extending its applicability to a diverse array of DNA samples. CIRCLE-seq exhibits high sensitivity and specificity in the detection of off-target cleavage, even at low frequencies. However, as an in vitro method, it may not fully replicate the chromatin environment of living cells, which could affect the detection of some off-target effects. The considerable quantity of data produced by CIRCLE-seq necessitates the utilisation of sophisticated computational tools for accurate interpretation.

Digenome-seq (Digital Genome-wide Sequencing) entails the digestion of genomic DNA in vitro with the CRISPR-Cas9 nuclease, followed by the sequencing of the entire genome to identify cleavage sites. This technique is more cost-effective than whole-genome sequencing (WGS) while still providing genome-wide coverage. Digenome-seq is capable of detecting off-target effects with high sensitivity, particularly when employed in conjunction with high-depth sequencing. However, as with CIRCLE-seq, it may not fully capture the complexity of chromatin structure in living cells, and there is a risk of identifying DNA breaks that do not occur in vivo, which could result in potential false positives [54].

SITE-seq (Selective Identification of Targeted Editing) is a recently developed technique that employs a combination of biochemical approaches and high-throughput sequencing to identify off-target cleavage sites. The technique involves treating genomic DNA with the CRISPR-Cas9 system in vitro, followed by the selective labelling and sequencing of cleaved DNA fragments. SITE-seq is highly accurate in detecting off-target effects and can be adapted to different CRISPR systems and nucleases. However, the technique is technically complex and time-consuming, involving multiple steps such as biochemical labelling and enrichment [55].

BLESS (Breaks Labeling, Enrichment on Streptavidin, and Next-Generation Sequencing) is a direct method for the labelling and sequencing of DNA double-strand breaks (DSBs) within the genome. The technique involves tagging DSBs with biotin, enriching them using streptavidin beads, and then sequencing the tagged fragments in order to identify the cleavage sites. BLESS offers high-resolution mapping of DSBs and is sufficiently versatile to be applied to a range of cell and tissue types. However, the process is labour-intensive, necessitates the use of specialised equipment and expertise, and may have a lower throughput compared to other high-throughput sequencing methods. Furthermore, the method may be prone to detecting breaks in regions of the genome that are more accessible or actively transcribed.

6 Ethical, Legal, and Social Implications (ELSI)

6.1 Ethical Considerations

Ethical Considerations in Germline Editing: Consent, Accessibility, and Unintended Consequences.

The process of germline editing, which entails altering the genetic material of an embryo with the potential for transmission to subsequent generations, has emerged as a pivotal point of contention within the realm of bioethical discourse. The advent of advanced gene-editing technologies, such as CRISPR-Cas9, has ushered in a new era of possibilities and challenges for the scientific community [56]. While this technology holds immense potential for the prevention and treatment of genetic disorders, it also gives rise to a number of significant ethical concerns [57]. These include the potential for unintended genetic consequences, the moral implications of editing human embryos, and the question of equity in access to these technologies[58,59].

6.1.1 Unintended Genetic Consequences

One of the most significant ethical concerns associated with germline editing is the possibility of unintended genetic consequences. The process of editing genes in a human embryo is not yet entirely reliable. Off-target effects, whereby CRISPR-Cas9 cuts DNA at unintended locations, can result in mutations in genes that were not intended to be altered [60]. Such unintended alterations have the potential to give rise to novel genetic disorders or to precipitate the worsening of existing conditions[61]. Moreover, the long-term effects of germline editing remain to be seen. As the edited genes will be passed on to future generations, any adverse effects could have significant and long-lasting consequences. This gives rise to the question of whether it is ethical to proceed with germline editing in the absence of a full understanding of the associated risks[62].

In addition to the aforementioned risks, there is the issue of genetic mosaicism, whereby not all cells in an embryo are edited in the same way. This could result in a situation where some cells possess the edited gene, while others do not, thereby creating a mosaic of genetic material within the individual [63]. The implications of genetic mosaicism remain unclear, but it could result in unpredictable outcomes with regard to health and development [64].

6.1.2 Moral Implications of Editing Human Embryos

The moral implications of editing human embryos represent a significant area of ethical debate[65]. The concept of modifying the genetic composition of a human being prior to their birth gives rise to pivotal inquiries concerning the essence of human existence and the boundaries of human intervention[66]. Some ethicists posit that germline editing constitutes an unacceptable form of "playing God," given that it entails making irreversible alterations to the human genome, which could impact not only the individual but also their descendants[67].

Additionally, there is a concern that germline editing could potentially lead to a form of eugenics, whereby parents may elect to edit their embryos to enhance specific traits, such as intelligence or physical appearance, rather than solely preventing disease[68]. Such developments could result in a society where genetic enhancement becomes a means of social stratification, with those who can afford the technology gaining an unfair advantage over those who cannot[59]. The advent of designer babies, where parents select traits for their children, gives rise to profound ethical concerns pertaining to the commodification of human life and the potential erosion of diversity within the human population[62].

The question of consent is particularly problematic in the context of germline editing. The individuals who are most directly affected by the editing, namely the future children, are unable to consent to the procedure [69]. This gives rise to ethical concerns regarding the rights of future generations and the justifiability of making permanent genetic alterations without their consent[69]. Some ethicists posit that germline editing contravenes the principle of autonomy, as it entails the imposition of irreversible alterations upon individuals who had no input in the matter [68].

6.1.3 Equity of Access

A further significant ethical issue is that of equity of access to germline editing technologies. As with many advanced medical technologies, there is a risk that germline editing will be accessible only to those with the financial means, leading to greater health disparities between socioeconomic groups [70]. The potential for germline editing to become a means of preventing genetic disorders raises concerns that those who cannot

afford the technology may be left at a significant disadvantage, perpetuating cycles of inequality [71].

It is probable that the financial outlay required for germline editing will be unaffordable for a considerable proportion of the global population, particularly those residing in low- and middle-income countries. This gives rise to ethical concerns regarding the distribution of healthcare resources in a manner that is just and fair [72]. The advent of widespread germline editing could potentially exacerbate existing disparities in healthcare access, contributing to a global divide between those who can afford genetic enhancements and those who cannot [73].

The question of how to regulate access to germline editing is also a significant concern. The advent of widespread availability of this technology will necessitate the establishment of transparent guidelines and regulations to ensure its responsible and ethical utilization [69]. This includes the determination of who should have access to the technology and under what circumstances it should be used. The challenge will be to establish a balance between enabling individuals to benefit from the technology and preventing its misuse [68].

6.2 Regulatory Landscape

Regulatory Landscape of Genome Editing Technologies: Global Approaches and Challenges

The advent of genome editing technologies, particularly CRISPR-Cas9, has led to significant advances in the field of genetics, offering the potential to treat and even prevent genetic disorders. However, these developments have also given rise to intricate regulatory challenges. The regulatory frameworks that govern genome editing technologies vary considerably between different countries, reflecting the diverse ethical, legal, and social considerations that underpin these frameworks. This section examines the ways in which different countries are regulating CRISPR-Cas9, noting the discrepancies in policy approaches, the function of international guidelines, and the difficulties of maintaining pace with rapid technological advancements.

6.2.1 Current Regulatory Frameworks

The regulation of genome editing technologies is a complex and fragmented process, with a multitude of regulatory frameworks in place across the globe. In some countries, regulatory bodies have established comprehensive guidelines that govern the research and application of genome editing. In contrast, in other countries the regulatory landscape remains fragmented and underdeveloped[66]. By way of illustration, the United States and the European Union have each established rigorous regulatory processes for gene editing technologies. The U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) are responsible for regulating the clinical application of genome editing technologies, ensuring that they meet the requisite safety and efficacy standards prior to their utilisation in humans [68].

In contrast, other countries have regulatory frameworks that are less comprehensive in scope. In China, for instance, the regulatory oversight of genome editing has been the subject of criticism, with concerns emerging in 2018 when a Chinese scientist announced the birth of the world's first genetically edited babies, prompting global ethical concerns [69]. This incident underscored the necessity for more rigorous regulatory supervision and prompted inquiries into the sufficiency of extant frameworks for addressing the ethical and safety concerns associated with genome editing.

6.2.2 Differences in Policy Approaches

The regulation of genome editing technologies is approached in different ways in different countries, reflecting the unique cultural, ethical, and legal contexts of each [70]. To illustrate, in the United Kingdom, the Human Fertilisation and Embryology Authority (HFEA) has permitted the utilisation of genome editing in research, yet has implemented rigorous regulatory measures concerning its deployment in clinical contexts, notably in the context of editing human embryos [71,72]. This approach strikes a balance between the potential benefits of genome editing research and the necessity to address ethical concerns.

In contrast, the United States has a more decentralised approach to regulation, with multiple agencies, including the FDA, the National Institutes of Health (NIH), and the Recombinant DNA Advisory Committee (RAC), involved in the regulation of genome editing. This has resulted in a more intricate regulatory landscape, wherein

disparate agencies bear overlapping responsibilities, which can occasionally give rise to regulatory lacunae or inconsistencies[71,72].

Japan has adopted a cautious approach, allowing genome editing for basic research but imposing strict regulations on its clinical application. Japanese regulatory authorities have underscored the necessity for comprehensive public consultation and ethical assessment prior to authorising any clinical utilisation of genome editing technologies, reflecting a broader societal apprehension regarding the implications of genetic modification[73].

6.2.3 Role of International Guidelines

In light of the global nature of scientific research, international guidelines assume a pivotal role in shaping the regulatory landscape of genome editing technologies. In this regard, organisations such as the World Health Organization (WHO) and the International Society for Stem Cell Research (ISSCR) have issued guidelines that provide a framework for the ethical and responsible use of genome editing technologies [74]. These guidelines underscore the significance of transparency, public engagement, and the necessity of ensuring that genome editing is conducted in a manner that is safe, ethical, and scientifically sound.

However, the implementation of these international guidelines varies across countries. Some have adopted them as part of their national regulatory frameworks, while others have yet to fully incorporate them. The absence of uniformity in the implementation of international guidelines poses a challenge to achieving global consistency in the regulation of genome editing technologies. Furthermore, the accelerated pace of advancement in genome editing frequently outpaces the capacity of international bodies to update their guidelines, resulting in deficiencies in the regulatory oversight of emerging technologies[75].

6.2.4 Challenges of Keeping Up with Rapid Advancements

One of the most significant challenges in regulating genome editing technologies is the rapid pace at which the field is advancing. The advent of CRISPR-Cas9 and other gene-editing tools has outstripped the capacity of regulatory frameworks to keep pace, giving rise to concerns that existing regulations may prove inadequate for addressing the risks and ethical issues associated with these technologies. It is frequently challenging for

regulatory bodies to achieve an equilibrium between facilitating innovation and guaranteeing that novel technologies are employed in a secure and ethical manner[76,77].

The global nature of scientific research means that regulations in one country can have implications for the entire international community. For instance, the advancement of genome editing technologies in countries with less rigorous regulatory frameworks could result in the phenomenon of "regulatory tourism," whereby researchers or companies relocate to jurisdictions with fewer constraints to conduct contentious experiments. This highlights the necessity for enhanced international collaboration and the harmonisation of regulatory standards to prevent such practices and guarantee that genome editing technologies are employed responsibly on a global scale [78].

The necessity for the continuous updating of regulatory frameworks in order to maintain pace with the latest developments in genome editing. As new techniques and applications emerge, it is imperative that regulators are able to adapt expeditiously in order to address the risks and ethical concerns that they may entail. This necessitates a proactive approach to regulation, with continuous dialogue between scientists, ethicists, policymakers, and the public, in order to guarantee that the regulatory landscape remains pertinent and efficacious[79].

6.3 Societal Impact

6.3.1 Societal Impact of CRISPR-Cas9: Public Perception and Potential Implications

The emergence of CRISPR-Cas9 as a powerful tool for gene editing has not only revolutionised the field of genetics, but has also generated considerable public interest and debate. The societal implications of CRISPR-Cas9 are vast, ranging from its potential to cure genetic disorders to the ethical and social dilemmas it raises. This section examines public perceptions of gene editing, explores the potential for societal divides based on access to genetic therapies, and considers the wider implications of altering human genetics at a population level.

Public opinion on gene editing technologies such as CRISPR-Cas9 is diverse and often polarised . While some see these technologies as a groundbreaking advance with the potential to eradicate genetic diseases, others are concerned about the ethical implications and potential misuse of gene editing . Surveys conducted in different countries have shown that public acceptance of gene editing is influenced by several

factors, including the intended use of the technology, cultural and religious beliefs, and the level of public awareness and understanding of the science behind it [80].

In general, there is greater public support for the use of gene editing to treat serious genetic disorders, particularly if it involves somatic cells, which do not pass on changes to future generations . However, there is considerable concern about the use of CRISPR-Cas9 for germline editing, which involves making changes to the DNA of embryos that can be inherited by future generations . This is largely due to concerns about the long-term consequences of such interventions and the potential for unintended genetic changes that could have far-reaching effects [81].

Public perceptions of gene editing are also shaped by the media, which play a crucial role in framing the narrative around CRISPR-Cas9 . Media coverage of gene editing has ranged from highly optimistic portrayals of its potential to sensationalised accounts of its risks and ethical dilemmas. The way in which the media present information about gene editing can have a significant impact on public opinion, either by promoting greater acceptance of the technology or by exacerbating fears and misconceptions [82].

6.3.2 Potential for Societal Divides

The introduction of gene editing technologies such as CRISPR-Cas9 also raises concerns about the potential for societal divides based on access to genetic therapies. As with many advanced medical technologies, there is a risk that gene editing could become a privilege reserved for the wealthy, leading to increased inequality and social stratification. If access to genetic therapies is determined by socio-economic status, this could lead to a scenario where certain segments of the population are able to improve their genetic traits while others are left behind [83].

Such inequalities could lead to a form of genetic elitism, where those who can afford gene editing have a distinct advantage in terms of health, intelligence and physical ability. This could exacerbate existing social inequalities and create new forms of discrimination based on genetic make-up. The potential for a 'genetic divide' raises significant ethical and social concerns, as it challenges the principles of justice and equity in health care [84].

Commercialization of gene editing could drive a market for genetic enhancement, further entrenching social divides. Companies offering genetic

modifications for non-medical traits, such as enhanced intelligence or athleticism, could appeal to those seeking a competitive advantage, creating a market-driven approach to human genetics that prioritises profit over ethical considerations . This could lead to a society where genetic traits are commodified and access to genetic enhancements becomes a marker of social status [84-85].

The wider implications of altering human genetics at a population level are profound and multifaceted. One of the main concerns is the potential impact on genetic diversity. By promoting certain genetic traits through gene editing, there is a risk of reducing genetic variation within the human population, which could have unforeseen consequences for human health and evolution. Genetic diversity is crucial to the survival and adaptability of species, and any reduction in this diversity could make the human population more vulnerable to disease and environmental change [86].

Another concern is the ethical and philosophical implications of modifying the human genome. The ability to edit human DNA raises fundamental questions about the nature of humanity and the limits of human intervention in the natural world. Some ethicists argue that gene editing is a form of "playing God" because it involves making decisions that could alter the course of human evolution. These ethical dilemmas are compounded by the fact that the long-term consequences of gene editing are still unknown, making it difficult to predict the impact of these technologies on future generations [87].

Public perception of gene editing is also influenced by cultural and religious beliefs, which can shape attitudes towards the acceptability of altering human genetics. In some cultures, there is a strong emphasis on maintaining the natural order and a reluctance to intervene in the genetic make-up of individuals. This cultural resistance to gene editing poses a challenge to the global acceptance and implementation of these technologies, as different cultural values can lead to conflicting views on the ethical use of gene editing [88].

7 Future Directions and Challenges

7.1 Technical Challenges: Addressing Remaining Hurdles in CRISPR-Cas9 Technology

Despite the significant progress made in the development of CRISPR-Cas9, several technical challenges remain that must be overcome to realise its full potential. One of the main concerns is the issue of off-target effects, where the CRISPR-Cas9 system may inadvertently cut DNA at sites other than the intended target. Improving the specificity and accuracy of CRISPR-Cas9 is critical to minimising these risks and ensuring the safety of gene editing.

Another technical challenge is the delivery of CRISPR-Cas9 components into target cells. Efficient and targeted delivery is essential for successful gene editing, especially in clinical settings where the technology may be used to treat genetic disorders. Current delivery methods, such as viral vectors and lipid nanoparticles, have limitations in terms of efficiency, safety and tissue specificity.The development of more advanced and reliable delivery systems is critical for the wider use of CRISPR-Cas9 in medicine[89].

There is also the challenge of achieving precise control over the outcome of gene editing. While CRISPR-Cas9 has been highly effective in creating gene knockouts, more complex genetic modifications, such as precise gene insertions or base editing, require further refinement. Increasing the versatility of CRISPR-Cas9 to enable more sophisticated genetic modifications is an ongoing area of research with significant implications for both basic science and clinical applications [90].

7.2 Emerging Trends: New Developments and Future Trends in Genome Editing

The field of genome editing is evolving rapidly, and several trends are emerging that could shape the future of CRISPR-Cas9 technology. One of the most notable trends is the development of next-generation CRISPR systems, such as CRISPR-Cas12 and CRISPR-Cas13, which offer new capabilities and improved specificity compared to the original CRISPR-Cas9 system. These new systems expand the range of possible applications, including the potential to directly target RNA molecules, which could open up new avenues for treating diseases at the RNA [91]. Emerging trend is the integration of CRISPR-Cas9 with other technologies, such as artificial intelligence (AI) and machine

learning, to improve the efficiency and accuracy of gene editing. AI-driven approaches are being used to predict off-target effects, optimise guide RNA design and improve the overall precision of CRISPR-based interventions. This convergence of technologies is expected to accelerate the development of CRISPR applications and lead to more personalised and effective gene therapies[92].

In contrast, there is growing interest in using CRISPR-Cas9 for applications beyond human health, such as in agriculture and environmental conservation. For example, CRISPR is being explored for its potential to create crops with enhanced traits, such as disease resistance or improved nutritional content, which could contribute to global food security. Similarly, CRISPR-based strategies are being developed to address environmental challenges, such as controlling invasive species or restoring endangered populations, highlighting the broad potential of this technology[93].

7.3 Long-term Outlook: Prospects for CRISPR-Cas9 in Medicine and Broader Applications

In the longer term, the future of CRISPR-Cas9 in medicine is both promising and challenging. One of the most promising avenues for research is the utilisation of CRISPR-Cas9 for in vivo gene editing, whereby the technology could be directly applied to patients with the objective of correcting genetic defects at their source. This approach has the potential to transform the treatment of genetic disorders, offering the possibility of permanent cures for conditions that are currently incurable [94].

Nevertheless, the extensive implementation of CRISPR-Cas9 in clinical practice will necessitate the surmounting of considerable regulatory and ethical challenges. It is imperative to guarantee the safety and efficacy of CRISPR-based therapies, particularly with regard to the minimisation of off-target effects and the management of immune responses, in order to secure regulatory approval and public acceptance. Furthermore, the ethical implications of gene editing, particularly in the context of germline modifications, must be carefully considered to prevent potential misuse and ensure the responsible use of this technology[95].

The potential applications of CRISPR-Cas9 extend well beyond the field of medicine. Its ability to precisely edit genes offers a unique opportunity to address a number of global challenges, including improving crop yields, developing sustainable

biofuels and mitigating the effects of climate change. As the technology continues to evolve, it is likely to become an integral part of many scientific and industrial processes, driving innovation across multiple sectors[95].

8 Conclusion

8.1 Summary of Key Points

This thesis has conducted an exhaustive examination of the transformative potential of CRISPR-Cas9 technology in the treatment of genetic diseases, with a particular emphasis on cystic fibrosis (CF) and Huntington's disease (HD). The CRISPR-Cas9 system enables precise genome editing, thereby offering the potential for the correction of the specific genetic mutations that are the underlying cause of these diseases. In the case of cystic fibrosis, CRISPR-Cas9 has the potential to repair the ΔF508 mutation in the CFTR gene, which is essential for restoring normal chloride ion transport and alleviating the severe respiratory and digestive complications associated with the disease. In the case of Huntington's disease, CRISPR-Cas9 has demonstrated potential in targeting the expanded CAG repeats in the HTT gene, with the possibility of slowing or halting the neurodegenerative process that defines the condition. Despite the significant promise, the technology faces challenges, including ensuring specificity, overcoming delivery barriers, and addressing ethical concerns.

8.2 Concluding Remarks

The significance of CRISPR-Cas9 as a genetic instrument for the treatment of ailments such as cystic fibrosis and Huntington's disease cannot be overstated. The underlying genetic mutations of both conditions are well-defined, thereby rendering them optimal candidates for CRISPR-Cas9 intervention. In cystic fibrosis, where the disease arises from a mutation in a single gene (CFTR), CRISPR-Cas9 provides a direct method to correct this mutation at its source, offering the potential for a cure rather than merely symptomatic relief. The capacity to repair the CFTR gene has the potential to transform the lives of patients by improving lung function, reducing chronic infections, and enhancing overall quality of life.

Similarly, Huntington's disease is a monogenic disorder where a single, identifiable mutation leads to the production of a toxic protein that causes neurodegeneration. The capacity of CRISPR-Cas9 to target and edit the HTT gene offers a groundbreaking opportunity to mitigate or even prevent the progression of Huntington's disease, which currently has no cure.

Nevertheless, the implementation of CRISPR-Cas9 in these disorders is not without its inherent difficulties. It is of the utmost importance to achieve precision in order to avoid off-target effects that could result in unintended genetic consequences. Furthermore, the effective delivery of CRISPR components to the appropriate tissues, such as the lungs in CF or the brain in HD, represents a significant technical challenge. Furthermore, ethical considerations, particularly those pertaining to potential germline modifications, necessitate meticulous deliberation and rigorous regulatory oversight.

Notwithstanding these challenges, CRISPR-Cas9 represents a highly promising tool for the treatment of cystic fibrosis and Huntington's disease. Its capacity to directly address the genetic aetiology of these conditions renders it a superior alternative to traditional therapies that primarily manage symptoms. As research progresses, the potential for CRISPR-Cas9 to become a fundamental element in the treatment of these and other genetic disorders is considerable. This could signal the advent of a new era in personalised medicine, with the capacity to significantly alter the landscape of genetic disease treatment.

9 References

1. Mani I. CRISPR-Cas9 for treating hereditary diseases. In: Progress in Molecular Biology and Translational Science, vol. 181. New Delhi: Department of Microbiology, Gargi College, University of Delhi; 2021. p. 166-177. doi:10.1016/bs.pmbts.2021.01.017
2. Davies JC, Alton EWFW, Bush A. Cystic fibrosis. BMJ. 2007 Dec 15;335(7632):1255-9. doi: 10.1136/bmj.39391.713229.AD.
3. Andhale R, Shrivastava D. Huntington's Disease: A Clinical Review. Cureus. 2022 Aug;14(8):e28484. doi: 10.7759/cureus.28484.
4. Zhou W, Yang J, Zhang Y, Hu X, Wang W. Current landscape of gene-editing technology in biomedicine: Applications, advantages, challenges, and perspectives. MedComm. 2022;3:e155. doi: 10.1002/mco2.155.
5. Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X. Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. Signal Transduct Target Ther. 2020;5:1. doi:10.1038/s41392-019-0089-y.
6. Macdonald, M. (1993). Cell, 72(6), 971-983.
7. Bates, G. P., Dorsey, R., Gusella, J. F., et al. (2015). Nat Rev Dis Primers, 1, 15005.
8. Wild, E. J., & Tabrizi, S. J. (2017). Lancet Neurol, 16(10), 837-847.
9. Ross, C. A., & Tabrizi, S. J. (2011). Lancet Neurol, 10(1), 83-98.
10. Vonsattel, J. P., & DiFiglia, M. (1998). J Neuropathol Exp Neurol, 57(5), 369-384.
11. Plotkin, J. L., & Surmeier, D. J. (2015). Curr Opin Neurobiol, 33, 53-62.
12. Vonsattel, J. P., Myers, R. H., Stevens, T. J., Ferrante, R. J., Bird, E. D., & Richardson, E. P. Jr. (1985). J Neuropathol Exp Neurol, 44(6), 559-577.
13. Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X. Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances, and prospects. Signal Transduct Target Ther. 2020;5(1):1.
14. Zhou J, Shen B, Zhang W, Wang J, Yang J, Chen L, et al. One-step generation of different immunodeficient mice with multiple gene modifications by CRISPR/Cas9 mediated genome engineering. Int J Biochem Cell Biol. 2014;46:49-55.

15. Yin H, Kauffman KJ, Anderson DG. Delivery technologies for genome editing. Nat Rev Drug Discov. 2017;16(6):387-99.
16. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol. 2014;32(4):347-55.
17. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-23.
18. Gostimskaya I. CRISPR–Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing. Biochemistry (Moscow). 2022;87(8):777-788. doi:10.1134/S0006297922080090.
19. Misganaw Asmamaw Mengstie, Belay Zawdie Wondimu. Mechanism and Applications of CRISPR/Cas-9-Mediated Genome Editing. Biologics: Targets and Therapy. 2021;15:353-361. doi:10.2147/BTT.S326422.
20. genOway. CRISPR/Cas9 Editing Technology: All about this solution. Disponível em: https://www.genoway.com/technologies/crispr-cas9-gene-editing. Acesso em: 20 jun 2024.
21. Li C, Mei H, Hu Y. Applications and explorations of CRISPR/Cas9 in CAR T-cell therapy. Brief Funct Genomics. 2020;19(3):175-182. doi:10.1093/bfgp/elz042.
22. Sun J, Hu H, Cheng Y, Lu X. CRISPR in medicine: applications and challenges. Brief Funct Genomics. 2020;19(3):151-153. doi:10.1093/bfgp/elaa011.
23. Reuven, N., & Shaul, Y. (2022). Selecting for CRISPR-Edited Knock-In Cells. International Journal of Molecular Sciences, 23(11919), 1-16. https://doi.org/10.3390/ijms231911919.
24. Agudelo, D., Duringer, A., Bozoyan, L., Huard, C. C., Carter, S., Loehr, J., ... & Maresca, M. (2021). Marker-free coselection for CRISPR-driven genome editing in human cells. *Nature Biotechnology, 39*, 61-68. https://doi.org/10.1038/s43586-021-00093-4.
25. Reuven, N., & Shaul, Y. (2022). Selecting for CRISPR-Edited Knock-In Cells. International Journal of Molecular Sciences, 23(11919), 1-16. https://doi.org/10.3390/ijms231911919.
26. Agudelo, D., Duringer, A., Bozoyan, L., Huard, C. C., Carter, S., Loehr, J., … & Maresca, M. (2021). Marker-free coselection for CRISPR-driven genome editing in human cells. Nature Biotechnology, 39, 61-68. https://doi.org/10.1038/s43586-021-00093-4.

27. Lu, X., Zhang, M., Li, G., Zhang, S., Zhang, J., Fu, X., & Sun, F. (2023). Applications and Research Advances in the Delivery of CRISPR/Cas9 Systems for the Treatment of Inherited Diseases. International Journal of Molecular Sciences, 24(13202), 1-19. https://doi.org/10.3390/ijms241713202.
28. Mengstie MA. Viral Vectors for the in Vivo Delivery of CRISPR Components: Advances and Challenges. Front Bioeng Biotechnol. 2022;10:895713. doi: 10.3389/fbioe.2022.895713.
29. Yip BH. Recent Advances in CRISPR/Cas9 Delivery Strategies. Biomolecules. 2020;10(6):839. doi: 10.3390/biom10060839.
30. Alkanli SS, Alkanli N, Ay A, Albeniz I. CRISPR/Cas9 Mediated Therapeutic Approach in Huntington's Disease. Mol Neurobiol. 2023;60:1486-1498. doi: 10.1007/s12035-022-03150-5.
31. Ensinck M, Mottais A, Detry C, Leal T, Carlon MS. On the Corner of Models and Cure: Gene Editing in Cystic Fibrosis. Front Pharmacol. 2021;12:662110. doi: 10.3389/fphar.2021.662110.
32. Tsuchida CA, Brandes N, Bueno R, Trinidad M, Mazumder T, Yu B, et al. Mitigation of chromosome loss in clinical CRISPR-Cas9-engineered T cells. Cell. 2023 Oct 12;186(20):4567-4582. doi: 10.1016/j.cell.2023.08.041.
33. Frangoul, H., Locatelli, F., Bhatia, M., et al. (2022). Efficacy and Safety of a Single Dose of Exagamglogene Autotemcel for Severe Sickle Cell Disease. Blood, 140, 29-31.
34. Locatelli, F., Lang, P., Li, A., et al. (2022). Efficacy and Safety of a Single Dose of Exagamglogene Autotemcel for Transfusion-Dependent β-Thalassemia. Blood, 140, 4899-4901.
35. Fu, B., Liao, J., Chen, S., et al. (2022). CRISPR-Cas9-mediated gene editing of the B
1. CL11A enhancer for pediatric β^0/β^0 transfusion-dependent β-thalassemia. Nat. Med., 28, 1573-1580.
36. Bak, R.O., Dever, D.P., & Porteus, M.H. (2018). CRISPR/Cas9 genome editing in human hematopoietic stem cells. Nat. Protoc., 13, 358-376.
37. Cromer, M.K., Camarena, J., Martin, R.M., et al. (2021). Gene replacement of α-globin with β-globin restores hemoglobin balance in β-thalassemia-derived hematopoietic stem and progenitor cells. Nat. Med., 27, 677-687.

38. Ertl HCJ. Immunogenicity and toxicity of AAV gene therapy. Front Immunol. 2022 Aug 12;13:975803. doi: 10.3389/fimmu.2022.975803.
39. Riley RS, June CH, Langer R, Mitchell MJ. Delivery technologies for cancer immunotherapy. Nat Rev Drug Discov. 2019 May;18(3):175-196. doi: 10.1038/s41573-018-0006-z.
40. Naldini L. Gene therapy returns to centre stage. Nature. 2015 Jun 4;526(7573):351-60. doi: 10.1038/nature16144.
41. Chakraborty S, Al-Tannak NF, Woolard J, Sen D. Precision Medicine with CRISPR: Hopes and Challenges. Int J Mol Sci. 2020 May 13;21(10):3483. doi: 10.3390/ijms21103483.
42. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, et al. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature. 2017 Nov 23;551(7681):464-471. doi: 10.1038/nature24644.
43. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 2016 Apr 7;533(7603):420-424. doi: 10.1038/nature17946.
44. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. 2019 Oct;576(7785):149-157. doi: 10.1038/s41586-019-1711-4.
45. Wang X, Li Y, Li J, Wang Y, Feng R, Ling C. High-fidelity and high-frequency CRISPR off-target detection via HIFI sequencing. Nucleic Acids Res. 2021 Mar 31;49(5). doi: 10.1093/nar/gkab009.
46. Frangoul H, Altshuler D, Cappellini MD, Chen YS, Domm J, Eustace BK, et al. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. N Engl J Med. 2021 Feb 18;384(3):252-260. doi: 10.1056/NEJMoa2031054.
47. Graphite Bio, Inc. Graphite Bio Provides Update on CEDAR Study of GPH101 in Sickle Cell Disease. Press Release. 2023 Jan 25. Available at: https://www.graphitebio.com/news-releases/news-release-details/graphite-bio-provides-update-cedar-study-gph101-sickle-cell
48. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014 Jun 5;157(6):1262-1278. doi: 10.1016/j.cell.2014.05.010.

49. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol. 2013 Sep;31(9):822-6.
50. Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, et al. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol. 2014 Jun;32(6):569-76.
51. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. Science. 2016 Jan 1;351(6268):84-8.
52. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature. 2016 Jan;529(7587):490-5.
53. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014 Jun 5;157(6):1262-78.
54. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 2016 May 19;533(7603):420-4. doi: 10.1038/nature17946.
55. Shen MW, Arbab M, Hsu JY, Worstell D, Culbertson SJ, Krabbe O, et al. Predictable and precise template-free CRISPR editing of pathogenic variants. Nature. 2018 Nov;563(7733):646-51.
56. Lanphier E, Urnov F, Haecker SE, Werner M, Smolenski J. Don't edit the human germ line. Nature. 2015 Mar 12;519(7544):410-1. doi: 10.1038/519410a.
57. Doudna JA, Sternberg SH. A crack in creation: Gene editing and the unthinkable power to control evolution. Boston: Houghton Mifflin Harcourt; 2017.
58. Hurlbut JB, Jasanoff S, Saha K, Benjamin C, Coller BS, Etchegary H, et al. Building capacity for a global genome editing observatory: Conceptual challenges. Trends Biotechnol. 2018 Oct;36(10):879-881. doi:10.1016/j.tibtech.2018.05.009.
59. Gyngell C, Savulescu J, Douglas T. The ethics of germline gene editing. J Appl Philos. 2017 Aug;34(4):498-513. doi:10.1111/japp.12249.
60. Baylis F. Altering genes, altering minds. Hastings Cent Rep. 2019 May-Jun;49(3):3. doi: 10.1002/hast.1011.

61. Kohn DB, Porteus MH, Scharenberg AM. Ethical and regulatory aspects of genome editing. Blood. 2016 Nov 17;128(20):2603-2607. doi: 10.1182/blood-2016-05-718232.
62. Ishii T. Germline genome-editing research and its socioethical implications. Trends Mol Med. 2017 Aug;23(8):710-719. doi: 10.1016/j.molmed.2017.06.001.
63. Lander ES. The code of life: The potential and peril of genome editing. Foreign Affairs. 2015;94(3):26-35.
64. Nuffield Council on Bioethics. Genome editing and human reproduction: social and ethical issues [Internet]. London: Nuffield Council on Bioethics; 2018 [cited 2024 Aug 21]. Available from: https://www.nuffieldbioethics.org/publications/genome-editing-and-human-reproduction
65. National Academies of Sciences, Engineering, and Medicine. Human genome editing: Science, ethics, and governance. Washington, DC: The National Academies Press; 2017.
66. Araki M, Ishii T. International regulatory landscape and integration of corrective genome editing into in vitro fertilization. Reprod Biol Endocrinol. 2014 Jul 7;12:108. doi: 10.1186/1477-7827-12-108.
67. Cohen IG, Adashi EY. The FDA is prohibited from going germline. Science. 2016 Feb 12;351(6271):561-2. doi: 10.1126/science.aaf5725.
68. Cyranoski D. The CRISPR-baby scandal: What's next for human gene-editing. Nature. 2019 Jan;566(7745):440-442. doi: 10.1038/d41586-019-00673-1.
69. Ishii T. Germline genome-editing research and its socioethical implications.
2. Trends Mol Med. 2017 Aug;23(8):710-719. doi: 10.1016/j.molmed.2017.06.001.
70. Cavaliere G. A 14-day limit for bioethics: The debate over human embryo research. BMC Med Ethics. 2017 Dec 4;18(1):38. doi: 10.1186/s12910-017-0198-5.
71. Evans JP, Berg JS. Next-generation DNA sequencing, regulation, and the limits of paternalism: The next challenge. JAMA. 2011 Apr 20;305(15):1507-8. doi: 10.1001/jama.2011.450.
72. Ledford H. US health agency to weigh gene-editing moratorium. Nature. 2015 Dec 22;528(7583):319-20. doi: 10.1038/528319a.
73. Tashiro S, Matsubara Y. Regulatory aspects of gene therapy in Japan. Curr Gene Ther. 2014;14(2):112-7. doi: 10.2174/1566523214666140307195643.

74. Baylis F. Altering genes, altering minds. Hastings Cent Rep. 2019 May-Jun;49(3):3. doi: 10.1002/hast.1011.
75. Hurlbut JB, Jasanoff S, Saha K, Benjamin C, Coller BS, Etchegary H, et al. Building capacity for a global genome editing observatory: Conceptual challenges. Trends Biotechnol. 2018 Oct;36(10):879-881. doi: 10.1016/j.tibtech.2018.05.009.
76. Lander ES. The code of life: The potential and peril of genome editing. Foreign Affairs. 2015;94(3):26-35.
77. Ledford H. CRISPR, the disruptor. Nature. 2015 Jun 4;522(7554):20-4. doi: 10.1038/522020a.
78. Isasi R, Knoppers BM. Oversight of human inheritable genome editing. Nat Biotechnol. 2019 Jan;37(1):36-38. doi: 10.1038/nbt.4313.
79. National Academies of Sciences, Engineering, and Medicine. Human genome editing: Science, ethics, and governance. Washington, DC: The National Academies Press;
80. Scheufele DA, Xenos MA, Howell EL, Rose KM, Brossard D, Hardy BW. U.S. attitudes on human genome editing. Science. 2017 Jul 14;357(6348):553-554. doi: 10.1126/science.aan3708.
81. McCaughey T, Sanfilippo PG, Gooden GE, Budden DM, Fan L, Fenwick E, et al. A global social media survey of attitudes to human genome editing. Cell Stem Cell. 2016 May 5;18(5):569-572. doi: 10.1016/j.stem.2016.04.011.
82. Bosley KS, Botchan M, Bredenoord AL, Carroll D, Charo RA, Charpentier E, et al. CRISPR germline engineering—the community speaks. Nat Biotechnol. 2015 May;33(5):478-486. doi: 10.1038/nbt.3227.
83. Nerlich B, McLeod C. The dilemma of raising awareness "responsibly." EMBO Rep. 2016 Feb;17(2):163-166. doi: 10.15252/embr.201541113.
84. Ishii T. Germline genome-editing research and its socioethical implications. Trends Mol Med. 2017 Aug;23(8):710-719. doi: 10.1016/j.molmed.2017.06.001.
85. Toth-Fejel T. Equity and access in public health ethics. In: Jennings B, Arras JD, Barrett DH, Ellis BA, editors. The Encyclopedia of Bioethics. New York: Macmillan Reference USA; 2014. p. 926-930.
86. Oye KA, Esvelt K, Appleton E, Catteruccia F, Church G, Kuiken T, et al. Regulating gene drives. Science. 2014 Aug 8;345(6197):626-628. doi: 10.1126/science.1254287.

87. Isasi R, Knoppers BM. Oversight of human inheritable genome editing. Nat Biotechnol. 2019 Jan;37(1):36-38. doi: 10.1038/nbt.4313.
88. Lanphier E, Urnov F, Haecker SE, Werner M, Smolenski J. Don't edit the human germ line. Nature. 2015 Mar 12;519(7544):410-1. doi: 10.1038/519410a.
89. Jasanoff S, Hurlbut JB, Saha K. CRISPR democracy: Gene editing and the need for inclusive deliberation. Issues Sci Technol. 2015;32(1):25-32.
90. National Academies of Sciences, Engineering, and Medicine. Human genome editing: Science, ethics, and governance. Washington, DC: The National Academies Press; 2017.
91. Lino CA, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: A review of the challenges and approaches. Drug Deliv. 2018 Jan;25(1):1234-1257. doi: 10.1080/10717544.2018.1474964.
92. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 2016 May 19;533(7603):420-4. doi: 10.1038/nature17946.
93. Chen JS, Doudna JA. The chemistry of Cas9 and its CRISPR colleagues. Nat Rev Chem. 2017 Sep;1(10):0078. doi: 10.1038/s41570-017-0078.
94. Ma J, Köster J, Qin Q, Hu S, Li W. CRISPR-DO for genome-wide CRISPR design and optimization. Bioinformatics. 2016 Aug 15;32(16):2618-20. doi: 10.1093/bioinformatics/btw180.
95. Waltz E. Gene-edited CRISPR mushroom escapes US regulation. Nature. 2016 Apr 21;532(7599):293. doi: 10.1038/nature.2016.19754.
96. Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Castellanos Rivera RM, et al. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. Science. 2016 Jan 22;351(6271):403-7. doi: 10.1126/science.aad5143.
97. Ishii T. Germline genome-editing research and its socioethical implications. Trends Mol Med. 2017 Aug;23(8):710-719. doi: 10.1016/j.molmed.2017.06.001.
98. Esvelt KM, Smidler AL, Catteruccia F, Church GM. Emerging technology: Concerning RNA-guided gene drives for the alteration of wild populations. Elife. 2014 Jul 17;3:e03401. doi: 10.7554/eLife.03401.
99. Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S., & Gregory, P. D. (2010). Genome editing with engineered zinc finger nucleases. Nature Reviews Genetics, 11(9), 636-646.

100. Bogdanove, A. J., & Voytas, D. F. (2011). TAL effectors: customizable proteins for DNA targeting. Science, 333(6051), 1843-1846.
101. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science, 337(6096), 816-821.
102. Abudayyeh, O. O., Gootenberg, J. S., Essletzbichler, P., Han, S., Joung, J., Belanto, J. J., ... & Zhang, F. (2017). RNA targeting with CRISPR-Cas13. Nature, 550(7675), 280-284.
103. Gaudelli, N. M., Komor, A. C., Rees, H. A., Packer, M. S., Badran, A. H., Bryson, D. I., & Liu, D. R. (2017). Programmable base editing of A• T to G• C in genomic DNA without DNA cleavage. Nature, 551(7681), 464-471.
104. Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., ... & Liu, D. R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. Nature, 576(7785), 149-157.
105. Silva, G., Poirot, L., Galetto, R., Smith, J., Montoya, G., Duchateau, P., & Paques, F. (2011). Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy. Current Gene Therapy, 11(1), 11-27.
106. Cutting, G. R. (2015). Cystic fibrosis genetics: from molecular understanding to clinical application. *Nature Reviews Genetics, 16*(1), 45-56.
107. Bell, S. C., De Boeck, K., & Amaral, M. D. (2020). New pharmacological approaches for cystic fibrosis: Promises, progress, pitfalls. *Pharmacology & Therapeutics, 210*, 107517.
108. Kaur, M., & Gope, S. (2024). *Pulmonary Complications and Advancements in the Treatment of Cystic Fibrosis*. Bioscene: Journal of College Biology Teaching.
109. Nanda, S. R., & Sharma, P. (2024). *Targeted mutagenesis using TALENs: A newly developed technique*. Research and Reviews in Plant Science.
110. Kleinstiver, B. P., et al. (2016). "High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects." Nature, 529(7587), 490-495. DOI:10.1038/nature16526
111. Zhou, J., et al. (2019). "CRISPR/Cas9-mediated genome editing in plants: Current status and future prospects." Frontiers in Plant Science, 10, 1-12. DOI:10.3389/fpls.2019.00101

112. Nishimasu, H., et al. (2018). "Crystal structure of Cas9 in complex with guide RNA and target DNA." Cell, 156(5), 935-949. DOI:10.1016/j.cell.2014.02.001
113. Huang, X., et al. (2019). "CRISPR/Cas9: A new tool for genetic engineering." Molecular Biotechnology, 61(1), 1-12. DOI:10.1007/s12033-018-0221-0
114. Rosenberg, S. A., et al. (2016). "Adoptive cell transfer for the treatment of patients with metastatic melanoma: the role of tumor-infiltrating lymphocytes." Nature Reviews Clinical Oncology, 13(6), 309-320. DOI:10.1038/nrclinonc.2016.12
115. You L, Tong R, Li M, Xue J, Lu Y. Advancements and obstacles of CRISPR-Cas9 technology in translational research. Mol Ther Methods Clin Dev. 2019;13:359-370.
116. Zhang F, et al. CRISPR-Cas9: A New Era in Drug Discovery. Nat Rev Drug Discov. 2020;19:1-20. doi:10.1038/s41573-019-0050-0.
117. Shalem O, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science. 2014;343(6166):84-87. doi:10.1126/science.1247005.
118. Lazar NH, Celik S, Chen L, Fay MM, Irish JC, Jensen J, et al. High-resolution genome-wide mapping of chromosome-arm-scale truncations induced by CRISPR–Cas9 editing. Nat Genet. 2024;56(7):1482-93. doi:10.1038/s41588-024-01758-y (s41588-024-01758-y (1)).
119. Shen MW, Arbab M, Hsu JY, Worstell D, Culbertson SJ, Krabbe O, et al. Predictable and precise template-free CRISPR editing of pathogenic variants. Nature. 2018;563(7733):646–51.
120. Zou Y, Wang L, Cai Y, Dan J, Chen J, Lan Y, et al. CRISPR/Cas9-mediated gene editing reveals the synergistic effects of β-catenin and NF-κB signaling on odontogenic differentiation of dental pulp stem cells. Stem Cell Res Ther. 2022;13(1):140. doi: 10.1186/s13287-022-02794-1.
121. Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotechnol. 2015;33(2):187–97. doi: 10.1038/nbt.3117.
122. Sosnay PR, et al. "Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene." *Nat Genet*. 2013;45(10):1160-7.
123. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*. 2019;576(7785):149-57.

124. Lander ES. The Heroes of CRISPR. Cell. 2016 Jan 14;164(1-2):18–28. Available from: http://dx.doi.org/10.1016/j.cell.2015.12.041

125. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-23 (PIIS0092867415017055).

126. King NM, Lord PC, Lemley DE. Editing the genome: Prospects, progress, implications, and cautions. *Curr Genet Med Rep*. 2017;5(1):35-43. doi:10.1007/s40142-017-0109-6.