HIGH SCHOOL EDITION
Journal of Student Research
Volume 11 Issue 3 (2022)

The Applications of the CRISPR/Cas9 Gene-Editing System in Treating Human Diseases

Akanksha Varanasi[1] and Elisabeth Wilson[#]

[1] Monte Vista High School, USA
[#]Advisor

ABSTRACT

In the early 2010s, scientists realized that CRISPR/Cas9, a bacterial immune defense system against viruses that involves the CRISPR-associated protein #9 (Cas9) endonuclease enzyme, single-guide RNAs (sgRNAs), and PAM recognition, could be used to intentionally manipulate genes, essentially changing gene expression and regulation in such a way that would allow for a customized genome. Since then, CRISPR technology has revolutionized medical research and the biotechnology industry, and its newfound capabilities have scientists asking if CRISPR can be used to modify genes in such a way that would cure or treat certain harmful or life-threatening diseases. There have been CRISPR-based clinical studies done to treat β-thalassemia (TDT), sickle-cell disease (SCD), the human immunodeficiency virus (HIV), and several other genetic and non-hereditary diseases, but there is still a long way to go before CRISPR can become a widespread treatment for many more such diseases (Ebina et al., 2013; Esrick et al., 2021; Frangoul et al., 2021). Currently, researchers are looking to see if CRISPR is an accurate, specific, non-harmful, and effective treatment for these diseases, which means addressing and eliminating potential concerns about its safety and efficacy through extensive pre-clinical and clinical research, as well as overcoming moral and social obstacles. In this review, I will look at how the CRISPR/Cas9 gene-editing system can be applied in humans to prevent, cure, or treat these diseases, as well as what needs to be done before the CRISPR/Cas9 system can be made publicly available as a medical treatment for diseases.

Introduction

Organisms belonging to the domains bacteria and archaea use RNA-guided CRISPR/Cas immune defense systems to protect themselves from foreign invaders, such as viruses and plasmids. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats, which are sequences of DNA that contain a series of consistently-occurring repeats that are 23 base pairs (bps) to 47 bps long. These repeats are isolated from each other in terms of genomic location, and they are distinguished by intervening unique spacer sequences, or pieces of the viral genome that become incorporated and transcribed by the host to recognize foreign genetic material (Jinek et al., 2012).

There are three known types of CRISPR/Cas9 systems: Type I, Type II, and Type III (Figure 1). Over the last several years, a specific Type II CRISPR/Cas system—CRISPR/Cas9 from the Streptococcus pyogenes (S. pyogenes) bacteria—has been extensively researched as a novel method for editing the human genome. CRISPR/Cas9 generally uses endonuclease enzymes from the CRISPR-associated protein #9 (Cas9) family, with the most commonly-used Cas9 nuclease in CRISPR/Cas9 originating from S. pyogenes (Figure 1) (Jinek et al., 2012)

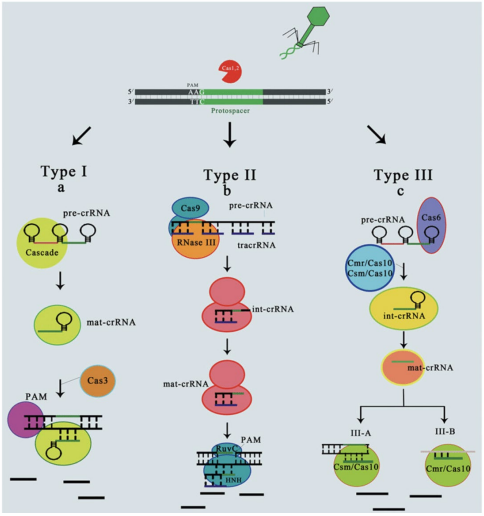


Figure 1. The Three Types of CRISPR/Cas Systems (Shabbir et al., 2016)

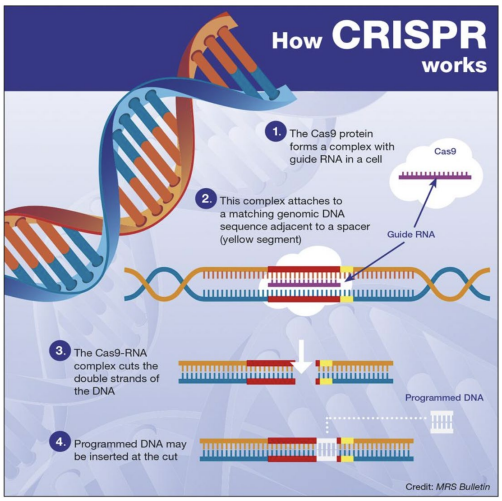
Cas9 is the preferred enzyme for CRISPR editing systems because of its ability to cleave both single-stranded RNA (ssRNA) and DNA, a result of its high-affinity binding to ssRNA corresponding to single-guide RNA (sgRNA). Cas9 is also versatile, harboring endonuclease activity for linear and supercoiled plasmids, which allows it to cleave a plasmid several times if it has been programmed with various different crRNAs (O'Connell et al., 2014).

While CRISPR/Cas9 has had a number of emerging roles in molecular biology, one of its more prominent applications is the induction of lesions, or permanent DNA-level changes that alter the way genes are expressed or regulated in various organisms. This process happens in a specific way in all CRISPR systems. The repeated spacers are first transcribed into precursor CRISPR RNAs (pre-crRNAs), which are then processed into mature CRISPR RNAs (crRNAs). In Type II CRISPR/Cas9 systems, trans-activating crRNAs (tracrRNAs) corresponding to the pre-crRNA short repeats are needed to initiate crRNA processing by the ribonuclease RNase III when the endonuclease enzyme Cas9 is present. Both tracrRNA and crRNA combine to form a dual, chimeric RNA structure known as single-guide RNA (sgRNA)—or simply guide RNA (gRNA) (Figure 2). In CRISPR/Cas9 systems, sgRNA complexes with Cas9 to form a ribonuclear protein that can effectively recognize, target, bind to, and cleave double-stranded DNA (dsDNA) of viral nucleic acids at specific, complementary sites (Figure 2) (Jinek et al., 2012).

Specifically, the Cas9-sgRNAs ribonuclear complex will recognize and bind to a complementary DNA sequence about 20 bps long, inducing site-specific cleavage of foreign DNA (DNA interference) (Figure 2) (Pattanayak et al., 2013). However, in order for Cas9 to properly work with the dual RNA structure, a protospacer adjacent motif (PAM) sequence needs to be recognized (Figure 4) (Jinek et al., 2012). In general, the PAM is a short sequence motif (2–6 bps) on the non-target strand of DNA that is near the corresponding region of the target DNA 3–4 bps after the site of Cas9 cleavage, and it is contained in the genome of the foreign invader (Figure 4) ("Importance of the PAM Sequence in CRISPR Experiments," n.d.). The *S. pyogenes* Cas9 nuclease most commonly recognizes the 5'–NGG–3' PAM sequence. The PAM sequence determines the relative cleavage location on both the complementary strand (relative to the crRNA target-binding sequences) and the non-complementary DNA strand, allowing the dsDNA to separate into different strands and the target DNA to bind appropriately (Figure 4) (Jinek et al., 2012).

Furthermore, the PAM sequence is critical for the formation of R-loops, or structures made up of RNA and DNA that are necessary to initiate genome editing, making it a defining characteristic of the genome-editing system. During R-loop formation, the sgRNA binds to complementary target DNA sequences adjacent to PAM

sequence sites, and it directs dsDNA to unwind in order to form a composite structure that involves both the



target DNA strand and the spacers of the crRNA, a hybrid DNA-RNA R-loop structure (Jinek et al., 2012).
Figure 2. How the CRISPR/Cas9 System Works (Ball, 2016)

This structure then interacts with the Cas9 complex, leading to the initiation of dsDNA cleavage 3–4 bps before the PAM sequence, through the Cas9 HNH and RuvC-like nuclease domains ("Importance of the PAM Sequence in CRISPR Experiments," n.d.). These R-loop protein-nucleic acid interactions cause the DNA strand non-complementary to the sgRNA—the displaced DNA strand—to be displaced and positioned near the Cas9 RuvC-like domain, where it will be cleaved by the homologous RuvC endonuclease. These interactions also place the Cas9 HNH nuclease domain near the complementary target DNA strand, which will be cleaved by the homologous HNH endonuclease (Jinek et al., 2012).

In conclusion, enzymes in the Cas9 family can be programmed with RNA to cleave DNA at specific sites, allowing for targeted dsDNA gene editing (Figure 2). In order for the DNA recognition by Cas9 and the cleavage reaction to happen, the presence of magnesium, the Cas9 enzyme, an sgRNA dual structure, and a PAM sequence are all necessary (Jinek et al., 2012). The ability to alter target DNA sequences makes the CRISPR/Cas9 system novel and broadly applicable, which has led scientists to state that CRISPR/Cas9 has significant potential as a gene-editing technology. The research done on CRISPR/Cas9 over the years has confirmed this idea, and now it is believed that the gene-editing system can be applied to certain human diseases as valid treatment approaches, a significant achievement for the biotechnology and health industries.

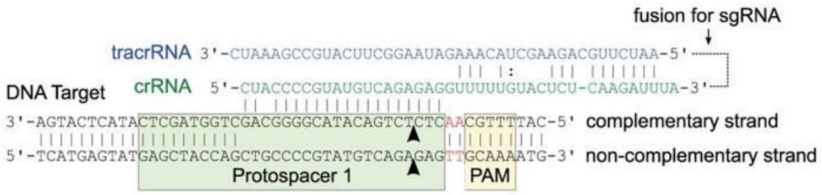
Accuracy, Efficiency, Specificity, and Safety of the CRISPR/Cas9 Gene-Editing System

Considerations in Designing the sgRNA and PAM Sequences

While the CRISPR/Cas9 gene-editing system has been successful overall, there are still many concerns with its accuracy, efficiency, and specificity. In particular, research has found the presence of off-target effects in CRISPR systems, which are the result of cleavage of the target DNA at unintended sites. For all the times that the CRISPR/Cas9 system has actually been applied for the purpose of genome editing, there has been a significant variance each time in how often off-target effects occur in relation to the occurrence of expected on-target activity, and this is an issue because the increased risk of off-target mutations could affect how other non-

targeted genes function (Doench et al., 2016; X.-H. Zhang et al., 2015). Before CRISPR/Cas9 becomes a widespread treatment for human diseases, these problems need to be recognized and addressed.

Overall, the efficacy of several aspects of the CRISPR/Cas9 system is vital in ensuring that CRISPR is a viable gene-editing strategy. In particular, one part of the CRISPR/Cas9 system that must be effective is the *S. pyogenes* 5'–NGG–3' (N = A, T, C, or G) PAM sequence—specifically the seed sequence (Figure 4)—which is necessary in order for Cas9 to bind to and cleave the DNA correctly, as well as for sgRNA to base-pair accurately (Figure 3). The two most prevalent types of PAM sequences in CRISPR are 5'–NGG–3' and 5'–NRG–3' (R = G or A), and both of these sequences have a different binding frequency—the first nucleotide



is often not conserved, while the second nucleotide usually is (X.-H. Zhang et al., 2015).

Figure 3. Target DNA, sgRNA & PAM Binding (Hongfan et al., 2014)

While an NGG PAM sequence on the target DNA sequence is 80% more effective at recruiting a Cas9 complex than an NRG sequence is, even the NGG sequence may not be the most effective for Cas9-sgRNA PAM recognition and DNA cleavage if the goal is to ensure precision, as it does not always lead to the specific induction of point mutations or insertions in the DNA. In Type II systems (*S. pyogenes*), the NGG and NRG sequences may not be optimal for every application. Other related Type II systems, like *Streptococcus thermophilus* and *Staphylococcusaureus*, are valid options, as they involve NGA and NAC PAM sequences, which can cause fewer off-target effects and thus are more effective (X.-H. Zhang et al., 2015). Ultimately, the PAM sequence needs to be carefully chosen to maintain the precision of CRISPR.

Additionally, the sequence content of the sgRNA in CRISPR/Cas9 systems is important, as different sequences imply different three-dimensional shapes, which impact protein-DNA interactions in ways that may lead to varying levels of on-target versus off-target activity. The 10–12 bp section at the 3' end of the sgRNA sequence—centered near the PAM part of the target DNA sequence that the sgRNA is bound to—is called the seed sequence (Figure 4). The seed sequence is critical to the function of Cas9, and it needs to be more accurate than other sequences of sgRNA because it determines how specific Cas9 is (Figure 4). Even 3–5 bp mismatches in the part of the sgRNA sequence furthest from the PAM can result in off-target cleavage of the target DNA (Figure 4) (X.-H. Zhang et al., 2015).

In order for DNA to be targeted accurately, the sgRNA needs to correctly base-pair to the DNA next to the PAM sequence, but it cannot do that if the seed section of the sgRNA sequence is not entirely accurate. The strength of the seed sequence can be determined through the method of chromatin immunoprecipitation and sequencing (ChIP-seq), as a ChIP-seq assay is capable of predicting sites of off-target activity in seed sequences. However, it can sometimes lead to over-predictions due to only measuring the seed sequence-DNA binding events and not the cleavage events (X.-H. Zhang et al., 2015). Thus, as it is difficult to determine the true strength of a seed sequence, it needs to be precisely engineered from the start, or CRISPR will have decreased accuracy, efficiency, and specificity.

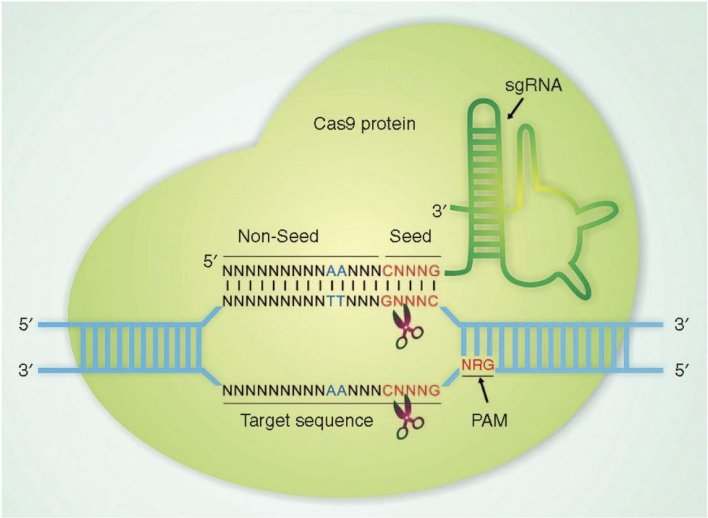


Figure 4. The sgRNA Seed Sequence (Zhang et al., 2015)

Considerations for the Function of the Cas9 Enzyme

Of course, the Cas9 enzyme itself needs to be efficient in ensuring that the CRISPR/Cas9 system works overall. In theory, Cas9 specificity should be controlled by the sgRNA and the PAM sequence next to the target sequence. However, if a methyl group is added to the DNA, Cas9 may have a decreased ability to bind efficiently to the DNA (X.-H. Zhang et al., 2015). Other than DNA methylation, other epigenetic mechanisms that can hinder the activity of CRISPR are demethylation, hydroxyl-methylation, histone modification, chromatin remodeling, gene imprinting, and non-coding RNA manipulation (Xie et al., 2018).

In addition, mutations in the PAM sequence motif have been found to greatly affect the Cas9-directed cleavage of the non-complementary DNA strand, which in turn negatively affects the formation of the Cas9 complex of crRNA and tracrRNA. The Cas9 DNA cleavage of the non-complementary strand is highly sensitive to these mutations, as they interfere with any subsequent cleavage and protein formation (Jinek et al., 2012). Furthermore, using smaller biological monomers that can epigenetically activate or repress certain Cas9 genome-editing and double-stranded break (dsb) repair pathways through DNA homology-directed repair is one way to improve the specificity and efficiency of Cas9 (X.-H. Zhang et al., 2015). In general, the individual aspects of Cas9—as well as its applications—are important to consider when trying to minimize off-target effects.

Several *in vitro* accuracy tests have been done on the CRISPR/Cas9 system to determine how accurate it truly is. Specifically, *in vitro* selection and DNA sequencing have been used to determine the tendency of Cas9 and sgRNA to cleave at off-target sites. Previous studies with small groups of off-target sites have suggested that the sgRNA needs to be complementary with the target DNA in the 3' end-most 7–12 bps next to the PAM sequence and in 2 bps of the PAM, though there can be mutations or mismatches on the 5' end. However, a new study indicates that Cas9 is not just specific to a 7–12 bp-long DNA sequence. Instead, Cas9 recognizes the target DNA at 18–20 base pairs and 2 bps of the PAM sequence—beyond a 7–12 base pair DNA sequence (Pattanayak et al., 2013).

The *in vitro* tests also found that sgRNA on-target activity and specificity have an inverse relationship, as larger and more active sgRNA is less specific than smaller and less active sgRNA. Essentially, off-target cleavage depends on the structure of the sgRNA and how they cleave DNA overall. Since sgRNA structure heavily influences Cas9 activity and specificity, increasing the number of active Cas9 endonucleases and sgRNAs ensures the cleavage of mutated off-target sites close to or in the PAM recognition sites, but also decreases

the specificity of DNA cleavage overall due to the greater abundance of Cas9 molecules engaging the genome. However, not all bases in the DNA are affected by the changes in sgRNA specificity, which is why it is necessary to understand how specific Cas9 and sgRNA are in cleaving DNA by extensively studying the Cas9 cleavage of more off-target sites (Pattanayak et al., 2013).

Safety and Toxicity of the CRISPR/Cas9 System

Another valid concern with CRISPR is its safety, and any potential consequences and adverse long-term effects that it might cause during disease treatment. For example, CRISPR as a gene therapy for lung cancer was found to have minimized off-target activity and a lack of side effects. However, it does have limitations, as the treatment results are different based on the gene and the patient, sgRNA must be selected very carefully, and the long-term effects of the treatment are not yet known (He, 2020). A method of CRISPR-based gene knockout, called base editing, is capable of addressing such limitations, although this approach has its own consequences when it comes to intentionally inducing mutations, such as what was done in the lung cancer study through Cas9 expression vectors (Carlaw et al., 2020; Lu et al., 2020).

Currently, scientists are in the process of developing newer, safer, and more accurate CRISPR-based cell-therapy methods. These new methods are favored because they do not induce double-stranded breaks—which would increase the risk of DNA damage—and they are currently being tested in clinical trials to determine how accurate, effective, and specific they are. Once these methods are fully developed, they will dictate the future of CRISPR-based cell therapy clinical trials (He, 2020).

Recently, a trial with the disease primary hyperoxaluria type I (PH1), a metabolic disease caused by the toxic accumulation of the metabolic substance hepatic oxalate, was conducted with mice to determine if CRISPR would be an effective treatment. Specifically, CRISPR has promised to be a successful substrate reduction therapy (SRT) that would reduce the concentration of toxic metabolites—for PH1, it targeted the nonessential enzyme glycolate oxidase to prevent the buildup of hepatic oxalate. The study found that the therapy was specific to certain tissues, and there were no off-target effects with the sgRNAs involved. The study also found that the livers of the animals who were given the CRISPR/Cas9 SRT were relatively normal. Based on the results of this trial, CRISPR could theoretically also be applied to other diseases that affect metabolism. Overall, although the data from the study strongly suggests that CRISPR is a safe treatment for diseases such as PH1, there are still risks with CRISPR-based therapy that need to be extensively studied, especially the consequences of off-target effects (Zabaleta et al., 2018).

As mentioned above, there are still concerns with CRISPR/Cas9 systems in regards to base editing, a form of CRISPR-based gene knockout therapy that has become popular because it has the potential to correct mutations that alter gene expression or gene product activity. Base editors are a hybrid of an inactive Cas9 nuclease that can initiate single-stranded breaks rather than double-stranded breaks and an enzyme that can modify bases, thereby correcting mutations that cause diseases in humans. Base editors are of interest to CRISPR scientists because they can accurately recognize and repair mutations in a gene without harming other genes. However, recent research has shown potential safety issues that come with base editing in addition to the concerning amount of off-target effects that it still causes, though there have been recent developments that promise to increase the safety and efficiency of base editors (Carlaw et al., 2020). Overall, it is crucial to identify and address all of these issues—no matter how minor—before advancing CRISPR/Cas9 disease treatments.

Completed and Ongoing Pre-Clinical Research, Case Studies, and Clinical Trials Evaluating the CRISPR/Cas9 Gene-Editing System

Clinical Trials with the CRISPR/Cas9 System

There have recently been many attempts to mitigate symptoms of certain diseases using the CRISPR/Cas9 gene-editing technology. Specifically, CRISPR as a disease treatment is currently being studied in many clinical trials that are in various phases, and they all look to measure its efficacy and safety (Table 1 & Figure 5). Sickle-cell disease (SCD) is one of the diseases being investigated through ongoing clinical trials. SCD is caused by a mutation in the β-globin gene (HBB), which produces a variant of adult hemoglobin (HbA) called sickle hemoglobin (HbS). Research has shown that high levels of fetal hemoglobin (HbF) can remedy the effects of SCD, as newborns and infants with SCD rarely manifest symptoms. Since the erythroid-specific transcription factor *BCL11A* is said to repress HbF production in erythroid cells, silencing *BCL11A* increases HbF concentrations, making CRISPR-based therapy a promising approach for treating SCD (Esrick et al., 2021).

Table 1. Clinical Trials with the CRISPR/Cas9 System (Esrick et al., 2021; Frangoul et al., 2021; Lu, 2020; Lu et al., 2020; Vertex Pharmaceuticals Incorporated, 2022b, 2022a; Williams, 2022)

Study & Status	Purpose & Goal of Study	Patient Demographics	Administration of Trial	Results Data & Outcome
Pilot and Feasibility Study of Hematopoietic Stem Cell Gene Transfer for Sickle Cell Disease Active, not recruiting	• Phase I Clinical Trial • The primary goal of the study was to determine how safe and practical a CRISPR-based *BCL11A* gene therapy was for SCD patients	• Patients in the first trial had to be 12+ years old • Patients had the following genotypes: HbSS, HbS/β^0, HbSD, or HbSO • Participants had to have severe clinically-defined SCD (2+ acute chest syndrome episodes and 3+ severe pain episodes in the past two years, and a need for transfusions)	• A lentiviral vector was transduced into CD34+ cells to silence a *BCL11A* enhancer through CRISPR-based erythroid knockdown • These cells were infused into patients intravenously • Patients were monitored for their reaction to the treatment for about 18 months	• HbF levels increased in all patients • Down-regulating *BCL11A* can induce HbF production • None of the patients had a vaso-occlusive crisis, acute chest syndrome episode, or stroke following the infusion • Previous symptoms (priapisms, necrosis) or necessary treatments (blood transfusions) have decreased
A Phase 1/2/3 Study to Evaluate the Safety and Efficacy of a Single Dose of Autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells	• Phase I/II/III Clinical Trial • The main objective was to increase fetal hemoglobin levels by blocking *BCL11A* in study participants in order to	• Patients in the second trial were between 18 and 35 years old • Patients had the following genotypes: βS/βS or βS/β^0 • Participants had to	• CRISPR was used to edit alleles of the *BCL11A* (a transcription factor that inhibits fetal hemoglobin expression in erythroid cells) erythroid-specific enhancer	• After a year, the patient with SCD had more fetal hemoglobin expressed than initially • The patient no longer had vaso-occlusive crises • Complications of

(CTX001) in Subjects With Severe Sickle Cell Disease (CLIMB SCD-121) Active, not recruiting	resolve the symptoms of SCD	have had 2+ severe vaso-occlusive crises per year for the past two years	• These edited genes were placed in erythroid cells that were given to two individuals (one of whom had SCD)	SCD (sepsis, cholelithiasis, abdominal pain) were eliminated by the treatment
A Phase 1/2/3 Study of the Safety and Efficacy of a Single Dose of Autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs) in Subjects With Transfusion-Dependent β-Thalassemia (CLIMB THAL-111) Active, not recruiting	• Phase I/II/III Clinical Trial • The main objective was to increase fetal hemoglobin levels by blocking *BCL11A* in study participants in order to resolve the symptoms of TDT	• Patients in the trial were between 18 and 35 years old • Patients who had been diagnosed with TDT and received 100ml+ transfusions in the past two years could participate	• CRISPR was used to edit alleles of the *BCL11A* (a transcription factor that inhibits fetal hemoglobin expression in erythroid cells) erythroid-specific enhancer • These edited genes were placed in erythroid cells that were given to two individuals (one of whom had TDT)	• After a year, the patient with TDT had more fetal hemoglobin expressed than initially • Complications of TDT (acute respiratory distress syndrome, headaches, and pneumonia) were eliminated by the treatment
A Phase I Clinical Trial of PD-1 Knockout Engineered T Cells Treating Patients With Advanced Non-small Cell Lung Cancer Completed	• Phase I Clinical Trial • The scientists wanted to explore the functions of the edited T-cells • The objectives of the study were to show that CRISPR-based T-cell therapy was safe and practical	• Patients in the study had non-small-cell lung cancer and failed the phase I clinical trial therapy • There were 22 patients (five without infusions, and another five that did not complete the entire study)	• CRISPR was used to edit checkpoint genes in the immune system in order to make T-cell therapy for late-stage lung cancer more effective • T-cells were engineered *ex vivo* with the Cas9 endonuclease and plasmids containing sgRNA, and were infused into the patients	• Only lower-grade symptoms (hypertension, anemia, etc.) occurred in participants of the study • None of the patients had cytokine release syndrome after the treatment • Patients who received the treatment had a survival rate without cancer progression of 7.7 weeks and a median survival of 42.6 weeks

A Phase I clinical trial study published in early 2021 highlights the use of a lentiviral vector—similar to the gene knockout strategy of CRISPR—to knock out the *BCL11A* transcription factor (Figure 5). The primary goal of this study was to determine how safe and practical the *BCL11A* gene therapy was for SCD patients. Patients in the trial had to be older than 12 years old and have the following genotypes: HbSS, HbS/β^0, HbSD, or HbSO. They also had to have severe clinically-defined SCD, which was stated as two or more acute chest syndrome episodes and three or more severe pain episodes in the past two years, as well as a need for transfusions (Table 1) (Esrick et al., 2021).

During the treatment, the lentiviral vector was transduced into CD34+ cells to silence a *BCL11A* enhancer through CRISPR-based erythroid knockdown, and these cells were infused into patients intravenously. Patients were monitored for their reaction to the treatment for about 18 months, and it was found that downregulating *BCL11A* can induce HbF production—as HbF levels increased in all patients—and reduce SCD symptoms, as none of the patients had a vaso-occlusive crisis, acute chest syndrome episode, or stroke following the infusion. Previous symptoms (priapisms, necrosis) and necessary treatments (blood transfusions) have also decreased since the treatment began (Table 1) (Esrick et al., 2021). Thus, CRISPR-mediated gene therapy appears to be a successful strategy for treating SCD.

Another 2021 paper discussed two simultaneous and similarly-administered Phase I/II/III clinical trials: one that also focused on SCD, and another that focused on transfusion-dependent β-thalassemia (TDT) (Figure 5). TDT, like SCD, is also caused by a mutation in HBB and thus can be treated by increasing HbF levels. The main objective of this study was to increase fetal hemoglobin levels by blocking *BCL11A* in study participants in order to resolve the symptoms of both SCD and TDT. Patients in both trials were between 18 and 35 years old, and in the SCD study, had the following genotypes: βS/βS or βS/β0. SCD trial participants also had to have had two or more severe vaso-occlusive crises per year for the past two years, and TDT trial

<table>
<tr><td colspan="5">CRISPR/Cas9 Trials
FDA Approval ⇩</td></tr>
<tr><td rowspan="2">Pre-Clinical</td><td colspan="4">Clinical</td></tr>
<tr><td>Phase I</td><td>Phase II</td><td>Phase III</td><td>Phase IV</td></tr>
<tr><td>• Feasibility</td><td>• Safety
• Dosing
• Side-Effects</td><td>• Safety
• Effectiveness</td><td>• Side Effects
• Effectiveness
• Comparisons
• Research</td><td>• Research
• Applications
• Risks
• Benefits</td></tr>
<tr><td rowspan="4"></td><td>SCD #1</td><td colspan="3" rowspan="2"></td></tr>
<tr><td colspan="2">SCD #2</td></tr>
<tr><td colspan="2">TDT</td><td colspan="2" rowspan="2"></td></tr>
<tr><td>Lung Cancer</td><td></td></tr>
<tr><td>HIV</td><td colspan="4" rowspan="6"></td></tr>
<tr><td>DMD</td></tr>
<tr><td>HD</td></tr>
<tr><td>Cataracts</td></tr>
<tr><td>Influenza</td></tr>
<tr><td>SARS-CoV-2</td></tr>
</table>

participants had to have been diagnosed with TDT and have received 100ml+ transfusions in the past two years (Table 1) (Frangoul et al., 2021).

Figure 5. Pre-Clinical & Clinical Trials with the CRISPR/Cas9 System (Blanchard et al., 2021; Dabrowska et al., 2018; Ebina et al., 2013 Long et al., 2018; Lu, 2020; "On Biostatics and Clinical Trials, "2019"; "Phase 3 Clinical Trials Opening for Hepatitis Delta Patients," 2019; Vertex Pharmaceuticals Incorporated, 2022b, 2022a; Williams, 2022; Wu et al., 2013)

The results of the trials highlight two specific patients: one with SCD and the other with TDT. Both studies involved *BCL11A*: specifically, CRISPR was used to edit the alleles of the transcription factor to repress it and stimulate HbF production, and these edited genes were placed in erythroid cells that were given to the two patients. After a year, both patients had more fetal hemoglobin expressed than initially, and the patient with SCD no longer had vaso-occlusive crises. In addition, both complications of SCD (sepsis, cholelithiasis, abdominal pain) and complications of TDT (acute respiratory distress syndrome, headaches, and pneumonia) were eliminated by the treatment, further indicating that the CRISPR-directed repression of *BCL11A* can treat both SCD and TDT by elevating HbF concentrations and mitigating the respective symptoms of each disease (Table 1) (Frangoul et al., 2021).

A third Phase I clinical trial study, referenced above, indicated the observed effects regarding safety, feasibility, and efficacy of CRISPR/Cas9 technology in advanced refractory non-small-cell lung cancer (NSCLC) (Figure 5). In particular, CRISPR was used to edit PD-1 T-cells and checkpoint genes in the immune system in lung cancer patients to improve the response of cytotoxic (killer) T-cells to cancerous cells and tumors. Patients in the study had late-stage lung cancer and failed the Phase I clinical trial therapy; there were 22 patients in total—five without infusions—and another five did not complete the entire study (Table 1) (Lu et al., 2020).

The scientists in the study made sure that the experiment was safe, practical, and effective before proceeding. During the study, the targeted T-cells were engineered *ex vivo* with the Cas9 endonuclease and plasmids containing sgRNA, and these cells were infused into the patients. As a result of the treatment, only lower-grade symptoms (hypertension and anemia) occurred in the study participants, and none of the patients had cytokine release syndrome after the treatment. Patients who received the treatment also had a survival rate without cancer progression of 7.7 weeks and a median survival of 42.6 weeks (Table 1). Overall, there were very few off-target effects throughout the experiment, which allowed the scientists to conclude that CRISPR was an optimal method to improve the efficacy of gene therapy for NSCLC (Lu et al., 2020).

Pre-Clinical Research with the CRISPR/Cas9 System

Outside of clinical trials, there has also been significant progress with pre-clinical research that evaluates the feasibility of CRISPR/Cas9 methods in treating certain diseases (Figure 5). For example, in a study involving the human immunodeficiency virus (HIV), a specific targeting system designed with CRISPR was used to detect the presence and inhibit HIV-1 gene expression by removing the HIV genes from the chromosome, thus permanently blocking latent provirus and eliminating the HIV infection (Figure 5). In the future, CRISPR may become a valuable tool in targeting and indefinitely removing HIV, but there are particular issues, like its vulnerability to off-target effects when editing HIV-related genes, that need to be addressed first (Ebina et al., 2013).

Additionally, another pre-clinical trial focused on using CRISPR/Cas9 to repair Duchenne muscular dystrophy (DMD) mutations; specifically, the repairing mechanisms occur as a result of the communications between the splicing sites and the PAM sequence in the CRISPR system (Figure 5). The scientists exploited these circumstances and engineered sgRNAs that would intentionally skip specific DMD exons (myoediting) to correct DMD mutations. Myoediting was done in DMD patients to fix the DMD gene in the engineered heart

muscle (EHM) to express dystrophin normally. The results of the DMD study show CRISPR can be used to correct DMD by repairing specific mutations in the DMD gene, and as the scientists behind the study have received approval to begin clinical trials, CRISPR may prove to be an effective treatment for DMD in the future (Long et al., 2018).

Furthermore, a study found that the CRISPR system can be used with Cas9 nickase to eliminate the CAG repeats in the huntingtin gene (HTT) that cause Huntington's disease (HD) (Figure 5). Removing these repeats prevents the HTT gene from further producing the huntingtin protein with HD-causing CAG repeats. This CRISPR treatment for HD is beneficial because Cas9 nickases are safe—as they specifically induce single-stranded breaks—and the treatment can be used for a few other neurodegenerative diseases as well (Dabrowska et al., 2018).

In a pre-clinical study involving mice, CRISPR was used to fix mutations in the Crygc gene in mice that caused cataracts (Figure 5). Specifically, CRISPR sgRNAs that detected specific mRNA sequences in the Crygc gene were created to identify the locations of the cataract-causing mutations as well as how they could be targeted and fixed, then CRISPR was inserted into the Crygc gene in the mice zygotes to correct the mutations, proving itself as an effective treatment for cataracts in mice (Wu et al., 2013).

Lastly, a recent study done with CRISPR shows its ability to treat influenza and a variant of the coronavirus (Figure 5). This study showed the efficacy of eliminating RNA viruses from controlled-growth cells in animals, mainly targeting influenza A in mice and SARS-CoV-2 in hamsters. Specific crRNAs were engineered and then selected based on their effectiveness at mitigating the viruses. The CRISPR system worked to deteriorate the influenza RNA in mice and prevented SARS-CoV-2 RNA from replicating. Thus, CRISPR can be used to target and degrade viruses that attack the respiratory system, making it a capable treatment for respiratory-based diseases (Blanchard et al., 2021).

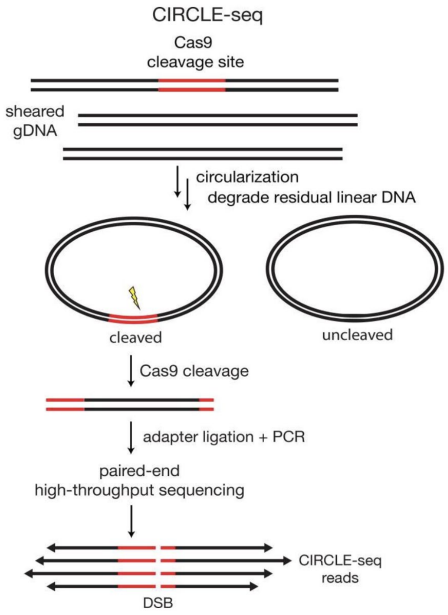
Overall, clinical and pre-clinical studies have shown the effectiveness and potential of the CRISPR/Cas9 gene-editing system as a genetic treatment for certain diseases through its gene knockout and silencing capabilities, as well as its ability to remove and correct disease-causing mutations (Esrick et al., 2021; Long et al., 2018; Wu et al., 2013). Through further clinical trials and testing, CRISPR/Cas9 can become a widespread and publicly-available method for treating many different types of diseases and disease-causing agents in the future.

Next Steps for the CRISPR/Cas9 Gene-Editing System in Disease Treatment

Validation Techniques for Detecting and Minimizing Off-Target Events

Several concerns need to be addressed before the CRISPR/Cas9 gene-editing technology can become a widely-utilized treatment option. First, off-target effects must be minimized, as any off-target activity can cause problems within the genome and for the disease. Currently, validation techniques exist to determine the rate or tendency of off-target activity—or the types, locations, and numbers of off-target effects—and to also cleave off-target or unindented mutations caused by CRISPR/Cas9 gene editing (S. Zhang et al., 2019).

Previously, the only existing *in vitro* CRISPR genomic verification method was Digenome-seq, which works by cleaving DNA, sequencing it, and identifying any off-target sites present on the DNA. However, Digenome-seq requires a deep sequencing of DNA to identify the frequency of off-target effects, which becomes even more critical in the case of rare off-target events. Similarly, sequencing and identification errors can occur in the absence of high sequencing coverage. Digenome-seq can also be costly—a potential barrier in patient treatment—and thus is not practical for frequent medical use (Kim et al., 2015). As such, a new validation technique called CIRCLE-seq (Circularization for *In Vitro* Reporting of Cleavage Effects by Sequencing) was developed to be a more specific and efficient *in vitro* way to determine off-target effects in CRISPR (Figure 6). CIRCLE-seq works similarly to Digenome-seq, though it has improvements that reduce the amount of DNA



reads needed and increase the chance of lower-frequency off-target effects being detected, making it vastly different from and better than other *in vitro* prediction technologies (Figure 6) (Tsai et al., 2017).

Figure 6. The CIRCLE-seq Off-Target Detection Method (Tsai et al., 2017)

Another issue with the CRISPR/Cas9 system is the potential for new DNA mutations to arise through mutagenesis. A method for detecting and cleaving these mutations—the enzyme mismatch cleavage method—was developed to eliminate any mutations that may occur as a result of CRISPR gene editing. It was tested in frog embryos with the T7E1 enzyme as well as other Surveyor Mismatch nuclease enzymes, and it was found that T7E1 shows greater efficiency than Surveyor in identifying mutations associated with gene editing. Specifically, T7E1 is more sensitive to frameshift deletion mutations, while Surveyor works better with point mutations, particularly single nucleotide substitutions. Regardless, both enzymes serve as an effective way to cleave CRISPR-induced mutations, though they have not yet been used to detect and fix off-target mutations in patients (Vouillot et al., 2015).

Additionally, bacterial CRISPR/Cas9 adaptive immune systems have evolved to self-contain checkpoints used in gene editing to ensure the accuracy of CRISPR. One of these, from the *Pseudomonas aeruginosa* bacteria, involves the endoribonuclease enzyme Csy4, which binds to and cleaves the repeating CRISPR sequences (spacers), and is also required for the formation of crRNAs. Csy4 then recognizes an RNA substrate and separates the cleaved sequences, ensuring that specificity and accuracy are prioritized over speed with CRISPR (Haurwitz et al., 2012)

Beyond specific CRISPR/Cas9 validation mechanisms, scientists have created sgRNA libraries and predictive algorithms for off-target activity for both the human and mouse genomes in order to minimize off-target effects and increase on-target activity for CRISPR overall. These libraries are necessary to compile the locations of off-target sites and the frequency of off-target activity occurrence, and they will be used to maximize the accuracy of CRISPR and minimize potential off-target effects in future genetic engineering projects (Doench et al., 2016).

Furthermore, as mentioned before, there are currently many ongoing clinical trials with the *S. pyogenes* CRISPR/Cas9 system in the context of gene therapies for various diseases. These trials have been consolidated in a review that also details advances and developments in both new and already-existing CRISPR systems, how CRISPR can be applied, and how ethical CRISPR is. Of course, more clinical trials must be conducted to monitor off-target effects through studies on both patient physical health and genomic stability as the next steps towards determining how accurate, effective, specific, and safe CRISPR is at treating and preventing certain diseases (Kick et al., 2017).

Moral and Social Considerations with the CRISPR/Cas9 System

Aside from potential health concerns, there are also social obstacles that need to be addressed. One of the biggest is the ethical and moral aspect of using the CRISPR/Cas9 system to edit the human genome, even if it is simply for the purpose of treating diseases. Although current research, clinical trials, and case studies have stuck to using CRISPR strictly as a disease treatment, in the future, the potential for CRISPR to be used purely for cosmetic purposes—or in human embryos to edit the genomes of future generations—is something which many are concerned with (Figure 7). CRISPR has commonly been associated with the idea of "designer babies," and the repercussions—both healthwise and ethical—of using CRISPR for this purpose have been heavily debated

in the scientific community (Figure 7) (Brokowski & Adli, 2019).

Figure 7. The Editing of Human Embryos with the CRISR/Cas9 System to Produce "Designer Babies" (Vyas, 2019)

The most notable instance of the unethical usage of CRISPR is the case of a Chinese doctor and scientist named He Jiankui, who used CRISPR to genetically modify the genomes of two twin girls—aliases Nana and Lulu. Although the babies were successfully treated by CRISPR and born healthy as non-identical twins, the Chinese government was not pleased with his study, and he was jailed and criticized for his actions (Greely, 2019). He's fate shows how the ethics regarding the use of CRISPR to intentionally alter one's genome are very nuanced, and it is unlikely that there will be a scientific consensus regarding the future of CRISPR anytime soon. Thus, until the ethical issues are settled, CRISPR cannot easily become an accessible medical treatment, as there are concerns that clearing the gene-editing system for public use might make it easier for it to potentially be used beyond somatic therapy (Brokowski & Adli, 2019).

In the end, the CRISPR/Cas9 gene-editing system still has a long way to go before it can become a publicly-available gene therapy for certain diseases. Off-target effects and mutations need to be minimized or even eliminated—which is one of the issues scientists are addressing in current CRISPR research (S. Zhang et al., 2019). In addition, the accuracy, specificity, and efficiency of CRISPR have to be ensured, and problems with its ethics have to be resolved. In the future, however, CRISPR/Cas9 has the potential to be used in health and medicine to treat diseases (Brokowski & Adli, 2019).

Conclusion

Overall, the *S. pyogenes* bacteria-derived CRISPR/Cas9 gene-editing system is a significant breakthrough in the scientific community—including the health and medicine industries—for treating and preventing diseases. Research has proven the gene-editing system to be largely safe and effective as well as accurate, as multiple aspects of the system, such as the Cas9 endonuclease enzyme, tracrRNA-crRNA sgRNA dual structure, and the PAM sequence, are largely very specific when precisely engineered (X.-H. Zhang et al., 2015). Additionally, current research on multiple genetic and non-hereditary diseases suggests that it can be viewed as a new form of illness treatment—though it is not yet available to the public, it has the potential to become a widespread treatment once effective ways to completely eliminate both off-target activity and unintended mutations are developed (S. Zhang et al., 2019). Both completed and ongoing human clinical trials and CRISPR case studies have primarily therapeutic purposes in living individuals or a group of similarly-affected patients (Blanchard et al., 2021; Dabrowska et al., 2018; Ebina et al., 2013; Esrick et al., 2021; Frangoul et al., 2021; Long et al., 2018; Lu et al., 2020; Wu et al., 2013). However, this could later lead to the eradication of many such diseases through the germline for all future generations. Despite its promising applications, however, the future of CRISPR cannot be determined until its ethics and morality issues are resolved (Brokowski & Adli, 2019). Until then, it is important to continue researching and applying the CRISPR/Cas9 gene-editing system in clinical studies in order to ensure its effectiveness as a medicinal therapy for certain diseases.

Acknowledgments

I would like to acknowledge Polygence for giving me the opportunity to conduct research online as a high school student. I am especially very thankful to my mentor for guiding me through my research on the CRISPR/Cas9 gene-editing system and how it can be used to prevent diseases.

References

Ball, P. (2016, November 17). *CRISPR: Implications for materials science*. Cambridge Core. https://www.cambridge.org/core/journals/mrs-bulletin/news/crispr-implications-for-materials-science

Blanchard, E. L., Vanover, D., Bawage, S. S., Tiwari, P. M., Rotolo, L., Beyersdorf, J., Peck, H. E., Bruno, N. C., Hincapie, R., Michel, F., Murray, J., Sadhwani, H., Vanderheyden, B., Finn, M. G., Brinton, M. A., Lafontaine, E. R., Hogan, R. J., Zurla, C., & Santangelo, P. J. (2021). Treatment of influenza and SARS-CoV-2 infections via mRNA-encoded Cas13a in rodents. *Nature Biotechnology*, *39*(6), 717–726. https://doi.org/10.1038/s41587-021-00822-w

Brokowski, C., & Adli, M. (2019). CRISPR Ethics: Moral Considerations for Applications of a Powerful Tool. *Journal of Molecular Biology*, *431*(1), 88–101. https://doi.org/10.1016/j.jmb.2018.05.044

Carlaw, T. M., Zhang, L.-H., & Ross, C. J. D. (2020). CRISPR/Cas9 Editing: Sparking Discussion on Safety in Light of the Need for New Therapeutics. *Human Gene Therapy*, *31*(15–16), 794–807. https://doi.org/10.1089/hum.2020.111

Dabrowska, M., Juzwa, W., Krzyzosiak, W. J., & Olejniczak, M. (2018). Precise Excision of the CAG Tract from the Huntingtin Gene by Cas9 Nickases. *Frontiers in Neuroscience*, *12*. https://doi.org/10.3389/fnins.2018.00075

Doench, J. G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E. W., Donovan, K. F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., Virgin, H. W., Listgarten, J., & Root, D. E. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nature Biotechnology*, *34*(2), 184–191. https://doi.org/10.1038/nbt.3437

Ebina, H., Misawa, N., Kanemura, Y., & Koyanagi, Y. (2013). Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Scientific Reports*, *3*(1), 2510. https://doi.org/10.1038/srep02510

Esrick, E. B., Lehmann, L. E., Biffi, A., Achebe, M., Brendel, C., Ciuculescu, M. F., Daley, H., MacKinnon, B., Morris, E., Federico, A., Abriss, D., Boardman, K., Khelladi, R., Shaw, K., Negre, H., Negre, O., Nikiforow, S., Ritz, J., Pai, S.-Y., … Williams, D. A. (2021). Post-Transcriptional Genetic Silencing of BCL11A to Treat Sickle Cell Disease. *New England Journal of Medicine*, *384*(3), 205–215. https://doi.org/10.1056/NEJMoa2029392

Frangoul, H., Altshuler, D., Cappellini, M. D., Chen, Y.-S., Domm, J., Eustace, B. K., Foell, J., de la Fuente, J., Grupp, S., Handgretinger, R., Ho, T. W., Kattamis, A., Kernytsky, A., Lekstrom-Himes, J., Li, A. M., Locatelli, F., Mapara, M. Y., de Montalembert, M., Rondelli, D., … Corbacioglu, S. (2021). CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. *The New England Journal of Medicine*, *384*(3), 252–260. https://doi.org/10.1056/NEJMoa2031054

Greely, H. T. (2019). CRISPR'd babies: Human germline genome editing in the 'He Jiankui affair'*. *Journal of Law and the Biosciences*, *6*(1), 111–183. https://doi.org/10.1093/jlb/lsz010

Haurwitz, R. E., Sternberg, S. H., & Doudna, J. A. (2012). Csy4 relies on an unusual catalytic dyad to position and cleave CRISPR RNA. *The EMBO Journal*, *31*(12), 2824–2832. https://doi.org/10.1038/emboj.2012.107

He, S. (2020). The first human trial of CRISPR-based cell therapy clears safety concerns as new treatment for late-stage lung cancer. *Signal Transduction and Targeted Therapy*, *5*(1), 1–2. https://doi.org/10.1038/s41392-020-00283-8

Hongfan, C., Choi, J., & Bailey, S. (2014). Cut Site Selection by the Two Nuclease Domains of the Cas9 RNA-guided Endonuclease. *The Journal of Biological Chemistry*, *289*, 13284–13294. https://employees.csbsju.edu/hjakubowski/classes/ch331/Lit_Learn_Module/LLM_CRISPR_Cas1_KEY.html

Importance of the PAM Sequence in CRISPR Experiments. (n.d.). In *How To Use CRISPR: Your Guide to Successful Genome Engineering*. Synthego. https://www.synthego.com/guide/how-to-use-crispr/pam-sequence

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. https://doi.org/10.1126/science.1225829

Kick, L., Kirchner, M., & Schneider, S. (2017). CRISPR-Cas9: From a bacterial immune system to genome-edited human cells in clinical trials. *Bioengineered*, *8*(3), 280–286. https://doi.org/10.1080/21655979.2017.1299834

Kim, D., Bae, S., Park, J., Kim, E., Kim, S., Yu, H. R., Hwang, J., Kim, J.-I., & Kim, J.-S. (2015). Digenome-seq: Genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nature Methods*, *12*(3), 237–243. https://doi.org/10.1038/nmeth.3284

Long, C., Li, H., Tiburcy, M., Rodriguez-Caycedo, C., Kyrychenko, V., Zhou, H., Zhang, Y., Min, Y.-L., Shelton, J. M., Mammen, P. P. A., Liaw, N. Y., Zimmermann, W.-H., Bassel-Duby, R., Schneider, J. W., & Olson, E. N. (2018). Correction of diverse muscular dystrophy mutations in human engineered heart muscle by single-site genome editing. *Science Advances*. https://doi.org/10.1126/sciadv.aap9004

Lu, Y., Xue, J., Deng, T., Zhou, X., Yu, K., Deng, L., Huang, M., Yi, X., Liang, M., Wang, Y., Shen, H., Tong, R., Wang, W., Li, L., Song, J., Li, J., Su, X., Ding, Z., Gong, Y., … Mok, T. (2020). Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. *Nature Medicine*, *26*(5), 732–740. https://doi.org/10.1038/s41591-020-0840-5

O'Connell, M. R., Oakes, B. L., Sternberg, S. H., East-Seletsky, A., Kaplan, M., & Doudna, J. A. (2014). Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature*, *516*(7530), 263–266. https://doi.org/10.1038/nature13769

On Biostatistics and Clinical Trials. (2019, August 21). https://pubrica.com/academy/statistical/on-biostatistics-and-clinical-trials/

Pattanayak, V., Lin, S., Guilinger, J. P., Ma, E., Doudna, J. A., & Liu, D. R. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature Biotechnology*, *31*(9), 839–843. https://doi.org/10.1038/nbt.2673

Phase 3 Clinical Trials Opening for Hepatitis Delta Patients. (2019, March 21). *Hepatitis B Foundation*. https://www.hepb.org/blog/phase-3-clinical-trials-opening-hepatitis-delta-patients/

Shabbir, M., Shabbir, M. A. B., Hao, H., Shabbir, M. Z., Hussain, H., Iqbal, Z., Ahmed, S., Sattar, A., Iqbal, M., Li, J., & Yuan, Z. (2016). Survival and Evolution of CRISPR?Cas System in Prokaryotes and Its Applications. *Frontiers in Immunology*, *7*. https://doi.org/10.3389/fimmu.2016.00375

Tsai, S. Q., Nguyen, N. T., Malagon-Lopez, J., Topkar, V. V., Aryee, M. J., & Joung, J. K. (2017). CIRCLE-seq: A highly sensitive in vitro screen for genome-wide CRISPR–Cas9 nuclease off-targets. *Nature Methods*, *14*(6), 607–614. https://doi.org/10.1038/nmeth.4278

Vertex Pharmaceuticals Incorporated. (2022a). *A Phase 1/2/3 Study of the Safety and Efficacy of a Single Dose of Autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs) in Subjects With Transfusion-Dependent β-Thalassemia* (Clinical Trial Registration No.

NCT03655678). clinicaltrials.gov. https://clinicaltrials.gov/ct2/show/NCT03655678

Vertex Pharmaceuticals Incorporated. (2022b). *A Phase 1/2/3 Study to Evaluate the Safety and Efficacy of a Single Dose of Autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (CTX001) in Subjects With Severe Sickle Cell Disease* (Clinical Trial Registration No. NCT03745287). clinicaltrials.gov. https://clinicaltrials.gov/ct2/show/NCT03745287

Vouillot, L., Thélie, A., & Pollet, N. (2015). Comparison of T7E1 and Surveyor Mismatch Cleavage Assays to Detect Mutations Triggered by Engineered Nucleases. *G3 Genes|Genomes|Genetics*, *5*(3), 407–415. https://doi.org/10.1534/g3.114.015834

Vyas, K. (2019, July 4). *Designer Babies: Gene-Editing and the Controversial Use of CRISPR*. https://interestingengineering.com/designer-babies-gene-editing-and-the-controversial-use-of-crispr

Williams, D. (2022). *Pilot and Feasibility Study of Hematopoietic Stem Cell Gene Transfer for Sickle Cell Disease* (Clinical Trial Registration No. NCT03282656). clinicaltrials.gov. https://clinicaltrials.gov/ct2/show/NCT03282656

Wu, Y., Liang, D., Wang, Y., Bai, M., Tang, W., Bao, S., Yan, Z., Li, D., & Li, J. (2013). Correction of a Genetic Disease in Mouse via Use of CRISPR-Cas9. *Cell Stem Cell*, *13*(6), 659–662. https://doi.org/10.1016/j.stem.2013.10.016

Xie, N., Zhou, Y., Sun, Q., & Tang, B. (2018). Novel Epigenetic Techniques Provided by the CRISPR/Cas9 System. *Stem Cells International*, *2018*, 7834175. https://doi.org/10.1155/2018/7834175

Zabaleta, N., Barberia, M., Martin-Higueras, C., Zapata-Linares, N., Betancor, I., Rodriguez, S., Martinez-Turrillas, R., Torella, L., Vales, A., Olagüe, C., Vilas-Zornoza, A., Castro-Labrador, L., Lara-Astiaso, D., Prosper, F., Salido, E., Gonzalez-Aseguinolaza, G., & Rodriguez-Madoz, J. R. (2018). CRISPR/Cas9-mediated glycolate oxidase disruption is an efficacious and safe treatment for primary hyperoxaluria type I. *Nature Communications*, *9*(1), 5454. https://doi.org/10.1038/s41467-018-07827-1

Zhang, S., Li, X., Lin, Q., & Wong, K.-C. (2019). Synergizing CRISPR/Cas9 off-target predictions for ensemble insights and practical applications. *Bioinformatics*, *35*(7), 1108–1115. https://doi.org/10.1093/bioinformatics/bty748

Zhang, X.-H., Tee, L. Y., Wang, X.-G., Huang, Q.-S., & Yang, S.-H. (2015). Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Molecular Therapy - Nucleic Acids*, *4*, e264. https://doi.org/10.1038/mtna.2015.37