Curative Therapy of Sickle Cell Disease using Gene Editing Technologies

ABSTRACT

Sickle cell disease (SCD) is a monogenic blood disorder caused by a single nucleotide mutation in the β-globin gene that swaps the hydrophilic glutamic acid at position six with the hydrophobic valine. Haematopoietic stem cell transplantation is the only curative treatment for SCD, but has major risks and problems. Autologous gene therapy, in which "all" gene copy is put into the patient's cells, a faulty gene is fixed, or genes are deactivated, has the advantage of not requiring the patient to find a perfect donor. Gene therapy has been shown to be curative in preclinical and clinical trials. LentiGlobin, a self-inactivating lentiviral vector encoding the human antiserpentine. Haemoglobin Subunit Beta, is now being tested in clinical trials. However, lentiviral vector-based gene therapy poses potential dangers such as Replication-Competent Lentivirus and insertional mutagenesis. Gene editing technologies allow for permanent modification of disease-causing genes, and HSPCs are the therapeutic product for autologous transplantation. CRISPR/Cas9 is a versatile and efficient class of programmable nucleases that use single-stranded guide RNA sequences and the Cas9 endonuclease to attach to a specific target in the genome. Base editors are generated by pairing a nucleotide deaminase with a Cas9 protein that is catalytically suppressed. All four gene editing strategies have been evaluated in HSPCs for the treatment of SCD, including correcting the sickle cell mutation in HBB, producing enough HbF to reverse the sickle shape of the RBC, focusing on the HbF transcriptional repressors, and introducing the advantageous HPF mutations. CRISPR/Cas9 gene editing has been used to treat SCD, including correcting the sickle cell mutation, producing enough HbF to reverse the sickle shape, focusing on HbF transcriptional repressors, and introducing HPFH mutations. Future challenges for the success of CRISPR/Cas9 gene editing include off-target effects, gene repair efficiency, and *in vivo* transplantation of gene-edited HSPC.

Keywords: CRISPR/Cas9, gene editing, gene therapy, β-globin gene, sickle cell disease, single nucleotide mutation.

1. INTRODUCTION

"Sickle cell disease (SCD) refers to a group of monogenic blood diseases that include mutations in the gene encoding the beta subunit of haemoglobin, and affects around 8 million people globally" [1]."It is most common in Africa, the Middle East, and India, and iscaused by a single nucleotide mutation in the β-globin gene that swaps the hydrophilic glutamic acid at position six with the hydrophobic valine. The understanding of the phenotypic expression of the disease is still limited. However, environmental factors such as cold weather and air quality, infections, fetal hemoglobin level, and other genetic subtypes play a role in the manifestation of the disease. Clinical manifestations are variable and affect multiple systems, and generally cause lower life expectancy"[2-4].

"The genetic mutation described causes polymerization of the Hemoglobin molecule that alters the erythrocyte shape and its ability to deform. There is an increase in adhesion of

erythrocytes followed by the formation of heterocellular aggregates, which physically cause small vessel occlusion and resultant local hypoxia. This process triggers a vicious cycle of increased HbS formation, the release of inflammatory mediators and free radicals that contribute to reperfusion injury. The jagged cells cause unpredictable attacks of intense pain and damage vital organs.Hemoglobin also binds to nitric oxide (NO), a potent vasodilator, and releases oxygen. Erythrocytes are more likely to sickle and become rigid in the presence of dehydration. This process is mostly caused by changes in cation homeostasis, especially increased potassium and water efflux caused by potassium-chloride co-transport and Gardos channels (calcium-dependent potassium channel) Other associated pathological events include increased neutrophil adhesiveness, nitric oxide binding, increased platelet activation, and hypercoagulability" [5].

"The histopathology in sickle cell disease is very variable and necrosis and ischemia are seen in the affected organ system, with liver, bone marrow, lungs, spleen, kidneys, and lungs being the commonly affected organs. The history and physical exam of sickle cell patients range from being asymptomatic to a broad range of presentations. Patients are asymptomatic for the first 6 months of life due to the presence of fetal hemoglobin, which gradually decreases, and HbS begins to predominate. The clinical presentation of SCD is variable depending on the type of complication and the body system affected. A few of the common presentations are Vaso-Occlusive Crisis, Acute Chest Syndrome, Infections, Pulmonary Hypertension, Cerebrovascular Accidents/Stroke, Pulmonary Embolism, Renal Complications, Eye Complications, Splenic Sequestration, Priapism, Cholelithiasis and biliary sludge, Osteonecrosis, Aplastic Crisis" [5].Despite being the first molecular illness with a genetic foundation discovered more than 60 years ago, therapeutic options for SCD are still quite limited and the average life expectancy of patients is very low.

"Autologous gene therapy, in which "all" gene copy is put into the patient's own cells, a faulty gene is fixed, or genes are deactivated, has the advantage of not requiring the patient to find a perfect donor.*Ex vivo* engineering of autologous haematopoietic stem and progenitor cells (HSPCs) followed by transplantation of genetically engineered cells offers a potentially permanent treatment that can be applied to patients regardless of the availability of suitable donors and without graft-host risk [6]. Sickle cells mature inefficiently and also have a shorter life compared to healthy RBCs, suggesting a selective advantage of gene-corrected HSPCs over SCD HSPCs *in vivo*. Only 2–5% of donor chimerism after allergenic transplantation is sufficient to alleviate SCD-related symptoms in patients, providing the basis for a gene therapy approach. Thus, successful gene addition or correction in relatively few haematopoietic stem cells (HSCs) can lead to clinically relevant levels of erythrocyte chimerism in peripheral blood. Gene therapy for SCD utilizing a lentiviral vector has been shown to be curative in preclinical and clinical trials throughout the last two decades. The first SCD patient whoreceived therapy with anti-sickle HBB induction in autologous HSCs was effective, with high therapeutic levels of anti-sickle-globin 15 months later"[6]."LentiGlobin, a self-inactivating (SIN) lentiviral vector encoding the human antiserpentine Haemoglobin Subunit Beta (HBB), is now being tested in clinical trials later" [7]. "However, the use of lentiviral vectors poses potential dangers such as the creation of Replication-Competent Lentivirus (RCL), which can infect non-target cells, and insertional mutagenesis, which can lead to genetic dominance and DNA damage"[8]. Recent lentiviral gene therapy clinical trial findings show promise for *ex vivo* creation of autologous HSPCs, lengthier follow-up is required to validate the safety and efficacy. Gene editing technologies, as opposed to traditional gene therapy approaches, allow for the permanent modification of disease-causing genes through precise repair, deletion, insertion and disruption of specified sequences. Gene-edited HSPCs from SCD patients (SCD HSPCs) are the therapeutic product for autologous transplantation. In recent preclinical research, several gene editing techniques have showed promise for SCD therapy, including:

i. Repair of the HBB-causing point mutation

ii. Induction of foetal haemoglobin (HbF) through disruption of γ-globin (Haemoglobin subunit gamma HBG) repressors;
iii. Induction of HbF through creation of advantageous hereditary persistence of foetal haemoglobin (HPFH) mutations at the β-globin locus [9].

"Haemoglobin molecules are made up of four subunits: two polypeptide chains and two non-polypeptide chains. The overall haemoglobin composition of a healthy adult is 97% of haemoglobin (HbA), 3% or less HbA_2, and not more than 1% of HbF. HPFH is a harmless disorder caused by mutations in the β-globin gene cluster, which results in increased HbF levels in adults. SCD patients with concurrent HPFH have better clinical outcomes, whereas higher HbF levels are associated with morbidity and mortality. With greater awareness of how the globin locus is regulated, there is an interest in developing ways to boost HbF expression for therapeutic treatments. HbF induction is accomplished either by silencing transcription factors such as B-cell lymphoma 11A (BCL11A), which mediate HBG silencing after birth, or by mimicking advantageous HPFH mutations. Furthermore, the identification of additional HbF regulatory factors is an important topic of research"[10].

Gene editing techniques employ engineered nucleases such as TAL Effector Nucleases (TALEN), Zinc Finger Nucleases (ZFN), and Clustered Regularly Spaced Short Palindromic Repeats (CRISPR)/Cas9 systems to generate a user-defined location for a DNA Double-Strand Break (DSB). Repair, deletion, insertion, and excision of certain sequences accomplished through targeted DSB creation which is followed by Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR) offers the possibility of permanent correction of disease-causing mutations. ZFNs and TALENs have different DNA binding domains, yet they both break DNA with the FokI endonuclease domain. However, programming these nucleases is difficult, time-consuming and requires a high level of knowledge. The CRISPR/Cas9 system has shown to be the most versatile and efficient class of programmable nucleases in recent years. CRISPR/Cas9 uses single-stranded guide RNA (gRNA) sequences and the Cas9 endonuclease to attach to a specific target in the genome. Similarity between the gRNA and target DNA sequences directs the Cas9 endonuclease to a specific target location. Though off-target effects are still a possibility, they can be considerably decreased through logical gRNA design or the use of high-quality Cas9 protein. Base editors are generated by pairing a nucleotide deaminase with a Cas9 protein that is catalytically suppressed. Base editors transform one base to another without causing DSBs, and so do not rely on HDR to repair point mutations in non-dividing cells. As a result, base editors are a promising technique for DNA editing and are preferred over the use of Cas9 nuclease, which can result in undesirable tiny insertions/deletions or indels, translocations or chromosomal rearrangements. All four gene editing strategies (ZFNs, TALENs, CRISPR/Cas9, and base editor) have been evaluated in HSPCs for the treatment of SCD. SCD mutations have been corrected by combining ZFNs or TALENs with a DNA donor template. Researchers have created ZFN and TALEN that target HbF transcriptional repressors or the repressor-binding site in order to induce HbF. A Phase-1/2 clinical trial using ZFN that targets the BCL11A locus has been conducted (BIVV003, clinicaltrials.gov).

This review concentrates on methods for treating SCD that use CRISPR/Cas9 gene editing, including correcting the sickle cell mutation in HBB, producing enough HbF to reverse the sickle shape of the RBC, focusing on the HbF transcriptional repressors, and introducing the advantageous HPFH mutations. Optimizing the genome editing method, including CRISPR Cas9/gRNA and donor template and delivery method, are very crucial for achieving high safety and efficacy. Small improvements at each step are key to clinical translation. Challenges in the translation of a gene editing strategy to the clinic include the possibility of off-target effects, the need to improve gene repair efficiency, and *in vivo* transplantation of gene-edited HSPC.

2. GENE TARGETING FOR THE TREATMENT OF SCD

Programmable nucleases produce double-strand breaks (DSB) at a particular genomic location, which are then repaired by either non-homologous end joining (NHEJ) or homology directed repair (HDR), which uses homologous sequences from sister chromatids, homologous chromosomes, or an extra chromosome DNA sequence from the donor that has been submitted for correction (Fig. 1). Three significant nucleases were first introduced for various genome editing uses five years ago, including mega nucleases, sometimes referred to as mould endonucleases CRISPR editing for SCD, zinc finger nucleases (ZFN), and TAL effector nucleases (TALEN). By modifying regulatory sequences such promoters or other regulatory sequences like BCL11A, KLF1, and MYB to reduce mutation severity in sickle HSPCs, these methods have been successfully employed to *ex vivo* repair SCD mutation and induce foetal β-globin. Although these nucleases are highly specific, which minimizes off-target effects (OTE), programming these enzymes is challenging, time-consuming, and knowledge-intensive. Clustering short palindromic repetitions at regular intervals (CRISPR)/CRISPR-related protein 9 (Cas9) is a novel genome editing technique[11]. In this method, a specific RNA (guide RNA) sequence identifies the DNA target region of interest and directs the effector Cas protein there for editing. The advantage of this strategy is that it is easily designed, highly efficient, and reasonably priced. DNA repair mechanisms are activated when CRISPR/Cas9-driven DSBs are added to an object. If HDR is engaged, this mechanism would either cause some additions/deletions (INDEL), which in theory results in the loss of function of a particular gene, or repair the DNA damage using homologous chains. The obstacles associated with adopting this technology still include efficiency, safety, and delivery. CRISPR/Cas9 technology can target SCD mutation correction or induce by altering foetal haemoglobin expression; chromosomal areas control its expression.

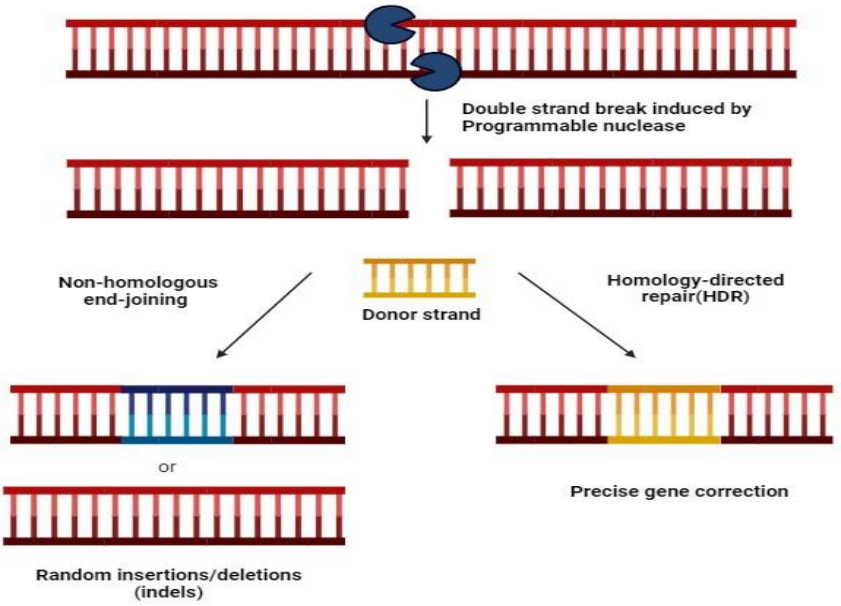

Fig. 1. DSBs caused by programmable nucleases and the two distinct DSB repair mechanisms (NHEJ and HDR) are used in genome editing.

2.1. Conventional Gene Targeting by Homologous Recombination

By utilizing homologous recombination between genomic DNA and an external targeting vector, gene targeting modifies a chromosome. The target vector, also known as ‘donor DNA’, comprises an insert DNA that has been inserted into a particular region of the genome. This insert is surrounded by sequences that are homologous to the target locus,

known as the 5' and 3' homology arms [12]. Homology-directed repair (HDR) of spontaneous DNA double-strand breaks (DSBs) is used for accurate integration of input DNA and normally uses the sister chromatin as a repair template. HR between chromosomal and foreign DNA can be employed to repair spontaneous DSBs after donor DNA has been transfected with sequences similar to the locus to be transformed. This results in the integration of the desired insert into the target site [13].

Gene targeting may be an effective strategy for treating SCD, since it would preserve the HBB gene's natural environment and only require HR to replace the incorrect mutant sequence. In mammalian cells, genes have been repaired using a variety of techniques. Only the DSB repair template with the repaired mutation is delivered into the cells in non-selective systems. For modest alterations, the repair template can take the shape of a plasmid or a brief single-stranded oligonucleotide. Adenoviruses, adeno-associated viruses, or Integration-Deficient Lentiviral Vectors (IDLV), among others, could also be employed as fresh sources of donor DNA. Another method for selecting cells through gene correction is to incorporate a positive selection marker with the corrected mutation location into the genome. In order to remove the selection cassette from the genome since the transgene's presence in the genome is unwanted, a second round of editing would be necessary. Given its potential application for gene repair in patients with SCD or thalassemia, the HBB locus was the focus of the first investigations to demonstrate HR between a chromosomal region and foreign DNA[13]. Gene targeting later showed promise in mouse embryonic stem cells (mESCs), and then was applied to mESCs carrying the human HbS gene. In human cells, HR-mediated alteration of the HBB locus has also been demonstrated.

The ineffectiveness of HR for gene targeting in human cells is an issue and depends on uncommon DSBs in the target area, which necessitates the employment of extensive homology branches to capture DSBs. Additionally, HR is not a significant route for mending DSBs in mammalian cells, resulting in a frequency of less than one event per 10^5 cells overall, which is too low to be employed and makes it challenging to locate precisely targeted clones. But multiple proof-of-concept studies indicated that introducing a DSB into a particular region of the genome can boost HR rates in the chosen location [14].

2.2. Use of programmable nucleases to mediate repair of sickle cell mutations by gene targeting

Programmable nucleases hold enormous promise as therapeutic agents for single-gene SCD because they can significantly increase the effectiveness of gene targeting. Genome editing methods include ZFNs, TALENs, and CRISPR/Cas9 can produce desired changes in a range of cell types and eukaryotic models. In order to create site-specific DNA DSBs, programmable nuclease-mediated gene editing relies on the recruitment of endonucleases to user-defined genomic locations. While the cellular DNA repair systems are repairing the DSB, the desired modification is incorporated into the genome. Non-homologous end joining (NHEJ), which is prone to errors, is one technique for fixing DSBs. The insertion/deletion mutations that result from this lead to gene knockouts. The damage is repaired by HR in the presence of donor DNA with the proper homology arms, enabling gene insertion, replacement, or deletion. Previous proof-of-concept investigations have shown that these three programmable nucleases have therapeutic potential [15].

2.2.1. Zinc Finger Nucleases (ZFN)

ZFNs are designed proteins having two functional domains: A FokI endonuclease domain and a eukaryotic zinc-finger transcription factor-based DNA-binding domain (DBD) [16]. Each zinc finger protein in the DBD is able to recognize three base pairs (bp) of DNA. The DBD consists of 3-6 tandem repeats of these proteins. Most of the three bp combinations can be recognized by zinc finger proteins. Therefore, a DNA sequence of interest between 9

and 18 bp can be recognized by each ZFN monomer and changed by replacing the ZFN zinc finger protein. The DNA-cleavage domain of the type IIS restriction endonuclease FokI is linked to the DNA-binding domain to introduce DSB at a particular locus. FokI can only be used when it is dimerized. For DSB induction, ZFNs cooperate in pairs for this reason. In order to enable FokI dimerization, the dimer recognizes a total sequence of 18–36 bp and binds to DNA in a tail–to–tail orientation using a spacer between each monomer's binding sites. In the space between the two ZFN binding sites, DSBs are generated[16, 17]. A ZFN was created that could target the area around the sickle mutation of the β-globin locus but not the γ-globin locus itself in one of the earliest examples of its application in mammalian cells. It was put to the test empirically rather than being probed. A study that used ZFNs to treat the sickle mutation in patient-derived iPSCs provided evidence of the therapeutic potential of ZFNs in SCD. Site-specific nucleases were used to target genes more effectively and to fix the sickle mutation in human iPSCs. Otherwise, it would have been exceedingly challenging to accomplish this. In order to facilitate the identification of the proper clone in a successful gene targeting event, a drug resistance cassette was additionally incorporated into the donor construct. An average targeting efficiency of 9.8% was attained using this technique. To create transgene-free cells, the selectable marker was excised using Cre-recombinase. The lox P sites left behind by this technique could have an impact on how β-globin expression is expressed. An encouraging result was found in a study by Sebastiano *et al*. [18]. Genome-wide analysis is more useful for identifying potential off-target mutations, despite the absence of off-target mutagenesis in potential off-target locations. Similar methods for gene repair were employed in a different research that also used iPSCs from SCD patients. In contrast, the repaired β-globin gene had decreased expression in erythroid cells [19]. They reported that one of the HbS alleles was corrected and iPSCs effectively differentiated into erythroid cells. Remaining lox P sites following the selectable marker's deletion may cause reduced expression. Despite the introduction of the hsv-TK negative selection marker to lower the frequency of false-positive clones caused by random integration of the entire plasmid, targeting efficiency was not high (1 in 300 drug-resistant clones). The HBB gene is quiet in iPSCs, and silent genes are often more challenging for HR to target, which may account for the low HR efficiency. Instead of iPSCs, ZFNs have recently been used with $CD34^+$ haematopoietic stem and progenitor cells (HSPCs). ZFN mRNA was electroporated with high cleavage efficiency between 35 and 65%. The IDLV capture methods thorough investigation of off-target DSBs revealed great specificity for ZFNs. This study's utilization of IDLV or short oligonucleotides as donor DNA is another unique feature. Short oligonucleotide donor templates are simple to utilize and reasonably priced, whereas lentiviral vectors have high transduction efficiency into stem cells. Immuno-suppressed mice might receive modified HSPCs, which could then develop both *in vivo* and *in vitro* into erythroid, myeloid, and lymphoid cells. Although the results were promising, bulk-edited human HSPCs displayed a level of gene alteration of 10–20 prior to transplantation, but the degree of gene modification in human cells from the mouse's spleen and bone marrow was substantially higher. Therefore, a significant barrier was still the low rate of gene correction in long-term repopulating HSCs.

2.2.2. Transcription Activator like Effector Nuclease (TALENs)

Like ZFNs, TALENs also bind to certain DNA sequences and work as heterodimers to cause DSBs at particular sites. A DBD is coupled to the FokI nuclease domain to form TALEN monomers. DBDs are produced by the plant pathogen Xanthomonas' transcriptional activator-like effectors (TALEs), which are injected into plant cells and function as transcriptional activators in the nucleus. 33–35 amino acid tandem repeats make up the DBD. The typical TALEN design calls for 14 to 31 iterations. Only the amino acid residues 12 and 13, also known as the repeat variable orientation (RVD), distinguish these almost identical repeats. A single nucleotide is recognized by each repeat, and RVD establishes base specificity. Adenine is frequently represented by the codes NI, thymine by NG, cytosine

by HD, guanine by NH, and adenine and guanine together by NN. Since TALENs also utilize the DNA cleavage domain of the FokI nuclease, TALEN pairs are necessary for the creation of site-specific DSBs [20]. There are many platforms for TALEN synthesis that all rely on repeats being arranged in a specific order. After TALEN was discovered, its therapeutic potential for SCD was also looked at in the initial investigation, DSBs were induced at regions proximal to mutations in the HBB locus using TALEN pairs with an optimized architecture and high effectiveness. Targeting sites without his leading 5'T, which is often a prerequisite of his TALEN bindings, was made possible by the new TALEN architecture. This choice provides more flexibility and expands the range of potential targets for TALENs. Following this investigation, drug-resistant clones displayed gene targeting efficiencies compared to those reported for ZFNs, targeting the endogenous HBB locus in patient-derived human iPSCs with >60% efficiency. The Cre-lox P technique was abandoned in favour of the PiggyBac transposon, which allowed for the seamless repair of the sickle mutant. It will be fascinating to see if the PiggyBac transposon can use the Cre-loxP system to solve the issue of β-globin expression [21]. Although it would require genome-wide investigation, the absence of off-target events at areas of significant sequence similarity was also encouraging. Another study used TALENs with high activity at the β-globin gene and reported a 19% targeting efficiency without drug selection. However, since K562 cells were used for this work, additional examination of the methods using clinically pertinent cells would be preferable. Human stem cells have a low HR rate; hence, a selection-free method might not work for these cell types. During the same year, another work demonstrated the utilization of TALENs in patient-specific iPSCs while using Cre-lox P system for selective marker ablation. Corrected iPSC-derived erythroid cells expressed the wild-type allele at 30–40% of total levels. Similar to prior research, only one allele was repaired, with the sickle mutation still present in the other allele. Due to the absence of illness symptoms in HbA and HbS heterozygotes, therapy may only require one allele to be corrected. It was verified that there were no off-target effects at comparable sites. The conclusion that TALENs cause few genetic changes in the genome was later validated by a more thorough examination of off-target effects using whole-genome sequencing of numerous gene-corrected human iPSC clones [22]. Overall, the data mentioned above imply that TALENs might be a useful and precise method for treating sickle mutations in iPSCs.

2.2.3. Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR)

Due to its simplicity of use and low cost, CRISPR/Cas9 is now the newest and most popular genome editing technology. CRISPR and CRISPR-associated (Cas) proteins were initially discovered to protect bacteria from viral or plasmid DNA invasion. The currently utilized CRISPR system is one of the six ones known. Unlike ZFN and TALEN, which recognize DNA based on protein-DNA interaction, CRISPR/Cas9 technology for gene editing relies on a type II system. The Cas9 endonuclease and guide RNA (gRNA) are the two main elements of CRISPR/Cas9. The Cas9 protein is connected to the gRNA scaffold and can be recruited to any DNA sequence by the gRNA, which has a user-defined target sequence of 20 nucleotides. The target sequence must be located immediately upstream of the protospacer flanking motif (PAM) in order for this system to function. The 5'-NGG-3' PAM sequence is required by the *Streptococcus pyogenes* Cas9 protein, which is the most frequently utilized Cas9 protein. When the target is connected, Cas9 causes a DSB three nucleotides before the PAM [23].

In comparison to ZFN or TALEN cells, the CRISPR/Cas9 method displayed higher excision effectiveness at the HBB locus, according to a recent study using human iPSCs [24]. In the same work, iPSCs from adult SCD patients were repaired using CRISPR/Cas9. The corrected allelic globin protein was created by the CRISPR-corrected stem cells, which could then develop into erythrocytes. Either the utilization of better culture conditions or the use of iPSCs produced from blood may be responsible for the efficient erythrocyte maturation and

expression of β-globin. Another study reported that CRISPR/Cas9 had a higher cutting efficiency than TALENs at the HBB locus, with a nuclease activity that was dose dependent and active in K562 cells after microinjection when co-injected with DNA that produced GFP, compared to 1.6% for TALEN [25]. However, a direct comparison of efficacy in clinically valuable cells would be more instructive, given that the efficiency of HR can differ from cell to cell. Targeting introns can have less off-target consequences than exons, of the HBB locus for rational design of acceptable CRISPR targets. So that the CRISPR generated is suited for more people, it has been suggested that they comprise fewer single nucleotides as better target polymorphisms (SNPs). Though that while this discovery may be accurate for the HBB locus, it's vital to keep in mind that for other loci, introns may not have as many SNPs as exons do. The application of CRISPR technology in human zygotes[26] has generated a great deal of ethical debate. Sadly, only 4 of 54 embryos had good HR performance at the zygotic HBB locus, and off-target alterations were found. Another problem was the preference for a short oligonucleotide donor inserted as a DSB repair template over the HBD locus with high sequence homology HBB gene. Subsequent investigations revealedthat numerous CRISPRs intended to target the HBB gene had considerable off-target alterations. This proof highlights the significance of CRISPR/Cas9 specification refinement before human application. Despite certain reservations, the use of CRISPR/Cas9 to repair HSPCs carrying the sickle mutation significantly increased the HDR efficiency in HSCs.

3. CRISPR CAS9 GENE EDITING

Due to its simplicity of usage and low cost, CRISPR/Cas9 has become the most widely used and current genome editing method. The prokaryotic genomes of bacteria and archaea contain DNA sequences known as the CRISPR family [27]. These sequences are obtained from various DNA segments of phages that have infected prokaryotes in the past. They are all employed in the subsequent infection to identify and eradicate phage-like DNA. Therefore, these sequences play a significant role in prokaryote antiviral defence systems and offer a wide range of acquired immunity. Around 50% of sequenced bacterial genomes and 90% of sequenced archaeal genomes contain CRISPR. Prokaryotic antiviral defence system using CRISPR The enzyme Cas9, also known as the CRISPR-associated protein 9, uses CRISPR sequences as a guide to identify and cut particular DNA strands that are complementary to the CRISPR sequence. CRISPR-Cas9 technology, which can be used to modify the genes of organisms, is made up of the Cas9 enzyme combined with CRISPR sequences. This editing procedure has a wide range of uses, including the development of biotechnology products and the treatment of diseases.

3.1. History behind formation of CRISPR CAS9

A group of Japanese researchers at Osaka University discovered an odd DNA sequence pattern in a gene related to an intestinal bacterium [28]. A short non-repetitive ‘speedster’ DNA sequence seemed to separate the gene's five short repetitive DNA regions. Different *Mycobacterium tuberculosis* strains contained various spacer sequences between the DNA repeats, according to research conducted in the Netherlands in 1993 under the direction of J.D. van Embden. Spoligotyping, or the classification of *M. tuberculosis* strains is based on their spacer sequences, which were subsequently found in numerous bacterial and archaeal genomes and referred to as CRISPRs [29]. Jansen *et al.* [30] discovered that a CRISPR sequence was always followed by another set of sequences. This second group of sequences was given the term Cas genes (CRISPR-associated genes). It emerged that the Cas genes encoded DNA-cutting enzymes. In 2005, three study teams independently came to the conclusion that the ‘spacer’ in question might represent a weapon in the bacteria's defence system. Some investigations showed that inserted viral DNA fragments into their spacer sequences, and that they lost their resistance each time the new spacer sequences were excised, provided evidence for the operation of the CRISPR/Cas9 system.

RNA molecules in bacteria to recognize incoming DNA from an incoming virus and instruct the Cas9 enzyme to cut it in order to block the virus. Bethesda, Maryland, information revealed for the first time how the CRISPR/Cas copies segments of its DNA and inserts them into their genome as 'spacers' between short DNA repeats in CRISPR. As Virginijus Siksnys of Vilnius University independently submitted a paper to Cell examining the potential of CRISPR-CAS9 for gene editing, a small team of researchers led by Jennifer Doudna from the University of California, Berkeley, and Emmanuelle Charpentier from Umea University published work in 2012 demonstrating how to use the natural CRISPR-Cas9 system [31]. A year later, other researchers working in various labs published papers demonstrating how CRISPR might be used to alter the human cell's DNA, increasing the precision and effectiveness of CRISPR-Cas9 technology. The invention of novel Cas9 fusion proteins that don't cleave DNA is a milestone.

3.2. Classification of CRISPR CAS9

Based on Cas protein structure and function, CRISPR/Cas systems can be divided into

- Class I (types I, III, and IV)
- Class II (types II, V, and VI).

Class II systems use a single Cas protein, while class I systems use complexes of many Cas proteins. The straightforward design of type II CRISPR/Cas-9 has been extensively investigated and employed in genetic engineering. *Streptococcus pyogenes* provided the Cas-9 protein, which was the first Cas protein to be employed for genome editing (SpCas-9). The three primary elements of CRISPR/Cas9 are

1) Target-specific guide RNA(gRNA): Consists of two segments: a trans-activating non-coding RNA sequence (tracrRNA) and a targeting sequence that contains complementary target RNA (crRNA).

2) Cas9 endonuclease: A gRNA-Cas9 complex is created when gRNA binds to the Cas9 enzyme, which then attaches to particular genomic targets and cleaves the appropriate genomic DNA target sequences.

3) Donor DNA template ("knock-in"): After Cas9 causes a DSB at the desired genomic region, repair DNA must be double-or single-stranded to apply genomic changes.

The scaffold-associated Cas9 protein can be attracted to any DNA sequence by the gRNA's unique 20-nucleotide targeting sequence. The target sequence must be located directly above the protospacer adjacent motif (PAM) for this system to function. Cas-9, often known as the genetic scissor, is a large (1368 amino acids) multidomain DNA endonuclease that cleaves target DNA to create double-stranded breaks. Cas-9 has nuclease (NUC) and recognition (REC) lobes. The NUC lobe is made up of RuvC, HNH, and protospacer adjacent motif (PAM) interaction domains, whereas the REC lobe is made up of REC1 and REC2 domains that bind guide RNA. The PAM-interacting domain imparts PAM specificity and is in charge of starting binding to target DNA, whilst the RuvC and HNH domains are used to cleave any single-stranded DNA. The trans-activating CRISPR RNA (tracrRNA) and the CRISPR-RNA (crRNA) are the two components that make up guide RNA. The target DNA is specified by the crRNA's 18–20 base pairing with the target sequence. TracrRNA, on the other hand, has a lengthy loop that acts as a scaffold for Cas-9 nuclease's binding. Guide RNAs are used to target viral DNA in prokaryotes, but as gene editing tools, they are created synthetically by combining crRNA and tracrRNA to generate a single guide RNA (sgRNA), making it possible to target virtually any putative gene sequence. SgRNA enlists the Cas9 endonuclease to produce double-strand breaks (DSBs) at specified locations in the genome during the genome editing process. Both the error-prone non-homologous end joining (NHEJ) route and the homology-driven repair pathway (HDR) are endogenous self-repair systems that can fix DSBs.

Because NHEJ is active for around 90% of the cell cycle and is unreliant on adjacent homologous donors, it is generally more effective than HDR. NHEJ has the potential to add random insertions or deletions (indels) at the site of cleavage, leading to frameshift mutations or premature stop codons inside the target gene's open reading frame (ORF), ultimately inactivating the target gene. As an alternative, HDR can introduce precise genomic alterations at target regions using homologous DNA repair templates. Furthermore, substantial fragment deletion and simultaneous knockout of numerous genes can be accomplished by utilizing multiple sgRNAs that target one or more distinct genes.

Microorganisms gain CRISPR immunity through (A) Adaptation or spacer acquisition, (B) CRISPR RNA (crRNA) biogenesis, and (C) Targeted interference.

During the adaptation stage, invasive DNA is cut up into tiny fragments and integrated into CRISPR loci as fresh spacers that act as an infection's memory protocol. The leader end of the CRISPR locus is predisposed to receive additional spacers in response to DNA infection. The "CRISPR motif" or "that" "flanking the protospacer motif" flanks the protospacer as "(PAM)" is depicted, according to analysis of protospacers (sequences inside the invasive nucleic acid that share sequence homology with spacer sequences). PAM sequences are necessary for Cas1 and Cas2 protein complexes to select and incorporate protospacers into CRISPR arrays. The CRISPR array is translated into a precursor CRISPR RNA (pre-crRNA) during the crRNA biogenesis stage. This precursor crRNA matures into a crRNA that contains certain spacer sequences bordered by short RNA sequences. The conversion of pre-CrRNA to mature crRNA depends on the presence of tracr RNA, RNase III, and Csn1 (Cas9). Cas9 and mature crRNA-tracrRNA hybrids continue to be closely linked, forming a complex for targeted interference. The Cas9-crRNA-tracrRNA-ribonucleoprotein (crRNP) complex binds with the relevant protospacer during the interference step to activate Cas9 to cleave both strands of the target to disclose the complementary sequence to recognize and eliminate (Fig. 2). Three bases upstream of the nearby protospacer motif, the Cas9 protein cleaves the protospacer. The absence of PAM sequences in the host locus prevents an 'autoimmune response' within the CRISPR locus, since PAM sequences are absolutely necessary for protospacer cleavage.

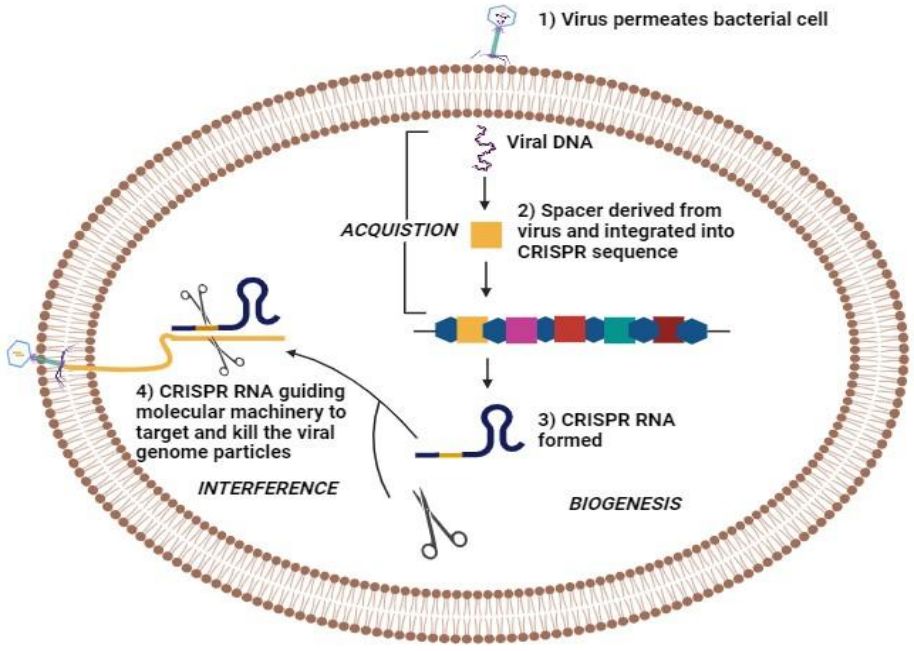

Fig 2. Mechanism of CRISPR CAS9 in Gene Therapy.

4. HOW DOES THE GENE EDITING VIA CRISPR CAS9 HAS BEEN DONE IN SCD PATIENTS?

This section concentrates on a few CRISPR/Cas9 gene editing strategies for treating SCD. Targeting the HbF transcriptional repressor produced enough quantities of his HbF to reverse SCD, resulting in beneficial HPFH. Specifically, the sickle mutation of HBB was repaired. A mutation is brought about. In a phase 1 clinical trial using CRISPR/Cas9 gene editing in patients with severe SCD (CTX001, clinicaltrials.gov), an erythroid lineage-specific enhancer designed to stimulate HbF expression and autologous $CD34^+$ HSPC expressing the BCL11A gene were employed as an example. The use of gene-editing-based techniques in clinical settings is fraught with difficulties, including the possibility of off-target consequences, the need to improve the effectiveness of gene editing, and the *in vivo* transplantation of gene-edited HSPCs. Achieving high levels of safety and effectiveness requires optimizing genome editing techniques, including CRISPR-Cas9/gRNA and donor templates, and delivery techniques.

4.1. Preclinical studies for *ex vivo* HSPCs gene editing

Ex vivo gene editing of human HSPCs followed by transplantation in an immuno-deficient mice model has been used in the majority of preclinical research. Since the viability of permanent HSC engineering is a need for the robustness of autologous HSCT, it intends to assess the long-term engraftment capacity of genetically altered HSCs.

4.1.2. Isolation of $CD34^+$ Cells

Umbilical cord blood, bone marrow, and mobilized peripheral blood can all be used to isolate human $CD34^+$ HSPCs. Most genome editing experiments have employed CD34 HSPCs from peripheral blood. Prior to gene editing, isolated $CD34^+$ cells are grown for a number of days in cytokine pre-stimulation media because it has been demonstrated that exposing the post-isolated culture to cytokines increases the effectiveness of gene editing [32]. However, achieving high editing efficiency requires balancing with of off-target mutations and immunogenicity brought on by persistent or over expressed CRISPR components.

When delivering RNPs directly into HSPCs, electroporation utilizing a nucleofection procedure is frequently chosen since it enables the RNP to enter the cell nucleus quickly and start cutting the genome right away. To obtain high editing efficiency and specificity in $CD34^+$ HSPCs with less cytotoxicity, RNP has been employed in the majority of genome editing research. By lessening the toxicity of $CD34^+$ HSPCs, chemical alterations of gRNAs increased the effectiveness of genome editing even more. Gene-edited HSPCs from SCD patients (SCD HSPCs) are the therapeutic product for autologous transplantation.
Recent preclinical studies have shown potential for several gene editing ideas to treat SCD, including:

(i) correction of the causal point mutation in HBB.
(ii) gene disruption of β-globin (HBG) repressors to induce foetal haemoglobin (HbF).
(iii) introducing advantageous hereditary persistence of foetal haemoglobin (HPFH) mutations on the β-globin locus to induce HbF

4.1.3. HbF Induction through BCL11A Gene Editing

HbF is the dominant globin type after the first trimester and replaced by HbA 6 months after giving birth. Switching from HbF to adult globin is predominantly regulated by a potent upstream enhancer known as a locus control region (LCR) that includes each globin promoter to activate their production, both HbA and HbF are retained on chromosome 11 [33]. The clinical severity of SCD is significantly influenced by HbF levels, and reactivating HbF by targeting the genes that control HbF is a viable therapeutic strategy. Numerous

causal loci have been discovered by genome-wide association studies (GWAS) of HPFH patients [34], and many transcription factors are believed to be indirectly implicated in the silencing of HbF. By working in conjunction with other DNA-associated factors at various sites within the globin locus, including direct repression of the HBG promoter by BCL11A increase, BCL11A is a key regulator of HbF levels and represses foetal haemoglobin expression. Therefore, the BCL11A-binding motif or loss of BCL11A suggests an appealing and distinct target for therapeutic gene editing to treat SCD.

4.1.3.1. *BCL11A gene deletion*

Using a TALE nuclease mRNA targeting the BCL11A coding sequence, Humbert *et al.* [35] verified BCL11A's role as a HbF repressor and carried out a proof-of-concept transplantation research in the NHP model. BCL11A, however, plays many roles in several hematopoietic lineages, and coding mutations in BCL11A are extremely harmful. Targeting the core sequence of the erythrocyte enhancer BCL11A in HSPCs provides a foundation for erythrocyte-specific therapeutic genome editing. Several investigations have confirmed that the erythrocyte enhancer BCL11A is the target for HbF induction. While BCL11A's capacity to promote HSC activities like differentiation, reconstitution, and long-term engraftment potential was maintained, disruption of the BCL11A enhancer resulted in levels of HbF reactivation comparable to those seen in BCL11A-encoding knockout (KO) mice. Wu *et al.*[36] showed highly effective therapeutic gene editing in HSPCs by disrupting GATA1-binding sites in the 58 BCL11A erythroid enhancer by CRISPR/Cas9. This led to a therapeutic induction of foetal β-globin upon engraftment of SCD-HSCs and an erythrocyte-specific decrease in BCL11A expression. The gRNAs that directly cleaved at the core of the 58 erythroid enhancers of BCL11A showed the highest HbF induction in erythroid progeny with high indel rates. Based on clonal examination of $CD34^+$ HSPCs edited with the BCL11A enhancer, γ-globin was substantially produced by biallelic alteration of the cleavage site. To investigate the effects of BCL11A enhancer editing on long-term transplanted HSCs, human designed SCD $CD34^+$ HSPCs were infused into immunodeficient NBSGW mice [36]. The genetic engineering of self-renewing HSCs was confirmed by NBSGW, which supported comparable engraftments with human myeloid, lymphoid, and erythroid cells in comparison to non-engineered cells. BCL11A enhancer editing had no negative consequences on stem cell activity, as evidenced by the fact that indels at the BCL11A enhancer remained after secondary transfer. Intriguingly, the indel spectrum of long-term transplanted HSCs was different from that of bulk HSPCs, indicating that transplanted HSCs may prefer NHEJ to Microhomology-Mediated End-Joining i.e. MMEJ repair. The ability of BCL11A enhancer-engineered cells to sustain suggests that a gene disruption strategy involving NHEJ may be more efficient than gene editing techniques based on HDR or MMEJ. This is due to the fact that NHEJ occurs preferentially in HSCs and is active throughout the cell cycle. This study reveals that BCL11A enhancer editing using CRISPR/Cas9 is a practical therapeutic approach to induce HbF at therapeutic levels in transplanted HSCs. In a phase 1 clinical trial (CTX001, clinicaltrials.gov) using CRISPR/Cas9 to modify the erythrocyte enhancer BCL11A to promote HbF expression in him, Vertex Pharmaceuticals and CRISPR Therapeutics have shown encouraging results.

4.1.3.2. *Base editing of BCL11A*

Zeng *et al.* [37] has established the viability of changing the treatment baseline in repopulating and self-renewing multilineage human HSCs. When compared to nuclease-based editing, base editing has the potential to produce better purity of gene-modified products. The Base Editor makes base modifications without triggering DSBs, avoiding undesirable indels and off-target effects produced by ineffective HDR and DSBs. As an RNP targeting the BCL11A erythroid enhancer in SCD-HSPCs, an A3A-BE3-based editor was presented. To eliminate the GATA1 motif, this base editor targets cytosines in the base editing window. Two cycles of electroporation boosted therapeutic base throughput, but

lowered viability and engraftment potential. Similar to nuclease editing, biallelic single-nucleotide editing in the BCL11A enhancer within the GATA1 motif resulted in significant HbF induction [36]. Base-editing frequency was lowered in transplanted HSCs relative to input HSPCs after transplantation into NBSGW mice. Multiple lineage rearrangements were seen in base-edited cells, with similar base-editing frequencies in each lineage. Erythroid enhancer disruption resulted in erythroid lineage-specific BCL11A knockdown. Both gRNA-dependent and independent off-target editing should be investigated for baseline editing.

4.2. HPFH mutations are brought about via HbF induction

BCL11A and leukaemia/lymphoma-related factor (LRF), which are the two main repressors of the foetal haemoglobin gene, bind specifically to the HbG promoter in areas around 115bp and 200bp upstream of the transcription start site, respectively [38]. Significant amounts of HbF are produced when the LRF or BCL11A binding site in HBG promoters is disrupted using CRISPR/Cas9. As a potential DNA target for genome editing, Traxler *et al.* [39] identified a naturally occurring 13-nucleotide HPFH deletion in the HBG promoter. Since the Cas9 cleavage site is flanked by 8-nt tandem repeats that facilitate MMEJ repair in edited progenitors that generated erythrocytes with increased HbF-levels that were sufficient to reverse sickling *in vitro*, the 13-nt deletion identical to the naturally occurring mutation predominates among other indels after CRISPR/Cas9 editing. The goal of this technique was to show high-throughput engineering of human HSCs capable of engrafting multi-lineage grafts following transplantation into immune-deficient mice without detectable off-target mutations or negative haematological consequences. An NHP autologous transplantation model illustrates the therapeutic potential of this strategy[40]. Because this region of HBG is conserved in both humans and rhesus macaques, previously verified CRISPR gRNA targets for human cells were employed as CRISPR targets. The homologous HBG1 and HBG2 genes' promoters contain GRNA targets [40]. Co-excision has been implicated in a remarkable number of massive deletions that delete the whole HBG2 gene plus a portion of the HBG1 promoter. Despite the fact that following transplantation in NHP, the frequency of large deletions was dramatically reduced, the underlying mechanism is still unknown, and the long-term therapeutic significance of the large deletion has not been proven.

4.2.1. HBG base editing

Base editing that results in a single nucleotide alteration at the HBG promoter's BCL11A-binding region is sufficient to prevent BCL11A binding and boost HBG expression [41]. Because the base editor mediates base conversions without triggering DSBs, the HBG copy number remained unaffected. This suggests that base editing might result in safer therapeutic applications without worsening the genome's DSB-induced damage. In HSC transplantation, the effectiveness of this strategy has not been evaluated. Independent of BCL11A, the transcription factor LRF inhibits the expression of HbF. They experimented with targeting LRF binding sites within the HBG promoter, since LRF degradation boosts HbF expression while delaying erythroid development. The SCD phenotype was exacerbated by CRISPR/Cas9 disruption of the LRF binding site. Upon repopulation of HSCs that develop into erythrocytes that express therapeutically relevant quantities of the HbF, editing of the HBG promoter is maintained. The simultaneous inhibition of LRF and BCL11A, with an additive effect on HbF, was made possible [42]. Base editing to concurrently disrupt LRF and BCL11A repressor binding sites in the HBG promoter is a promising tactic given the separate roles of LRF and BCL11A, and work by Zeng *et al*. [37] has demonstrated efficient multiple base editing. As a potential strategy for SCD treatment, several researchers have revealed proof-of-concept CRISPR/Cas9-mediated gene editing to recapitulate significant deletion HPFH mutations within the β-globin gene cluster. These methods concentrate on NHEJ rather than DSB to precisely produce big deletions that resemble the Corfu deletion of the naturally occurring Sicilian HPFH or the intergenic γ-δ region, which contains the δ-and

β-globin genes [43]. It has not been documented whether these alterations are effective in HSC transplantation. When large HPFH deletion mutations are introduced, two DSBs must be treated simultaneously and their distal ends must be joined. This increases the likelihood of off-target effects while decreasing the frequency of large deletions. Additionally, their clinical applicability is restricted by rival genome editing technologies, tiny indels, and the inversions linked to these deletions.

4.3. Correction of SCD Mutations

Correcting the SCD mutation appears to be the most difficult, but also one of the most viable and promising approaches to Cas9 cut sickle β-globin. This break can be repaired when a normal β-globin sequence flanked by arms of homology to the DSB is provided. The proper β-globin sequence can be provided without the requirement to activate the foreign transgene, suggesting that genotypic correction is attainable through specific location in the genome. Many scientists employ gene editing techniques to fix SCD mutations in many cell types, rising to assure appropriate repairs. The CRISPR/Cas9 method, which outperformed other gene editing technologies in terms of repair efficiency and OTEs, is used in the majority of these studies (Fig. 3).

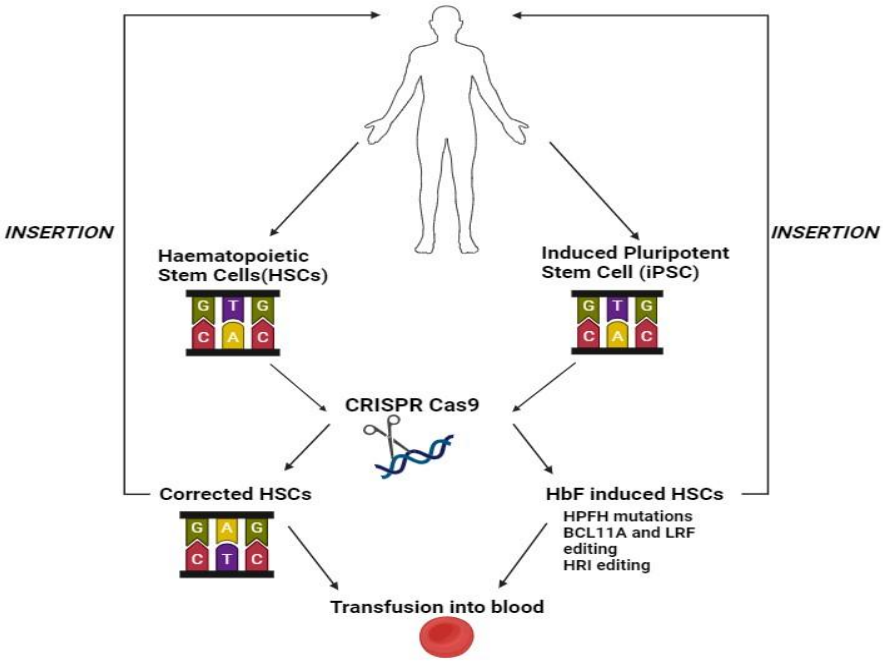

Fig 3. CRISPR/Cas9 treatment for SCD. The proof-of-concept studies have demonstrated the possibility of correcting SCD mutations, inducing foetal haemoglobin (HbF) in SCD-derived HSCs and iPSCs, and subsequently normal erythrocyte derivation for transfusion.

The majority of genome editing studies currently use bone marrow-derived $CD34^+$ HSPCs as the source of HSPCs, but due to safety concerns regarding the use of granulocyte colony-stimulating factor in SCD patients, newly peripherally mobilized $CD34^+$ HSPCs have shown promise. It is possible to modify these $CD34^+$ cells so that they are reinfused into the patient. Nevertheless, alterations in the cell cycle or the presence of particular nucleases that can affect the repair pathways cells adopt after DNA double-strand breaks confer a general resistance to effective gene editing. In these experiments, the most effective way to administer the adeno-associated virus (AAV)-6 vectors for the CRISPR/Cas9 delivery system with donor DNA is by electroporation [44].

Numerous studies examine gene editing at the DNA level, by targeted deep sequencing, or by nested digital PCR of droplets for the evaluation of SCD mutation correction, while others used more functional studies, like RNA sequencing or RNA expression levels, with three studies using High performance liquid chromatography (HPLC) to measure protein levels after correction of an SCD mutation in the β-globin gene [45]. Since the procedure to create human iPSCs from somatic cells was published in 2008, other groups have created protocols to differentiate iPSCs into various cell lineages, such as hematopoietic cells, which are emerging as another reliable source of autologous HSPCs. Hematopoietic cells produced from iPSCs are now immature rather than final hematopoietic cells, and therefore cannot be implanted in a mouse model using a xenograft. iPSCs can be differentiated into HSPCs using a variety of methods, but the majority of them replicate the primitive haematopoiesis that can be seen when HSPCs are produced mostly from erythroid cells that contain ε-globin and γ-globin and very little, if any, β-globin.

Using a gene editing technology already in existence to fix the SCD mutation in iPSCs. A suitable differentiation strategy is needed to produce transplantable HSPCs from iPSCs for therapeutic reasons. This therapy ultimately overcomes the two main barriers to allogeneic transplantation rejection and GVHD in transplant therapy, along with other autologous modifying methods. Furthermore, because cloning repaired cells from a large iPSC population would enable 100% of the cells to be repaired; low repair efficiency is an issue in HSPC studies. Even though several groups have demonstrated correction of SCD mutation at the DNA level using nested ddPCR or DNA sequencing, only one study in SCD presented a mutational correction to SCD-derived iPSCs at the RNA and protein levels by quantitative PCR and Western blot analyses, respectively. Although both underlying mutations in $CD34^+$ and iPSCs can be rectified, only a few immune-deficient mouse models have shown significant healing rates following ex-vivo transplantation of corrected human cells [19]. Even though immune-deficient mice are commonly used as transplantation models for human cells, it is unclear if the results of these studies accurately represent the clinical consequences of these methods. Larger animal models are required to investigate the application potential following the optimization of corrective techniques.

Although it is conceivable to alter $CD34^+$ cells, there are a variety of genotypic outcomes that could occur, and editing for the long-term transplantation of HSPCs has not yet been well investigated. Processing cells using CRISPR/Cas9 and a donor with β-globin can result in cells that are in their original state (uncorrected), have a scythe feature (one allele corrected), are healthy (both alleles corrected), have thalassemia major (both alleles disturbed), have a feature of β -thalassemia (one allele repaired but otherwise disturbed), or have sickle/β-thalassemia (one allele broken) the cell's NHEJ/HDR machinery. Prior to conducting clinical studies, accurate correction in the long term HSPCs are still not effective and efficient leads to reduction of transplanted HSPCs mixed culture might be clinically troublesome.

Table 1: Selected mutation correction studies in SCD using CRISPR/Cas9

Gene	Cell Types	Genome-editing tool	Outcome/Results	Mouse Transplantation Studies	Reference
HBB	iPSC	CRISPR Cas9	RNA expression and western blot can be used to identify the correction of SCD mutations.	ND	19, 24
HBB	$CD34^+$ Cell	CRISPR Cas9	Anti-sickling β-globin	Concentrated	44

	Mobilized		cDNA donor with 29% RNA expression level is used for SCD mutation correction.	$CD34^{+}$treated population long-term engraftment assessed by flow Femoral BM cytometry (4–30%)	
HBB	iPSC	CRISPR Cas9	Up to 67.9% of compensation effectiveness was examined sequentially. No functional studies	ND	45

4.4. Challenges

Traditional genome engineering techniques are pricy, time-consuming, labour-intensive, and call for knowledge of protein engineering to create particular nucleases. In comparison, the CRISPR/Cas9 genome editing method is far more widely utilized and is easier, cheaper, and more effective. This not only made it simpler and less expensive to produce knockout animal models, but also made easier to create genome-wide screening libraries that can find therapeutic genes or chromosomal areas that have a direct impact on the desired phenotype. Although there is a lot of interest in CRISPR/Cas9-based editing techniques on a global scale, cutting and editing efficiency (both NHEJ and HDR), precision and delivery techniques need to be improved. Finally, security has been addressed everywhere, particularly with OTE devices that have been improved and debated before this method was used to standard clinical treatment.

4.4.1. Editing Efficiency

The CRISPR/Cas9 system is dependent on Protospacer-Adjacent Motif (PAM) sequence close to the object, which is a limiting element for the varied application of knowledge. There aren't many options for guide RNA for various Cas proteins, similar to how SCD mutation repair studies must focus on a certain chromosomal area. In order to recognize diverse PAM orders, significant efforts have been undertaken to create various Cas-effector proteins. Despite the development of 19 variants of the CRISPR system, each of which has a distinct Cas-effector protein that can recognize a new PAM site and an expanded target genomic region, not all of them have been examined for their efficacy and safety. Established Cas types, such as Cas12a or Cas9 from *Streptococcus pyogenes,* are still used by researchers in their studies [46]. While some other Cas9 orthologs have been reported to require longer PAM sites, SpCas9 PAM recognition is 5' NGG 30. Although they differ from the traditional SpCas9 in certain ways, their longer PAM sites restrict their application despite maybe more effective delivery. For instance, smaller Cas effector proteins, such SaCas9 from *Staphylococcus aureus* [47] with the NNGRRT PAM site, are better at delivering viruses. Increase the target area's size such that mutations at neighbouring PAM DNA duplex residues can successfully change the PAM preference. Cas-effector subunit proteins were better understood, enabling PAM modification selectivity. *Streptococcus canis* Cas9 (ScCas9) was described as looking as 50-NNG-30 PAM and reported to have an 89.2% sequence similarity to SpCas9 in a recent study [48]. A structural investigation revealed that the specificity of a minimum PAM sequence is caused by two distinct mutational areas, positively charged insertions in the REC domain (367–376) and KQ insertions in the PAM interaction domain. Another group has recently developed Cas9 variants with various PAM compatibilities utilizing the PACE technique, or phage-assisted continuous evolution. However, more intriguingly, they showed better DNA specificity of Cas9 variants of canonical SpCas9, which has a smaller genome-wide target, extending the recognition of PAM Cas9 variants projected to have substantially more OTE. Sniper-Cas9, which has a high goal and few OTEs, was successfully obtained via the wizard development in the second strategy.

These investigations highlight the opportunity for and necessity of additional action improvements in genomic targets for various Cas-effector proteins. When using the methodologies in ordinary clinical applications, safety should also be taken into account in order to maximize recovery effectiveness.

4.4.2. Possibility of Editing Tools or Edited Cells Being Immunogenic

CRISPR technology's ultimate goal is to modify disease-related mutations or regulate disease-related gene expressions in patient-derived stem/progenitor cells. There are still a lot of questions about how CRISPR/Cas9 systems behave *in vivo*. Since 2019, there have been active clinical trials using CRISPR/Cas9 as a potential treatment for SCD, thalassemia, HIV-1, and several cancer types both domestically and internationally. Ex-vivo research suggests that there may be negative impacts of technology, even though these doctors have hope for future tests. Whether guide RNAs or Cas9 itself has an impact on the immune system is the initial query. Kim *et al*. [49] revealed which *in vitro* transcribed RNAs at 5-0-triphosphate group (50-ppp) caused cytotoxicity due to activation of the innate immune system in human and mouse cells in an effort to partially answer this question. The authors also note that the elimination of triphosphate resulted in a high mutation rate in the 45 CD4+ cells utilized in the first human CRISPR experiment for anaemia sickness, which allowed them to avoid detection by the innate immune system. Charlesworth *et al.* [50] demonstrated pre-existing anti-Cas9 antibodies in a small group of healthy volunteers in a recent preprint article. These antibodies were acquired from *Staphylococcus aureus* (79%) or *Streptococcus pyogenes* (65%). The prevalence of anti-SaCas9 antibodies and SpCas9 was shown to be 10% and 2.5%, respectively, in additional research using 200 blood samples [51]. Although the results are not shocking, activating the immune system with CRISPR/Cas9 most likely led to problems and injury in real life. Although extensive animal models and clinical studies are anticipated to address these discoveries and potential immune responses, Cas9 expression levels, delivery strategies, vector types for transduction routes, and target cell populations should be optimized in any way possible to lessen a severe immune reaction.

4.4.3. Editing Specificity

OTE are unquestionably one of the most demanding circumstances for the CRISPR/Cas9 technology, aside from potential immunological responses. OTE for a given manual RNA cannot be disregarded, since the Cas9-manual RNA complex can comprehend sequences with up to five mismatched nucleotides. Numerous improvements were made to increase the CRISPR/Cas system's specificity, but the manual RNA design is still the most crucial method for eliminating OTEs. There are excellent manual RNA layout tools available, and the most recent versions include additional algorithms that compare on-goal reduction performance in addition to selectivity for the goal. Additional modifications to the structure of the manual RNA during synthesis, including as the truncation of spacer RNA and chemical modifications, have been linked to increased Cas9 endonuclease specificity[52]. Additionally, by increasing the stability of manual RNAs in cells, chemical alterations using 20-O-methyl 30 phosphorothioate and 20-fluoro-ribose improve the modifying performance. Enhancing Cas9 specificity is the second essential element to reduce OTEs. Because two closed recognition sites inside the DNA are needed for a double strand break, a mutant form of Cas9 called nickase (Cas9n) can best reduce a single strand of DNA. As a result, OTEs are significantly decreased (50–1500-fold in human cells). However, this method also advanced with the development of a catalytically inactive Cas9 and Fok1 fusion protein, which can convert some single nicks to double strand breaks. In this method, the Fok1 enzyme is brought into close contact to the Fok1 nuclease, which is necessary for active dimerization. While those methods greatly reduced off-target concerns, the need for double recognition sites might result in substantially lower editing efficiencies, and the demand for double manual RNA utilization might limit viral delivery methods. Active nucleases are being designed for greater specificities to retain modifying performance that is excessive enough

for scientific application. The initial idea behind high specificity nucleases was to lessen Cas9's interactions with its DNA in order to diminish OTEs while maintaining enough power for on-goal popularity. In contrast to wild type nucleases, high constancy Cas9 and higher specificity Cas9 have no or much fewer OTEs while maintaining robust on-goal activity. In a recent post, Doudna claimed that SpCas9-HF1 and eSpCas9 are both bound to mismatched goals and locked in inactive regions, and that the non-catalytic region of Cas9, REC3, is responsible for goal popularity and the direction of nuclease activity. They were able to generate hyper-accurate Cas9 variations (HypaCas9) with broad genome specificity using those observations without compromising any discernible OTEs [53]. Numerous courses have recently brought up the pertinent issue of the CRISPR/Cas system's unintended consequences, which include significant chromosome deletions, insertions, and rearrangements when it is used in clinical trials. Although it's not always clear whether this uncertainty will be clarified or is clinically relevant, it is feasible to encourage further pre-scientific research addressing those valid safety concerns.

4.4.4. A Secure Delivery

Based on the specifications for the required amount of protein, exposure period, efficiency, and constraints for OTEs and other safety considerations, the application of the CRISPR/Cas9 system to a particular cell type, structure, and carrier components must be specified. Systematically, it can be (i) integrating/non-integrating virus vectors/plasmids expressing both mRNAs, guide RNA and Cas9 (ii) Cas9 mRNA and guide RNA and (iii) a ribonucleoprotein complex (RNP) that forms the Cas9 protein and guide RNA. After its discovery, the CRISPR/Cas9 system can be used in human cells for genome editing, virus constructions that provide continuous expression of Cas9 and guide RNAs were used to investigate this potential, although it can be useful for gene editing methods that require a long-standing expression, it was also recognized continuous expression of guide RNAs and Cas9 increased the possibility of mismatched pairs and OTEs [54]. Although viral problems, immune system-based systems, and insertional mutagenesis are still up for debate, the use of vector transfer in the lab is reliable and affordable. An alternate way of plasmids/vectors uses a primer mRNA for Cas9, which is transported into the cell and translated into an active protein. Although this technology can also be utilized only for genome editing techniques that allow for temporary Cas9 expressions, it does so by bypassing the time needed for Cas9 transcription conveyed with plasmids. The Cas9 protein reached its peak in mice at 6 hours after Cas9 administration mRNA and went undetectable at 24 hours [55]. Different distribution methods or chemical alterations for stability RNAs, as previously discussed in security, could be used to optimize effectiveness. In addition to the issue of whether a native there are significantly more foreign proteins for human cells immunogenic to suppress RNP potential of use, another option is the RNP complex, which forms native Cas9 protein and guide RNA one complex that is readily active when internal to a room. However, the biggest drawback of this application is that the Cas9 guide RNA structure is relatively a large complex. Other DNA and RNA systems as well as non-virus dissemination methods like electroporation, encapsulation, and delivery modified are popular. Long used as a non-selective delivery method, electroporation increases the number of cell membrane pores by applying a high electric field, allowing different DNA, RNA, and proteins to pass over the cell membrane. Despite the fact that this technique is quite effective once Cas9 and guide RNA are introduced into HSPCs. The purpose of electroporation in the clinical setting during interrogation is to fix the SCD mutation, the problem of toxicity, and the issue of long-term survivability. Large amounts of Cas9 protein may be needed in the clinical setting, and it is currently not economically feasible to clean Cas9 protein that is endotoxin-free. In order for this technique to be useful in a clinical environment, it is important to research more industrially feasible approaches to attain GMP in grade Cas9 production.

4.5. *In Vivo* Gene Modification

The expensive cost of *ex vivo* gene editing hinders the use of this therapy for SCD patients in locations with limited resources, despite the fact that it offers several benefits, including high editing efficiency and the ability to remove unedited HSPCs from the patient. Using non-integrating adenovirus, efforts have been made to construct an *in vivo* HSC transduction/selection method. *In vivo* editing of the HBG promoter by CRISPR/Cas9 was carried out in mice that were transgenic for YAC/CD46 [33]. In order to reactivate β-globin, a human CD46-targeted adenoviral vector (HDAd-HBG-CRISPR/mgmt) expresses CRISPR/Cas9 targeting the HBG promoter. The vector additionally has an O-6-methylguanine DNA methyltransferase i.e. MGMTP140K cassette enabling chemotherapeutic drug-based *in vivo* selection of transduced cells when viral transduction is directed by hematopoietic tissue, CD46 is universally expressed in HSPCs. Direct *in vivo* investigation of β-globin reactivation using an adenoviral vector targeting human CD46 is possible because the P-YAC/CD46 mice bearing the human β-globin gene locus, which express human CD46 at a level and pattern similar to that of humans. An *in vivo* HSPC transduction method includes mobilizing HSPCs from the bone marrow into the peripheral blood and then injecting an adenoviral vector (HDAd-HBG-CRISPR/mgmt) intravenously because direct transduction of BM-located HSPCs is ineffective. This led to the reactivation of human β-globin in adult animals' erythrocytes, which persisted even after a secondary HSPC transplant. This strategy might have long-lasting effects, since mobilized HSCs that were transduced into peripheral blood could locate to the bone marrow and regenerate [33]. Despite the promise of *in vivo* gene editing for the treatment of SCD, there remain numerous obstacles. SCD HSCs need to have high *in vivo* trafficking and editing efficiency, and target cell/tissue editing could be a concern. Although viral vector-based gene editing machinery delivery *in vivo* can be very efficient, it can also result in unregulated Cas9/gRNA expression, which can be genotoxic and trigger the immune system. Contrarily, nonviral *in vivo* delivery vehicles may be ineffective and widely biodistributed.

5. CLINICAL TRIALS PERFORMED BY CRISPR CAS9 MECHANISM

Seven clinical trials utilizing gene editing methods have been started in the last four years to treat SCD. Ex vivo delivery of editing agents to autologous HSCs is used in all of them. In five of these treatment strategies, BCL11A expression is suppressed in the erythroid lineage by destroying enhancer elements or altering BCL11A binding sites in HBG promoters. This is done in an effort to restore β-globin expression. The disease that is causing the mutation at the HBB gene by HDR is being addressed by two alternative methods.

5.1. Phase 3 Clinical Trial NCT05329649 CTX001

The experimental gene-edited cell treatment CTX001 is being tested in two Phase 3 trials, one in children with SCD and the other in people with transfusion-dependent β-thalassemia (TDT), by CRISPR Therapeutics and Vertex. Both Phase 3 studies will consist of a maximum of 12 kids between the ages of 2 and 11. In the study VX21-CTX001-151 (NCT05329649), children with severe SCD who are intolerant or unresponsive to hydroxyurea are being enrolled. Participants undergo a single intravenous infusion of CTX001, which is made up of bone marrow-derived cells that have been altered to create foetal haemoglobin, and are subsequently followed up for up to two years. In May 2026, both trials will come to a conclusion. CTX001 stimulates the formation of foetal haemoglobin in the blood cell progenitors of SCD patients by using the CRISPR-Cas9 gene editing technology. A type of haemoglobin created during foetal development called foetal haemoglobin transports oxygen more effectively than adult haemoglobin. In the case of a stem cell transplant, the altered cells are reinfused into the patient. CTX001 is anticipated to decrease the prevalence of VOCs in SCD patients and the requirement for ongoing transfusions in TDT patients by raising foetal haemoglobin levels.

5.2. NCT05477563, A Phase 3 Clinical Trial

A phase 3b trial to assess the safety and efficacy of a single dose of autologous CRISPR Cas9-modified $CD34^+$ human haematopoietic stem and progenitor cells (CTX001) in individuals with severe sickle cell anaemia or transfusion-dependent β-thalassemia. Participants in this single-dose, open-label research have severe SCD or transfusion-dependent β-thalassemia (TDT). The effectiveness and safety of autologous CRISPR-Cas9-modified $CD34^+$ human haematopoietic stem and progenitor cells (hHSPC) will be assessed in the subsequent investigation employing CTX001.

5.3. NCT04774536 for SCD by Drug CRISPR SCD001

Patients with severe SCD may get a transplant of CRISPR-modified haematopoietic progenitor stem cells (CRISPR_SCD001). NCT04774536. The CRISPR-Cas9-edited RBCs (also known as the therapeutic product CRISPR_SCD001) are used in the study to assess haematopoietic stem cell transplantation (HSCT). Adults make up the first six items. Three adolescents between the ages of 12 and 18 will be enrolled in the trial to continue collecting data on the safety of CRISPR SCD001 if it proves to be safe after the first six months.

5.4. Long-Term Follow-Up Study in Subjects Who Received CTX001 NCT04208529

This is a multi-site observational study to assess the long-term safety and efficacy of CTX001 in patients who had previously received CTX001 or VX21-CTX001-141 for transfusion-dependent β-thalassemia (TDT) or CTX001-121 or VX21-CTX001-151 for severe SCD in the study of CTX001-111 (NCT03655678).

5.5. Phase I/II Cedar Clinical Trial NCT04819841

A phase I/II clinical investigation, the CEDAR trial (NCT04819841), is being funded by Graphite Bio and will begin in 2021. GPH101, in contrast to the products previously mentioned, is HDR-based and depends on a superior CRISPR-Cas9 system coupled with an AAV6-based HDR model. The point mutation in HBB that causes SCD must be fixed [56]. 90% of erythrocytes in preclinical investigations on mice had normal HbA levels after approximately 20% of HSCs had their HBB locus repaired. Preclinical safety studies revealed no chromosomal translocations, aberrant haematopoiesis, or discernible side effects. Up to 15 patients are currently being tracked at various facilities across the USA, according to Graphite Bio, which recently announced the enrolment of its first patient, in midway through 2023; preliminary CEDAR study results are anticipated.

5.6. Phase I/II Clinical Trial Ruby NCT04853576

With about 40 people, Editas Medicine began the Phase I or II clinical trial RUBY (NCT04853576) in 2021 to assess the effectiveness and safety of EDIT-301. The final product is made from autologous HSC cells in which the HBG1/2 promoter regions have been disrupted using CRISPR-Cas12. In preclinical mouse models, HSCs generated with HBG1/2 have shown long-term engraftment. In cells from healthy donors (43%) and SCD patients (54%), large levels of HbF induction were linked to around 90-fold off-target alleles without any discernible off-target effects [57].

5.7. Gene Therapy (OTQ923) To Treat SCD

This study assesses a drug called OTQ923, which lowers the biological activity of BCL11A, boosts the generation of foetal haemoglobin (HbF), and lessens the issues associated with sickle cell anaemia. The blood-forming cells in OTQ923 are genetically altered, and physicians think this can lower BCL11A activity and raise foetal haemoglobin (HbF). The person will first get customary drugs to get the body ready to gather its own blood-forming cells. The next step is apheresis, which is a procedure for gathering, separating, and preserving blood-forming cells. Through gene editing, haematopoietic cells are processed in the lab to make OTQ923and own genetically altered blood-forming cells (OTQ923) will be

given to the person in a single intravenous (IV) infusion on the day of the transplant. To determine how well the treatment is working, a person will undergo a biopsy. The clinical trial doctors will continue to check on health for two years after the completion of the treatment. Then, a person might have the chance to have medical professionals oversee their health throughout a clinical trial for up to 15 years. The Food and Drug Administration (FDA) has not yet given OTQ923 its approval.

5.8. CTX001 Clinical Trial NCT03745287

The most recent trial is CTX001, which Vertex Pharmaceuticals and CRISPR Therapeutics created. The phase II/III clinical trial CLIMB-121 (NCT03745287), which began in 2018 with 45 SCD patients, is now testing it. CTX001 is given as a lineage-specific enhancer of the BCL11A gene that is disrupted by a CRISPR-Cas9-engineered equivalent HSC product. This modification decreases the expression of BCL11A in erythroid cells, which in turn promotes the expression of β-globin. High amounts of edited alleles are found in the stem cell compartment in the first two patients (one with SCD and one with TDT), according to published clinical data (69 and 80%, respectively). The HbF level of the SCD patient grew from 9.1 to 43.2% at 15 months following transplantation, while the HbS level reduced from 74.1 to 52.3%. Patients were said to be VOC-free and independent of transfusions [58].

A recent update of the effects of CTX001 infusion in 44 TDT and 31 SCD patients found that all patients maintained HbF increases (39.6-49.6%), improved mean Hb levels (>11g/dL) 3 years later, and eliminated volatile organic compounds. More than 80 alleles showed a persistent response to this treatment, according to bone marrow analyses (>12-month follow-up). However, after receiving injections of modified cells, some individuals had a number of serious adverse effects (SAEs), including VOC liver disease, sepsis, cholelithiasis, and haemophagocytic lymphohistiocytosis (HLH). There have also been reports of mild lymphopenia, possibly as a result of a delayed lymphocyte recovery.

Unveiling the Therapeutic Potential of CRISPR-Cas9 SCD for one sickle cell patient, Victoria Gray, who has suffered from this crippling disease her entire life, there is a chance to get rid of it once and for all, and a chance to protect her and gift to future generations through the new CRISPR Cas9 technology (clinicaltrial.gov). Much of her life was spent on trips to the emergency room due to pain, and her life and career goals were interrupted or disrupted. It has progressed, making it difficult for his heart to work, and strokes are always a constant threat. It is the most common inherited blood disorder. Alleviating the symptoms and burden of the disease with a bone marrow transplant remains the only option for many to this day, while many others have dark hopes for the future due to the rapid progression of the disease. As Victoria's doctors considered a bone marrow transplant for the same purpose, they suggested something else to treat her condition. He took a chance and became the first patient listed to be treated with the CRISPR-Cas9 genome editor. As of August 2020, a year after starting treatment, this method seems to work better than any other. The billions of genetically modified cells infused into his body seem to alleviate almost all the complications of his SCD. CTX001–clinical trial, although Victoria Gray's SCD is a genetic disease, the genetic intervention did not involve removing an entire copy of the β-globin gene and inserting it into her DNA. Instead, CTX001 is used to treat it. CTX001 increases the production of foetal haemoglobin HbF. It is a type of haemoglobin that is present at birth but stops being produced in adulthood. The principle of CTX001 is that the production of HbF can compensate for the defective Hb produced.

The steps involved are: 1. Collection of stem cells from the patient's bone marrow, 2. Delivery of CRISPR-Cas9 components to cells. and 3. Effect of CRISPR Cas9 deletion in the BCL-11A gene (it encodes a transcription factor that normally inhibits foetal haemoglobin synthesis).

Consequence of CRISPR-Cas9 disruption in the BCL-11A gene causes production of billions of such modified and improved stem cells. When the majority of stem cells enter the body and produce protein, it offsets the production of HbS and effectively controls the disease. Prognosis and outcome the patient's predicted HbF level was 20% at the start of therapy. In August 2020, the patient's HbF values far exceeded the doctors' expectations; the HbF level was 46% of the total haemoglobin. Additionally, 81% of body's bone marrow contains the stem cell-mediated genetic modification necessary to produce the foetal haemoglobin protein. This indicates that the engineered and modified stem cells remained in his body for a long time, much longer than expected. Quality of life after treatment from frequent trips to the emergency room and multiple blood transfusions needed to survive and continue living, to zero hospital stays and freedom from symptoms and complications after CRISPR-Cas9 gene therapy, Victoria Gray's life has improved. Although he was the first SCD patient to receive this experimental treatment, he is not the only one. In 2019, β-thalassemia patients also received CTZ001 treatment to increase foetal haemoglobin levels through stem cell transformation.

Although gene therapy can treat many, but not all, complications of the disease, it has not yet been considered a cure for some diseases. CTX001 is not without its drawbacks, like any other treatment. It is believed that the patient will undergo chemotherapy to remove their own bone marrow and make room for new cells. Skin chemotherapy for cancer patients and transplant patients, side effects remain the same due to fatigue, nausea, mouth ulcers, bleeding, and infections due to immunosuppression, hair loss and loss of appetite. Thus, gene therapy effectively controls the disease, but there are still side effects that the patient will inevitably encounter. Technical challenges of *ex vivo* genome editing approaches in HSCs are similar to LV-based approaches and include obtaining sufficient numbers of mobilized $CD34^+$ cells as starting material, sufficient editing efficiency in the LT-HSC compartment, lower *ex vivo* engraftment rates of edited cells and editing HSC reduced stemness (clinicaltrials.gov).

Table 2: Clinical Trials Performed by CRISPR CAS9

Clinical trial	Phase	Year started	Treatment name	Target gene	Delivery mode	Designer nuclease	Sponsors
NCT05329649	III	2022	CTX001	BCL11A	RNP Electroporation	CRISPR CAS9	Vertex pharmaceutical, CRISPR Therapeutics.
NCT05477563	III	2022	CTX001	BCL11A	RNP Electroporation	CRISPR CAS9	Vertex pharmaceutical, CRISPR Therapeutics.
NCT04774536	I & II	2022	CRISPR-SCD001	HBB	RNP Electroporation	CRISPR cas9	University of California
NCT04208529 {Long term follow-up activity}	-	2021	CTX001	BCL11A	RNP Electroporation	CRISPR cas9	Vertex pharmaceutical, CRISPR Therapeutics.
NCT04819841	I & II	2021	GPH 101	HBB	RNP Electroporation	CRISPR cas9	Graphite Bio.
NCT04443907	I & II	2020	QTQ923	BCL11A	RNP Electroporation	CRISPR cas9	Novartis Pharmaceutical, Intellia Therapeutics
NCT03745287	II & III	2020	CTX001	BCL11A	RNP Electroporation	CRISPR cas9	Vertex pharmaceutical, CRISPR Therapeutics.

6. CONCLUSION AND FUTURE PERSPECTIVES

Most SCD patients can now be cured by autologous transplantation of genetically modified haematopoietic stem cells due to developments in CRISPR/Cas9 technology. Because it is affordable, simple to use, and extremely successful, CRISPR editing is one of the leading choices for treating anaemia disease. The necessity for high editing efficiency and low off-target effects are just two of the obstacles involved in bringing a gene editing-based treatment approach for SCD to the clinic. For secure clinical applications, a quantitative comprehension of the genotypic and phenotypic effects of various mutations in CRISPR/Cas9-edited SCD $CD34^+$ cells are necessary. The development of engineering techniques that allow for high yields of polyclonal and highly proportionate genetically modified cells to be long-term repopulated in HSCs is still a difficulty. Additionally, little is known about how SCD pathophysiology affects HSPC viability and engraftment potential, particularly in patients who have endured years of chronic inflammation linked to SCD. Our understanding of the impact of chronic systemic inflammation and poor erythropoiesis associated with HSPCs from SCD patients is currently limited because the majority of *in vivo* transplantation research related to SCD has been carried out with cells from healthy persons. Gene editing results and engraftment potential can be strongly impacted by individual variations in the source of HSPCs and SCD pathophysiology, including patient status. The survivability and capabilities of SCD HSPCs are probably influenced by genetic and environmental variables. *Ex vivo* gene editing techniques currently used have certain drawbacks. HSCs often make up a very modest portion of $CD34^+$ cells in SCD patients. It is invasive to take HSCs out of the bone marrow. Low blood counts and infections are among the negative effects of chemotherapy that myeloablative chemotherapy patients also face. HSC pluripotency and engraftment potential are lost as a result of *in vitro* culture and gene modification. *Ex vivo* gene editing-based therapy may also be unaffordable for some patients because of the high expenses associated with the need for highly specialized facilities and the technological know-how necessary. Because *in vivo* treatment can be minimally invasive and cost-effective, it is more accessible in resource-poor locations and may be able to overcome the constraints of *ex vivo* gene editing. To achieve both high *in vivo* delivery and high editing efficiency, as well as to make *in vivo* gene editing a clinically effective method, there are considerable obstacles to overcome. A partnership between the NIH and the Bill and Melinda Gates Foundation is working to create *in vivo* gene editing therapy for SCD.

Two gene therapy treatments for sickle cell disease - exa-cel (CTX001) from CRISPR Therapeutics and Vertex Pharmaceuticals, and lovo-cel from Bluebird Bio are expected to be FDA-approved by December 2023 with a probability of success at 77.8% [59]. One concern is cost and complexity of the therapy requiring a bone marrow transplant and lengthy hospitalization which put it out of reach for those who need it most as well as in less affluent countries where the disease is most common. Reduction in cost of treatment and occurrence of off target mutations will be two future challenges to be addressed by researchers to bring this advance therapy safe and benefitthe patients.

Using this gene editing technique, a lot of cell line genotyping and phenotyping has already taken place. The therapeutic effects are vast, including inhibition of viral infection, restoration of handicapping disorders such muscular dystrophy, and tumour eradication in cancer models. Without a doubt, the CRISPR/Cas9 system will transform scientific investigation into the treatment of allergy and immunological illnesses. A safe and ethical use of developing technology for basic research and therapeutic reasons must be enabled by laws and regulations that have been approved by the relevant authorities. A remarkable accomplishment in treating diseases at their source, tolerates symptom relief and introduces fresh medicinal approaches. Scientists are still working on CRISPR-Cas9's proverbial ‘tip of

the iceberg'. Proteins produced by genes through their expression continue to serve both a metaphorical "weapon" and an environmental 'trigger' in biology.

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CONFLICT OF INTEREST

The authors declare that there is no Conflict of Interest.

AUTHORS' CONTRIBUTIONS

NL designed the study, managed the analyses of the study and wrote the first draft of the manuscript. PP managed the literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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