Characteristics of gene editing clinical trials from public trial registries

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CHARACTERISTICS OF GENE EDITING CLINICAL TRIALS FROM PUBLIC TRIAL REGISTRIES

Diploma thesis

Academic year:
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TABLE OF CONTENTS

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Michael Zlatin

1. INTRODUCTION

1.1. **The United States Food and Drug administration (FDA) and European Medicines Agency (EMA) provide regulatory framework for clinical trials in gene editing**

Gene editing (genome editing) is genetic engineering that involves DNA double strand breaks and repair mechanisms for incorporating site-specific modifications into a human cell's genome. If the mutant genes could be “knocked out”, and functional gene “knocked in”, or if the expression of overactive or underactive genes could be normalized, the disease could be treated at the molecular level, and potentially be cured (1). This concept seems particularly true for the treatment of diseases caused by mutations in a single gene.

Research involving modern gene editing techniques is done on both somatic cells (stem and differentiated) and germline cells. However, human clinical trials are done on somatic cells. Based on concerns about ethics and safety, there is currently an international moratorium on human germline cell and embryo genome editing (2).

Gene therapy products are classified as biologics, along with vaccines, blood and blood components, somatic cells, tissues, and recombinant therapeutic proteins. The regulatory guidelines for manufacturing, non-clinical assessment, clinical trials, and product approval requirements for cell gene therapy products and other biologics are provided by Food and Drug Administration (FDA) in US and European Medicine Agency (EMA) in EU. They can be found on their respective websites fda.gov (1) and ema.europa.eu.

All clinical trials registered with FDA are presented in the National Clinical Trials (NCT) registry at ClinicalTrials.gov website and follow strict guidelines outlined in collection of FDA documents (3).

FDA regulations on informed consent follow the principles of Nuremberg Code (1947) and Declaration of Helsinki (1964). These principles served as the basis for “International Ethical Guidelines for Biomedical Research involving Human Subjects” by WHO (1993), and are used by Ethics Committees (In US- Institutional Review Boards, IRB) in the trial approval process (4).

Primary outcomes were observations of Adverse Events (AE) and other safety data, usually for a period of 6-12 months. However, unlike regular drugs, gene therapy products are designed to achieve therapeutic effect through permanent or long-acting changes in the human body. To understand and mitigate the risk of delayed adverse events, FDA recommended that subjects in gene therapy trials are monitored for an extended period of time, which includes “long term follow-up” period of a clinical study and long term follow-up/surveillance period

post licensure (1). Not all gene therapy products will require long term observations; a risk assessment should be performed by a trial sponsor based on several factors as outlined in FDA guidance.

The US Food and Drug Administration Amendments Act of 2007 (FDAAA) requires that the "Responsible Party" for clinical trials registers with, and submits the results information to, the National Clinical Trials (NCT) databank at ClinicalTrials.gov. Clinical trials conducted in Europe are entered into EU Clinical Trials Register clinicaltrialsregister.eu. German clinical trial registry is DRKS.de. Chinese clinical trial database can be found at chictr.org.cn. World Health Organization (WHO) developed their own comprehensive protocol for reporting clinical trials, based on NCT protocol. It is called International Clinical Trials Registry Platform (ICTRP) and can be found at who.int/ictrp. WHO accepts any trial that is already registered in recognized primary registers (data providers). NCT and EU registries are two of the primary registers for WHO. At this time registration with WHO is optional, and few trials exercise this option.

International Committee of Medical Journal Editors (ICMJE) accepts for publishing data from trials registered in NCT, or any other primary register of the WHO ICTRP (5). A trial acceptable to NCT, ICTRP registries, and to ICMJE must include the minimum 24-item trial registration dataset (TRDS) at the time of registration and before enrollment of the first participant. The items are marked in Tabular View with subscript "icmje". Enrollment officially starts when the participant signs an informed consent form. The Ethics Committee (in US - Institutional Review Board, IRB) should conduct ethics review and approve protocol and informed consent forms before submission to Registries. If changes to the protocol happen during the trial, on NCT and WHO registries the previous version is archived and changes can be retrieved and investigated to determine if the study was manipulated to conceal unfavorable outcomes.

As stated by the ICMJE, "The purpose of clinical trial registration is to prevent selective publication and selective reporting of research outcomes, to prevent unnecessary duplication of research effort, to help patients and the public know what trials are planned or ongoing into which they might want to enroll, and to help give ethics review boards considering approval of new studies a view of similar work and data relevant to the research they are considering. Retrospective registration, for example at the time of manuscript submission, meets none of these purposes"(5).

1.2. Early gene editing techniques

Genetic engineering experiments in yeast began in the 1970s. Mario Capecchi, Martin Evans and Oliver Smithies were awarded Nobel Prize in Physiology and Medicine in 2007 "for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells"(6). Genetically modified transgenic (knockout) mice were created with genes disrupted by nucleases. The process was inefficient. Only one in a million cells or less were modified, which prevented the use of this technology beyond creation of knockout mice for research. While doing these early experiments an important observation was made that helped develop modern gene editing techniques: an intact DNA is inert and resistant to recombination, but if double strand breaks happen, DNA is activated for recombination. Restriction enzymes (nucleases) that could be used as "molecular scissors" to cut DNA backbone at specific sites were discovered in 1968 in John Hopkins University. An enzyme, called Hind11, was used by *Haemophilus influenzae* bacteria to cut and destroy DNA of bacteriophages. Hundreds of restriction nucleases were isolated from different bacterial species. They were effective at cutting DNA, but recognized and cut at multiple sites, which prevented their use for genetic engineering of larger mammalian genomes.

1.3. History of gene editing technology in clinical trials

Safety and ethical problems became obvious when gene editing trials in humans started. It was Jesse Gelsinger's death in 1999 that brought adverse events intrinsic to genetic engineering technology to the forefront. Jesse Gelsinger was an 18 year-old man with a mild form of ornithine transcarbamylase enzyme deficiency, causing ammonia build up. His form of the disease could be controlled by diet and medications. He was the eighteenth and final patient in the trial led by James Wilson, director of the University of Pennsylvania's Institute for Human Gene Therapy (IHGT). 3.8×10^{13} of adenovirus vector particles containing a gene to correct Jesse's genetic defect were injected into his hepatic vein. In the next four days Jesse's condition progressively worsened until he died from what was likely a severe inflammatory reaction to the adenovirus vector (cytokine release syndrome): disseminated intravascular coagulation (DIC) followed by liver, kidney, and lung failure. This was the first widely publicized death directly attributed to gene therapy. FDA suspended all gene therapy trials, and the Senate investigated. The investigations drew attention to wider problems in oversight of gene-therapy experiments and human research generally. For example, FDA revealed that 691

volunteers in gene-therapy experiments had fallen ill or died in the seven years before Jesse's death; only 39 of these incidents had been reported promptly as required. Informed consent documents given to Jesse did not disclose death of monkeys in animal studies and severe adverse events suffered by 2 other patients in his trial, even though the original consent form submitted to NIH for approval mentioned them. There were more violations with informed consent: different forms were presented to FDA and given to patients; false and coercive statements in recruitment of volunteers ("very low doses", "promising results"); and lead researcher James Wilson having financial stakes in gene editing technology company (7).

One of the main conclusions about technology failures was a statement that "Adenovirus vectors are unreasonably dangerous"**.** There is a possibility of severe inflammatory reaction and organ failure due to certain types of antibodies, now called binding antibodies, produced after previous exposure to adenovirus. This realization prompted the search for safer vectors (7).

Another widely publicized failure of early gene editing was development of cancer in some of the 11 children treated in France for Severe Combined Immunodeficiency Disorder (SCID) in 2002. Lymphocytes were edited *ex vivo* and reinfused. Some children showed signs of improved immune conditions. However, in the following months 4 children had leukemia-like symptoms, a lymphocyte proliferation disease. In those children "insertional mutagenesis" happened: retroviral vector inserted a therapeutic gene near oncogene in T cells, triggering oncogene expression. The trials were stopped (8).

Jesse Gelsinger's death and cancer in SCID children contributed to the impression that manipulation of human genome is dangerous. The field of gene therapy collapsed, taking its grandiose promises of miracle cures along with it. Biochemist Jennifer Doudna, who later discovered aspects of CRISPR-Cas9 gene-editing mechanism, remembers the shock waves, "We were all very much aware of what happened there and what a tragedy that was. That made the whole field of gene therapy go away, mostly, for at least a decade. Even the term "gene therapy" became kind of a black label. You didn't want that in your grants. You didn't want to say, 'I'm a gene therapist' or 'I'm working on gene therapy.' It sounded terrible."

1.4. Advancements in gene editing techniques

Methods such as ZFN, TALEN and CRISPR were developed which targeted specific genes or specific sites within an organism genome (9,10). Viral vectors were modified to make

them safer. Gamma retroviruses, lentiviruses and adenoviruses were replaced with recombinant adeno associated virus vectors (AAV) (11,12,13). Other delivery methods, such as Lipid Nanoparticles (LNPs) were developed. These new systems, while safer, still have unsolved technological problems.

Experts' and public attitudes moved toward accepting risks of gene editing in patients severely debilitated by genetic diseases, and gene therapy rebounded. "Contrary to hopes of human research reform spurred by Jesse Gelsinger's death, oversight has flattened and profit motives have become more entrenched in medical research", wrote O. Obasogie, a professor of bioethics at the University of California, Berkeley, in 2009 (7). In conclusion, the death of Jesse Gelsinger is a tragic reminder of medical risks involved in gene therapy and the importance of caution, transparency, and oversight in clinical trials.

1.5. Mechanism for gene editing

An efficient method for making breaks in DNA targeted to specific gene sequences was needed. Eventually, three novel gene editing techniques were developed that used programmable sequence-specific DNA-cutting nucleases, to make double strand DNA breaks at specific sites even in large genomes such as humans: Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN), Clustered Regularly Interspaced Palindromic Repeats (CRISPR) with CRISPR associated enzymes (Cas) (9).

All three techniques use complex restriction enzymes, artificially created by fusing DNA recognition (binding) domain to a DNA cleavage domain (a nuclease capable of cutting sugar phosphate backbone between nucleotides).

1.5.1. Zinc Finger Nuclease (ZFN)

ZFN was the first technique developed. From 2009 to 2014 first clinical trial with ZFN was conducted to treat HIV. Favorable results helped bring gene editing back into clinical trials.

Zinc finger is a protein structure with folds stabilized by zinc ions in the shape of alpha helix finger-like protrusions that can fit the grooves of DNA helix. Each natural zinc finger recognizes a set of three bases, on the DNA molecule. By stringing three or four fingers together, researchers can generate artificial proteins that match a particular site. Two sets of zinc fingers (recognition domain) are attached to a nuclease (cleavage domain) that cuts DNA

in between the 2 sites matched by the fingers. Specificity is changed by constructing different zinc finger combinations.

Recognition domain: Zinc Finger proteins

Cleavage domain: Nuclease

Disadvantage: construction of unique ZFNs specific to each target site requires expertise and is expensive. Most work with ZFNs is still done by Sangamo Therapeutics.

1.5.2. Transcription activator-like effector nucleases (TALEN) technique

TALENs are restriction enzymes that share many properties with Zinc Finger Nucleases, but are considered easier to construct and more effective than ZFNs.

Recognition (binding) domain: Transcription Activator Like Effector (TALE) proteins, fused to DNA cleavage domain (nuclease).

One recognition module, made of 34 aminoacids, binds to 1 DNA base pair. Different combinations of many aminoacids provide for robust code with high specificity (higher than CRISPR and ZFN).

1.5.3. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with CRISPR associated enzymes (Cas)

CRISPR-Cas complex restriction enzyme evolved in nature as an “immune system” in bacteria to destroy DNA of bacteriophages and was adapted for gene editing (14).

The best description of a long journey of CRISPR discovery by young scientists, whose groundbreaking research was rejected by prestigious journals, but eventually led to creation of modern gene editing technology, can be found in a review written by Eric S. Lander “The Heroes of CRISPR” (10).

CRISPR recognition domain: small guide RNA molecule, sgRNA.

DNA cleavage domain: complex enzyme Cas (CRISPR associated protein), which consists of helicase and nuclease. Cas enzymes are transcribed and translated from Cas genes attached to CRISPR array. CRISPR array consists of many identical(palindromic) repeats with unique spacers between them. Guide RNA is transcribed using unique sequences in spacers that in nature were pieces of DNA from previously invading bacteriophages, conserved to enable bacterial cell recognize the bacteriophage when it invades again, and quickly respond to attack with Cas restriction enzyme. The fact that recognition domain is formed not by proteins (as in ZFN and TALEN), but by RNAs, gives CRISPR technology immediate advantage. Guide RNAs can be easily constructed by synthetic biology techniques, no need for

expensive production of unique ZFN recognition proteins. CRISPR arrays are found in approximately 50% of bacterial genomes and nearly 90% of archaea.

It is now possible not only knock out and knock in genes with CRISPR, but change gene expression: to activate or repress the gene. With this purpose CRISPR interference (CRISPR*i*) and CRISPR activation (CRISPRa) have been developed. Cas9 has a remarkable feature: its ability to bind to DNA is independent from its ability to cleave. Because of this, point mutations can render nuclease domains inactive, creating a dead Cas9 (dCas9) without the capability to cleave. dCas9 can be tagged with repressive or activating effectors to modulate expression of the targeted gene (15).

1.5.4. DNA repair as the critical step of gene editing

Cells respond to double strand breaks in DNA made by CRISPR, ZFN or TALEN with 2 main repair mechanisms:

1. Homology directed repair (HDR) is assumed to be error free because of the use of a template for precise repair of broken DNA strands. In nature homologous DNA sequence from homologous chromosome or sister chromatid serves as a template. Homology directed repair maintains genomic stability and prevents cancer. In gene therapy templates with genes are delivered into cells for targeted insertion by HDR;

2. Non-homologous end joining (NHEJ) happens when there is no homologous template for directing repair. NHEJ directly ligates DNA from either side of a double-strand break where there is very little or no sequence overlap for annealing (pairing by hydrogen bonds to form a double-stranded DNA). *NHEJ* may result in errors in the genome via insertion, deletion, or chromosomal rearrangement; any such errors may render the gene products coded at that location non-functional. Cancer may result.

Target gene deletions can be made by non-homologous end-joining (NHEJ). Targeted insertion of new sequences is done via HDR with a repair template delivered into the cell**.**

The major problem in gene editing is that in many cell types error prone NHEJ is more prevalent than HDR.

1.5.5. Challenges in gene editing

While the molecular mechanism of CRISPR-Cas9 and other nucleases are getting the most attention from researchers, the delivery of nucleases and gene templates into the cells is

the most challenging part, and probably accounts for most of cytotoxicity, such as severe inflammatory reactions or allergies.

Many techniques or their combination are used for delivery of components of gene editing technology into cells. Review of multiple delivery technologies can be found on thermofisherscientific.com, a leading manufacturer of delivery systems. In summary, most common delivery methods are:

1. DNA plasmids;
2. Viral Vectors carrying gene cassettes (used *in vivo* and *ex vivo*). Transfection with viral vectors is the most popular technique in gene editing. Homologous recombination occurs immediately after DNA cleavage by Cas9 using virally delivered gene for knock-in mutation of host DNA (11;13);
3. Lipid nanoparticles are non-viral vectors with small carrying capacity. They are used to deliver mRNA;
4. Electroporation and other physical methods are used *in vitro* and *ex vivo.* Electric shocks open pores in cell and nuclear membranes through which foreign DNA and proteins can enter. (A number of pharma companies working on experimental COVID DNA based vaccines are considering electroporation *in vivo* at injection site to deliver DNA coding for COVID spike protein into cells, as a variation of *in vivo* human gene editing technique where inducing immunogenicity is a goal, rather than undesirable adverse event) (16).
5. Microinjection with tiny needles can be used in cultured cells, in gametes, or in early embryonic cells in germline gene editing.

For *ex vivo* transfer of genome editing tools designer nucleases are usually transferred either as a manufactured CRISPR/Cas complex or in the form of synthetic messenger RNA (mRNA), which induces ribosomes to produce desired nucleases inside the cell (ZFN, TALEN, Cas9). If gene addition by HDR is the goal, an additional genetic sequence to be copied into on target site is provided as part of HDR template. Commonly an HDR template is provided as part of non-integrating viral vectors. For clinical *in vivo* genome editing adeno associated virus (AAV) vectors have been employed almost exclusively to deliver DNA coding sequence. AAV is also used to deliver mRNA coding sequence for Cas enzyme (13). Vectorbiolabs.com offers AAV vectors of 4.7 kilobase length into which they insert 3 kb Cas9 coding sequence of *Staphylococcus aureus* origin.

1.6. Possible unintended consequences of gene editing

Modern techniques, designed for site specific gene editing, (ZFN, TALEN, CRISPR/Cas) were declared remarkably precise and efficient, able to overcome major problems of older methods, such as insertional mutagenesis and inefficiency. Therefore those techniques were considered suitable for medical applications in humans.

However, multiple studies discussed further show that new techniques are not as precise as initially described and often cause unintended mutations.

1.6.1. Off target mutations

Gene editing can induce double-strand DNA break at a site other than intended, resulting in deletions, insertions or chromosomal translocations. As a result, tumor suppressor genes may be down regulated or oncogenes may be activated. Multiple off target mutations can occur. Persistent nuclease expression in targeted cells can produce more off target mutations. Each nuclease has a pattern of specificity for off target sites. Only complete sequencing of edited genome at single cell level can detect all off target site mutations. There is no means of predicting long term biological consequences of off target mutations, which makes adverse events monitoring and long term follow up especially important (17,18,19).

1.6.2. Unintended mutations at target site (on target mutations)

Deletions or rearrangements at targeted site can arise from DNA repair processes following gene editing induced double strand DNA break. Recent research by Tuladhar et al., 2019 (20) in an article called “CRISPR-Cas9-based mutagenesis frequently provokes on-target mRNA misregulation” describes unanticipated gene expression in target genes after CRISPR editing. ”By examining the mRNA and protein products of CRISPR targeted genes in a cell line with presumed gene knockouts, we detect the production of foreign mRNAs or proteins in ~50% of the cell lines”. The proteins were internally or terminally truncated.

1.6.3. Target knockout genes may still be expressed at various levels.

Confirming and expanding a study by Tuladhar *et al.* (20), a new “Heidelberg Study” by Stanford University and GlaxoSmithCline, Smits *et al*. in 2019 (21), characterized protein expression of 193 genetically verified deletions, generated by CRISPR, in 136 distinct genes. Authors observed residual protein expression for about one third of the targets, at variable levels from low to original. There were truncated proteins with partially preserved function,

and proteins with internal sequence deletions. Authors conclude that "systematic characterization of residual protein expression or function in CRISPR–Cas9-generated knockout cell lines is necessary, in addition to DNA sequencing." The findings of this latest study, as well as the earlier observations by Tuladhar *et al.(*20), show that mutant protein production takes place after the editing event and is not dependent on the gene-editing precision. There is no way of avoiding these issues because they arise from the innate properties of the basic molecular biology of gene expression.

In addition, use of some viral vectors (adenovirus, lentivirus) as gene delivery methods may result in unintentional and random integration of vector backbone DNA into the targeted genome (11), which also causes mutagenesis.

In summary, residual expression of genes at "on target" sites produces truncated proteins with unknown properties, leading to altered biochemistry.

1.6.4. Cytotoxicity causes most adverse events in clinical trials

Insertional mutagenesis, off target and on target mutations and introduction of gene editing technology components: ZFN, TALEN, Cas nucleases, mRNAs, viral vectors, lipid nanoparticles, can work synergistically to cause adverse events monitored in clinical trials.

Cytotoxicity can cause systemic inflammation and allergic reactions described as Cytokine Release Syndrome. It presents as an abnormal inflammatory response: high fever, chills, fatigue, myalgia, headache, confusion, delirium, tremor, seizures, and loss of coordination. CRS may progress in a positive feedback loop to anaphylaxis, sepsis, DIC (Disseminated Intravascular Coagulation), capillary leakage, and lung, kidney, liver and heart failure. This happened to Jesse Gelsinger as a reaction to adenovirus vector, and happens to various degree after infusion of *ex vivo* gene edited CAR T cells, and sometimes after administration of other biologics, such as blood transfusions or vaccines. Autoimmune diseases and allergies may result from delayed cytotoxicity.

Acute liver injury is listed as the most serious adverse event in "black box warning" that FDA placed on their first approved (2020) gene therapy product Zolgensma for pediatric patients with spinal muscular atrophy. It was attributed to high amount of adeno associated (AAV) vector used in therapy. Early presentation is jaundice and elevation of liver enzymes.

Biodistribution of AAV was across many organs, highest in the liver (23). Tumorogenicity is another adverse event that should be assessed in a long term follow up**.**

European Network of Scientists for Social and Environmental Responsibility (ENSSER) issued a statement on September 27, 2017, signed by many prominent scientists, urging precautionary principle in the application of new genetic modification techniques, such as CRISPR. The statement emphasized many unintended and unknown consequences of new gene editing technologies (24).

1.7. Primary candidates for gene editing

Single gene disorders targeted in clinical trials have 2 main characteristics: the disease should be severe enough with no (or limited) conventional treatment; and cells should be easily reached by delivery systems carrying genes and other components.

This makes Hematopoietic Pluripotent Stem Cells (HPSCs), carrying genes for blood diseases, such as sickle cell disease, beta-thalassemia, and hemophilia A and B, ideal candidates for gene therapy. T lymphocytes, in patients suffering from HIV and cancers, are also suitable candidates. Third type of cells are hepatocytes, targeted at albumin site for disorders connected to liver enzymes.

Conferring resistance to HIV virus with targeted editing of patient's T cells was one of the earliest interventions with Zinc Finger Nuclease (ZFN) technique, treating HIV as a kind of genetic disease. It was known from the "Berlin patient" case that a certain (delta 32) mutation to CCR5 gene, coding for integral membrane protein that is chemokine receptor, confers resistance to HIV infection. HIV virus uses CCR5 receptor to enter cells. In patients, homozygous for mutant (nonfunctional) CCR5 gene, HIV may not progress to AIDS, virus may become undetectable, and the amount of CD4 T cells increases (22). HIV patients' cells were genetically modified *ex vivo* with Zinc Finger Nuclease (ZFN) to carry the CCR5-Δ32 trait, then reintroduced into the body as a potential HIV treatment. Results reported in 2014 were promising, and helped bring the return of gene editing technology into clinical research.

Another major category of gene editing trials is immunotherapy of B cell cancers using Chimeric Antigen Receptor T cells (CAR T cells), autologous (patient's own) or allogeneic (from healthy donor). CD19 is a transmembrane protein that is expressed on the surface of all B cells, both normal and neoplastic, as a B cell marker. T cells can be collected, genetically

modified *ex vivo* to express CD19 specific CAR (Chimeric Antigen Receptor), and cultured (expanded). Edited CAR T cells then are infused into patient and interact with CD19, expressed on B cells in acute lymphoblastic leukemia (ALL) and B cell lymphomas. T cells proliferate, cytokines directed at B cells are produced and neoplastic B cells undergo lysis. This is a form of immunotherapy for cancer. The patient undergoes lymphocyte depletion chemotherapy prior to the introduction of the engineered CAR T cells, which helps to promote the expansion of the infused engineered CAR T cells.

Though the initial remission rate in CAR T cell therapy is as high as 90% in patients with leukemia and lymphoma, long term survival is low because of "antigen escape" when cancer cells stop expressing targeted receptor CD19.

Most common AEs of CAR T treatment are Cytokine Release Syndrome **(CRS)**, anaphylaxis and neurotoxicity. CRS occurs in all patients, and its severity is connected not with effectiveness of treatment but rather with disease burden. Neurotoxicity usually is presented with delirium and seizures, but a few deaths from cerebral edema happened in the past trials.

CD34 is a transmembrane protein, encoded by CD34 gene and expressed on Hematopoietic Stem Cells (HSCs). CD34 is also important adhesion molecule for T cells that facilitates their migration. As such, CD34 gene in HSCs and T cells is often targeted for editing.

Hemoglobinopathies include transfusion resistant beta-thalassemia and sickle cell disease. Both of them are caused by mutations in the gene HBB, which codes for a beta-globin chain of hemoglobin. This results in defective hemoglobin and anemia. The purpose of gene therapy is to replace defective HBB gene with functional transgene. In addition to HBB, BCL11A gene plays a role in development of hemoglobinopathies, enabling transition from fetal to defective adult hemoglobin. Fetal hemoglobin HbF reactivation through knockout of BCL11A has been offered as a strategy to treat β-thalassemia.

1.8. Forbidden gene editing therapies

The separate problem that requires ethical and legal attention is the fact that modern techniques made human germline modification possible.

US National Institute of Health and international group of researchers, including developers of CRISPR, called for a moratorium on clinical use of human germline editing until its safety is better investigated and acceptable uses agreed on (2).

Experiments on using CRISPR/Cas in human embryos started in 2015. Previous germline editing experiments on mice, rats and monkeys showed that off-target mutagenesis and mosaicism can happen.

The need to establish rules for germline editing research, and mechanism for detecting rogue unethical experiments became urgent when existing bans did not prevent Chinese scientist He Jiankui from implanting the first genome-edited human babies in 2018, in IVF clinic in China. He learned germline CRISPR techniques as a post doc in US. When MIT Technology Review published the story in November 2018 based on information from NCT registry, He Jiankui made an announcement, at a summit on Human Genome Editing in Hong Cong, that healthy twins were already born in October 2018. His unpublished manuscript was obtained and analyzed by MIT Technology Review magazine (25). Interviewed experts were highly critical of technical and ethical aspects of He's work. He entered his trial into ChiCTR (#1800019378, withdrawn, as shown in Table 1) and WHO registry (withdrawn), claiming that the embryos were edited at their CCR5 gene by CRISPR in an attempt to confer genetic resistance to HIV. In his manuscript and report at the summit He declared absolute success: "No off target mutations, large deletions and pathogenic cancer gene mutations were observed. We here bring a novel therapy to enable acquired immunity to HIV, and to control HIV epidemic". The statement assumed that large amount of people will be genetically edited as embryos in a vaccine like campaign to prevent HIV epidemic. He Jiankui was arrested by Chinese government and sentenced to 3 years in prison and heavy fine. He and his collaborators were found guilty of having "forged ethical review documents and lying to doctors". The twins are now under supervision of Chinese government and their condition is unknown to scientific community. It is claimed that one twin is homozygous and another heterozygous for small mutations in CCR5 gene, different (smaller) than natural typical CCR5 Δ32 mutation. It is difficult to say if the girls are indeed resistant to HIV, as there is little chance they will be ever exposed to this disease. It is also not clear what method He used to deliver CRISPR/Cas into embryonic cells. If he used microinjection, it could potentially increase chance of bad health outcomes, including autism, similar to outcomes of another microingection technique, ICSI variant of IVF.

Reportedly, He Jiankui planned to open a "designer baby" clinic with a prominent Chinese American owner of New York based fertility clinic, and this financial motivation could be a reason for his reckless behavior.

Potential adverse events connected to gene editing of somatic, and especially germline, cells remind of the importance of transparency in Clinical Trial Registry reporting and in publishing results.

"What are societal implications of technology that is so enabling for making targeted changes to DNA, truly a profound thing? It gives humans power to control evolution of organisms, and our own evolution. It is exciting, enabling, it gives a sense of awe. It also gives a feeling that we need to proceed with caution and respect for this powerful technology."
Jennifer Doudna, one of discoverers of CRISPR

"Some would call it kind of irrational exuberance. The hope exceeds the science, and expectations are not met."
James Wilson, pioneer in the field of human gene therapy, former Director of the University of Pennsylvania's Institute for Human Gene Therapy

2. OBJECTIVES

The aim of this thesis were:

The overall aim of the study was to analyze technological and ethical aspects of selected clinical trials involving modern gene editing therapies.

Specific aims of the study were:

1. To identify single gene diseases targeted for gene therapy;
2. To determine what modern gene editing techniques (CRISPR, ZFN, TALEN), *ex vivo and in vivo,* were used for sequence specific targeting;
3. To determine what viral vectors were used as delivery system for transgenes (knock-in genes) and if their safety profile was the primary reason in their choice;
4. To determine the status of the trials, with Terminated, Withdrawn and Unknown status indicating challenges;
5. To investigate safety aspects of gene editing: most common adverse events; and if modern methods for genetic sequencing and protein expression were used to find expected off target and on target mutations;
6. To evaluate transparency of the results, based on availability and quality of publications, submission of results to the registry, completeness of data presented to the registry database, and allowing access to Individual Patient Data (IPD). Make recommendations on increasing transparency of the trials.

3. METHODS

World Health Organisation's (WHO, who.int) Primary Registries were searched.

- US National Institute of Health National Clinical Trial (NCT) Registry at ClinicalTrials.gov. Trials are assigned National Clinical Trial database NCT number;
- EU Clinical Trials Registry, EU-CTR, ema.europa.eu;
- German Clinical Trials Registry, DRKS.de;
- Chinese Clinical Trial Registry, ChiCTR.org.cn;
- Australian New Zealand Clinical Trials Registry;
- Brazilian Clinical Trials Registry (ReBec);
- Clinical Research Information Service (CRiS), Republic of Korea;
- Clinical Trials Registry - India (CTRI);
- Cuban Public Registry of Clinical Trials (RPCEC).

The databases was downloaded on December 1, 2019. Search terms "gene editing" and "genome editing" were applied.

Data extraction sheets were compiled with columns corresponding to items in WHO and NCT (ClinicalTrials.gov) dataset (TRDS), such as trial number in the registry, dates (submitted, posted, completed, *etc*), titles, descriptions, primary and secondary outcomes, phase, design, conditions, status, publications, investigators, sponsors, *etc.*

I extracted the data and another researcher, Dr. Diana Jurić, checked the included trials and extracted data for all included trials

For this observational study "gene editing" was defined as genetic engineering or molecular biology techniques that involve DNA double strand breaks and repair mechanisms for incorporating site-specific modifications into a human cell's genome. Trials that were not consistent with this definition and duplicate trials registered in 2 or more registries were excluded from analysis. Search of FDA website and Sangamo Therapeutics website, developer of Zinc Finger Nuclease technology, yielded additional trials.

The results are presented as descriptive analysis. As this was a descriptive cross-sectional study, no attempt of statistical analysis was made.

4. RESULTS

Search results retrieved 55 studies (42 from ClinicalTrials.gov, 5 EUCTR, 2 DRKS, 6 ChiCTR). 9 trials were not gene editing. 2 trials were hybrid human to mice studies: human bone marrow cells were edited with unspecified technique and injected into mice. Four trials were listed in both ClinicalTrials.gov and EU registries. Those were considered duplicates and analyzed once, using data from ClinicaTrials.gov registry. Overall, 15 trials were excluded.

The search of WHO registry produced 3 trials, all from China, that were already extracted from the primary registries.

One trial was added from FDA.gov website for the first gene therapy drug being approved by FDA: Zolgensma drug for Spinal Muscular Atrophy (NCT 02122952) (23). One trial, on Hemophilia A, was added after searching Sangamo Therapeutics website (NCT02576795, EUCTR2014003880-38 GB).

The number of trials used in the final analysis was 42.

The search and selection of eligible trials is presented in Figure 1.

Figure 1. Flow-chart of the search and selection of eligible clinical trials

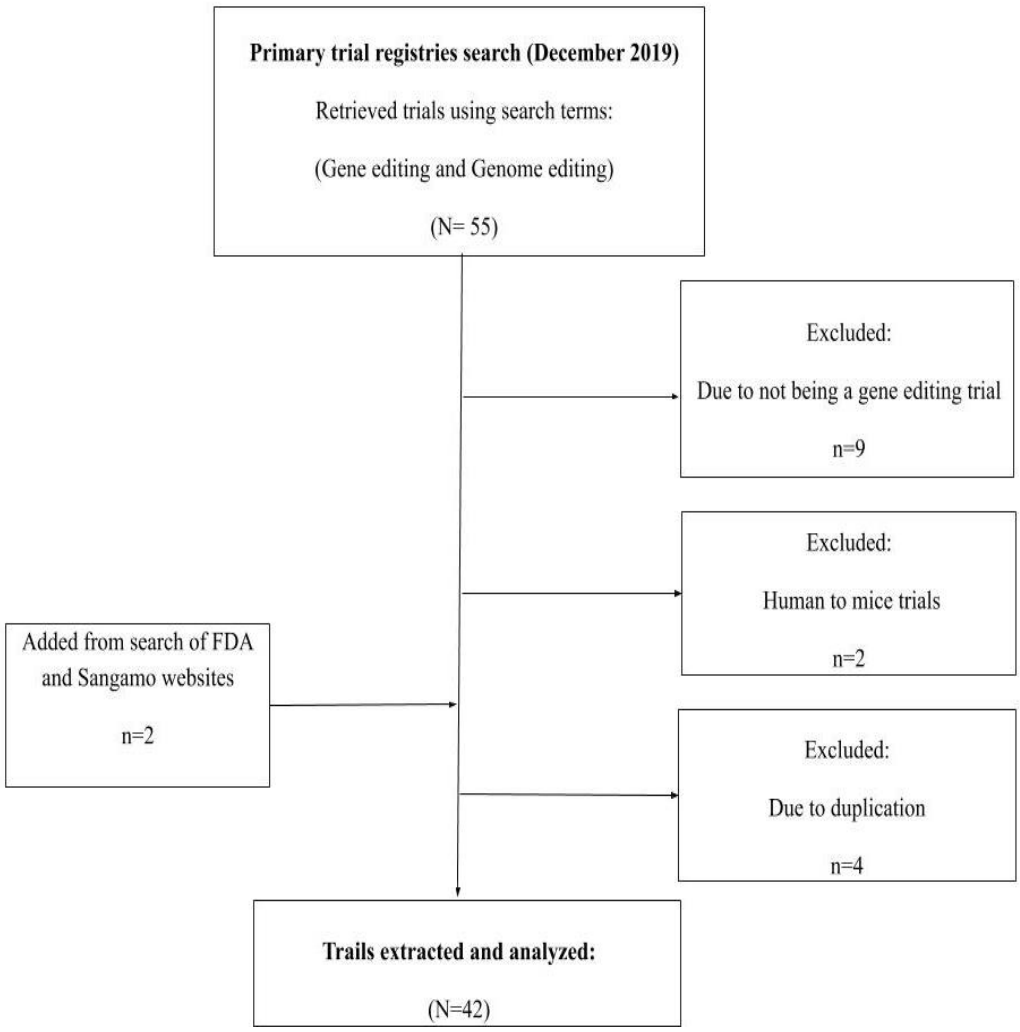

Table 1. Characteristics of clinical trials involving gene editing

Condition and gene	ID Number and Country	Technology*	Status+	Publications/ Results report+
HIV (CCR5)	NCT03666871 US	ZFN (T cells CD4 ex vivo autologous), r/db	R	N/ N
HIV (CCR5)	NCT00842634 US	ZFN (T cells CD4 *ex vivo* autologous)	C	Y/ N
HIV (CCR5)	NCT01044654 US	ZFN (T cells CD4 *ex vivo* autologous)	C	N/ N
HIV (CCR5)	NCT03164135 China	CRISPR/Cas9 (HPSC CD34 *ex vivo* allogeneic)	R	Y/ N
HIV (CCR5)	NCT02500849 US	ZFN (HPSC CD34 autologous after busulfan)	A, not R	Y/ N
HIV (CCR5)	NCT02225665 US	ZFN (T cells CD4, autologous, after cyclophosphamide)	C	N/ N
HIV (CCR5)	NCT02388594 US	ZFN (T cells CD4, autologous, after cyclophosphamide)	C	N/ N
HIV (CCR5)	NCT01252641 US	ZFN (T cells CD4, autologous)	C	N/ N
HIV (CCR5)	NCT03617198 US	ZFN (CCR5, T cells CD4, autologous)	R	N/ N

HIV (CCR5)	ChiCTR 1800019378	CRISPR embryo (germline) editing (He Jiankui study) which later resulted in birth	W	N/ N
Leukemia B cell Lymphoma B cell	NCT03398967 China	CAR T cell CD 19,20,22, ex vivo allogeneic)	R	N/ N
Leukemia B cell Lymphoma B cell	NCT03166878 China	CRISPR, lentivirus (T CAR T cells CD19 allogeneic),	R	N/ N
Leukemia, ALL Lymphoma (Burkitt)	NCT03298828 China	CAR T cells CD19, PD-1, autologous	Not yet R	N/ N
Leukemia (ALL) Lymphoma (Non Hodgkin)	NCT03229876 China	CAR T cells CD19, allogeneic, after cyclophosphamide, fludarabine)	R	N/ N
Lymphoma Diffuse Large B-cell	NCT04026100 China	CAR T cells CD19 allogeneic	Not yet R	N/ N
Leukemia, ALL Lymphoma, r/r	ChiCTR 1900023500	CAR T cells CD19	A?	NA
Leukemia, r/r lymphoma B cell	ChiCTR 1900025089	CAR T cell CD19 allogeneic, after cyclophosphamide, fludarabine	A?	NA
Multiple Myeloma, BCMA	NCT03752541 China	CAR T cells anti B cell Maturation Antigen, allogeneic	R	N/ N
CD7+ T/NK cell hematology malignancies	ChiCTR 1900025311	CAR T cells	R	NA

EBV Malignancies, PD-1	NCT03044743 China	CRISPR/Cas9 in plasmids, electroporation ex vivo , autologous PD-1 knockout lymphocytes	R	Y/ N
Gastrointestinal Epithelial Cancers CISH-2	NCT03538613 US	CRISPR inhibited cytokine induced SH-2 gene encoding intracellular immune checkpoint in Tumor Infiltrating Lymphocytes, Autologous + immuno/s drugs	R	Y/ N
Advanced Esophageal Cancer PD-1	NCT03706326 China	Anti-MUC1 CAR-T cells Combined with PD-1 knockout T cells, autologous.	R	N/ N
Metastatic Renal Cell Carcinoma, PD-1	NCT02867332 China	CRISPR/Cas PD1 knockout T cells, autologous	W	Y?/ N
Solid Tumors PD-1, TCR	NCT03545815 China	CRISPR/ knockout Cas CAR T cells	R	N/ N
Solid Tumors, PD-1	NCT03747965 China	CRISPR/Cas CAR T cells with PD-1 knockout + drugs	R	N/ N
Invasive Bladder Cancer, PD-1	NCT02863913 China	CAR T cells, PD-1 knockout + drugs	W	Y?/N
Multiple Myeloma Melanoma Synovial Sarcoma Liposarcoma TCR, PD1 (first in humans, 3 participants)	NCT03399448 US	T Cells Engineered with lentivirus vector delivery to express NY-ESO-1 TCR after CRISPR knocked out endogenous TCR (NYCE T Cells), autologous, + 2nd CRISPR (PD1)+ drugs,	T	Y/ N

Prostate Cancer, PD-1	NCT02867345 China	T cells, PD 1 knockout autologous	W	Y?/ N
Metastatic Non-small Cell Lung Cancer, PD-1	NCT02793856 China	CRISPR, T cells PD-1 knockout, autologous + drugs	A, not R	Y/ Y
HPV 16/18 cancer E7 oncogene	NCT02800369 China	ZFN oncogene knockout,, *in vivo*, suppository	U	Y/ N
HPV 16/18 cancer E7, E6 oncogenes	NCT03057912 China	CRISPR/Cas9, TALEN plasmids in gel, *in vivo*, topical	U	Y/ N
HPV 16 cancer E7, E6 oncogenes	NCT03226470 China	TALEN *in vivo*, suppository	U	Y/ N
Thalassemia, HBB knock-in	NCT03728322 China	*Ex vivo* CRISPR/Cas gene correction in iHSC, autologous	Not yet R	N/ N
Thalassemia, Sickle cell Enhancement (knockout) of BCL 11 A gene	NCT03655678 EUCTR2017-003351-38 (GB and DE) US, UK, Canada, Germany, Italy	CRISPR/Cas modified CD34+ Hematopoietic Stem and Progenitor Cells (HSPCs) (CTX001 product) , autologous *ex vivo*, injected IV single dose., after myeloablative drug busulfan.With long term (18 years) of follow up as a second study	R	N/ N

Sickle Cell Disease BCL11 A	NCT03653247 US	ZFN edited HSPC CD34, *ex vivo* autologous, reinfused by IV, after busulfan	R	N/ N IPD share Y
1) Intermittent Claudication + 2) Arteriosclerosis, transcription factor for VEGF	NCT00080392 US	*In vivo,* ZFN and DNA plasmid vector to produce transcription factor for VEGF gene expression. *G*ene knock-in. Randomized, double blind, placebo control, dose escalation	C (2011)	Y?/ N
Hemophilia B Factor 9 gene knock-in	NCT02695160, US, EUCTR2017-004805-42-GB	*In vivo,* ZFN with AAV 2/6 vector delivery of DNA coding for Factor 9 IV into albumin locus on hepatocytes.	A not R	N/ N
Hemophilia A, Factor 8 gene knock in	NCT02576795 UK EUCTR2014003880-38 GB	*In vivo,* ZFN AAV5 (capsid proteins) carrying gene for Factor 8, IV, single dose	A not R	Y/N
Mucopolysaccharidosis I, MPS I IDUA enzyme gene, knock-in	NCT02702115 US	*In vivo*, ZFN editing with DNA coding for IDUA gene delivered by viral vector AAV2/6 into albumin locus on hepatocytes, IV, single dose.	A not R	Y/ N
Mucopolysaccharidosis II, MPSII IDS enzyme gene, knock-in	NCT03041324 US	***In vivo***, ZFN editing with DNA coding for IDS gene delivered by viral vector AAV2/6 into albumin locus on hepatocytes, IV.	A not R	N/ N

Spinal Muscular Atrophy SMN (survival motor neuron)	NCT02122952 US (Zolgensma drug, first gt drug approved by FDA)	*In vivo,* AAV9 viral vector carrying SMN gene under control of CMV-cytomegalovirus, IV single dose	C	Y/Y
Major genetic disease", unspecified, CCR5 and PCSK9 genes as targets	ChiCTR 1800018955	Investigator He Jiankui planned germline editing in 400 embryos using CRISPR with Cas9 protein or Cas9 RNA, or base editing. As preparation for ChiCTR 1800019378	T	N/ N

Total = 42

Abbreviations: Clustered Regular Inter Spaced Palindromic Repeats (CRISPR), Zinc Finger Nuclease (ZFN), Transcription Activator Like Effector Nuclease (TALEN);

Viral vectors delivery systems: Adeno Associated Virus (AAV) and lentivirus;

A - active. C - completed. R- recruiting. T- terminated. U- unknown. W- withdrawn.

N- no. Y -yes.

T**able 2**. Techniques used in gene therapy trials

Technique	**CRISPR**	**ZFN**	**TALEN**	**Viral vector**	**Unknown**	**Total**
Ex vivo, HIV, 8 autologous, 1 allogeneic, 1 germline	2	8				10
Ex vivo, various cancers, 7 autologous, 3 unknowns	7			Lentivirus used with CRISPR in 1 trial	3	10
Ex vivo, hematologic malignancies, 1 autologous, 6 allogeneic, 2 unknowns	1				8	9
Ex vivo, hemoglobinopathies, autologous	2	1				3
In vivo, HPV, topical, knockout	1	1	1 (+1)			3
In vivo, Hemophilia A, B, MPS 1, MPS 2, Atherosclerosis, SMA, IV, knock-in		5 (ZFN+AAV)		1 (AAV) with unknown editing		6
Germline (embryos) unspecified condition	1					1
Total	**14**	**15**	**1 (+1)**	**1**	**11**	**42**

Table 1 and **Table 2** show that the disease most often targeted for gene therapy was HIV (10/42 trials, 24%). The gene CCR5 was targeted for knockout in CD4 T cells in 7 trials, and in hematopoietic stem CD34 cells in 2 trials. HIV virus uses CCR receptor to enter the T cells, as was discussed in "Introduction", 1.7 (22). All of HIV trials, except one, were done ex vivo, mostly as personalized treatment in autologous CD4 T cells with Zinc Finger Nuclease (ZFN) technique, which is more sequence specific and precise than CRISPR. One trial was done in allogeneic (donor) cells, which increases chance of immunogenicity adverse events, but paves the way to less expensive production of "off the shelf" drugs. 5 out of 10 HIV studies were completed. One trial was withdrawn: infamous "CRISPR babies" experiment, where CCR5 gene was knocked out in germline (embryo) cells. The trial, ChiCTR 1800019378, is discussed in "Introduction" 1.8. It was done by He Jiankui with major ethical violations (25). As shown in Table 1, it was preceded by his similar "preparation of the crime" study ChiCTR 1800018955, that proclaimed "major genetic disease" as an unspecified grandiose goal, at the time of registration. This evasiveness should have been a "red flag" for ethics committee, but it was not. Presumably preparatory study did not end up with live birth of genetically edited babies.

Various cancers formed another big group. Identification of good target on solid tumors had always been challenging: such genes must be highly expressed in the majority of cancer cells, but largely absent on normal tissues. Most trials in this group used CRISPR to knock out PD-1 gene (Programmed Death gene). The name "programmed death" is misleading. Tumor cells express PD-1 immunoglobulin on their surface to suppress T cell activity, ensuring their protection from immune system surveillance and their survival (26). The better name in this case would be "self tolerance" gene. Knockout of PD-1 in tumor cells would increase anti-tumor activity of T cells, in theory. It will also promote autoimmune diseases. 10 trials were counted (2 in US and 8 in China), which is 24% of total trials. Of those, 3 Chinese trials were withdrawn (prostate, renal and bladder cancers), and 1 US trial, NCT03399448, was terminated with only 3 participants (18 were planned). This trial and possible reasons for its termination are reviewed in "Discussion" section.

The third most common group was hematologic malignancies, such as B cell acute lymphoblastic leukemia (ALL), B cell lymphomas, and multiple myeloma. 9 trials, all based in China, all gave very limited information on techniques used, even when data were submitted to ClinicalTrials.gov database. This points to the need for better oversight of submitted gene therapy trials by ClinicalTrials.gov registry and better design for Chinese registry. In all B cell leukemias and lymphomas Chimeric Antigen Receptor (CAR) T cells, mostly allogeneic, were modified *ex vivo,* so that they would target CD19 marker on neoplastic B cells. CAR and CD19 were discussed in "Introduction" 1.7. In one case PD1 gene was mentioned. In one case CRISPR was mentioned. None of the studies were completed.

Group 4 (3 trials, 7%) included hemoglobinopathies: thalassemia and sickle cell disease. They were done in multiple countries in *ex vivo* autologous hematopoietic stem cells (HSC) modified by CRISPR (2 trials) or ZFN (1 trial). HBB gene, coding for beta globin subunit in adult hemoglobin, was targeted for knock-in. In 2 trials BCL11A gene that is expressed on HSC34 during transition from gamma to beta globin in fetal to adult hemoglobin switch, was targeted for "enhancement", which was actually knockout, contrary to the meaning of the word. Replacing some of defective adult hemoglobin with fetal hemoglobin was expected to improve anemia. No one trial in this group was completed.

In summary, trials in 4 groups described above (74% of the trials) were performed *ex vivo,* most often with autologous T cells or hematopoietic stem cells, edited and expanded in culture, then reinfused back into patient. Chimeric Antigen Receptor (CAR) T cells were targeted for treatment of B cell cancers (leukemia and lymphoma). CRISPR was a technique most often used for *ex vivo* editing, being the least expensive and easy to learn. Some were done with cyclophosphamide and busulfan to bring down the number of unedited cells with defective gene.

In the next 2 groups cells were edited *in vivo,* (21% of the trials).

HPV 16/18 infected cells were edited *in vivo*, topically, using suppository (3 trials, 7% of total trials, all based in China). Oncogenes E7 and E6 were targeted for knockout. Trials were designed the same way, but with 3 different techniques: ZFN, TALEN and CRISPR +

TALEN. While it would be interesting to compare 3 modern editing techniques *in vivo*, the status of these trials is unknown.

In vivo intravenous delivery of genes for knock-in , by Adeno Associated Virus (AAV) vector, with Zinc Finger Nuclease (ZFN), was expected to correct several diseases: Hemophilia B (Factor 9), Hemophilia A (Factor 8), Mucopolisaccharoidosis MPS 1 (IGUA gene), MPS 2 (IDS gene), Spinal Muscular Atrophy (SMN gene), and Atherosclerosis/ Intermittent Claudication (transcription factor for VEGF). Total 6 trials (14%); 4 were active, 2 completed, and one of them, for Spinal Muscular Atrophy, was the first gene editing drug that got FDA approval, under the name Zolgensma (23). One completed trial, on Atherosclerosis/ Interermittent Claudication, had randomized, double blind, placebo controlled design, a rarity in studies in biologics, where open label, no placebo control trials are most common. These studies were done in US, or in US and EU, none of them in China. It should be mentioned that the most difficult, *in vivo*, intervention produced studies that were of the best quality,

Most of the gene editing trials were in Phase1 or Phase 1/2, assessing safety, tolerability and efficacy in small numbers of participants, from 3 to 80, with 12 to 18 participants being the most common. 40 studies out of 42 are open label. Only 2 were randomized double blind, placebo controlled.

5. DISCUSSION

It should be noted that FDA requirements for clinical trials and approval of biologics are less stringent than for other drugs, where the gold standard is randomized, double blind, placebo controlled study. For biologics, non-randomized, open label, no placebo trials are acceptable, and in fact, almost all reviewed gene editing trials had this design.

The characteristics of diseases targeted for gene editing can be summarized as follows: severe with no (or limited) conventional treatment; affected by single gene; and cells should be easily reached by delivery systems carrying genes and other components.

Several diseases have these characteristics and were chosen for clinical trials: HIV, hematological malignancies and various other cancers, sickle cell disease and thalassemia, hemophilia A and B, mucopolysaccharoidosis (MPS 1 and 2), atherosclerosis, and spinal muscular atrophy.

Among all trials, CRISPR and ZFN were the most used techniques: 33% and 36% of the trials mentioned them. Only one trial (on HPV) used TALEN, and another used both CRISPR and TALEN for oncogene knockout (Table 2). Trials based in China often did not specify techniques. It is obvious that for *in vivo* IV infusions, where sequence specificity and reducing off target mutations is especially important, the choice of the technique for gene editing was determined by safety considerations: Zinc Finger Nuclease (expensive but more sequence specific than CRISPR), combined with recombinant Adeno Associated Virus (AAV) vector carrying transgene template. Non integrating recombinant AAV is considered the safest of all viral vectors, even though it can carry less genetic material than adenovirus or lentivirus (11,13). Gene therapy drug approved by FDA, called Zolgensma, for spinal muscular atrophy, used safer ZFN + AAV technology *in vivo* by IV for functional gene knock-in, and the price for single injection is more than 2 million dollars. In the future gene therapy drugs likely will be in the same range (27). Only 14% of the trials were in the *in vivo* IV category, but those were of the best quality.

Only 17% of trials were completed. Most of the trials remain in Phase 1 or1/2 for years, often not updated within last 12 months. Relatively high number of withdrawn, terminated and of unknown status trials (21%), especially in various cancers group, points to safety and tolerability challenges in use of gene editing technology and biologics in general, but especially for cancer treatment. The NCT00399448, the only one terminated trial, deserves reconstruction and detailed analysis. The approach was opposite to Zolgensma. The choice of technique in

this trial was not about safety, but about lower cost (CRISPR) and effectiveness of delivery at the expense of safety (lentivirus). It used CRISPR-Cas complex to knockout endogenous T cell Receptor gene (TCR) *ex vivo*, and another CRISPR-Cas to knock out PD-1 gene on tumor cells (presumably *in vivo,* since solid tumors were targeted). Then lentivirus vector delivered a code for transgenic TCR, specific for a certain tumor associated antigen, called NY-ESO-1. Resulting edited autologous T cells, called NYCE T cells, were expanded, injected into participants, and were expected to kill tumor cells, such as myeloma, sarcoma and melanoma, that are expressing this particular tumor associated antigen. The trial originally planned 18 participants, aiming at complete response and remission after single injection. This terminated trial is a cautionary tale, that complex design with ambitious goals, while possible in theory, in practice produce unexpected outcomes when safety is neglected. The researchers, though, did not want to acknowledge negative results. It has to be noted that lentivirus is a retrovirus, in the same family as HIV, and injecting retrovirus infected T cells into humans, especially immunocompromised cancer patients, is dangerous. Cancer patients may have unexpected immune reactions to gene therapies, and biologics in general, and this problem should be further investigated.

2 of the studies were performed in China on germline (embryo) cells by the same researcher, with multiple ethics violations. One of them resulted in live birth (25) and reminded of the need for better oversight and strict international rules for germline editing (2).

Transparency as assessed by completeness of data, posting results of completed trials to Registry and in publications, and willingness to share Individual Patient Data with other researchers, was low. NCT ClinicalTrials.gov database was the most comprehensive and transparent in their reporting. Chinese trials, presented in the ChiCTR Registry gave only the basic information, and there was no section that allowed to post results or publications to this registry. Chinese trials posted to US registry also often lacked information on techniques used. WHO Registry is currently optional and practically not functional. WHO announced on 26 August 2019 that a special Registry for gene editing trials, both somatic and germline, will be open within its ICTRP Registry. If WHO follows on their decision the quality of trial data may greatly improve. NCT registry should also pay more attention to completeness of submitted data, especially techniques used.

Publications should be checked against content of the trial. It was not uncommon to fill "Publication" part of the dataset with articles published before the trial was submitted to the registry (4 cases), therefore the publications could be only an introduction to the topic, not discussion of results. They are denoted with "?" sign next to Y in publication column in Table 1. Excluding those, only 13 trials (31%) had published results. In some cases publications did not adequately reflect content of the trial. For example, publication for NCT02702115 trial for Mucopolysaccharoidosis 1, still ongoing with only 3 participants, *in vivo*, mentioned "low transgene expression level". The rest of the article talked about studies in mice.

Only one trial posted results to ClinicalTrials.gov registry, giving information on adverse events. It was Zolgensma trial, NCT02122952 (23), the first gene therapy drug approved by FDA. The company was obligated to provide results as part of their approval process. Analysis of their results shows that success was moderate, adverse events were frequent and unpleasant, and the 2 million dollar price (27), was exorbitant. One more study, NCT02793856, submitted results, but clarifying information was requested that they were not able to provide.

Only one trial out of 42 was willing to share Individual Patient Data (NCT03653247). Analyzing IPD may help to uncover bias, selective reporting, and other questionable practices. When in the past controversies around gene editing trials prompted official investigations (Jesse Gelsinger, He Jiankui trials), individual patient level data pointed to violations and reckless decisions. Informed consent forms are part of IPD. In both mentioned controversies official informed consent forms were different than forms given to participants. In He Jiankui case the consent form was coercive, demanding large amount of money from participants if they did not complete the trial. In Gelsinger case, University of Pennsylvania also used misleading language in their consent form and advertisements to recruit participants. One way to prevent this type of ethical violations and protect participants would be create one more required item (# 25) in Trial Registry Data Sets (TRDS), with a link to official informed consent form, and patients should be able to download their form from the link before enrollment. Changes to the form should be saved in "archived version". Investigator should be required to disclose financial conflicts of interest on consent form the same way they do it in publications. These two rules will greatly reduce coercive recruitment and increase safety in trials.

Interestingly, knowledge accumulated in development of the gene therapies for genetic diseases made it possible to develop novel vaccines for COVID 19, which can be considered a simplified, less expensive variation of gene editing technology, For example, Cas (CRISPR associated) protein may be produced in the cell from code delivered by messenger RNA (mRNA). Moderna's "mRNA-1273", a leading vaccine announced for release in US on November 1, 2020, uses injected mRNA coding for COVID spike protein, in order to stimulate production of antibodies. The delivery system for mRNA sequence is lipid nanoparticles. Russian Sputnik V vaccine uses Adenovirus vectors, Ad5 and Ad26, by IM injection, to deliver COVID spike protein code into the cells. It is now in Phase 3 (NCT04437875 and NCT04436471, not part of this review). "Oxford" vaccine, a European leader, uses chimpanzee adenovirus ChAdOx as a vector (28). mRNA and DNA based COVID vaccines have some features common with *in vivo* gene editing in analyzed clinical trials. They are injected intramuscularly or intradermally, not intravenously. The main differences between novel adenovirus based COVID vaccines and gene therapies analyzed in this review: restriction enzymes such as CRISPR are not used, which greatly reduces costs but brings technology back into "random insertion" era, when insertional mutagenesis caused cancers; and in vaccines immunogenicity is a required outcome, while in gene therapies it is an adverse event (16).

6. CONCLUSION

We analyzed 42 trials on gene editing posted on US National Institute of Health ClinicalTrials.gov, EU-CTR and Chinese registries, and on FDA and Sangamo Therapeutics websites.

1. Severe diseases connected to single genes with no effective reasonably priced treatment were targeted: HIV, B cell leukemia and lymphoma, various other cancers, sickle cell disease, thalassemia, hemophilia A and B, mucopolysaccharoidosis 1 and 2, atherosclerosis, and spinal muscular dystrophy. All trials, except two, were done on somatic cells. Chinese trial (internationally criticized "CRISPR babies study", now withdrawn for major ethics violations) did germline editing in early embryo cells with the purpose of HIV prophylaxis, and resulted in live birth (25). All trials in HIV and cancer patients (which comprise majority of the studies) were done *ex vivo,* on autologous or, less often, allogeneic T cells or hematopoietic stem cells (HSC). Edited Chimeric Antigen Receptor (CAR) T cells targeting CD19 marker on B cells were often used to treat B cell malignancies: leukemia, lymphoma, and myeloma. Edited cells were expanded and reinjected, often after treatment with drugs to suppress native, unedited cell population. Amount of withdrawn, terminated or of unknown status trials in cancer group points to challenges in using gene therapy techniques in cancer patients.

2. Advancements in gene editing techniques such as the use of complex restriction enzymes: Zinc Finger Nucleases (ZFN); Clustered Regularly Inter Spaced Palindromic Repeats (CRISPR) with CRISPR associated enzymes (Cas); Transcription Activator Like Effector Nucleases (TALEN), allowed to induce double strand DNA breaks at specific targeted locations, rather than random locations, as was the case with earlier gene editing techniques (9). Less expensive CRISPR techniques was used ex vivo more often than ZFN.

3. In trials done *in vivo* by a single intravenous injection, safety was a priority over cost. More precise (and more expensive) ZFN technique was used, with functional gene delivery for knock-in by non-integrating recombinant Adeno Associated Virus vector, the safest of all viral vectors. These group of trials, while small (14%), was of the best quality. One of them became the first gene editing drug that got FDA approval, under the name Zolgensma (23), as a treatment for spinal muscular dystrophy in pediatric patients.

4. Despite claims of precision and safety of modern techniques, off target and on target site mutations with expression of altered proteins were reported in the literature, but no trials planned to do whole genome sequencing studies to detect them. Zolgensma study confirmed protein expression of targeted SNM gene (23), but no other trial did protein expression research. Safety was assessed by monitoring adverse events for 6 or 12 months, and in some cases, long term. Biochemical tests were also used.

5. The most common adverse event of gene editing is cytotoxicity. While gene editing techniques, CRISPR and ZFN, are getting most attention, viral vectors are primarily responsible for cytotoxicity, presented as Cytokine Release Syndrome (fever, chills, vomiting, fainting, etc). FDA put a "black box" warning on Zolgensma about hepatotoxicity. Autopsy of 2 children, who died during the trial, showed that the highest amount of Adeno-Associated vector accumulated in the liver, though all major organs had it. On the positive side, spinal neurons were expressing the targeted gene.

6. Transparency level of the trials was low, especially for trials based in China. With some exceptions, lack of results posted to registries, incomplete data, lack of publications that reflect content of the trial, unwillingness to share Individual Patient Data (IPD), were characteristics of selected trials. Chinese registry required only the basic information, but even NCT registry allowed posting of incomplete descriptions, where techniques were not mentioned. Measures to increase transparency and quality of posted results, for example, proposed by World Health Organization creation of special international registry dedicated to gene therapy trials, are needed. Posting a link to informed content form as an additional required item (# 25) in Trial Registry data set can help protect participants from unethical behavior of investigators that in the past gene editing trials resulted in death and severe adverse events.

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8. SUMMARY

Title: CHARACTERISTICS OF GENE THERAPY CLINICAL TRIALS FROM PUBLIC TRIAL REGISTRIES

Objectives: To analyze technological and ethical aspects of studies involving modern gene editing therapies registered in public trial registries: genetic diseases targeted for gene therapy; technologies used (CRISPR, ZFN, TALEN, viral vectors); status; if modern methods for off target and on target mutations were used as part of safety assessment: transparency of the trials, based on availability of publications, results posted to registry, IPD sharing and completeness of data.

Methods: Registries were searched for "gene editing" and "genome editing" and eligible trials were analyzed.

Results: 42 trials for single gene diseases were analyzed, and their characteristics were presented in tables. Zinc Finger Nuclease (ZFN) with adeno associated virus vector (AAV) was used for gene editing *in vivo*. CRISPR and ZFN techniques were used for *ex vivo* editing (autologous and allogeneic), mostly in Hematopoietic Stem Cells and CAR T cells. Most of the trials were open label, Phase 1 or 1/2, with 17% completed, one of them getting FDA approval. 21% withdrawn, terminated or of unknown status. Two of the studies, done in China, did editing on germline cells with major ethics violations, and one of them resulted in birth of genetically edited babies. Almost all trials did not post results to registry, did not share Individual Patient Data, and most lacked publications that reflected content of the trials.

Conclusion: Advancements in gene editing techniques such as ZFN, CRISPR/Cas9, and use of safer viral vectors (AAV) as delivery system decreased the risk of insertional mutagenesis and cytotoxicity, and made possible genetic therapy of single gene disorders in humans, with various cancers, HIV, sickle cell disease, and thalassemia being the most often investigated.

First gene therapy drug, approved by FDA for spinal muscular atrophy, Zolgensma, sets the standard for gene therapies, including high price and FDA warning for liver toxicity.

Lack of results posted to registries, lack of publications that reflect content of the trial, unwillingness to share IPD, and incomplete data show the low level of transparency in gene editing trials. Measures to increase transparency, for example, proposed by World Health Organization creation of special registry dedicated to gene therapy and gene editing trials are needed.

8. CROATIAN SUMMARY

Naslov:

KARAKTERISTIKE KLINIČKIH ISPITIVANJA GENE TERAPIJE IZ JAVNIH KLINIČKIH REGISTRA

Ciljevi: Analizirati tehnološke i etičke aspekte studija koje uključuju moderne terapije uređivanja gena registrirane u javnim registrima ispitivanja: genetske bolesti ciljane za gensku terapiju; korištene tehnologije (CRISPR, ZFN, TALEN); transparentnost.

Metode: Pretraženi su registri i odabrana i analizirana prihvatljiva klinička ispitivanja.

Rezultati: Analizirana su 42 ispitivanja za niz bolesti, uključujući rak i HIV, a njihove su karakteristike prikazane u tablicama. CRISPR i ZFN tehnike korištene su za ex vivo uređivanje (autologne i alogene), uglavnom u matičnim stanicama hematopoeze i CAR T stanicama. ZFN s adeno povezanim virusnim vektorom (AAV) korišten je za uređivanje gena in vivo. Većina pokusa bila su otvorene, faza 1 ili 1/2, sa 17% dovršeno, od kojih je jedno dobilo odobrenje FDA; i 21% povučeno, ukinuto ili nepoznatog statusa. Dvije studije, rađene u Kini, uređivale su stanice zametnih linija s velikim kršenjem etike, a jedna od njih rezultirala je rođenjem genetski uređenih beba. Gotovo sva ispitivanja nisu objavila rezultate u registru, nisu dijelila pojedinačne podatke o pacijentima, a većini su nedostajale publikacije koje su odražavale sadržaj ispitivanja.

Zaključci: Napredak u tehnikama uređivanja gena kao što su ZFN i CRISPR / Cas9 i upotreba sigurnijih virusnih vektora (AAV) kao sustava isporuke, smanjili su rizik od insercijske mutageneze i citotoksičnosti te omogućili genetsku terapiju poremećaja jednog gena kod ljudi. Prvi lijek za gensku terapiju, koji je odobrila FDA, Zolgensma, postavlja standarde za genske terapije, uključujući vrlo visoku cijenu.

Nedostatak rezultata objavljenih u registrima, nedostatak publikacija koje odražavaju sadržaj ispitivanja, nespremnost za dijeljenje IPD-a, pokazuju nisku razinu transparentnosti u ispitivanjima uređivanja gena. Potrebne su mjere za povećanje transparentnosti, na primjer, koje je predložila Svjetska zdravstvena organizacija, stvaranje posebnog registra posvećenog genskoj terapiji i ispitivanjima uređivanja gena.

10. CURRICULUM VITAE

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I am a sixth year Medical Student at the University of Split School of Medicine, Split, Croatia.

I was a volunteer with NeuroSplit student organization (2016-2020) and participated in the International Practical Knowledge for Students Conference as part of this organization.

In summer of 2010 I worked in the University of Maine School of Marine Science (Orono, Maine, USA) helping to develop technique for extracting DNA from soil samples (from microorganosms and forensic materials).

In summers of 2009 & 2013 I worked as a lifeguard for Massachusetts Department of Conservation and Recreation, doing ocean rescues and administering First Aid until arrival of paramedics.

From September of 2011 to May of 2013 I worked as an intern film editor in Lynn Community Television Station, filming and editing with Final Cut Pro editing program.