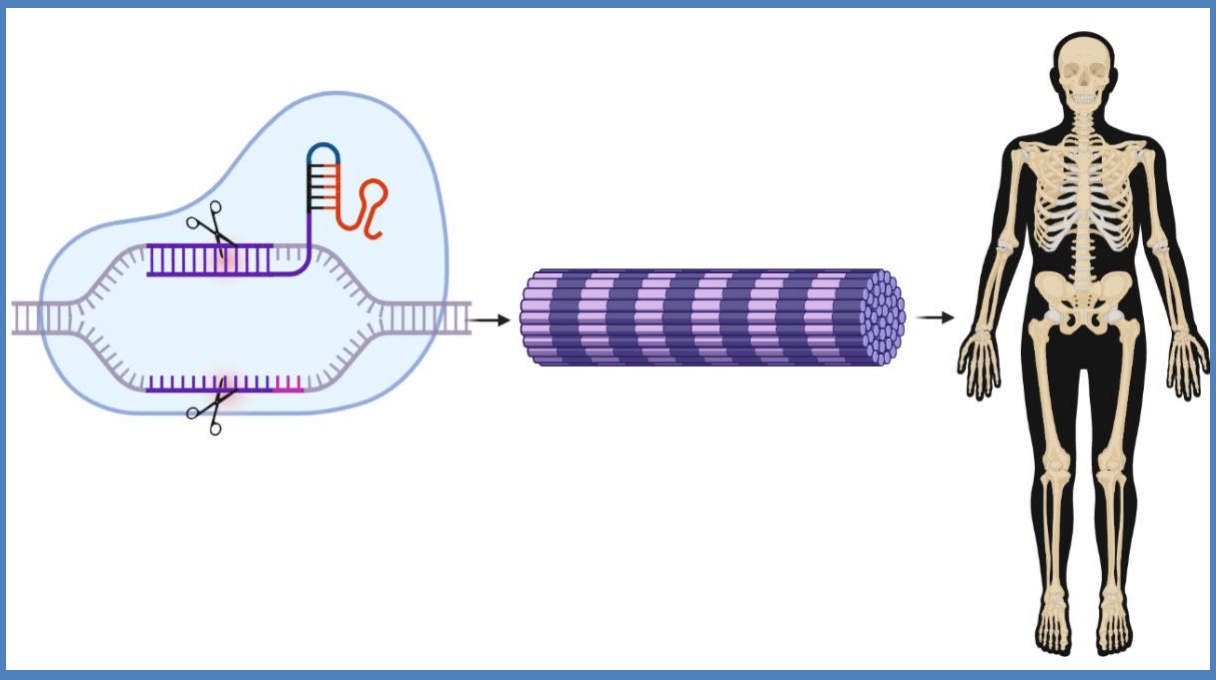
# Osteogenesis Imperfecta,disease analysis and the opportunities and challenges of CRISPR/Cas9 genetic editing treatment.



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# 1. Osteogenesis imperfecta

Osteogenesis imperfecta also known as brittle bone disease is a genetic disorder which undermines the structure of collagen or the ‘’glue” which holds together the structure of the human body. Osteogenesis Imperfecta (OI) is an autosomal genetic disorder predominantly caused by a defect in the COL1A1 or COL1A2 genes which subsequently affects the production of collagen type 1 (Tournis & Dede, 2018). As a direct result, OI patients experience varying degrees of bone fragility and deformity within a broad phenotypic range determined by the specific genetic context (Fig.1). The incidence of OI is about 1 in 15-20.000 births (Balasubramanian et al., 2017) and there are currently no curative options. OI affects both males and females equally (Forlino & Marini, 2016).

There are four distinct types of OI phenotypes, classified in 1979 by David Sillence into type I, II, III and IV, based on clinical degrees of severity (Sillence et al., 1979). The symptoms of these subtypes range from mild to life threatening (van Dijk & Sillence, 2014). This range is attributed to the underlying mutation type. A mutation silencing a single allele, effectively causing the halving of collagen synthesis, generally causes relatively mild symptoms in affected individuals. Point mutations however, alter the protein structure and are associated with more severe OI symptoms

Over the last years clustered regularly interspaced short palindromic repeats-CRISPR associated systems (CRISPR/Cas9) has become increasingly popular, cheaper and more effective (Uddin et al., 2020). It is currently the most promising genetic editing tool which is able to precisely cleave DNA strands at user-specified loci and insert or remove genes in a plethora of organisms. The curative potential of clinical CRISPR/Cas9 adaptations is currently being studied in many different genetic diseases (Li et al., 2020).

With the continued development of CRISPR/Cas9 technology and the accompanying future prospect of curative treatments for genetic disorders, OI could be made treatable via two distinct genetic editing pathways, which will be looked at in depth in this paper.
These two similar therapeutic options can be implemented either at the embryonal stage or when the disorder has already developed within the individual; late onset. Correcting or inactivating the mutated gene can potentially alleviate or remove symptoms altogether in OI affected individuals (Jung et al., 2021). The mechanisms, pitfalls and potential of CRISPR/Cas9 treatment will be discussed alongside implementation of its use in the context of OI.

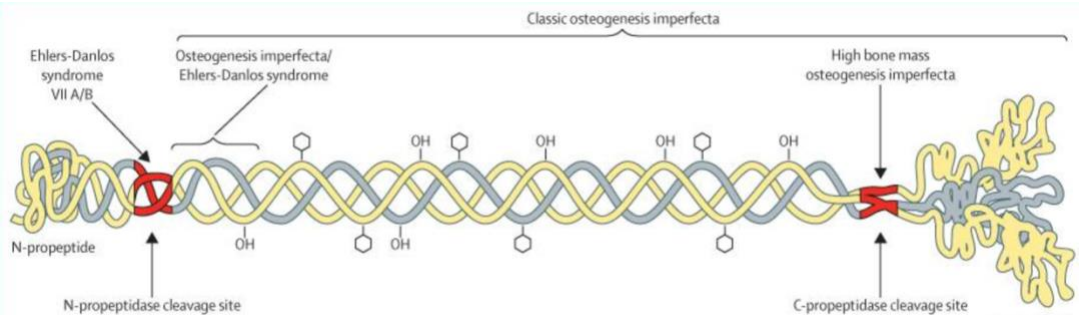



*Figure 1: Winding triple helix structure: Different mutations can cause distinct phenotypes (Source Forlino et al.)*

# 2. OI phenotypes

Understanding the different phenotypes of OI is crucial before attempting any type of genetic editing intervention.
As previously mentioned, there are 4 main types of OI, with OI type I being the most common and type II being the most debilitating variant (Steiner & Basel, 2005). All types of OI have analogous symptoms of skeletal abnormalities; such as bone fractures and deformities, low bone mineral density (BMD) and loose ligaments. Associated phenotypic features include blue sclerae (outer layer of the eye), caused by collagen defects which make the sclera thinner, to reveal the underlying choroidal pigment. Other phenotypic abnormalities can include abnormal spine development resulting in scoliosis or kyphosis, hearing loss, blood vessel fragility and dental problems in the form of dentinogenesis imperfecta (van Dijk et al., 2011). Besides physical therapy there are few options for OI patients. Rodding surgery (inserting metal rods into the long bones) is only used with regards to exceptional bone deformity and fractures. Medication is limited to biphosphate infusions, which are commonly used as treatment to reduce bone fractures and increase BMD (Ralston & Gaston, 2020).

Even though OI patients generally have lower BMD, OI patients have abnormally high levels of bone matrix mineralisation spanning across all 4 mutation types. The relative mineral volume fraction was around 12% higher in OI patients compared to non-OI control groups (Fratzl-Zelman et al., 2014). This number is increased due to a larger amount of mineral crystal compacted in the bone matrix. The size and shape of these hydroxyapatite crystals (the target of bisphosphonate therapy) does not seem altered in OI patients. A denser packing of the mineral particles results in a higher mineral content in OI bone. This increased mineral volume fraction impacts the quality of the bone tissue i.e. higher stiffness and increased brittleness (Currey, 1990)

## 2.1 Osteogenesis Imperfecta Type I

Type I OI is the mildest form of OI. Type I OI stems from a null mutation of the *COL1A1* gene that reduces the amount of collagen produced in the body. The protein structure of collagen remains however, unchanged. This result in a reduction of bone strength, caused by the halving of available collagen production (Forlino & Marini, 2016). The bone fragility is lessened, however not to the same degree as the other types of OI in which the protein structure of collagen has fundamentally changed. OI type I patients will generally experience growth abnormalities causing a reduction of height, bone-mass, an enlarged head (macrocephaly) and a thicker skull (hyperostosis). Abnormal outward curvature of the upper spine (scoliosis) and lateral curvature of the upper spine (Kyphosis) are common manifestations of type I OI. Dentinogenesis and hearing loss does occur in some cases, but is more rare (Joan C. Marini & An N. Dang Do, 2020). Affected infants rarely have life-threatening complications at birth and generally do not suffer many fractures, which can result in OI remaining undiagnosed for some time. Children with OI type I often have problems with developing motor skills, require physical therapy, a personalized diet and exercise routine to avoid bone fractures and stimulate muscle and joint strength.

## 2.2 Osteogenesis Imperfecta Type II

As previously mentioned OI type II is the most debilitating variant of Osteogenesis Imperfecta. In contrast to type I, type II is a perinatal lethal type of OI which causes

affected infants to experience life-threatening complications at birth. This is partially due to the increased fragility of the bones, which can already be fractured in utero (van Dijk & Sillence, 2014). The ribs and lungs of the infant are often underdeveloped which can cause serious breathing issues or death. Infants are also frequently underweight and undersized, with extreme deformations of the short and long bones as well as broad and beaded ribs. The combination of the abovementioned manifestations result in 90% of affected infants dying around the 4th week after birth (Marini et al., 2017).

## 2.3 Osteogenesis Imperfecta Type III

Individuals with type III will also have underdeveloped and extremely fragile bones which break easily, but type III OI is characterized by severe progressive skeletal deformity. Low BMD, blue sclera and dentinogenesis imperfecta are common manifestations (Mueller et al., 2018). OI type III patients are generally underweight, undersized and suffer from a progressive kind of kyphoscoliosis which can in turn cause breathing problems. The tension on the muscles during rest is also largely reduced, resulting in poor reflex generation, posture and balance. A triangular face along with fractures occurring from little to no trauma are a common phenomenon from the moment of birth. This consequently causes bones to progressively malform throughout the life of a type III affected individual (Forlino & Marini, 2016).

## 2.4 Osteogenesis Imperfecta Type IV

Type IV OI is the most variable type of OI, as the symptoms of type IV can range from mild to severe. Individuals with OI type IV also suffer fragile bones with frequently occuring fractures. Individuals with type IV generally have mild bone malformations and are shorter in stature. Spinal malformations like kyphosis and scoliosis are common occurrences in type IV OI. A triangular face is a phenotypic manifestation of type IV, similar to type III OI (van Dijk & Sillence, 2014). Besides the blue sclera, hearing impairments and dentinogenesis imperfecta are common.

# 3. Pathology Etiology

The classic OI etiology has changed in the last decade with the discovery of autosomal recessive variants of OI. In these cases it is not a glycine molecule which is substituted, but regulatory molecules involved in modification, secretion, processing and other interactions with post-translational collagen are mutated (Lim et al., 2017). molecules involved with bone mineralisation, collagen modification, ER hydroxylation and glycosylation, folding enzymes, chaperones and osteoblast-differentiation proteins have all been identified to be connected with OI phenotypes which are autosomal recessive. These autosomal recessive phenotypes of OI make up about 5-10% of all OI cases while the classic autosomal dominant phenotypes account for around 90-95% (Gajko-Galicka, 2002)

A recently discovered example of an autosomal recessive mutation causing an OI phenotype are mutations in the prolyl 3-hydroxylase complex (Fig 2). This complex is responsible for changing a proline of the α1 chain into a 3-hydroxyproline in type I procollagen in the endoplasmatic reticulum (ER) (Homan et al., 2014). Mutations in this complex can lead to dysfunctional collagen production and OI phenotypes with severe symptoms.

The underlying mechanisms of classic OI in which a single mutation of single gene causes such detriment to bone structure, can be understood by looking at the structure and significance of the collagen protein. Collagen is an abundant protein, which makes up ¼ of all proteins in the human body (Tzaphlidou, 2008). Type I collagen is encoded in the *COL1A1* gene on chromosome 17, and the α2 chain, which is encoded in *COL1A2* on chromosome 7. These genes translate for alpha collagen chains. This makes up type I collagen; containing 2 α1 chains and one α2 chain, which are tightly wound within a triple helix structure (Fig.3.) (Augusciak-Duma et al., 2018).

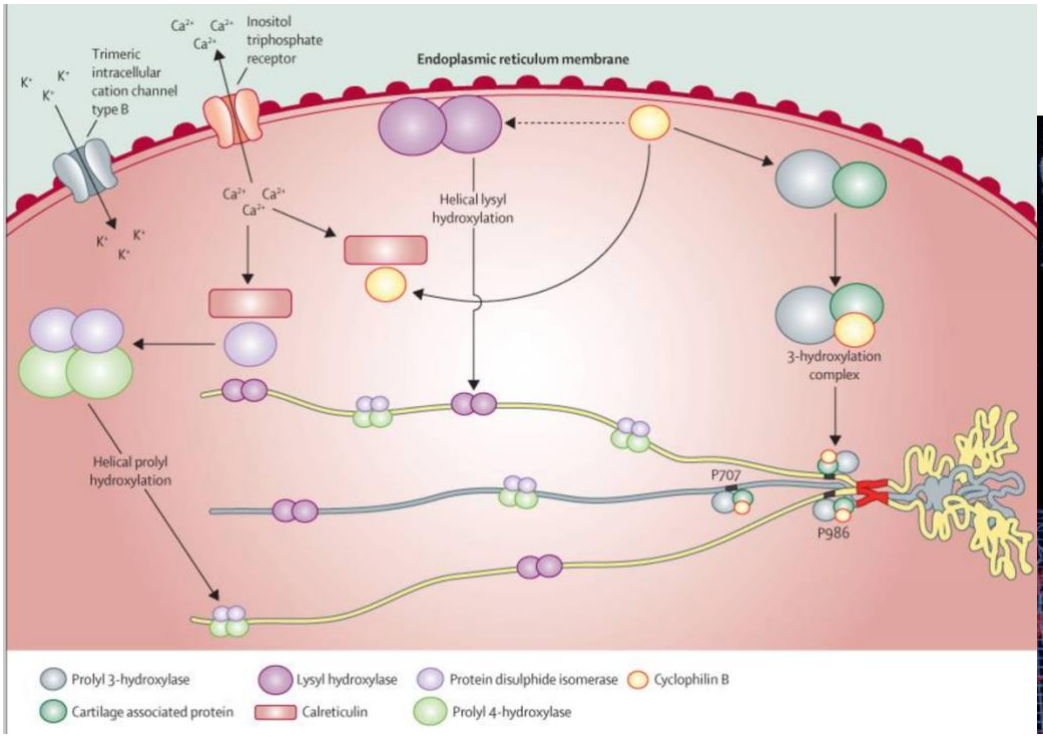
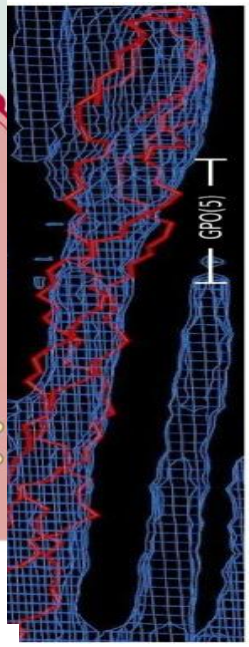



*Figure 1: Mutations in the prolyl 3-hydroxylase complex causes post-translational modification and folding in the ER(Source: Marini et al.)*




*3: Helical and non-organization of type I collagen.*

*COL1A1* and *COL1A2* are genetically similar genes, they both generally have around 50 exons. These two α1- polypeptide chains

contain a 338 repeats long sequence of gly-x-y (Marini et al., 2007). Glycine is attached to x, which often is a proline amino acid and y usually being a hydroxyproline. The glycine molecule has a crucial position for the formation of this triple helix; it is located on the interior of the triple helix which is only possible due to glycine being the smallest amino-acid.

The unique size of glycine makes it an extremely important amino acids in many fibrous structural proteins. The side chain in the molecular structure of glycine consisting of a single hydrogen atom causes steric hindrance effects to be minimal (Gautieri et al., 2009). Any other amino-acid will be too large to fit within the interior of the helix, as the available room is just sufficiently spacious for glycine's single hydrogen atom.

While in type I OI, a null mutation causes quantitative effects, the structure of collagen type I remains unaffected. Type II, III and IV however, are all more severe forms because their mutations structurally change the collagen a1 chains. This structural change occurs through substitution of the glycine amino acid, which will, by definition, always be replaced by a larger amino acid. The new amino acid will be located in the sterically restricted interior of the triple helix. These larger amino acids will cause helix folding and formation to be disrupted. It also exposes the collagen chains to be over-modificated (over-hydroxylated and glycosylated). which causes further helix instability (Shoulders & Raines, 2009).

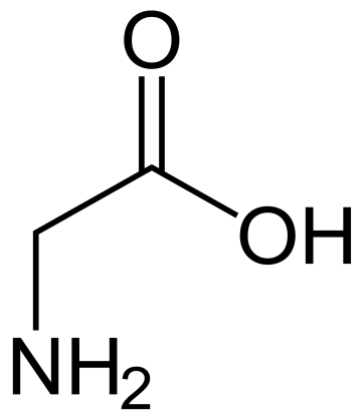



*Figure 4: A Glycine molecule, containing. A single hydrogen as side chain (source: NEUROtiker)*

## 3.1 Mutation variance and discrepancies

Clinical evidence suggests that glycine substitutions in the α1 part of the collagen chains are associated with OI with severe symptoms, contrasted by mutations in the α2 chains, which generally show less severe symptoms. Lethal substitutions are similarly more common in α1 collagen chains with around 33% causing type II OI, while in the α2 chain this is around 20% (ben Amor et al., 2011). The underlying mechanism behind this observed phenomenon, remains unexplored.

Within the α1 chain the same mutation can also have completely different phenotypic effects; with the same mutation causing type III in some type IV OI in others. Some glycine substitutions have been found to be lethal in some patients while non-lethal in others. This effect is even noticeable between family members with the exact same mutation (Forlino et al., 2011). The reason why severity can be variable might be attributed to genetic variance in collagen modifying mechanisms, but is poorly understood.

Another interesting manifestation of OI is observed in Aga2 mice, where mutations in the C-propeptide are induced. This part of the propeptide part of the inactive protein and is cleaved before translocation of collagen into the matrix (Barnes et al., 2019). This suggests that the generated protein does not have a glycine substitution, however moderate to lethal forms of OI are clinically observed. A possible explanation for these symptoms might be the triggering of the unfolded protein response in osteoblasts, caused by intracellular retention of the atypical collagen chains. This cellular stress response is caused by misfolded protein within the ER, which results in cellular apoptosis.

# 4. Pharmacological treatment

As mentioned in the introduction, there are currently no curative treatment options available for patients of any type of OI. The treatments available at the time of writing are of a palliative nature, catered to the varying degrees of disease severity through patient-specific treatment methods. This usually constitutes maintaining mobility, ameliorating symptoms and strengthening the bone and muscle tissues (Tauer et al., 2019).
Biphosphate infusions are commonly used as treatment to inhibit bone resorption and consequently reduce bone fractures and increase bone mineral density (BMD) (Dwan et al., 2016).
The previously mentioned surgical option of inserting metal rods in the long bones to strengthen bones and prevent fractures is reserved for those OI patients with serious bone deformations.

Protective braces are frequently used as a preventive measure which allows some freedom of movement for the patient. Besides metal rods, surgery is occasionally necessary in patients with severe dental problems caused by dentinogenesis imperfecta and in patients with severe types of scoliosis or kyphosis (Weintrob, 1995). OI patients are also regularly monitored on their hearing abilities as the inception of hearing impairment is usually around the third decade of life (Pillion et al., 2011). Besides hearing abilities, changes in bone density over time are also monitored closely. Those OI patients with a short stature and those with severe deformities of the ribs or spine regularly have their pulmonary function monitored as well. Respiratory infections can be especially detrimental and seasonal flu vaccines are recommended for this specific group.

Exercise and movement therapy are a staple of OI treatment as it reduces bone fracture frequency by training specific muscles and improving the physical state of the patient. With exercise often being uncomfortable and painful for OI patients; hydrotherapy has been proven a way to circumvent the physical discomfort of exercise (Ralston & Gaston, 2020).
Further therapies involve information on preventing fractures, maintaining a healthy diet and exercise routine. This is especially important as lack of exercise and physical activity can further weaken the muscles and bones. This can cause an unhealthy vicious cycle of increased discomfort with continued lack of exercise. As weight increases as consequence of lack of exercise, the frequency of fracture incidents increase as a result of the added physical stress on the bones, which in turn can cause additional physical limitations (Silverwood, 2001).

## 4.1 Bisphosphonate treatment

As previously mentioned, Biphosphate administration is a commonly used treatment which inhibits calcification and bone resorption and consequently causes an increase in bone quantity. The mechanisms behind this treatment involving the specific targeting of osteoclasts without targeting osteoblasts should be reviewed.

Bisphosphonates are administered orally or through intravenous infusion. When administered orally, bisphosphonates or partially absorbed through active transport in the stomach, duodenum and ileum. A somewhat common side effect of oral bisphosphonate intake: gastric ulcer and nausea. During the absorption phase, bisphosphonates have been shown to be damaging to epithelial layers of the ileum and duodenum (Russell, 2007). After absorption in- and transport through the bloodstream, the bisphosphonates are either immediately expunged through the kidneys or travel to calcified bone tissue. The fraction that travels to bone binds to hydroxyapatite crystals within the bone. Hydroxyapatite is a calcium phosphate mineral and is largely present/exposed in areas with a high bone turnover rate. After binding to hydroxyapatite, bisphosphonates are ingested through endocytosis by osteoclasts during the process of bone resorption (Drake et al., 2008). The nitrogen-group of bisphosphonates actively disrupts the intracellular mechanisms of osteoclasts and causes them to become inactive and ultimately leads to apoptosis. The mechanism behind this apoptosis pathway stems from nitrogen-containing bisphosphonates binding and inhibiting pyrophosphate synthase in osteoclasts.This regulatory enzyme plays important roles in the maintenance of homeostasis in osteoclasts by regulating cholesterol and isoprenoid lipid levels (the mevalonic acid pathway). This in turn causes post translational modification of different proteins including critical Guanosine triphosphate (GTP) - binding proteins to be disrupted. As these GTP-binding proteins help regulate essential roles of osteoclast cellular activities, the osteoclast initiates apoptosis (Itzstein et al., 2011). After ingestion, the bisphosphonates can be released and re-enter circulation upon apoptosis of the osteoclast. Thereafter, the previously described pathway can occur once more in a different osteoclast (Luckman et al., 1998). This might explain why years after termination of bisphosphonate treatment, bisphosphonate is still detectable in the urine of the patients. This described mechanism effectively causes bone formation rates to be preponderant to bone resorption rates. It however, does not change the structure of the bone, which is still fragile in OI patients. Only the quantity not the quality of bone has been affected, but intrinsically the bone remains unchanged.

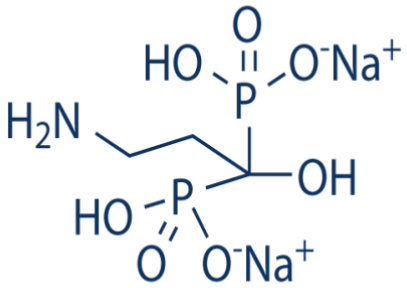



*Figure 5: An example of a Bisphosphonate molecule*

## 4.2 Potential Treatment options

Despite the lack of curative options, different promising drugs are actively being studied, promising potential benefits in novel treatments for OI patients. Such drugs include the anti-rank ligand, which has been observed to improve BMD in OI types I, III and IV. There is however a potentially dangerous side effect of altered calcium homeostasis (Joan C. Marini & An N. Dang Do, 2020)Other promising drugs include recombinant human parathyroid hormone analog (PTH), and is currently only given to postmenopausal women and men with severe osteoporosis, as well as OI patients whom exhibit allergic reactions to standard bisphosponate treatments (Ellegaard et al., 2010). Another potentially promising drug which is being actively researched is growth hormone. Growth hormone increases both muscle strength and mass while improving the generally impaired linear growth in all subtypes of OI (Antoniazzi et al., 1996; Doro et al., 2010).

## 4.3 Stem cell treatment

A potential (partially) curative option is combining stem cell transplantation with CRISPR/Cas9 gene editing. This treatment would entail an a priori editing of a patients' stem cells with CRISPR/Cas9, removing or repairing the mutation and transferring the edited cells back into the patient. This avoids possible graft failure caused by receiving a transplant from a HLA-mismatched donor (Tian et al., 2015). The transfer of healthy cells occurs after high-intensity radiation treatment of the patients' bone marrow to remove as many mutated OI stem cells as possible. Stem cells are more sensitive to high-intensity radiation, as they are rapidly dividing cells. They are more vulnerable because during cell division, DNA is divided into the vulnerable single-stranded DNA pairs. These single stranded DNA pairs are more vulnerable to radiation and result in cell replication processes to be disrupted, which generally leads to cellular apoptosis (Thrall, 1997). The temporary absence of stem cells subsequently allows the newly edited cells to engraft into the bone marrow of the patient. After a successful transplant the genetically edited cells will be producing a healthy collagen protein variant. This process is highly similar to the stem cell transplantation which is administered in patients with leukemia. A downside of this treatment is the high levels (5-11%) of graft failure (Mattsson et al., 2008). A graft failure can have different determinants; a low count of initial engraftment, depletion of T-cells and agranulocytosis, a dangerously low level of white blood cells. The potential consequences of graft failure are severe: internal bleeding, iron overload and extreme vulnerability to a variety of infections. The substantial chance of failure and radio- and chemo-therapy associated toxicity makes stem cell treatment a high risk treatment option for the majority of patients.

## 4.4 Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) can and have been used to disrupt mutant *COL1A1* RNA (Laptev et al., 1994). These ASOs help degrade the mutant version of the RNA and aid its degradation by binding to the mutant RNA which eventually leads to cleavage by RNAse H (Kuijper et al., 2021). Using this method, type IV OI was attempted to be changed to type I OI, by removing the amount of mutated *COL1A1/COL1A2* RNA. The study was able to reduce mutant protein levels 40-50% compared to the control group (Wang & Marini, 1996)The specificity of ASOs is however its downfall in this example, as regular collagen proteins were also targeted and reduced. This is likely a result of the structural similarities between the mutant and non-mutant variants; which only differ by a single glycine mutation. The collagen genes are highly repetitious (as explained in the etiology section) and ASOs seem unable to accurately distinguish by 3-7 mRNA nucleotides.

## 4.5 RNAi

Another treatment option, though not curative, is using RNA interference (RNAi) to treat more severe OI subtypes like type III and IV. RNAi is dependent on small dsRNAs which can inhibit the post transcriptional translation of the mutated *COL1A1* or *COL1A2* gene. The inhibition of the translation is caused by dsRNAs binding to *COL1A1*/*COL1A2* mRNAs. After a RNA induced silencing complex (RISC) is formed, the mRNA is cleaved and gene expression is inhibited (Kim & Rossi, 2008).

A RNAi study using OI cells treated with siRNAs, designed to silence mutated *COL1A1* mRNA, found that *COL1A1* mRNA levels were reduced by 65% and 78% compared to the control group (Lindahl et al., 2013) This suggests that a severe subtype III or IV OI can be reduced to a milder Type I OI. This technique does have several downsides, because of the many different possible mutations causing OI, each siRNA has to be specifically designed for each mutation. Another issue with RNAi is the need for continued treatments through lentiviral administration. The specificity of RNAi has also been called into question with several research articles finding siRNAs can have sequences similar to non-target genes, making harmful off-target effects a serious unresolved issue (Aagaard & Rossi, 2007; Dillin, 2003; Pecot et al., 2011).

## 4.6 Monoclonal antibodies

An alternative to Bisphosphonates could be in the form of monoclonal antibodies which targets the Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL). RANKL which in turn induces improved osteoclast proliferation, is now reduced. This mechanism follows a similar trend as bisphosphonates, which inhibit osteoclast activity. An upside of monoclonal antibodies over bisphosphonates is the short half-life, which allows increased bone turnover after approximately one month after discontinuing treatment, contrasted by the previously discussed 1-2 year half-life of bisphosphonates (Varenna & Gatti, 2011). Furthermore, monoclonal antibodies can be delivered via subcutaneous injections, contrasted by intravenous bisphosphonate injections. This might prove more comfortable to the patient. It would also circumvent the previously mentioned gastrointestinal side effects associated with oral bisphosphonate intake.

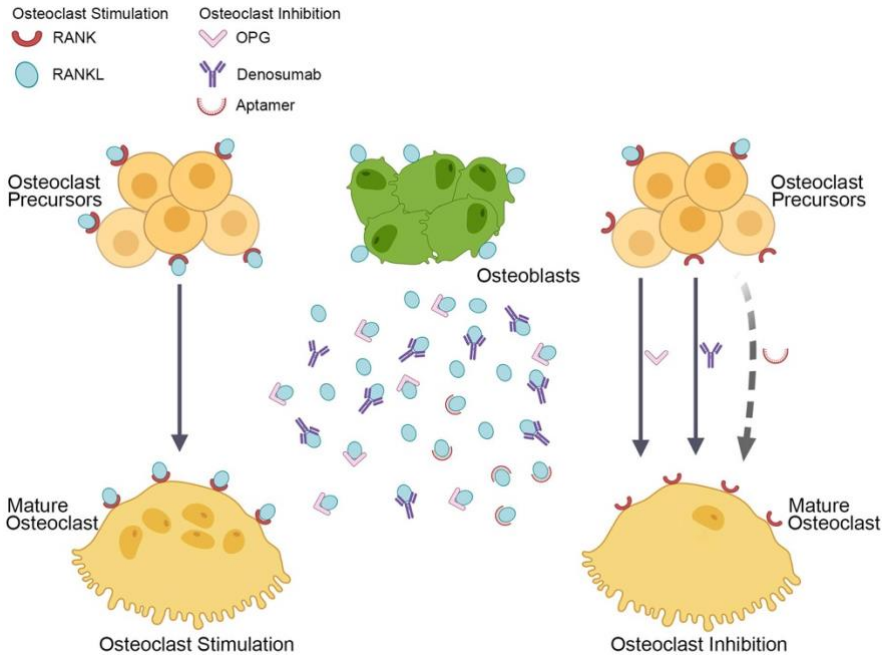



*Figure 6: Monoclonal antibody treatment, schematic overview (Source: Zhang et al. 2020)*

# 5. CRISPR/Cas9 therapy

A promising addition to the previously mentioned therapeutic potential and is a method using a genetic editing tool called CRISPR/Cas9. CRISPR/Cas9 is composed of two components: firstly the CRISPR part, which is a component of a Bacterial and Archaeal defense mechanism. This mechanism was used to cleave nucleic acids from bacteriophages entering the cell. The method of cleaving is performed through the integration of foreign bacteriophage-DNA onto a Crispr locus. A PAM sequence which is adjacent to the bacteriophage-DNA allows the bacterium to only target the specific bacteriophage-DNA (Hille & Charpentier, 2016).

This bacterial defense mechanism has been modified for use in humans and utilises Cas9 to target specific genes. This nuclease cleaves DNA strands at the specified locus by the guide RNA (gRNA) and establishes Double stranded breaks (dsbS) at specific target locations.

The target locations are specified within the custom made gRNA and also a short PAM sequence. The gRNAs contain a trans-activating CRISPR RNA (tracrRNA) part containing a scaffolding sequence which the Cas protein and CRISPR RNA (crRNA) bind to. This complex together defines the genomic target. The PAM is only a few BPs long and follows the Cas9 DNA sequence. These created dsbS are then repaired via non homologous end joining or homology directed repair (Jiang & Doudna, 2017). At this stage a modification, deletion or addition is possible. Specific mutations to OI can be removed or interchanged by creating appropriate gRNAs for the patient's genetic mutation. This complex is subsequently introduced into the patient's cells where the DNA should be altered. The transfer of CRISPR/Cas9 machinery to the patient's cells can be performed via viral, physical or chemical factors. This mechanism allows for an individualised mutation-specific approach in OI.

## 5.1 Genetic screening

Before CRISPR/Cas9 mediated treatment is achievable, a specific OI diagnosis needs to be made. There are currently over 1400 distinct mutations known to cause OI in an autosomal dominant form and over 150 in an autosomal recessive form (Götherström & Walther-Jallow, 2020). The exact type of mutation or mutations need first be affirmed before any genetic editing can be performed. The screening methods for the exact mutation are different between embryonic and late onset OI. For the late onset OI diagnosis; clinical evaluation is the main method. A biopsy of the skin will be needed to examine collagen structure. Subsequently, exome sequencing is used to find the exact mutation of interest. Embryonal diagnosis of OI can be performed via echoscopy, ultrasounds, amniocentesis or chorionic villus sampling (CVS) (Gay-Andrieu et al., 2016). Ultrasound can be used to detect fractures or malformation in long bones. Amniocentesis entails the removal and analysis of the fluid surrounding the fetus. CVS involves a sample being taken from the placenta or embryonal fluid. Subsequently, genetic and chromosomal screening is used on the embryonal fluid or placenta sample, after which the presence of specific mutations in *COL1A1* and/or *COL1A2* are determined.After the specific mutation is established, CRISPR/Cas9 can be used to edit the mutated sequence, which will generally entail replacing a mutated amino acid codon with a glycine codon, which is encoded by every codon starting with a double GG sequence.

## 5.2 CRISPR/Cas9 administration

A crucial aspect of this proposed treatment is proper administration of CRISPR/Cas9 to the target cells. Another aspect is that the expression of the CRISPR/Cas9 machinery should be brief, as not to cause off-target mutations or initiate an immune response (Khambhati et al., 2020). These issues pose a significant challenge for any in vivo applications of CRISPR/Cas9. In our proposed therapy for instance, high cell-specificity is required. If CRISPR/Cas9 is going to be used in human subjects, the maximum specificity and safety is paramount. At the time of writing, all methods of in vivo delivery of the CRISPR/Cas9 machinery are prone to affect non-targeted cells.

Although the problem of off-target mutations remains, recently developed genome-scale algorithms allow for highly accurate off-target predictions of unintended DSBs (Lin & Wong, 2018). The number of off-target mutations is largely dependent on the specific sgRNA used, with off-target mutation frequencies ranging from 0.03% to 87% (Wu et al., 2014). Low off-target rates would still be problematic for in vivo CRISPR/Cas9 treatment if e.g. tumor driver genes are affected. Besides off target mutations; unwanted immune activation through a lentiviral vector, a widely used gene delivery vehicle, also seems problematic (Henderson, 2021). An activated immune system can disrupt the genetic editing process and potentially cause the destruction of already transduced cells. (Charlesworth et al., 2018)Off-target mutations and immune activation are enormous obstacles to overcome, but several new options hold potential.

One of these options is utilising an adeno-associated virus (AAV). An AAV is a non-enveloped virus that can be engineered to deliver CRISPR/Cas9 to target cells(Yu & Wu, 2019). It is possible to create recombinant AAVs, which only moderately activate the immune system as they do not contain viral genes. AAVs integrate only in specific sites of the host genome, giving it an important advantage. Retroviruses as vectors for CRISPR/Cas9 are often unpredictable, as they integrate randomly in the genome. This unpredictable integration can have disastrous consequences like random deleterious insertions or cancer development. Using AAVs seems to be the safest method of CRISPR/Cas9 delivery due to their low cytotoxicity and immunogenicity (Hanlon et al., 2019).

Another option would be the introduction of a tissue-specific (TS) - promoter. This promoter is only activated in specific cell types (Zheng & Baum, 2008). A TS-promoter has already been used alongside CRISPR/Cas9 in Drosophila to restrict mutagenesis to specific cells(Meltzer et al., 2019). Using a TS-promoter in the context of OI, means only those collagen producing cells will be edited by the CRISPR/Cas9 machinery. The further development of TS-promoters seem invaluable in achieving specificity within CRISPR/Cas9 therapy.

Another strategy which relies on similar cell-specific signals to modulate the activity of the CRISPR/Cas9 machinery is the use of microRNAs. Many tissue-exclusive microRNAs have been catalogued and could be used to selectively activate CRISPR/Cas9 (Hirosawa et al., 2017)This would involve engineering CRISPR/Cas9 by integrating cell-specific microRNAs at binding sites into the 3′ untranslated region (3′ UTR) of CRISPR/Cas9. This strategy could potentially be used in conjunction with TS-promoters to achieve maximum specificity.

Assuming the issues of immune activation and cell-specificity are sufficiently dealt with, off-target cleavage and precise engineering is still a glaring complication. A recent development

in chemically induced Cas9 modulation could be the answer to this problem. A recent study has switched Cas9 on and off using 4-hydroxytamoxifen (4-HT(Liu et al., 2016)This was accomplished by integrating a hormone binding domain of ERT2, an estrogen receptor, to the Cas9 domain. This final product showed low endonuclease activity without 4-HT, and could efficiently induce endonuclease activity by adding 4-HT. This method significantly decreased off-target cleavage of CRISPR/Cas9.

## 5.3 Post-treatment assessments

Monitoring the production of normal *COL1A1* and *COL1A2* post-treatment in OI patients can be performed through check-ups, where periodically quantitative competitive reverse transcription-polymerase chain reaction (RT-PCR) analysis is performed of the *COL1A1* and *COL1A2* mRNA levels. This method would be similar to an already existing method for examining Lesch–Nyhan disease (LND) gene expression regulation(Torres et al., 2012). Venous blood is required for this method of analysis, which is relatively non-invasive.

Alternatively, Gel electrophoresis could be used to analyze the size (and charge) of formed proteins. This method would require culturing of patient cells. After running a gel electrophoresis it can be established if the formed collagen proteins have changed size after CRISPR/Cas9 treatment (Lee et al., 2012).

Another option could be periodically checking the BMD of patients. DXA- scans can be used to establish differences in BMD before and after CRISPR/Cas9 treatment. DXA-scans target the patient's bones using two different X-ray intensities and measures the difference in tissue absorption (Blake & Fogelman, 2007)Usage of DXA scans is already widely used in osteoporosis risk assessments. If the BMD is measured over a longer period, an improvement should be visible after CRISPR/CAS9 treatment.

## 5.4 Mosaicism

Another hurdle in CRISPR/Cas9 editing in vivo is the occurrence of genetic mosaicism. Mosaicism is a phenomenon in which one individuals' genome contains more than one genotype. In the case of OI, this would entail that a percentage of cells are successfully corrected after CRISPR/Cas9 treatment, while others still produce a mutated form of collagen. Before CRISPR/Cas9 treatment can be performed in humans, mosaicism rates must be minimized. The frequency at which mosaicism occurs varies greatly (Mehravar et al., 2019; Tu et al., 2017), the extent of variability is likely a result of the properties of the target gene and associated gene locus (Khambhati et al., 2020). The generation of a mosaic genome can lead to an unwanted phenotype of OI, in which brittleness and fractures still occur if only less frequently. It is theorized however, that due to increased stability and reduced turnover of functioning collagen (selective advantage), bones will eventually contain more functioning collagen than the mutated, less stable variant (Zhang et al., 2019). Mosaic variances are also known to occur at the zygote stage. When the zygote DNA is not corrected by CRISPR/Cas9 until after replication occurs, several daughter cells inherit uncorrected DNA still containing the OI mutation (Lamas-Toranzo et al., 2019). This will consequently still lead to OI development, only resulting in less severe symptoms. Both in vivo and germline mosaicism should be prevented, before CRISPR/Cas9 can be used in any clinical treatment.

# 6. Conclusion & Discussion

Osteogenesis imperfecta is caused by mutations in the *COL1A1* or *COL1A2* gene that result in detrimental changes of the collagen structure. There are 4 main variations of OI with disease severity and effectiveness of the limited treatments largely determined by the type of mutation (Sillence et al., 1979). The main consequences of these mutations include a lower BMD, high risk of fractures, blue sclerae, thinner collagen fibres and a hypermineralized bone matrix. In OI patients, these symptoms and their interactions can be altered to varying degrees of severity, underlining the need for patient-specific treatment. The currently available treatments for OI focus on reducing bone turnover, fracture rate and overall discomfort. Appropriate treatment which fundamentally changes not the quantity, but quality of the bone is currently completely absent. However, our knowledge of genetic editing has been ambitiously accumulating over the last few decades and has already been demonstrated to contain potential as therapeutic strategies for different genetic disorders. Among the more effective of the editing tools is CRISPR/Cas9. CRISPR/Cas9 has been successful by virtue of its accessibility, effectiveness, cost and malleability. The principle of a single gRNA which targets the Cas9 nuclease to the target DNA locus, and the high specificity of the generated DNA breaks have made CRISPR/Cas9 the most popular genetic editing tool to date. CRISPR/Cas9 certainly has the potential to become a reliable tool for repairing certain genetic disorders, after some important issues are addressed. Before any human treatment options are considered, performance improvements must be made i.e. off-target effects, mosaicism, delivery methods and specificity. Different solutions like AAVs, TS-promoters, microRNAs, 4-HT activation and many others are currently being researched and developed to deal with these issues. A study from 2014 has already successfully corrected a genetic disorder (Type I tyrosinemia) in vivo by using CRISPR/Cas9 genome editing (Yin et al., 2014). Despite real progress in the field of genetic editing with CRISPR/Cas9, many uncertainties remain and should be addressed. As of the time of writing, it can be concluded that CRISPR/Cas9 treatment for OI is currently not viable, but a promising and ambitious future prospect.

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