

The intersection between Age-related Macular Degeneration and CRISPR-Cas9 Therapeutics

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Age-related macular degeneration (AMD) has genetic, environmental, and pathophysiological factors causing the disease. Two prominent genes involved in AMD and its progression are VEGF and CFH, both of these which have been identified to have genetic mutations and linkages to AMD. As there is currently no cure for AMD, CRISPR-Cas9 systems could provide a potential tool to work toward a cure. While CRISPR is not being used as a treatment for patients, there have been studies done using CRISPR on AMD-associated genes, which have been both successful and suggestive for further improvements. There are current treatments to slow the progression of the disease, but this is not preventing complete vision loss in patients. As there are genetic aspects to the disease, CRISPR-Cas9 has been used in labs on both the VEGF and AMD genes, as well as on genes for other retinal diseases. By doing a literature review via PubMed and Google Scholar, this paper highlights what the previous use of CRISPR-Cas9 on VEGF and CFH could suggest as to whether base editing, AAV, or lipid nanoparticle delivery methods is the most efficient and safe. Finally, there are major limitations to studying the VEGF gene at this point and the potential unwanted mutations CRISPR-Cas9 could solve. Such limitations include molecular limitations like the efficiency, safety, and delivery method of CRISPR. Non-molecular limitations include the social and ethical concerns with the widespread use of CRISPR. By taking into account all of these factors, the studies collected and reviewed have suggested that CRISPR-Cas9 is still a potential realistic therapeutic for AMD.

Introduction to Age-related Macular Degeneration

AMD, or age-related macular degeneration, is a degenerative disease that occurs in the macula, or the center of the retina¹, and specifically affects photoreceptor cells responsible for absorbing and processing light. While photoreceptors in the macula degenerate as a result of AMD, making central vision blurry or nonexistent², patients retain their peripheral vision as this disease is only located in and affects the center of the retina, or the macula¹. There are also retinal pigment epithelial cells, or RPE cells, which absorb light and help a person to see². As macular degeneration progresses, more cells degenerate, including both photoreceptor and RPE cells, which in turn limits the vision of the patients. If the disease progresses too far, it could make the patient lose vision completely³. As seen in Figure 1, eye scans can tell doctors whether a patient has macular degeneration or not based on the look of the cells present. The white spot in the Figure shows that the cells have degraded, leading to a thin layer of photoreceptors in the macula. Therefore, it is essential that there is a way to manage the progression of this disease.

In the United States, approximately 11 million people have AMD, and approximately 170 million people worldwide are diagnosed with the disease⁴. The total number of people with

AMD is expected to rise by 100 million people in just 18 years. For patients older than 55 years old, AMD is the most prevalent cause of vision loss⁵, with AMD being the cause for vision loss in 54% of white Americans in 2008⁶. AMD is most common in European and other Western populations in comparison to Asian and African populations. Due to the combination of genetic, environmental, and other factors, it is currently not recommended to conduct genetic testing as a way to predict AMD diagnosis. Some common risk factors are ethnicity and age because of genetic and molecular lineages. Some environmental habits/factors that can increase the chances of developing AMD include smoking, hypertension, high cholesterol, obesity, and UV exposure⁶.

AMD has two main subtypes: wet AMD and dry AMD. According to Chung et al., wet AMD is when the abnormal growth in the choroidal blood vessels under the retina causes a loss of central vision while dry AMD is when a buildup of waste under the macula causes vision loss at a lesser degree to wet AMD⁷. Additionally, with wet AMD, angiogenesis, the formation of new blood vessels, is especially dangerous because it causes the blood vessels under the macula to leak blood and fluid⁷.

AMD is a complex disease as its etiology consists of both genetic and environmental factors. It is difficult to prove which is the greater cause and hence it is difficult to predict

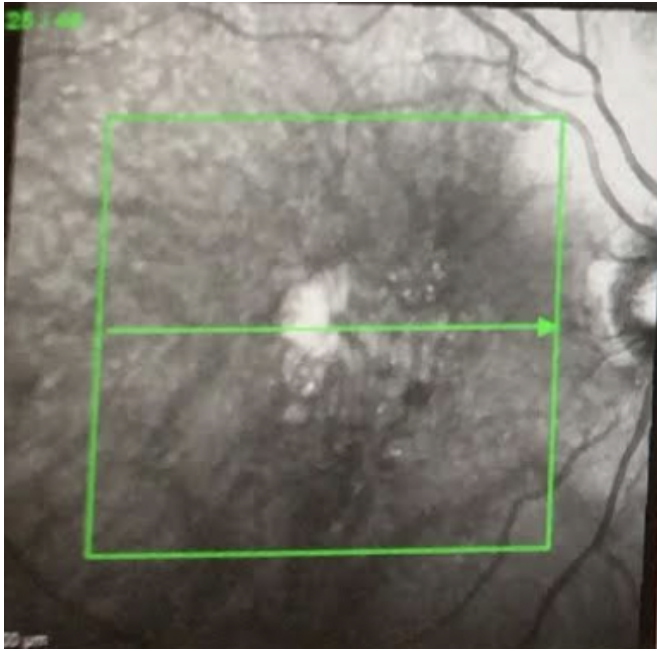


Fig. 1 Eye Scan of Patient with AMD. AMD can be observed as the white spot in the middle. The white area represents the thin layer of photoreceptors and other eye cells due to degeneration. Dr. Philip Laird, personal communication, July 14, 2023.

who will develop the disease⁸. Since there is a genetic aspect, AMD can be inherited, but this is not a rule. For this reason, doctors do not suggest doing genetic testing as it will not confirm any diagnoses (Dr. Philip Laird, personal communication, July 14, 2023). Other than age, some examples of environmental factors include smoking, diet, and in some studies, gender⁹. Mohamad et al. (2019) found that males have high smoking rates, increasing their risk of AMD. However, it is also mentioned that some studies recorded a higher risk in females. As concluded by Mohamad et al.'s study⁹, there was not a significant difference in control variables compared to experimental variables, meaning gender was not determined as a factor.

Smoking is a major factor to diagnosing AMD. Several studies, including the Beaver Dam Eye Study, Rotterdam Study, and the Blue Mountains Eye Study, all concluded that there were high rates of AMD in smokers. Additionally, the Blue Mountains Eye Study claims that approximately 20% of all blindness in Australia is due to smoking, and 14% of AMD cases. As a follow-up on the Beaver Dam Eye Study, the Beaver Dam Offspring study identified 3.4% of its patients with AMD. Smoking can cause molecular and pathological changes to the body, making the environment ideal for AMD development. Such change includes oxidative damage, inflammation, toxic damage, and more¹⁰. Therefore, there

is a strong connection between AMD and smoking, which is something patients and doctors should focus on when diagnosing AMD.

AMD can also have a pathophysiological aspect to it which involves RPE cells. As the extracellular matrix (ECM) experiences enzyme imbalance due to aging RPE cells, the body's Vascular endothelial growth factor (VEGF) immune response activates⁵. VEGF is a protein that stimulates angiogenesis, the formation of blood vessels⁷. VEGF stimulates blood vessel development from the Bruch's membrane, and these blood vessels rupture and cause leakage, leading to AMD. This type of response is an inflammatory response⁵. Since the RPE cells are still aging, it is expected that this response cycles, causing more degeneration of vision.

While there is currently no cure for AMD, there are various treatments and medical procedures that help slow the progression of both wet AMD and dry AMD¹¹. Both types of AMD have their own treatment (Dr. Philip Laird, personal communication, July 14, 2023), both of which are anti-VEGF injections¹¹; however, most treatments are directed towards wet AMD and the VEGF family⁴. VEGF is a protein that stimulates angiogenesis, which is the formation of blood vessels⁷. The formation of blood vessels, as previously discussed, only makes the disease worse, particularly wet AMD. Therefore, anti-VEGF intravitreal injections can slow down the amount of blood vessels that can leak, ideally slowing down the progression of the disease¹¹. Some examples of these injections are bevacizumab and ranibizumab⁹. However, these are just current treatments to help slow the progression of the disease, which indicates that new treatments should be developed to have a bigger effect on the disease and release the burden from the patients^{11,12}.

Genes with Relation to AMD

As mentioned previously, one gene to consider when researching AMD is the VEGF gene. With the neovascular form of AMD, or nAMD, VEGF expression is increased⁷, making it important to regulate the expression of VEGF such that AMD does not progress as rapidly. However, it is important to note that while VEGF is an unwanted gene for patients with AMD, it might not be the cause of AMD. The VEGF gene stimulates the growth of new blood vessels, which is beneficial for those without retinal degenerative diseases such as AMD⁷. For people without AMD, angiogenesis is helpful as it heals wounds, and supplies oxygen to the rest of the body¹. However, for patients suffering from AMD, the presence of angiogenesis can become detrimental. These new blood vessels also start to leak and cause a further build-up under the macula, increasing the degeneration of central vision¹³.

The VEGF gene family is a family of 7 genes that all cause to angiogenesis. When referencing VEGF in relation to AMD,

the most common gene is the VEGF-A gene. VEGF-A plays a key role in ocular development; however, mutations in the gene can cause pathological conditions to be altered. VEGF-A will bind to VEGFR1 and VEGFR2 (VEGF receptors), which stimulates angiogenesis¹⁴. If there are mutations in this protein, it could cause problems with binding and therefore stimulation of angiogenesis. More recently it has been determined that VEGF-A also plays a role in retinal vascular permeability, causing the blood vessels to leak¹⁴.

The VEGF gene's association with AMD is controversial among studies for the genotypes associated with and without the disease. In a meta-analysis by Barchitta and Maugeri (2016), it was confirmed that the C allele and CC genotype are indicators of an increased risk of AMD. However, the meta-analysis discovered mixed opinions on whether the TT genotype is associated with increased risk or decreased promoter activity, which would potentially decrease the risk of AMD¹⁵. Therefore, this meta-analysis, or statistical analysis of several studies, only confirmed that the C allele and CC genotype are a commonality between the VEGF gene and AMD. An experiment by Mohamad et al.⁸ had the same findings about the CC genotype, that it was an indicator of an increased risk of AMD. In addition, it was also determined that the GC genotype is related to an increased risk of AMD in certain ethnic groups such as Chinese and Tunisian⁸. Therefore, both of these studies have signified the correlation between the CC genotype and AMD; however, the other genotypes such as GC and TT need to be researched more.

Another gene that is relevant to AMD is the complement factor H (CFH) gene. CFH is one protein in the complement system which is a part of the body's immune system and protects it from infections and diseases. The gene encoding CFH is on chromosome 1q31, a locus that also has been proven to have connections to AMD¹⁶. CFH follows one of the three complement pathways called the alternative pathway¹⁷, which is triggered by a specific molecule binding to the cell surface¹⁸. The other two pathways are the classical pathway and the lectin pathway, which are used by other proteins in the complement system. In comparison to the alternative pathway, the classical pathway involves an antibody-antigen activation, and the lectin pathway gets activated by a polysaccharide binding to a microbial surface¹⁸. It has been identified that there are genetic variations in the DNA that could cause abnormalities in this pathway, increasing the risk of AMD¹⁸. CFH holds a key role in regulating inflammatory responses. The Y402H polymorphism is a single nucleotide polymorphism (SNP), therefore it is the change of one nucleotide. This mutation causes a change in amino acids, from tyrosine to histidine¹⁹. Because it is a change in amino acids, this has the potential to be a harmful mutation, meaning it is important to find a solution.

Baird et al. (2008)¹⁷ conducted a study that helped identify

the genotypes within the Y402H gene that are associated with both more progressive AMD and less progressive AMD. The benefit of identifying a safe genotype is to know how gene editing might be a potential factor in treating AMD in the future. The Y402H polymorphism located on the CFH gene has been identified to have a strong correlation to AMD²⁰. The Gangnon et al. (2012)²⁰ results discovered that those with the CC genotype had more progressed cases of AMD in comparison to those with CT and TT genotypes. The CC genotype had a 48.8% linkage to progressed cases of AMD¹⁷. In comparison, the CT genotype had a 32.7% linkage to progressed AMD cases, and the TT genotype had a 26.4% linkage to progressed AMD cases¹⁷. The studies therefore identify the CC genotype as having a stronger linkage to AMD than CT or TT. In a different study by Gangnon et al. (2012)²⁰, both the CC and CT genotypes were strongly associated with AMD²⁰. While it still appears unclear whether the CT genotype is associated with AMD, multiple studies have found that the CC genotype seems to have a strong correlation with the disease.

CRISPR-Cas9 Systems

Clustered regularly interspaced short palindromic repeats, or CRISPR, are short pieces of repetitive DNA that are spaced by unique intervening segments of DNA. Cas nucleases are CRISPR-associated nucleases²¹ that can cut specific parts of genomic DNA, which makes them essential tools in the genetic manipulation of an organism's genotype²². The CRISPR-Cas9 system is naturally found in bacteria. When a virus infects the bacteria cell, it injects its DNA into the bacteria. However, the viral DNA needs to be integrated into that of the bacteria to have an effect. This is accomplished by using CRISPR-Cas9. The main components of CRISPR are the Cas9 protein and the guide RNA (gRNA). There are many types of the Cas9 protein, such as *Streptococcus pyogenes* (SpCas-9) protein, and the *Staphylococcus aureus* (SaCas-9) protein²³. The Cas9 proteins are responsible for the cutting of the DNA. However, other components are needed to identify the region the Cas9 protein needs to cut. Cas9 proteins contain two regions: the recognition (REC) lobe which is responsible for binding to the gRNA, and the nuclease (NUC) lobe interacts with the DNA²⁴. The other component, the gRNA, is binded to the Cas9 and identifies the region where the cas9 protein needs to cut based on the nucleotide sequence it is complementary to²⁵. A final component of CRISPR is the protospacer-adjacent motif (PAM) region, which is adjacent to the region that the gRNA identifies²⁶. This PAM region needs to be 5'-NGG-3', N standing for any nitrogenous base followed by two guanine nucleotides²⁷. The cas9 nuclease along with the protospacer-adjacent motif (PAM) sequence identifies the part of the DNA that the viral DNA should be integrated into, and then cuts the bacterial DNA based on its

nucleotide sequence²⁷. As a result, the viral DNA can be consolidated into the bacterial DNA. The reason it is part of the defense (immune) mechanisms of the cell is because the bacteria has identified the viral DNA, allowing it to easily ward it off in the future^{22,28}. It has been identified that this CRISPR-Cas system does not only naturally occur within bacteria, but also in other types of prokaryotes²⁹.

Two of the tasks that CRISPR technology can perform are knock-ins and knock-outs. Both knock-in and knock-out mechanisms use the same components which is the CRISPR-Cas9 ribonucleoprotein (RNP). The RNP contains the gRNA and the cas9 protein. This is where knock-out and knock-in differ. After cutting the DNA, the DNA repair system is triggered. In this case, the DNA repair system is non-homologous end joining (NHEJ). This will knock out the gene of interest because it is cut out of the DNA which allows the DNA will repair itself. However, this is prone to indels, or random insertions and deletions. While the repair process does effectively knock-out the gene of interest, it is also prone to off-target errors and mutations²⁹. The knock-in mechanism is different as the RNP has a donor template of DNA attached to it. The cas9 will cut the DNA and this donor template will be added into the target DNA, therefore knocking-in the gene of interest. The DNA will be repaired using homology-directed repair (HDR), inserting the gene of interest²⁹. HDR will fill in the gap created by the Cas9, whereas NHEJ will stick the ends of the DNA strand back together, which as seen in Figure 2, has the potential to cause more mutations, suggesting that NHEJ is more harmful than HDR. Whilst NHEJ is more common in knock-outs and HDR is more common in knock-ins, the cell-type and loci also determine which DNA-repair mechanism will be activated³⁰. Another observation by Miyaoka et al. (2016)³⁰ is that Cas9 induces more NHEJ than HDR, but also a high level of HDR, while Cas9-nickases induce a significantly higher level of HDR than NHEJ. Evidently, there are many factors that contribute to the DNA-repair mechanism activated for each repair. In consensus with other studies, Miyaoka et al. (2016)³⁰ report that HDR is the preferred mechanism to NHEJ, as HDR is more precise and NHEJ is extremely error-prone.

Knock-ins and knock-outs are also very different in their precision. Performing knock-outs is reportedly much more feasible, as the PAM sequences are easy to allocate; whereas for knock-ins, there is a limited number of gRNAs that could work, making the task much more difficult³².

Whilst most CRISPR-Cas9 gene editing methods require double-stranded breaks (DSBs) which trigger DNA repair systems such as NHEJ and HDR, there are methods that do not require DSBs, a DNA template (for knock-ins), or DNA repair systems. These systems are considered safer to use as there are safety concerns with off-target mutations and indels that DSBs can cause²⁹. One example is base editors. As in

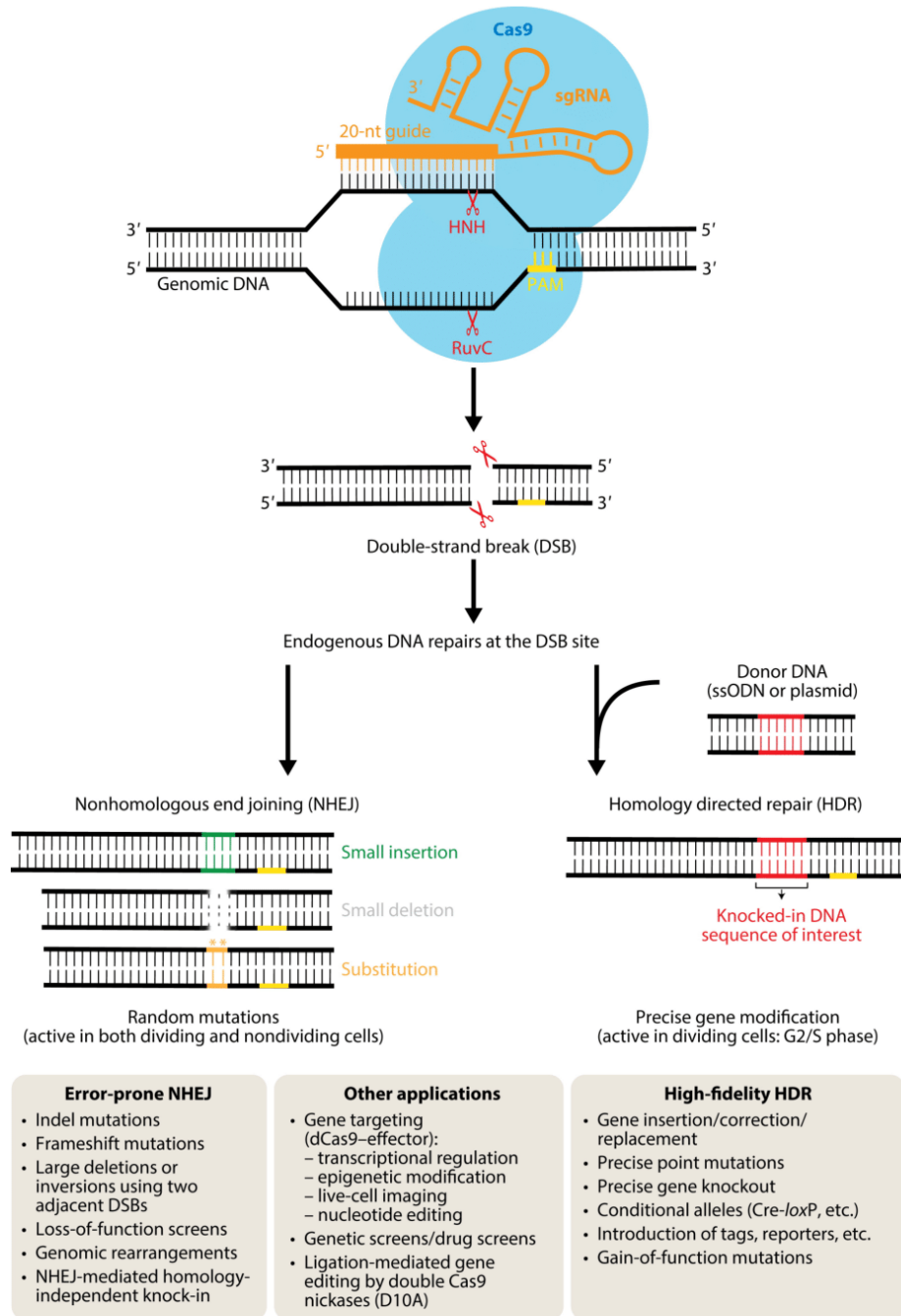
the name, base editors edit single nucleotides, or bases. There are two types of base editors: cytosine base editors (CBEs) and adenine base editors (ABEs). CBEs will convert a C:G base pair to a T:A base pair. Similarly, ABEs will convert an A:T base pair to a G:C base pair. Base editors use inactivated CRISPR-Cas9 nucleases, therefore not triggering the DSBs, and instead connect an enzyme that can cause single-stranded breaks so the single nucleotide can be edited. This is therefore much safer as it reduces the risk of indels and other unwanted mutations³³.

Prime editors, unlike base editors, handle small insertions and deletions with genome editing. Prime editors have a cas9-nickase like base editors do, but unlike base editors, they also have an engineered reverse transcriptase region²⁹. Prime editors will identify the target DNA location by using a prime editing guide RNA, or pegRNA. The 3' end of the pegRNA will act as the primer for reverse transcription, which will integrate the section of the pegRNA into the target genome³³. This allows CRISPR-Cas9 to do knock-ins and knock-outs without generating DSBs.

CRISPR use on VEGF and CFH Genes

In a study by Chung et al. (2022)⁷, single and paired gRNAs were compared to observe which was a better method of CRISPR-Cas9 editing to suppress VEGFA, a variant of the VEGF gene. Both single and paired gRNAs were in adeno-associated vector (AAV)-mediated CRISPR-Cas9 systems. The study determined that there was no significant difference in the efficacy of single and paired gRNA systems. Additionally, the study stated that suppressing the VEGF gene may be dangerous as it maintains vessel growth and photoreceptor maintenance, which is a benefit for both those with and without AMD. Another concern mentioned is the risk of off-target mutations caused by long term expression by Cas9. Through the experiment, researchers discovered that AAV delivery of CRISPR-Cas9 caused few off-target mutations. Even only one or two off-target mutations have the potential to be extremely harmful, which is something to be cautious about. Additionally, while there were few immediate off-target mutations, it was discussed that the Cas9 being expressed for long periods of time could cause harmful mutations. Another limitation relating to gene editing is the risk of chromosomal translocations³⁴. While the study did not identify much danger with using AAV, there are many risks that need to be analyzed. Therefore, Chung et al. (2022)⁷ suggest that if CRISPR-Cas9 is used, an RNP delivery system using methods such as lentiviral or lipid nanoparticle methods could be a much safer solution⁷.

Another study focusing on the use of CRISPR-Cas9 editing on the VEGF-A gene is a study by Ameri et al.'s (2020)³⁵, where they performed an experiment utilizing CRISPR-Cas9



Jiang F, Doudna JA. 2017. Annu. Rev. Biophys. 46:505–29

Fig. 2 CRISPR-Cas9 System. CRISPR uses the Cas9, sgRNA, and PAM sequence to activate gene editing. Once it finds the locus, the DNA will be cut. Subsequently, there are two possible routes: one follows NHEJ to repair the DNA, and the other inserts the donor DNA and uses HDR³¹.

RNPs via lipofectamine CRISPRMAX (LCM). LCM is a method of CRISPR-Cas9 delivery using lipid nanoparticles³⁶. The lipid nanoparticles contain a lipid bilayer which will allow the CRISPR-Cas9 to travel to the host DNA and then continue its function. In Ameri et al.'s (2020)³⁵ study, both Muller cells and RPE cells were utilized, both of which have been previously identified to have a relationship to AMD. The study concluded that while it was successful at disrupting the VEGF-A expression, several unexpected, off-target mutations occurred. Additionally, the VEGF-A expression and mRNA expression in RPE cells were difficult to detect, giving inconclusive results. Ameri et al. (2020)³⁵ suggest that while CRISPR-Cas9 editing methods are plausible, more experiments need to be done to identify the safest and most efficient method of doing so. Ameri et al. (2020)³⁵ did identify a limitation of the LCM, that the Muller cells were more dense than the RPE cells, so if LCM were to be used again, the target cells would need to be Muller cells instead of RPE cells. This study claims that lipid nanoparticles are a good method to use as they are nonspecific to cell targeting, meaning that they can target several cell types simultaneously³⁵. However, this is something to be cautious about, because according to Park et al. (2023)³⁷, using cell-type-specific methods can prevent unwanted, off-target mutations, increasing the safety of the editing system³⁷.

Park et al. (2023)³⁷ looked at RPE cells specifically with the aim of developing a CRISPR-Cas9 method for RPE cells. To do so, Park et al. (2023)³⁷ moderated Cas9 endonuclease expressions by modifying specific promoters. The VMD2 promoter which is specific to human RPE cells effectively allowed Park et al. (2023)³⁷ to develop a RPE-specific CRISPR-Cas9 system. Additionally, this system was so successful that no unwanted mutations and knock-outs were formed. By changing the promoter sequence, Park et al. (2023)³⁷ developed a cell-type-specific CRISPR-Cas9 editing system³⁷. However, this might not be the best solution when considering AMD as RPE cells are not the only cells involved with the disease. Other cells include photoreceptor cells and Muller cells. Because of this, all of the cells need to be targeted simultaneously, which is why nonspecific cell-type targeting may be a better option^{3,35}.

A study conducted on the CFH gene by Tran et al. (2019)³⁸ observed the alternative pathway that CFH follows and focused on CRISPR-Cas9 editing on a single nucleotide polymorphism (SNP) associated with a dysregulation in the pathway. The SNP is the rs1061170 variant, also known as the Y402H polymorphism. This polymorphism occurs when a cytosine replaces a thymine, causing an amino acid substitution. To correct this mutation, the CRISPR-Cas9 method used was base editing. The experiment was effective at editing the SNP and there were no off-target mutations or indels detected. The study reported that a difficulty was identifying the safest and most effective single-guide RNA (sgRNA)

as some were proven to be ineffective while others were effective. However, it was also mentioned that the base editors used were extremely precise in performing their task, leading to no off-target effects. Overall, the study was successful and researchers believe that base editing could be a potential future therapeutic against the genetic aspects of AMD, especially in the rs1061170 variant of CFH³⁸.

CRISPR use on other Retinal Diseases

Sorsby fundus dystrophy (SFD) is a disease similar to AMD as it causes loss of central vision in the macula through RPE cell loss or choroidal neovascularization (CNV)³⁹. However, it is different to AMD as it has an earlier onset and it is an inherited autosomal dominant disease. This means that it is important for the families of patients with SFD to get genetic testing, while AMD is not necessarily an inherited disease⁴⁰. The autosomal dominant gene causing SFD is a mutation in the TIMP3 gene which is expressed in both RPE and choroidal endothelial cells. The mutation causes the Bruch membrane to thicken, and metabolites and nutrients to become less permeable across the Bruch membrane, ultimately leading to vision loss⁴¹. Elsayed et al.'s (2022)³⁹ study decided to focus on wet AMD and conducted genetic testing on a 35-year-old Caucasian male who had developed AMD at the early stage. This is related to SFD because of the early onset and because the diagnosis had bilateral CNV, which is a diagnosis of SFD. The patient carried the TIMP3 mutation, which is common in nAMD patients. The CRISPR technology used was base editing for the benefit of no DSBs. One limitation of base editing was mentioned, as the specific requirements for the PAM site need to be met, meaning to use base editing, very minor details need to be met to ensure no off-target mutations. The SFD mutation studied was an SNP of an adenine converted to a thymine. The wild-type sequence of the SFD gene had AGC encoding a serine amino acid, whereas the mutation codon is TCC which encodes a cysteine. This changes the structure and function of the protein synthesized. What could be done is to use a GBE to encode a serine residue, however, there is no PAM site for the GBE that would target the mutation³⁹. It is additionally determined that base editing is the best solution in this study due to the variety between the two studies utilizing base editors. In the study by Elsayed et al. (2022)³⁹, glycosylase base editors (GBE) were used. In the study by Tran et al. (2019)³⁸, 5 different base editor constructs were used to correct a cytosine to thymine mutation: BE3, SaBE3, SaKKH-BE3, VQR-BE3, and Target-AID. Therefore, it has been proven that there are a wide variety of applications of base editors for different mutations, making it a versatile tool.

Similar to AMD, retinitis pigmentosa (RP) is a group of diseases that cause photoreceptor degeneration. Unlike AMD, RP is inherited and causes blindness in younger people. Pre-

mRNA processing factor 31 (PRPF31) is one of the many genes encoding splicing factors that have known mutations linking to RP. This mutation can be a frameshift, missense, nonsense missense, replication, or large indels. This study used subretinal, intravitreal, and systemic injections to deliver an AAV-mediated CRISPR-Cas9 knock-out vector that will attempt to replicate RP in mice models. The subretinal injections caused the outer nuclear layer and retinal vessels to thin, resulting in RP. The intravitreal injections caused changes to the inner retina, which could be photoreceptor degeneration. RP is more known in patients with PRPF31-RP, which is why further studies need to be conducted to determine whether this can be studied more for RP. Finally, systemic injections caused stunted development and the death of the mice within the first 4 weeks, which could replicate PRPF31 impacts across all tissues. When using AAV-mediated gene augmentation, it prevented RP forming in PRPF31 and maintained the structures of photoreceptors, RPE cells, and other retinal structures. These mice models could transfer to use of CRISPR on humans because mice are similar to humans genetically, physiologically and anatomically⁴². This means that while the next step is human trials, mouse models give scientists a good understanding of whether the treatment could work or not. Mice and humans are 85% similar in the protein-coding segments of the genome, and 50% similar in the non-coding segments⁴³. Additionally, the general gene regulation system is similar in mice and humans (Comparing the Mouse and Human Genomes, 2015), which is why for genetic experiments, mice are good models to use. Xi et al., (2022)⁴⁴ showed how CRISPR-Cas9 knock-out links to RP and that AAV-mediated gene augmentation therapies could prove to be useful in treating the degenerative disease⁴⁴.

The RHODOPSIN (RHO) gene is another gene involved with RP. P23H, a common dominant mutation in the RHO gene, causes rod photoreceptor degeneration and retinal impairment, leading to vision loss. AAV was the method of interest for the retina as the retina is easily accessible, has a small size, and is compartmentalized. Gianelli et al. (2018)²⁵ experimented with inactivating an SNP in the P23H RHO variant using CRISPR-Cas9 via plasmid DNA transduction and AAV. The results of this study include successful targeting, and increased efficacy of CRISPR throughout the whole process. However, there were some limitations which included the risk of systemic inflammation. To combat this, Gianelli et al. (2018)²⁵ suggested AAV delivery via IV. Additionally, it was identified that AAV cannot be delivered in one dose, and more than one AAV is necessary to completely deliver the CRISPR constituents. This limitation was said to be overcome due to a new SaCas9 which can have a small package size with AAV. AAV delivery of CRISPR was deemed a plausible method of gene editing, but more research needs to be done as there needs to be more than one type of AAV to accurately

deliver the CRISPR²⁵.

Stargardt disease (STGD1) is another prevalent retinal disease that is inherited through an autosomal recessive matter. STGD1 causes vision loss, similar to AMD. The ATP-binding cassette (ABC), a group of membrane transporters, can be mutated and the ABCA4 gene's mutation can cause STGD1. This study used human induced pluripotent stem cells (hiPSCs) to observe the effects of CRISPR-Cas9 gene editing. The type of CRISPR editing was with single-stranded oligodeoxynucleotides (ssODNs) which triggers HDR DNA repair to add in the template. This repair system is called ssODN-mediated repair. Within the ABCA4 gene, two mutations were studied: a cytosine to thymine SNP, and an insertion of a GT, which was assumed to cause a frameshift mutation. Therefore, with more research conducted, it is predicted that this is a plausible future treatment for ABCA4 mutations causing STGD1. It is important that this study activated HDR and inhibited NHEJ to make the editing more efficient. Additionally, Siles et al. (2023)²⁶ noted that finding the distance in base pairs between the cutting site and editing site will make the editing more precise. To highlight the limitations, there are possibilities of Cas9 re-cutting and off-target mutations that future experiments need to be cautious about which is why more research needs to be done using CRISPR-Cas9 gene editing before this becomes a real treatment²⁶.

A final experiment reviewed focused on a mutation in the myocilin gene (MYOC) which causes glaucomas⁴⁵. Glaucomas are similar to AMD as they involve the degeneration of retinal cells, except for glaucomas, the specific cells are ganglion cells⁴⁶. It has been identified that the mutations in the MYOC gene are gain-of-function⁴⁵, meaning the product from the mutation will have a different level of gene expression or the function will be abnormal⁴⁷. This mutation has various harmful effects, including activating the unfolded protein response, which causes misfolding of proteins, and stress to the endoplasmic reticulum. To help decrease the MYOC mutation, Jain et al. (2017)⁴⁵ decided to experiment with CRISPR-Cas9 systems on the mutation to help find a more permanent solution. By experimenting on mice, the experiment successfully prevented glaucoma development. However, it is suggested that this might not be as permanent as expected, as the data does not address the lifetime of the solution. A concern mentioned was about the use of NHEJ repair, as there is the possibility of mutations creating different protein structures. Whether harmful or not, this is still a concern that needs to be addressed. Based on previous research, if a solution could be developed that uses HDR instead of NHEJ, the use of CRISPR for glaucomas could be less of a safety issue³⁰). However, the overall conclusion was that CRISPR-Cas9 is a beneficial future therapeutic despite the lifetime it might have⁴⁵.

Discussion

The studies in this literature review analyzed genes that have been proven to correlate to age-related macular degeneration and current CRISPR-Cas9 technologies. By analyzing what has already been done with CRISPR on AMD-related genes and other similar retinal diseases, we can conclude whether or not CRISPR technology is a good option for future AMD therapeutics. This review has collected many sources, all of which conclude that CRISPR has the power to help patients with AMD and other retinal diseases^{7,25,37-39,44}. However, we must be careful about which mutations are being edited and what types of CRISPR delivery methods are being used. The latter is the biggest point to think about as errors with CRISPR can cause detrimental unwanted consequences^{7,26,29,37}.

Based on the studies focusing on genes related to AMD and to other retinal diseases, base editing, AAV, and lipid nanoparticles are three of the most common methods to perform CRISPR-Cas9 editing. Base editing has been reported as the safest option as DSBs are not required, and Tran et al. (2019)³⁸ reported no off-target mutations. Based on these studies, it is important to factor in off-target mutations as they could be extremely harmful. Additionally, it was reported that AAV delivery was less efficient in the need for several AAVs²⁵, and lipid nanoparticles might be too nonspecific³⁵, potentially causing unwanted mutations (park), becoming a limitation to certain studies. Therefore, it depends on the research being conducted for lipid nanoparticles, but base editing was reported in these studies to be the safest option when focusing on AMD and other retinal diseases. However, base editing can only be used on SNPs⁴⁸. This is a major limitation because not all mutations are SNPs. Researchers have made efforts to widen the editing window for base editors, spanning up to four base pairs; however, this is still a major limitation in gene editing³⁴. Another limitation mentioned by Yee Wert (2022)³⁴ is the need to develop base editors' efficiency in hard-to-reach regions of the genome. Similar to base editing is prime editing which no studies reported using²⁹. Therefore, we cannot assume that prime editing is a plausible, effective, and safe solution, but its similarity to base editing does open up many potentialities for future research⁴⁸.

The two studies that were reviewed that utilized base editing in the methodology section focused on different genes. Both of them used base editing, but the study by Tran et al. (2019)³⁸ focused on the Y402H gene, found on chromosome 1²⁰, and the study by Elsayed et al. (2022)³⁹ focused on several genes, including the TIMP3 gene found on chromosome 22. This only supports the argument for the use of base editing since both studies were successful, showing the versatility and efficiency of base editing.

Next, this paper examined AAV-mediated CRISPR delivery. AAV delivery was more controversial in the results of

the studies. Xi et al.'s (2022)⁴⁴ experiment was the only one out of three AAV-mediated CRISPR studies reported in this literature review that concluded with safe and positive results. Chung et al. (2022)⁷ reported off-target mutations, which can lead to potentially harmful results, and Gianelli et al. (2018)²⁵ reported an increase in inflammation. A final limitation to AAV is the size. The packaging size for AAV is capped at 4.7 kb, only allowing AAV to be applicable for some mutations³⁴. Therefore, it is suggested to focus on other CRISPR delivery systems, as AAV might not be the safest based on what has previously been researched. As suggested by Chung et al. (2022)⁷, lentivirus or lipid nanoparticle methods should be researched instead of AAV.

This leads to the next method studied, lipid nanoparticle delivery. Chung et al. (2022)⁷ suggest lipid nanoparticles over AAV as it is a safer option. The study by Chung et al. (2022)⁷ did not focus on lipid nanoparticles, but it was recommended, which indicates that it should be looked into further. In another study, it was concluded that lipid nanoparticles are beneficial as they are nonspecific in cell targeting. This indicates that within the macula, lipid nanoparticles can target RPE cells and Muller cells simultaneously³⁵. Hence, in the case of AMD, it could be a good system to research further as all of these cell types can be edited in a more efficient manner. However, other studies have proven differently. A study focusing on the transthyretin gene resulted in a 97% decrease in serum transthyretin levels from the use of lipid nanoparticle delivery of CRISPR⁴⁹. Also stated by Wilbie et al. (2019)⁴⁹, with weekly or monthly injections of lipid nanoparticles, CRISPR has a higher efficiency rate. Therefore, lipid nanoparticles do appear to be a better choice than AAV, since it does have a high success rate, but it also shares the limitation of only being able to be used in certain cases like base editing. There are also more limitations, such as the size of the nanoparticles being less than 10nm, and how lipid nanoparticles need to use HDR instead of NHEJ for safer editing⁵⁰. In comparison with base editing, which minimizes the use of NHEJ, it has been proven that HDR is safer than NHEJ in relation to off-target mutations. For such reasons, base editing is the better option for treating AMD with CRISPR.

There are many other factors to AMD that need to be considered. For example, AMD is a degenerative disease, meaning it needs to be identified as early as possible to potentially stop the disease⁵¹. Even if the disease progresses and therapeutics are developed that can stop the disease, the degeneration that has already taken place cannot be undone. Therefore, the patient will still have some loss of vision. Ideally, the genetic mutations should be detected as early as birth so diseases such as AMD do not have the chance to develop. This introduces mosaic CRISPR. Mosaic CRISPR occurs when CRISPR is used on embryos. Embryos are rapidly dividing and always changing, so when CRISPR is injected into an em-

bryo, it is not as effective as reaching all the cells. Additionally, it can have different effects on embryonic cells in their different stages of progression. This could lead to further mutations in the embryo (Mehrvan et al., 2019)⁵², creating even more potentially harmful effects from CRISPR. CRISPR editing on cells is supposed to result in two alleles; however, when cells are rapidly dividing, this can produce more than two alleles in the organism. Mosaicism occurs when DNA replication occurs before the CRISPR injection, meaning there are more sets of DNA⁵³. Mosaic CRISPR could cause an organism's cells to have differently edited genes, causing a major issue. Therefore, before CRISPR is used on a single organism during development, it is crucial for more research to be done on how to edit the whole organism's cells the same way. Lamas-Toranzo et al. (2019)⁵³ have suggested some ways to reduce mosaicism, including early delivery of CRISPR and early microinjections. Therefore, it is crucial to use CRISPR before the S phase, the phase of DNA replication, to ensure a reduced rate of mosaicism.

Another factor of AMD is the multifaceted nature of the disease. Since AMD can be genetic, environmental, and pathophysiological^{5,6}, CRISPR therapeutics do not ensure the disease will not be diagnosed. CRISPR can help prevent the disease from developing from the genetic side, but studies have identified environmental factors like smoking and pathophysiological factors like eye cells and VEGF to develop the disease.

Finally, while the VEGF gene may not be as much of a cause of AMD as the CFH gene, it is still a factor to consider with the disease. This is due to the fact that where the CFH gene mutation has been linked to causing AMD¹⁶, the VEGF gene seems to be more prominent in emphasizing the symptoms of AMD, such as vision loss. VEGF stimulates angiogenesis, so having a wild-type genotype for VEGF will cause angiogenesis, increasing the vision loss symptom of AMD¹³. Whilst it is stated by Chung et al. (2022)⁷ that it may be dangerous to suppress the VEGF gene, a mutation in the VEGF gene that potentially stops angiogenesis could theoretically be beneficial for patients with AMD. Therefore, it is seen that the VEGF gene needs to be turned off so the symptoms of AMD are stopped, which the injections are attempting to do¹¹. Due to these uncertainties, more research should be conducted on the relationship between the VEGF gene and AMD to better conclude whether VEGF needs to be addressed more or not.

Additional limitations of editing the VEGF gene include the risk of infection, the short lifespan of the injection requiring frequent doses, and the limited efficacy of the treatment. While the need for frequent injections might not sound like a limitation, it can cause other problems such as retinal detachment and change in pressure in the eyes. Additionally, anti-VEGF treatments are a tremendous burden on the older population affected with AMD. There have also been cases with the

current treatment of Brolucizumab causing ocular inflammation⁵⁴. Not only are there genetic concerns with the necessity of anti-VEGF treatments, but there are other responses to the treatment that might not make it the safest and most effective treatment possible.

There are also considerations to think about with CRISPR therapeutics. CRISPR is a beneficial therapeutic as seen through the various studies mentioned before^{7,38,39,44}. It has been proven, through various delivery methods, to effectively edit diseased genomes. However, CRISPR is prone to off-target mutations, especially with AAV delivery^{7,25}, DSBs²⁹, and NHEJ³⁰. These off-target mutations can be harmful due to the unknown edit. These mutations could cause harmless edits, but they could also cause unwanted indels²⁹. Solutions to maximizing the safety of CRISPR has been suggested. Some examples are reducing the use of NHEJ and increasing the use of HDR³⁰. Another example is using base editors or Cas9 nickase instead of Cas9 to induce SSBs instead of DSBs. By deleting only one nucleotide, there is a lowered risk of part of the genome being 'lost', ensuring a reduced risk of random mutations³³. A final solution would be to use cell-type-specific methods as they are proven to reduce the amount of unwanted mutations produced, increasing the overall safety of CRISPR³⁷. CRISPR has been identified by many professionals to be a future therapeutic; however, to become a future therapeutic, CRISPR should be developed more to ensure maximum safety.

In addition to the molecular limitations of CRISPR, there are also real-world implications of CRISPR. A benefit of CRISPR in the real world is the agricultural effects, such as performing genetic modifications and breeding for cheap prices⁵⁵. This could make healthier, stronger, and more crops, helping the agricultural field. However, there are also limitations in the genetic editing field. When thinking of gene editing, a major concern is designer babies. This is when people change the genes of their babies so they will have the traits the parents desire. Additionally, when genetic editing is done on germ cells, this will make the new trait heritable, and the issue is that this is possible⁵⁶. With the increased use of genetic editing tools like CRISPR, it does hold benefits with cost and treating disease, but there are also many ways society can misuse it.

Conclusion

AMD is an extremely complex disease that has both genetic and environmental factors. While CRISPR-Cas9 technology has the potential to solve many genetic mutations within the case of AMD, it is uncertain if the application of this technology will be sufficient. Such molecular uncertainties include the efficiency, safety, and best delivery method to correct the targeted genetic mutation. There are also other non-molecular

uncertainties surrounding CRISPR technology, including social and ethical dilemmas that need to be taken into account when creating a widespread use of CRISPR. There have been studies conducted on two prominent AMD genes: VEGF and CFH. There have also been studies conducted on diseases with similar symptoms to AMD, such as RP, SFD, and STGD1. All of these studies used a range of CRISPR-Cas9 delivery methods which could help us understand which will be most effective and safe. The three main CRISPR delivery methods identified were base editing, AAV, and lipid nanoparticles, with the conclusion that base editing is the safest. Base editing introduces single-strand breaks instead of DSBs, decreasing the risk of unwanted mutations both on and off-target. However, with CRISPR, there are always limitations and risks that need to be taken into account. One limitation is the risk of random mutations, which could lead to more harm. Other limitations include inflammation and mosaic CRISPR. In applying CRISPR to AMD, a further limitation might be if CRISPR can realistically be used to stop the progression of the disease, as there is no definitive genetic mutation identifying the development of AMD. Even though there are these limitations, the researchers all came to the conclusion that with more research and experimentation done, CRISPR has the potential to be a solution in the fight against AMD. Future studies that should be done are more applications of different CRISPR delivery methods, such as base editing and prime editing, on the Y402H and VEGF genes. With more studies and evaluations done, there can be a better understanding of the implications and possibilities of CRISPR use on AMD. A key research question should be if base editing is feasible and successful on the Y402H gene. Another research question could be looking into prime editing for non-SNP mutations and how this could compare in efficiency and safety to the other more commonly used CRISPR delivery methods. To mitigate off-target mutations and other harmful effects of CRISPR, it is suggested that there be developments of ways to use CRISPR with HDR instead of NHEJ, reducing the off-target mutations. With the implementation of safer CRISPR systems, this could apply to the treatments of AMD in the future and potentially create a more permanent solution to the disease, allowing patients to preserve as much of their vision as possible and not cause complete vision loss.

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