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Treating Alzheimer's and ATTR using CRISPR-Cas9 to Target Amyloidosis

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ABSTRACT

Approximately 50 million people live with Alzheimer's (Alz) and 50,000 people are living with Transthyretin Amyloidosis (ATTR) worldwide. Both Alz and ATTR are types of amyloidosis, a disease where amyloid protein builds up in the body's organs. Alz and ATTR are conditions with no known cures and similar etiologies but different manifestations in the brain and heart, respectively. Alzheimer's disease is irreversible, which causes lifelong suffering for patients and their families. ATTR is a potentially fatal disease that causes heart problems for patients. Amyloidosis arises due to genetic mutations in the amyloid precursor protein (APP) or proteins involved in the generation of APP such as *BACE1*, *PSEN1*, *PSEN2, APOE* and transthyretin (*TTR*) genes, which result in the overexpression and accumulation of amyloid protein. CRISPR-Cas9 is a breakthrough gene editing technology that is capable of editing mutations in multiple genes with high specificity. This technology has the capability of treating both Alz and ATTR through targeted gene editing of the mutations that contribute to these diseases. This review will focus on *BACE1, PSEN1, PSEN2, and APOE* as targets for Alz and TTR as a treatment for ATTR through the use of CRISPR-Cas9. Various delivery mechanisms and current clinical trials will be discussed to identify the best delivery route of these treatments for the respective disease . A CRISPR-Cas9 derived treatment would bring new hope for patients with these diseases and could propel cures for other types of amyloidosis.

Background

Amyloidosis is a disease caused by misfolded protein aggregates that can damage cells and organs. Protein misfolding can damage cells due to deposition and accumulation of aberrant protein fibrils (Wechalekar et al., 2016; Vaxman et al., 2020). There are various types of amyloidosis, including localized and systemic forms, each of which is caused by a unique combination of protein subunits. Localized amyloidosis is focused in one specific area of the body, while systemic amyloidosis occurs in the entire body. Alzheimer's disease (Alz) and Transthyretin Amyloidosis (ATTR) are the most prevalent forms of systemic amyloidosis (Vaxman et al., 2020).

Alzheimer's Disease

In 2018, it was estimated that 50 million people worldwide had Alzheimer's disease, and this number is expected to triple by 2050, with two-thirds of cases occurring in developing nations (Patterson, 2018). It is anticipated that the number of cases in Europe will double by 2050 (Scheltens et al., 2021). Additionally, Alz tends to be more prevalent in women than in men, though the reasons underlying this are unclear (Rajan et al., 2021). There are two distinct forms of Alzheimer's disease: early onset and late onset, where the disease can occur due to genetic determinants. This paper will focus on the genetics of Alz with a late onset phenotype. After a diagnosis of dementia, which is any decline in condition significant enough to interfere with a person's daily function, the average survival time in the United States is three to four years, while in Europe it is six years (Gale et al., 2018; Mayeda et al., 2017; Rhodius-Meester et al.,

2019). Also, the prevalence of dementia is found to be three times higher in patients over 85, with the disease becoming more prevalent as people age (Jack Jr et al., 2019; Scheltens et al., 2021). The risk of developing Alz increases after the age of 65, likely due to the higher load of tau protein (van der Lee et al., 2018).

In 60-80% of all Alzheimer's cases, the presence of one *APOE4* allele is the most important risk factor (van der Lee et al., 2018). In addition, 40 distinct risk alleles have been identified, which can be used to calculate a polygenic risk score with a 75-85% degree of accuracy (Escott-Price et al., 2017). Rare protein-damaging variants in genes such as SORL1, ABCA7, and TREM2 are additional risk factors (Scheltens et al., 2021), and carriers of the *APOE2* allele have a twofold increased risk of Alzheimer's disease. However, certain genetic variants, such as the Ala673Thr Icelandic protective mutation of *APP*, the Pro522Arg amino acid change in the PLCG2 gene, and the *PSEN1* mutation and the klotho longevity gene, have been associated with improved cognitive health and may provide protection against Alzheimer's (Proft et al., 2012; Sims et al., 2017; Arboleda-Velasquez et al., 2019; Belloy et al,. 2020; Scheltens et al., 2021). This paper will focus on BASE1, PSEN1 and PSEN2, and APOE genetic determinants of Alz, as will be discussed below.

Historically, Alzheimer's was diagnosed solely based on pathological characteristics such as amyloid plaques and neurofibrillary tangles during autopsy. However, technological advancements have led to the use of scans for earlier detection, although they are not yet perfected. The most established scans for neurodegeneration are medial temporal lobe atrophy on MRI and posterior cingulate and temporoparietal hypometabolism on 18FDG-PET (Frisoni et al., 2017). Amyloid-PET is used to measure amyloid beta deposition in the cortex (Rhodius Meester et al., 2020), the relevance of which will be described below. National Institute on Aging and Alzheimer's Association have established diagnostic criteria for Alzheimer's disease, which include abnormal brain imaging or cerebrospinal fluid for preclinical cases, unexplained mild memory impairment, severe memory impairment, and dementia (Mckhann et al., 2011; Scheletens et al., 2021).

Alzheimer's symptoms are associated with changes in specific brain regions and receptors, which can be identified using imaging techniques such as fluorodeoxyglucose positron emission tomography (PET) (Sultzer et al., 2003). There are correlations between delusions and decreased glucose metabolism in specific brain regions, and altered glucose metabolism has been linked to non-cognitive psychiatric symptoms (NPS) such as anxiety, apathy, agitation, and disinhibition citation). Mild cognitive impairment (MCI) and other symptoms including apathy, anxiety, irritability, and depression may be present in the early stages of the disease (Hashimoto et al., 2006; Lyketsos et al., 2011).

The Alzheimer's disease mechanism of action involves numerous cellular modifications and processes. Alzheimer's preclinical phase is characterized by changes in the brain in various cell types such as neurons, microglia, and astroglia as well as the presence of neuroinflammation, blood vessel changes, and glymphatic system dysfunction (De Strooper et al., 2016; Venegas et al., 2017; Sweeney et al., 2019; Plog et al., 2018). APP is a transmembrane glycoprotein that is cleaved by γ- secretase and β-secretase to form Aβ40 and Aβ42 (Rukmangadachar et al., 2022). Amyloid plaques consist of Aβ40 and Aβ42 that have been abnormally folded and are byproducts of *APP* metabolism (Serrano-Pozo et al., 2011). The *APOE* and *TREM2* genes contribute to the overgrowth of microglia, which are specialized brain-resident immune cells, believed to be due to its response to amyloid β plaques (Keren-Shaul., 2017). In this response, APOE binds to Aβ plaques and Alz variants of TREM2 bind less with APOE (Yeh et al., 2016). Microglia's role in Alz pathogenesis must be researched further as many different gene risks converge at microglial pathways to better identify what causes amyloid β plaque formation (Sierksma et al., 2020). In addition, neurofibrillary tangles are another manifestation of Alz in the brain. Neurofibrillary tangles are caused by the formation of tau fibrils. Together with amyloid plaques, it is predicted that these neurofibrillary tangles may contribute to Alzheimer's symptoms, although further research is needed to define the role that each may play in disease etiology or progression. In older patients, the presence of amyloid plaques and neurofibrillary tangles is associated with neurodegeneration, synaptic and neuronal loss, and can be accompanied by vascular disease and Lewy bodies (Lane et al., 2018). Alzheimer's disease proliferation via amyloid plaques is thought to begin in the isocortex and spreads to subcortical structures, whereas the development of neurofibrillary tangles from hyperphosphorylated tau is thought to begin in the allocortex

of the medial temporal lobe and spreads to the associative isocortex (Serrano-Pozo et al., 2011; Scheltens et al., 2021; Lane et al., 2018).

Current Alzheimer's treatments include lifestyle modifications such as improving diet and physical activity, as well as intensive blood pressure control (Williamson et al., 2019). It has been shown that lifestyle-based interventions that target multiple cognitive domains reduce the risk of cognitive impairment in at-risk populations (Williamson et al., 2019). Inhibitors of cholinesterase and the antagonist of N-methyl-D-aspartate receptors, memantine, are also used as treatments through targeting the glutamate site of the N-methyl-D-aspartate receptor to inhibit glutamate (Scheltens et al., 2021; Atri et al., 2018). 5-HT6 receptor antagonists are used as symptomatic treatments for Alz to block the 5HT6 receptor, increasing the release of glutamate, but they are only used to treat symptoms and cannot cure the disease(Andrews et al., 2018). In a basket clinical trial, it was demonstrated that the 5-HT2A receptor inverse agonist pimavanserin is effective for treating psychosis associated with dementia (Cummings et al., 2018). Brexpiprazole, citalopram, and nabilone were prescribed for the treatment of agitation, whereas escitalopram, prazosin, dextromethorphan, and Lemborexant were prescribed for irregular sleep-wake rhythm disorder (Liu et al., 2016; Janto et al., 2018). Today, monoclonal antibodies such as Aducanumab, BAN2401, and gantenerumab are used to slow disease progression by removing amyloid oligomers and plaques (van Dyck., 2018; Scheltens et al., 2021). Alzheimer's disease is too prevalent to ignore new treatments and utilizing CRISPR could be one way of treating it.

ATTR

Transthyretin Amyloidosis is a rare genetic disorder that causes amyloid deposits to form in a variety of tissues, including the nerves, heart, and leptomeninges, and is found in more than 10% of people over the age of 80 in postmortem studies (Cornwell et al., 1983). Familial Amyloid Polyneuropathy (FAP), Familial Amyloid Cardiomyopathy (FAC), and Familial Leptomeningeal Amyloidosis (FLA) are the three main phenotypes of the disease and will be considered throughout this review (Cornwell et al., 1983; Westermark et al., 1990). The most common phenotype is FAP, with many patients coming from Portugal, Sweden, and Japan (Sekijma et al., 2005). FAP can appear early or late in life, and the specific *TTR* mutation can influence the age of onset (Sekijima et al., 2005). FAC is distinguished by amyloid deposits in the heart, which cause heart dysfunction (Sekjima et al., 2005). FLA is the rarest phenotype marked by amyloid deposits in the leptomeninges, the membranes that surround the brain and spinal cord (Maia et al., 2015). Wild-type ATTR, a fourth type of the disease that occurs in people who do not have a known *TTR* mutation, is also being recognized as a distinct phenotype (Ueda et al., 2011; Sekijima et al., 2011). These individuals traditionally show symptoms such as congestive heart failure, atrial fibrillation and intractable arrhythmia (Sekijima et al., 2011)
Transthyretin amyloidosis (ATTR) is diagnosed through a series of steps that confirm the presence of amyloid deposits and the type of amyloid protein. Initial diagnosis occurs through finding clinical symptoms and analysis of amyloid deposition in biopsy samples, when appropriate (Simmons et al., 1993; Koike et al., 2004). The *TTR* gene, which will be further discussed below, can also be used to confirm inherited forms of ATTR. *TTR* gene analysis is used to identify disease-causing mutations in hereditary ATTR (Sekijima et al., 2006). Although invasive, endomyocardial biopsy can provide tissue samples for histopathological analysis (Simmons et al., 2006; Koike et al., 2004). The use of Congo red staining and immunocytochemical studies on amyloid-positive tissue can also aid in determining the type of amyloid protein, as appropriate (Simmons et al., 2006; Koike et al., 2004). This test, however, is rarely used because of how intricate and time consuming it is. Abnormal uptake of the radioisotope 99mTc in myocardial scintigraphy can suggest ATTR cardiac amyloidosis, but histopathological confirmation is still required (Ikeda et al., 1982). Immunocytochemical or proteomic analysis of amyloid-positive tissue in the case of Wild Type ATTR can confirm the type of amyloid protein (Ikeda et al., 1982). An alternative histopathological test is a surgical skin biopsy, and tenosynovial tissues obtained during carpal tunnel surgery can also be an indicator of ATTR amyloidosis (Sekijima et al., 2006).

ATTR symptoms can vary depending on the age of onset and the type of the condition. Early-onset ATTR is characterized by peripheral neuropathy, autonomic neuropathy, and cardiomyopathy, whereas late-onset ATTR is characterized by ophthalmopathy and leptomeningeal amyloidosis (Sekijima et al., 2011). Early-onset ATTR

peripheral neuropathy is distinguished by loss of superficial sensation, including nociception and thermal sensation, as well as significant autonomic dysfunction (Sekijima et al., 2011). Late-onset ATTR is often characterized by amyloid cardiomyopathy and male predominance, and it can result in heart failure, atrial fibrillation, and intractable arrhythmias (Sekijima et al., 2005). More than 10% of people over the age of 80 have wild type ATTR, which is a common aging-related phenomenon (Ueda et al., 2011; Cornwell et al., 1983). ATTR can cause congestive heart failure, autonomic dysfunction, and central nervous system dysfunctions like spastic paralysis, ataxia, convulsions, and dementia (Maia et al., 2015).

TTR is a homotetrameric protein complex of 55 kDa that is synthesized in the liver and secreted into the bloodstream (Blake et al., 1974). It is in charge of transporting thyroxine (T4) and the retinol binding protein-vitamin A complex (Blake et al., 1974). ATTR amyloid fibrils are formed by the dissociation of *TTR* tetramers into monomers, which is followed by partial misfolding and mis-assembly into ATTR amyloid fibrils (Colon et al., 1992; Lai et al., 1996). *TTR* mutations can destabilize native quaternary and tertiary structures, promoting tetramer dissociation and monomer unfolding (Sekijima et al., 2006). ATTR amyloid fibril formation is also aided by a decrease in TTR cell secretion efficiency, which irreversibly forms protein aggregates (Hurshman et al., 2004). TTR protein terminal fragments produced by trypsin proteolysis, as well as extracellular factors such as sulfated glycosaminoglycans like heparin, can all contribute to the formation of ATTR amyloid deposits (Mangione et al., 2014; Bergstrom et al., 2005; Ihse et al., 2013; Bourgault et al., 2011).

The *TTR* gene mutation causes the most common form of ATTR, for example, the Val30Met mutation causes familial amyloid polyneuropathy (FAP) (Sekijima et al., 2006). The presence of amyloid deposits in the heart characterizes familial amyloid cardiomyopathy, in addition to the Val122Ile mutation (FAC), whereas Asp18Gly, Ala25Thr, and Tyr114Cys mutations cause familial leptomeningeal amyloidosis (FLA) (Sekijima et al., 2006). The accumulation of wild-type TTR protein in systemic organs, without genetic mutation, causes wild-type ATTR (ATTRwt), though the underlying cause of amyloid protein buildup is unknown (Sekijima et al., 2006). ATTR is currently treated with drugs such as Diflunisal and Tafamidis, which worked to stabilize the TTR tetramer and prevent the formation of amyloid fibrils in recent clinical trials (Sekijima et al., 2006; Tojo et al., 2006). Another option for treatment is liver transplantation, which may replace the source of TTR production and prevent further amyloid formation (Tsuchiya et al., 2008). This, however, is a difficult, risky, and invasive procedure. Antisense oligonucleotides (ASOs), small interfering RNA (siRNA), and combination therapies using Doxycycline and tauroursodeoxycholic acid are among the other therapeutic approaches being studied (Benson et al., 2006; Coehlo et al., 2013; Obici et al., 2012; Sekijima et al., 2006). Immunotherapy is also being investigated as a possible treatment option for ATTR. These treatments aim to reduce the formation and accumulation of amyloid aggregates, improve affected individuals' symptoms, and slow disease progression (Terazaki et al., 2006; Su et al., 2012). More research is needed, however, to improve treatment of ATTR and to develop safe and effective ATTR therapies and possibly even cure the disease.

CRISPR-Cas9

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) was discovered in the genome of Escherichia coli in 1987 as a series of short direct repeats interspersed with short sequences (Ishino et al., 1987; Doudna et al., 2014). Initially, it was defined to be an adaptive defense system for bacteria that served as a memory of previous attacks (Makarova et al., 2011). The first evidence of adaptive immunity in lactic acid bacteria using CRISPR was reported in 2007, and it was discovered in 2008 that CRISPR RNA could be used as a guide to interfere with virus formation (Barrangou et al., 2007; Brouns et al., 2008).

CRISPR-Cas systems are divided into three types (I, II, and III), each of which employs a unique molecular mechanism for nucleic acid recognition and cleavage (Koonin et al., 2019; Makarova et al., 2006). The CRISPR-Cas system has been used in biotechnology and is commonly used in genome editing in biology research. The system cleaves DNA using HNH and RuvC-like domains, and the sgRNA directs the Cas9 protein to the correct location via base pairing (Bolotin et al., 2005; Makarova et al., 2006; Jinek et al., 2012). Breaks in DNA are more likely to occur

at protospacer adjacent motif (PAM) sites that are partially complementary to the guide RNA sequence (Mojica et al., 2005; Horvath et al., 2008).

CRISPR has become a valuable tool for genome editing due to the ability to introduce specific double-stranded breaks in DNA at a target site and subsequent repair by the cellular repair mechanisms (Choi et al., 2014). Furthermore, CRISPR can multiplex, or edit multiple sites in the genome at the same time, which has made it a popular choice for a wide range of biotechnological applications (Cong et al., 2013; Mali et al., 2013). However, the most serious issue with CRISPR is off-target effects and chromosomal translocations (Sternberg et al). Regardless, CRISPR-Cas9 has been used to replicate tumor-associated chromosomal translocations and to study gene functions in mammalian cells (Choi et al., 2014).

CRISPRi is a catalytically deactivated version of Cas9 (dCas9) that has been repurposed for targeted gene regulation (Qi et al, Sternberg et al). dCas9 has been used to repress multiple target genes at the same time, and effects are reversible (Qi et al., 2013; Zhao et al., 2014; Gilbert et al., 2013). It can also block transcriptional elongation, RNA polymerase binding, and transcription factor binding (Qi et al., 2013). As a result, CRISPRi has become a valuable tool for studying gene function and regulation and in the future could be applied to disease treatment by modifying gene expression rather than gene knock-out or correction.

Applying CRISPR-Cas9 and CRISPRi for diagnosis and treatment Alzheimer's disease

Late onset Alzheimer's disease is the most common type of dementia that develops after the age of 65 (Lu et al., 2021). It is a progressive disease with no cure that affects memory, thinking, and behavior (Lu et al., 2021). A combination of genetic, environmental, and lifestyle factors contribute to late-onset Alzheimer's, however, CRISPR-Cas9 technology has emerged as a potential treatment through gene editing.

The *BACE1* gene is thought to be important in contributing to Alz because it codes for a type I transmembrane protein involved in the errant production of Aβ (Hampel et al., 2021; Zhu et al., 2018; Haniu et al., 2000). The *BACE1* protein is widely expressed in the brain, and pancreas, and cleaves the APP protein β-site (Willem et al., 2015). The A673T mutation in the *APP* gene has been linked to 20-30% lower levels of soluble APP in comparison to controls, and carriers have 28% lower levels of Aβ40 and Aβ42 in plasma (Jonsson et al., 2012; Martiskainen et al., 2017). This protective effect is due to the BACE1 failing to cut APP to contribute to Alz pathology. Several BACE1 inhibitors have been developed, but their effectiveness has been limited by the large active site on the BACE1 protein and regulation via posttranslational modifications such as phosphorylation and ubiquitination (Moussa-Pacha et al., 2020; Citron et al., 2002; Walter et al., 2001; Kang et al., 2010). An intraperitoneal injection of 8 mg/kg of a BACE1 inhibitor resulted in a 30% reduction in Aβ40 brain levels in transgenic mice after 4 hours, according to in vivo studies (Moussa-Pacha et al., 2020; Ghosh et al., 2007). Although CRISPR-Cas9 technology provides a novel approach to targeting the *BACE1* gene, crossing the blood-brain barrier remains difficult since it is not very permeable and difficult to penetrate causing many drugs to fail (Yuan et al., 2013). Additional research is required to resolve this common obstacle for brain targeting drugs. Cas9 nanocomplexes containing sgRNAs targeting *BACE1* were successfully delivered into mouse primary neurons, with no off-target effects. Injection of the nanocomplexes into 6-month-old Alz mice resulted in a 34% decrease in *BACE1* expression and *APP*-β-cleavage products (Park et al., 2015; Lu et al., 2021). Further reduction of *BACE1* expression was achieved by adding an amphiphilic R7L10 peptide to the Cas9-sgRNA complexes, which aids in reducing *BACE1* expression to treat Alz (Park et al., 2015; Lu et al., 2021). Here, CRISPR could be used to eliminate *BACE1* gene expression through using an sgRNA and potentially treat or halt progression of Alz by preventing the incorrect cleaving of APP.

The CRISPR-Cas9 system was used to investigate the genes Presenilin-1 (*PSEN1*) and Presenilin-2 (*PSEN2*), which are transmembrane proteins that are essential components of the γ-secretase complex (Hutton et al., 1997). *PSEN1* and *PSEN2* share 60% similar genetic sequences, which gives good indication that treatments have a chance

to work for both (Park et al., 2019). The differences however, provide an indication about why *PSEN2* is less pathogenic compared to *PSEN1,* since PSEN1 lacks the sorting motif that PSEN2 has (Escamilla-Ayala et al., 2020). The researchers eliminated the background of endogenous γ-secretase, which produces Aβ, by knocking out the *PSEN1* genes in N2a cells, a mouse neural cell line (Sun et al., 2017). The L226F variant in PSEN1 TM5 showed the greatest increase in Aβ40 production (Sun et al., 2017; Lu et al., 2021). Exogenous addition of recombinant wild-type protein, on the other hand, reduces Aβ42 and Aβ40 production (Sun et al). Increased levels of Aβ were detected in converted neurons from mutant homozygous and heterozygous iPSCs than in isogenic controls derived from Alz patients (Paquet et al., 2016). This advances our understanding of the molecular mechanisms of Alzheimer's disease and could lead to earlier and accurate diagnosis. Using the CRISPR/Cas9 system, a novel V97L missense mutation in the PSEN1 protein was established in a Chinese familial Alz pedigree, and the level of Aβ42 was significantly elevated in the mutation type SH-SY5Y cells (Fang et al., 2006). Similarly, in the case of the PSEN2 N141I mutation, cell lines showed an increase in the Aβ42/40 ratio in iPSC-derived neurons, but this was normalized after the PSEN2 mutation was corrected using CRISPR/Cas9 (Ortiz-Virumbrales et al., 2017). Increased production of Aβ42 was observed in transgenic mice and neural cell lines, with a doubling of the Aβ42/40 ratio, a 50% increase in secreted Aβ40, and a 150% increase in Aβ42 (Ortiz-Virumbrales et al., 2017). CRISPR-Cas9 could be used to knock out *PSEN1* or *PSEN2* to treat Alzheimer's disease.

Apolipoprotein E (*APOE*) is a gene that has been linked to an increased risk of late-onset Alzheimer's disease, also known as sporadic Alz (SAD) (Huang et al). *APOE* is available in several alleles, including *APOE2, APOE3, APOE3r*, and *APOE4* (Serneels et al). The *APOE4* isoform is especially troublesome because it is a known genetic risk factor for SAD (Huang et al., 2017; Lu et al., 2021). Researchers attempted to address this issue in mouse astrocytes by using CRISPR-Cas9 to convert *APOE4* to *APOE3r*. This was accomplished by changing the C at position 158 to T, resulting in the conversion of R158C. The conversion rate of total DNA sequencing reads was 58-75%, with 36-50% editing at the third position of codon 158 and 38-55% editing at the first position of Leu159 (Komor et al., 2016). Wadhwani et al also used CRISPR-cas9 to convert the E4 allele into an E3/E3 genotype in Alzheimer's patients' induced pluripotent stem cells (iPSCs) (Wadhwani et al., 2019). This correction has been shown to reduce cytotoxicity, tau phosphorylation, and has the potential to ameliorate multiple Alzheimer's disease-related pathologies (Wadhwani et al., 2019; Lu et al., 2021). Thus, CRISPR could be used to target *APOE,* as prior research provides good indications towards future development of treatments. More research is needed to see if CRISPR could be used to target these various genes and treat Alz in the future.

Applying CRISPR-Cas9 for diagnosis and treatment of Transthyretin Amyloidosis

Transthyretin Amyloidosis is a debilitating disease characterized by the overgrowth of amyloid fibrils in various organs of the body due to protein malfunction. The use of CRISPR-Cas9 gene editing technology is already being explored in the clinic as a potential treatment option for this condition (Finn et al., 2018). Clinical trials have been conducted using NTLA-2001 to target the transthyretin (*TTR*) gene responsible for the disease (Finn et al., 2018). The trials involved administering the treatment intravenously to patients between the ages of 18 and 80 who had hATTR amyloidosis (with or without cardiomyopathy), with a body weight of 50-90 kg (Gillmore et al., 2021). The study was conducted in two initial dose groups (0.1 mg per kilogram of body weight or 0.3 mg per kilogram of body weight) between November 2020 and April 2021(Gillmore et al., 2021).Results of the drug NTLA-2001 showed a significant reduction of *TTR* in primary human hepatocytes, with reductions of up to 90% achieved (Gillmore et al., 2021). A limitation of this study is the small number of patients, but with further trials and testing, NTLA-2001 could prove a worthwhile treatment to pursue further.

Cynomolgus monkeys and transgenic mice also showed nearly complete elimination of serum *TTR* expression, with a 91% decrease of mRNA in cells and a 95% decrease of protein in cells (Gillmore et al., 2021; Finn et al., 2018). In preclinical studies, seven loci were identified as possible editing sites in noncoding regions, with a mean *TTR* reduction of 52% and 87% for the lower and higher dose groups, respectively (Gillmore et al., 2021).

Other options for targeting the *TTR* gene using CRISPR include using a biodegradable lipid nanoparticle-based delivery system to carry a single-guide RNA for in vivo editing (Finn et al., 2018). This approach induced a dose-dependent increase in DNA editing in the liver with decreases of up to 97% in serum TTR levels for 12 months in animal models (Habtemariam et al., 2021). These findings suggest that CRISPR-Cas9 technology could be a promising treatment option for transthyretin amyloidosis, though further research is necessary to determine the viability of this treatment on a larger scale.

Delivery Methods

Choosing the right delivery method for gene knockout is crucial to providing adequate care towards both diseases. Each delivery method has its own pros and cons, and by identifying these, it will provide the best chance to utilize the delivery method suited for treatment. There are two kinds of delivery methods, viral vectors, and non-viral vectors.
Viral vectors are one of the most important mechanisms for delivering genetic material into cells in gene therapy. Lentivirus is a common viral vector that involves modification to remove pathogenicity (Li et al., 2015). This modified virus would carry genetic material packaged into viral particles that are introduced into the body and efficiently infect host cells (Jost et al., 2017). One advantage of this method is it permanently integrates into the host genome, which enables the change to be passed down generations (Li et al., 2015). Adenosine Associated Virus (AAVs) is another commonly used viral vector (Hardcastle et al., 2018). It is an uncoated single-stranded linear DNA virus with a broad host range and low pathogenicity (Hardcastle et al., 2018). This vector also has a long period of expression, making it suitable for long-term gene therapy (Hardcastle et al., 2018). Using promoters to drive transgenes, on the other hand, can result in non-ideal ectopic expression (Lykken et al., 2018). Furthermore, the AAV limited 4.7 kg base packaging capacity limits the use of genes larger than 3.5 kb, which can be a challenge in certain gene therapy applications (Wu et al., 2010). AAV's have been used before as a mini-cytomegalovirus promoter/enhancer with SpCas9 successfully corrected a muscular dystrophy mutation in mice (Long et al., 2016). Nonetheless, viral vectors continue to be a promising tool in the field of gene therapy, and they are being developed and optimized for a variety of applications.

Another important approach in gene therapy is non-viral vector delivery mechanisms. One such mechanism is nanoparticles, which encapsulate drugs and allow them to pass through the cell membrane via endocytosis before releasing the drug into the organism (Verma et al., 2019). Another delivery approach involves liposomes or lipid nanoparticles (LNPs), which are ultrafine particles with diameters ranging from several nanometers to several micrometers formed by phospholipid alignment (Lino et al., 2018). The inner and outer surfaces of the liposome are formed by the hydrophilic head, while the bilayer structure is formed by the lipophilic tail (Lino et al., 2018). To deliver the drug, liposomes bind to the cell membrane (Lino et al., 2018). Another form of delivery involves lipoplexes/polyplexes which are easy to synthesize, safe, non-immunogenic, and widely used for drug delivery because they rely on electrostatic action for intracellular delivery (Li et al., 2017). Some examples of lipoplexes include FuGENE-6 reagent, which was used to deliver cas9 and sgRNA to inactivate human papillomavirus (Kennedy et al., 2014).

Microinjection is when plasmid DNA encodes both the Cas9 protein and sgRNA to be directly injected into a cell through a needle (Yang et al., 2013). Microinjection has an infinite loading capacity and is extremely precise, making it ideal for in vitro applications (Horii et al). However, only one injection per cell works, and it is less efficient in vivo (Horii et al., 2014). Another method is electroporation, which involves the temporary appearance of micropores in the cell membrane as a result of a pulsed electric field, which speeds up drug absorption (Tröder et al., 2018). In vitro delivery methods, such as electroporation, are more applicable than in vivo delivery methods (Tröder et al., 2018). For in vivo delivery, hydrodynamic delivery involves rapidly injecting drugs into veins with syringes (Suda et al., 2015). A dCas9 system was successfully delivered to mice via dynamic tail vein injection, resulting in transgenic mice that could transcribe and activate multiple genes simultaneously and accurately (Zhou et al., 2018). This method

has the potential for large-scale gene transfer and may be useful in the treatment of diseases that require gene transfer to a large number of cells or tissues (Zhou et al., 2018).

While non-viral vector delivery mechanisms have many advantages, they also have some drawbacks. Non-viral mechanisms, physical methods such as electroporation for example, can cause cell damage and inflammation, and some delivery methods may not be suitable for delivering large genes (Frangoul et al., 2021). Non-viral vectors are still being developed and optimized for various gene therapy applications, and they represent a promising alternative to viral vectors for certain types of gene therapy (Li et al., 2015).

CRISPR-Cas9 gene therapy approaches for treating Alzheimer's disease have shown promise. One such approach involves using Adeno-associated viral (AAV)-1 vectors to target the amyloid precursor protein (APP) gene, resulting in decreased production of *APP* and Aβ (Gyorgy et al., 2018). Another strategy employs the px330 plasmid to target the 3′-UTR region of the *APP* gene, resulting in decreased APP and Aβ production (Nagata et al., 2018). Micelle, which is a type of polyplex delivery method, has also been used to target the beta-site amyloid precursor protein cleaving *BACE1* in order to reduce memory impairment and Aβ levels (Park et al., 2019). Another potential target is the γ-secretase activating protein (GSAP), which can be targeted with a plasmid to reduce GSAP activity and Aβ (Wong et al., 2019). Finally, electroporation with three episomal plasmids was used to target *APOE*, a gene linked to Alzheimer's disease (Wadhwani et al., 2019). This method resulted in more *APOE4* being converted to *APOE3*, resulting in less hyper-phosphorylation of the Tau protein and less amyloid deposition (Wadhwani et al., 2019). While more research is needed to fully understand the safety and efficacy of these approaches, CRISPR-Cas9 targeting of these genes offers a potential therapeutic avenue for treating Alzheimer's disease.

A promising treatment strategy for transthyretin amyloidosis involves the use of a lipid nanoparticle (LNP) delivery system to deliver CRISPR-Cas9 gene editing machinery to target *TTR* gene which will impact transthyretin production (Gilmore et al., 2021). Since plasma TTR is almost entirely produced by hepatocytes, they are an ideal target for gene editing. An LNP-based delivery system for messenger RNA for the Cas9 protein and a single guide RNA targeting *TTR* has been developed (Finn et al., 2018). The LNP is covered by APOE in the bloodstream and binds to the low-density lipoprotein (LDL) receptor on the hepatocyte membrane, allowing active endocytosis of the entire delivery system (Finn et al., 2018). This enables highly efficient and targeted gene editing in hepatocytes, potentially resulting in lower TTR production and better outcomes for patients with transthyretin amyloidosis (Aimo et al., 2022; Gilmore et al., 2021; Finn et al., 2018). More research is needed to fully understand the safety and efficacy of this approach, but using LNPs for targeted gene editing offers a promising avenue for treating this debilitating disease.

Discussion

Alzheimer's disease and transthyretin amyloidosis are types of systemic amyloidosis caused by protein misfolding, which causes harm to cells and organs (Wechalekar et al., 2016; Vaxman et al., 2020). Alzheimer's disease is expected to triple by 2050, with developing countries accounting for two-thirds of cases (Patterson., 2018; Scheltens et al., 2021). Alz is caused by genetic factors, with the presence of one *APOE4* allele being the most important risk factor (van der Lee et al., 2018). Current Alzheimer's disease treatments include lifestyle changes and medications, but neither can cure or slow disease progression (Williamson et al., 2019). A lack of a concrete cure makes it all the more important for scientists to explore treatments like CRISPR that could provide this heavily needed cure. In mice and neural cell lines, CRISPR-Cas9 technology has shown promising results in reducing *BACE1* expression and *APP*-cleavage products, as well as correcting *PSEN1* and *PSEN2* mutations (Ortiz-Virumbrales et al., 2017). CRISPR-Cas9 was used to convert *APOE4* to *APOE3r* in mouse astrocytes, which could be a potential strategy for treating Alzheimer's disease (Komor et al., 2016).

Diagnosis of transthyretin amyloidosis entails confirming the presence of amyloid deposits as well as the type of amyloid protein via biopsy samples and *TTR* gene analysis (Simmons et al., 1993; Koike et al., 2004; Sekijima

et al., 2011). ATTR symptoms differ depending on the age of onset and the type of condition (Sekijima et al., 2011). Transthyretin (TTR) is a protein that is produced in the liver and secreted into the bloodstream in order to transport thyroxine (T4) and the retinol binding protein-vitamin A complex (Blake et al., 1974). *TTR* mutations can cause native structure destabilization, promoting the formation of amyloid fibrils (Sekijima et al., 2006). ATTR treatments currently on the market aim to stabilize the TTR tetramer and prevent the formation of amyloid fibrils (Sekijima et al., 2006; Tojo et al., 2006). Though other approaches like antisense oligonucleotides, small interfering RNA (siRNA), and immunotherapy are being considered, CRISPR-Cas9 gene editing technology in particular is being investigated for treatment of transthyretin amyloidosis. NTLA-2001, an intravenous CRISPR drug, reduced *TTR* expression by up to 90% in primary human hepatocytes (Gillmore et al., 2021). More research is needed to investigate CRISPR-Cas9 technology for the diagnosis treatment, and long-term implications on Alzheimer's disease and transthyretin amyloidosis.

Limitations

There are multiple limitations for treating amyloidosis. For one, treating Alzheimer's when it is in the later stages does not result in clinical improvement (Poon et al., 2020). Therefore, early diagnosis and screening will serve as preventative measures for reaching such a point and increase the chances of successful treatment for patients. Another issue includes the difficulty of crossing the blood-brain barrier. Many BACE1 inhibitors are unable to cross the blood-brain barrier, and in fact no drugs targeting BACE1 are FDA approved that can pass this barrier (Moussa-Pacha et al., 2020). Finding a way for a CRISPR application to cross the blood-brain barrier will definitely present a challenge and requires further research to overcome. A potential limitation of BACE1 inhibition is the gene's potent expression in the pancreas in addition to the expression in the brain (Fagerberg et al., 2014). When targeting BACE1, off-target effects could be found in treatments that could affect the normal function of the pancreas. Additionally, a major limitation for AAV delivery is the carrying capacity of only 4.7 kb, which is very small for encapsulating larger genes that exceed this capacity (Liu et al., 2016). Other forms of gene editing delivery systems should also be considered, such as lipoplexes and hydrodynamic delivery. A major concern over CRISPR-Cas9 treatment is off target gene editing. However, the NTLA-2001 clinical trial showed no off-target effects which is a good sign for future treatments of amyloidosis in the body, especially for treating ATTR (Gilmore et al., 2021). Ultimately, improvement in CRISPR specificity and long-term follow up times need to be further researched in order to be applied widely in the clinic.

Conclusion

With the success of these treatments, there are many future implications for potential therapies. Specifically, a working delivery method for Alzheimer's that is able to cross the blood brain barrier would enable researchers to utilize that delivery method to potentially treat other brain diseases that cause dementia like Parkinson's Disease. As touched on previously, early screening and diagnosis of Alz helps in providing treatment of Alzheimer's prior to irreversible damage being done. Applying CRISPR-Cas9 to treat Alz and ATTR through AAV's and LNP delivery methods is a potentially groundbreaking approach that could cure or improve symptoms of those afflicted by this disease.

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