Chapter

Amyotrophic Lateral Sclerosis

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Abstract

The term motor neuron disease refers to several diseases affecting the motor neurons and is sometimes used interchangeably to refer to amyotrophic lateral sclerosis (ALS), which is the most common motor neuron disease. This chapter will focus on ALS. A complex combination of molecular pathways and cell interactions cause ALS. About 10% of ALS cases are genetic, although it has been hypothesized that as more genes are discovered to contribute to the disease, a larger percentage of cases will be quoted. This chapter discusses in detail the most common genetic forms of ALS and current research on targeted treatments.

Keywords: motor neuron disease, antisense oligonucleotide, amyotrophic lateral sclerosis, primary lateral sclerosis, novel therapies, genetics, trials

1. Amyotrophic lateral sclerosis

1.1 History of ALS

The earliest known description of motor neuron disease was in 1824 by Charles Bell, although it may have been described even earlier. The term amyotrophic lateral sclerosis was coined by Jean-Martin Charcot in his paper in 1874, where he described the condition and its connection to underlying neurological problems [1]. In 1886, Alfred Vulpian described the flail arm presentation of ALS. In 1918, Pierre Marie and his student, Patrikios, described the flail leg presentation of ALS. In 1945, the US Navy reported ALS concomitant with dementia and parkinsonism in Guam. Later, in 2011, we would know that this is due to the C9ORF72 mutation [2]. In the 1969, electrodiagnostic criteria were established for the diagnosis of ALS and updated in 2008 [3]. In 1990, the El Escorial criteria for diagnosis were established at the World Federation of Neurology meeting [4]. The first mutation related to ALS identified was the SOD1 mutation in 1993. As a result of this discovery, mouse models were created and the first medication for ALS was developed, riluzole. In 2015, the second ALS medication, edaravone, was approved [2].

1.2 Epidemiology

The most common motor neuron disease is ALS with an incidence of up to 8 per 100,000 people worldwide [5]. On average, the age of onset is 56 in sporadic ALS and 46 in familial ALS. Men are more likely to develop ALS than women. Disease duration has been quoted to be 3 years, on average; however, this is extremely variable, and it is impossible to predict the rate of decline. Cause of death is usually respiratory failure [2].

1.3 Symptoms

There are several clinical phenotypes that describe the symptom onset of amyotrophic lateral sclerosis. The classic (Charcot) phenotype is characterized by limb onset with pyramidal signs, which are not predominant [6]. This includes patients who have onset in the proximal legs. The flail arm phenotype has progressive proximal weakness and wasting in the arms for at least 12 months before the involvement of the legs or bulbar [6]. By contrast, patients with the flail leg phenotype have progressive distal onset of weakness and wasting in the legs and feet for 12 months before the involvement of the arms or bulbar. These patients are more likely to have an SOD1 mutation, with an odds ratio of 3.75 [6].

Predominantly upper motor neuron (UMN) ALS has pyramidal signs, such as severe spastic para- or tetraparesis [6]. Upper motor neuron signs, such as Babinski, Hoffmann and hyperactive reflexes or jaw jerk, are present [6]. These patients also have dysarthria or pseudobulbar affect and must show clear signs of lower motor neuron disease to differentiate them from primary lateral sclerosis [2]. This is indicated by muscle weakness or wasting, or by the presence of denervation on EMG in at least 2 different muscles [3]. These patients are more likely to have a TARDP mutation, with an odds ratio of 2.65 [6].

The bulbar phenotype has a bulbar onset of disease, characterized by dysarthria or dysphagia. They have wasting of the tongue with fasciculations on examination, and they seem to spare the limbs for at least 6 months [6]. This phenotype is more typically seen in patients with the C9ORF72 mutation with an odds ratio of 2.39 [6]. Finally, in the respiratory phenotype, prevalent respiratory impairment is apparent at onset. This may include orthopnea or dyspnea on exertion or at rest. Upper and lower motor neuron signs in this subgroup are mild in the first 6 months of disease [6].

1.4 Diagnosis

The El Escorial criteria are used to make the diagnosis of ALS [2]. They are broken down into possible, probable or definite ALS. The criteria require progression of upper and lower motor neuron deficits [4]. The signs can be clinical or electrodiagnostic (laboratory-supported ALS has been incorporated into the other categories) [3]. Definite ALS is defined by the combination of upper and lower motor neuron signs in three regions of the body, including limbs (each limb is a region) and bulbar. In probable ALS, only two regions are required, although at least one upper motor neuron sign should be more rostral. In possible ALS, only one region of both upper and lower motor neuron signs is needed. Alternatively, two regions of the upper motor neuron signs are caudal to the lower motor neuron signs [4].

Electrodiagnostic criteria of ALS have been established [3]. CMAPs should be no less than 75% of their normal value and reduction of amplitude between two points of stimulation should not be more than 30%, as this would constitute a conduction block [3]. Motor latencies and durations should be normal, or not more than 1.5 times the upper limit of normal [3]. F-response latencies should not be more than 1.3 times the upper limit of normal [3]. There should be no conduction block, as this is a sign of multifocal motor neuropathy [2]. Sensory evoked potentials should be normal in ALS [2]. On electromyography, there should be positive sharp waves, fibrillation potentials and/or fasciculation potentials in at least 2 regions [3]. Chronic neurogenic changes, such as motor unit configuration of increased duration/amplitude, polyphasia, early/reduced recruitment and increased envelope amplitude of interference pattern, are expected to be present [3].

The differential diagnosis of ALS is broad and includes infectious, inflammatory, paraneoplastic and toxic/metabolic causes [7]. Benign fasciculation syndrome is a common differential to ALS. The fasciculations in benign fasciculation syndrome are exacerbated by exercise, anxiety, caffeine, thyrotoxicosis and alcohol [7]. Only a small subset of patients who present with fasciculations progress to include other motor neuron signs. Calf fasciculations are particularly benign in nature. On electromyography, the fasciculations in ALS can double, are shorter in duration, have polyphasia and have a higher firing rate than those in benign fasciculation syndrome [4].

Multifocal motor neuropathy with conduction block has a prevalence of 0.6 per 100,000 people, which is 10 times rarer than ALS. Compared with ALS, there is a slower progression and younger onset age, and it tends to be more distal with minimal wasting [7]. Wrist drop or finger drop is a common presentation. Sensory system is not involved, unless this is a rarer form, such as MADSAM [2]. Reflexes are variable and can be brisk in up to 20% of patients. There is no bulbar or respiratory involvement [7]. The presence of conduction block on motor nerve conduction studies or triple stimulation technique (TST) is significant. Multifocal motor neuropathy and its subtypes are treatable with IVIG [2, 7].

Chronic inflammatory demyelinating polyradiculopathy has a motor predominant form that can mimic ALS [2]. The disease is symmetric with a relapsing and remitting course, which distinguishes it from ALS [7]. CSF protein elevation is important in its diagnosis and treatment is IVIG [2, 7].

Inclusion body myositis is a myopathy that mimics the anterior horn disease. Involvement of specific muscle groups, including quadriceps, wrist and finger flexors, is suspicious for this disease [2]. About 5% of these patients can have overactive reflexes and up to 40% can have fasciculations [7]. To further complicate things, electrodiagnostic studies of these patients can look more neurogenic than myogenic [7]. Quantitative motor unit analysis of the quadriceps is most sensitive in revealing a myogenic pattern with short duration units [7]. Muscle biopsy is diagnostic [2]. There is no treatment for inclusion body myositis [2].

Spinobulbar muscular atrophy is a different motor neuron disease caused by an x-linked polyglutamine mutation with CAG repeats in the androgen receptor gene [2]. This makes the androgen receptor less functional and causes atrophy and weakness in bulbar and limb girdle muscles [2]. Endocrine signs, such as gynecomastia, diabetes mellitus and testicular atrophy, differentiate this from ALS [7]. There is no treatment for spinobulbar muscular atrophy [2, 7].

Primary progressive multiple sclerosis can mimic ALS; however, this is easily excluded with MRIs of the neuroaxis or the presence of oligoclonal bands in the CSF [7]. Myasthenia gravis, particularly MUSK, may mimic ALS [7]. Myasthenia gravis is characterized by fatigable weakness, differentiating it from the weakness of ALS [2]. Serum antibody testing can differentiate myasthenia gravis from ALS [7].

Infectious causes of motor neuron mimic syndromes include human T-lymphotropic virus (HTLV) and West Nile virus, as well as post-poliomyelitis syndrome [7]. Polio infections affect the anterior horn cells [2]. West Nile virus has an associated myelitis, among other neurologic symptoms [2]. HTLV causes a demyelinating upper motor neuron disease called tropical spastic paraparesis [7]. Bladder dysfunction and sensory changes differentiate this from ALS [7].

1.5 Current treatments

The mainstay of treatment at this time is riluzole [2]. Edaravone is also approved for the treatment of ALS; however, its intravenous administration and requirement of a port leads to complications [8]. An oral form of Edaravone is being developed.

A third approved drug, a combination of dextromethorphan and quinidine, has been beneficial for pseudobulbar affect and other bulbar dysfunction in ALS [2].

Respiratory function is monitored using forced vital capacity (FVC) every 3 months [9]. It is more accurately done lying down [9]. Unfortunately, FVC is not a good measure of early respiratory decline and can be confounded by the inability to create an adequate seal on the mouthpiece [9]. Respiratory failure in ALS is best treated with the use of noninvasive ventilation (NIV) [2]. These methods include cough assist early on and then may advance to BiPAP [9]. Invasive ventilation, such as intubation and tracheostomy, are options for emergent respiratory support or severe respiratory failure in ALS; however, there will be a difficult decision to withdraw invasive respiratory support, should the patient worsen. Early discussion of advanced directives (before dementia or inability to communicate) is essential to prevent unwanted invasive procedures in an emergency [9].

Physical and occupational therapy can help improve function by training patients in compensatory skills and providing assistive devices for every step of the way. A study that looked at exercise in ALS showed there is no risk of worsening disease with moderate exercise [10]. Low-impact aerobic exercises can improve cardiovascular health and decrease depressive symptoms [10]. Speech-language pathology is important for tracking bulbar dysfunction and giving advice on how to speak more clearly or modify foods and drink to prevent choking [10].

Maintaining weight using a high-calorie diet has been shown to improve quality of life and survival in patients with ALS [11]. More studies on which macronutrients are most beneficial are needed, although current studies show that high-fat and high-cholesterol diets are beneficial [11]. If bulbar dysfunction progresses to the point where the patient cannot eat or drink without choking, or if the patient loses more than 5% of his or her body weight between visits (3 months), a feeding tube (usually a percutaneous endoscopic gastrostomy, or PEG) is recommended [11]. Unfortunately, there is weak evidence that PEG tubes prolong survival, despite benefits of reducing weight loss, preventing dehydration and administration of medications [11].

The future of directed treatments for ALS is bright. Later in this chapter, I will discuss research into the treatment of genetic ALS.

2. Genetic amyotrophic lateral sclerosis

About 10% of ALS cases are genetic [2]. Most are autosomal dominant, although they can be recessive or X-linked [2]. Over 30 genes related to ALS have been discovered so far. The most common of these are C9ORF72 (about 30% in Europeans and 2.3% in Asians), SOD1 (14.8% in Europeans and 30% in Asians), TAR DNA-binding protein (4.2% in Europeans and 1.5% in Asians) and fused in sarcoma (2.8% in Europeans and 6.4% in Asians) [12]. Ubiquilin2 (UBQLN2), ALSIN, senataxin (SETX), spatacsin, vesicle-associated membrane protein-associated protein B (VAPB), angiogenin (ANG), factor-induced gene 4 (FIG 4), optineurin (OPTN) and "other unknown genes" account for the rest [2]. Only a few of these, such as C9ORF72, are causal. The rest are disease-modifying genes [12].

2.1 C9ORF72 and SETX

C9ORF72 is a protein differentially expressed in normal and neoplastic cells, which modulate (via Rab or Ras GTPase) endosomal trafficking and autophagy in primary neurons [12, 13]. The gene is located on chromosome 9p21.2 and is a hexanucleotide repeat of GGGGCC [2]. In a healthy person, there are 20–30 repeats;

however, someone with the mutation can have hundreds of repeats [13]. Although anticipation is shown in trinucleotide repeat disorders, it has not been demonstrated in hexanucleotide repeat disorders [14]. Repeats are typically expanded in multiples of 3 to preserve the genetic reading frame [15]. Most repeat disorders do not cause catastrophic frame shift mutations, unless a stop codon is the triplet added [15]. There is a transitional number between the normal number of repeats to the permutation and finally to the number of repeats that determine a mutation, although exact numbers are currently not agreed upon in ALS [13].

Typically, after inheritance of a repeat expansion, it remains dormant in the cell [13]. As the cells divide, the repeats tend to continue to expand when more repeats are copied onto the daughter strands during replication [13]. Repeat DNA is more susceptible to damage [15]. Fibroblasts and lymphocytes from patients with Huntington's disease, ALS, Alzheimer's disease and Parkinson's disease all have DNA that is relatively sensitive to ionizing radiation and chemical mutagens [15]. When the damaged DNA is repaired, more repeats are created during DNA repair [15]. Mismatch and base-excision repair cause somatic expansion of repeated sequences of trinucleotide repeat disorders [15]. As this in more cells, the organism eventually reaches a critical point at which a significant number of cells meet the threshold number of repeats to produce disease [13, 15]. Progression of disease occurs when more cells reach this threshold and enter a pathologic state [13, 15].

Repeat expansion mutations in the C9ORF72 gene, such as the hexanucleotide repeat seen in ALS, lead to the formation of R-loops in the DNA [16]. R-loops are a hybridization of mRNA with dsDNA with looped intron sequences, which have been spliced out of the mRNA during the transcription process [16]. R-loops occur naturally in several cellular processes, including mitochondrial DNA replication, and in the transcription bubble [16]. R-loops have been thought to rarely occur as transcriptional by-products but are more common than once thought [16]. Others believe that R-loops are natural intermediates of transcription that are eliminated by Senataxin [17].

RNA:DNA hybrids are more stable than dsDNA [16]. High G-content (like in the C9ORF72 hexanucleotide repeat) encourages and stabilizes R-loops by facilitating the opening of the transcriptional bubble while DNA strands are still separated [16]. SETX is one of the genes involved in terminating transcription and senataxin depletion (such as in SETX ALS) correlates with the accumulation of RNA:DNA hybrids [17]. The mRNA would usually move out of the nucleus and not interact with the dsDNA as much; however, this mRNA becomes sequestered in the nucleus [16]. This enlarges the nucleolus and recruits the cell DNA damage response [16]. If unsuccessful, the DNA damage response will signal for apoptosis of the neuron [16].

Haploinsufficient proteins form from translated GGGGCC introns that are not degraded after splicing [18]. These are exported out of the nucleus by an unknown mechanism and translated in the cytoplasm [18]. The resulting haploinsufficient C9ORF72 protein forms toxic dipeptide aggregates that accumulate in the neuron [18]. These haploinsufficient proteins may have properties of prions [18].

2.2 SOD

SOD (superoxide dismutase) is a cytoplasmic enzyme of 153 amino acids: one copper atom for function and one zinc atom for structural stability [18]. It converts oxygen radicals into peroxide and oxygen [18]. The cell is then able to turn the peroxide into water and oxygen with catalase [18]. This is an extremely stable protein, but can unfold from dimer to two unfolded monomers via a folded monomer

intermediate step [18]. A complex combination of molecular pathways and cell interactions cause ALS [2]. Oxidative stress, aberrant RNA processing and protein misfolding/insoluble proteins have all been implicated in motor neuron degeneration in ALS [2]. This is an example of an oversimplified mechanism of SOD1-mediated ALS. Microglia secrete cytokines, stimulating inflammation by recruiting astrocytes [18]. Astrocytes come and release nitric oxide and prostaglandin E2 [18]. SOD comes to convert NO into peroxide and oxygen [18]. In ALS patients, the SOD that arrives is mutated and aggregates, leading to endoplasmic reticulum (ER) stress [18]. The ER helps with endosomal trafficking, so defective endosomal trafficking causes organelle disruption, including mitochondrial disruption, and activates apoptosis of the neuron [18]. ER stress also causes dysfunction in axonal transport, leading to axonal loss [18].

The SOD1 gene is located on chromosome 21q22 [2] and contains 5 exons [18]. About 150 mutations of this gene have been identified, which are predominantly missense mutations, but also include nonsense mutations, insertions and deletions [2]. Notable mutations in this group include SOD1A4V-ALS for its rapid clinical progression, SOD1A89V-ALS for its sensory neuropathy, SODI113T-ALS for its diversity of phenotype and SOD1G93A-ALS for its use in transgenic mice [2, 18]. The SOD1D90 mutation is recessive in Scandinavians, but dominant in other groups [18]. SOD1 knockout mice do not develop clinical ALS [18]. Instead, they develop age-dependent distal motor neuropathy, suggesting a toxic gain of function in the SOD [18].

2.3 FUS and TAR DNA-binding protein

FUS is a DNA- and RNA-binding protein that repairs damage and breaks, especially double-strand breaks in DNA [19, 20]. Loss of function mutations in FUS gene (16p11.2) lead to impairment of the poly(ADP-ribose) polymerase (PARP)-dependent DNA damage response, leading to increased DNA damage, especially in neurons [19]. The mutated FUS aggregates [19]. In ALS, the mutations are located in the nuclear localization sequence in the c-terminus [19]. This causes FUS to lose its ability to stay in the nucleus and the mutated FUS will instead aggregate in the cytoplasm [19]. ALS due to the TARDBP gene has a similar mechanism also with cytoplasmic aggregates [20]. Inclusion bodies that are FUS positive, TDP-43 negative may be found [19]. The typical phenotype for FUS-ALS is a lower motor neuron predominant syndrome without bulbar involvement [2]. They may have frontotemporal dementia as well [2]. The typical age at onset ranges from as young as the mid-twenties to as late as the eighth decade of life [2]. Mean duration of this form of ALS is 33 months [2].

3. Genetic treatments

3.1 CRISPR

CRISPR (clustered regularly interspaced short palindromic repeats) was discovered in 1987 in Osaka by Ishino et al. in *E. coli*, although their significance was unknown [21]. The CRISPR/Cas system is a part of the prokaryotic immune system, allowing resistance to foreign genetic data from bacteriophages [21]. Archaea and bacteria use CRISPR/Cas to find bacteriophage DNA that has been entered into its genome and remove it [21].

There are three steps of CRISPR/Cas-mediated immunity [21]. The first step is adaptation [21]. Prokaryotes place protospacers in their DNA made up of pieces

of foreign DNA (from phages and plasmids that previously invaded) attached to palindromic repeats [21]. The second step is expression with maturation [21]. Transcription of the protospacers and repeats yields a precursor CRISPR-RNA, which will mature into the guide RNA (gRNA) [21]. The third step is interference [21]. Once mature, the guide RNA is used in the CRISPR/Cas system to recognize and create a double-strand break by foreign DNA [21].

Pieces of foreign DNA are stored by the prokaryote in the form of a genetic library of phages and plasmids, which have previously invaded [21]. Cas (CRISPR-associated protein) scans bacterial DNA for bacteriophage DNA that matches the guide RNA attached to CRISPR and cleaves it, when found [21]. Cas is a DNA endonuclease that allows for unwinding of DNA, checking for sites complementary to the guide RNA (20 BP spacer region) [21]. Once a match is found, it cleaves both strands of the DNA [21]. When the cell attempts to repair the break, mutations are often introduced, deactivating the viral gene [21]. The repair can be done via nonhomologous end joining (NHEJ), which can be done at any point in the cell cycle. In certain stages of the cell cycle, homology-directed repair (HDR) occurs, allowing for more precise DNA repair [21]. Researchers have been working on enhancing this type of repair for high-fidelity CRISPR/Cas-mediated gene editing [22]. This process can be manipulated for gene inactivation or insertion of foreign DNA [22]. Mammalian cells predominantly rely on nonhomologous end joining for DNA repair, which is error-prone, resulting in insertion and deletion (indels) mutations [21]. Specifically in neurons, which are terminally differentiated post-mitotic cells, homology-directed repair is limited, but nonhomologous end joining is easier [22].

3.2 Use of CRISPR in eukaryotes

In 2012, Jennifer Doudna from UC Berkeley and Emmanuelle Charpentier from Umea University in Sweden demonstrated the use of CRISPR/Cas for human controlled genetic editing [21]. She fused CRISPR RNA (crRNA) with trans-activating CRISPR RNA (tacrRNA) to form a chimeric single-guide RNA (sgRNA) to allow for site-specific gene editing in a eukaryote [21]. This method is popular due to its low cost and ease of production in a lab [21]. Since then, research in CRISPR has expanded to include every species: from attempts to drive malaria-carrying mosquito species to extinction to combating antibiotic resistance to agriculture, making crops hardier [21].

Unfortunately, the CRISPR/Cas9 system is not specific enough to prevent it from cleaving nontarget DNA [21]. DNA does not have to fully match the guide RNA (can tolerate 3–5 mismatches) for it to introduce a double-strand break, which leads to unpredictable mutations [21]. Researchers have been working to increase the specificity of CRISPR/Cas9 systems by using two guide RNAs or shorter (truncated) guide RNAs [22]. Decreasing the GC content of guide RNAs also helps specificity [23]. Adding a short-lived ribonucleoprotein to the CRISPR-Cas9 system decreases off-target effects by allowing the complex to break down after a short period of activity [22]. This allows for more locally acting gene editing. There is also the possibility of introducing the Cas protein instead of the Cas gene into a subject, so the effect on the genome is shorter-lived [22].

There are other downsides, including immune attacks on the system, due to its bacterial origin [21]. PEGylation, the modification of biomolecules by adding polyethylene glycol (PEG), a nontoxic, nonimmunogenic polymer, is one method to circumvent immune attack [21]. Humanization of the proteins is another method of circumventing this problem [22].

3.3 Cas modification and types

The Cas protein may be modified in many different ways, including deactivating one or both cutting domains and adding deaminases, transcriptional activators or blockers (dCas-sgRNA) [22]. Deactivating one of the cutting domains, creating a 'nick' instead of a 'break,' prevents unwanted damage in off-target sites [22]. One may opt to deactivate both cutting domains (dead Cas or dCas) and attach other enzymes to the complex, such as deaminases, which cause point mutations [22]. These point mutations may include stop codons to prevent transcription of a disease gene (CRISPR interference CRISPRi), or they may change a disease-causing mutation to a healthy gene [23]. Transcriptional activators, such as VP64 or MS2 coat protein, may be added to recruit transcription machinery and promote transcription of specific genes (CRISPR activation or CRISPRa) [21]. CRISPRi is accomplished by adding a Kruppel-associated box (KRAB) domains to inactivate transcription by recruiting factors that physically block the gene [21].

Alternatively, one can use a different type of Cas. Each bacterial species has its own Cas protein, or multiple types of Cas [22]. Strep. pyogenes Cas9 (spCas9) was found first and is most commonly used in a CRISPR type II system, may be directed by two guide RNAs [22]. Cas9 cuts double-stranded DNA that matches the guide RNA [21]. Staph. aureus Cas9 (saCas9) is small, which allows it to fit inside adeno-associated virus, making it a convenient choice for that vector [24]. Strep. thermophilus Cas9 (stCas9) is more specific, requiring a match to not only the guide RNA but also a protospacer-adjacent motif (PAM) (a specific sequence next to the viral DNA) [24]. This prevents unwanted off-target effects. CasX is the smallest known Cas so far and less immunogenic [25]. Jennifer Doudna discovered CasX, found in ground-dwelling bacteria, which are unfamiliar to the human immune system and nonpathologic, decreasing the chance of immunogenicity [25]. Cas12 cuts double-stranded DNA that matches the guide, as well as all single-stranded DNA in a cell in a nonspecific way [21]. Cas13 cuts all single-stranded RNA in a cell [21]. Cas14 is found in Archaea and is very small [22]. It cuts all single-stranded DNA in a cell in a more specific way, with a system (DETECTR) that detects infectious organisms and genetic mutations [22]. Cpf1 is an endonuclease that leaves an overhang on one side of the double-strand break (DSB), which promotes nonhomologous end joining in neurons [21].

Delivery methods for the CRISPR/Cas system include viral vectors, nanoparticles, lipofectamine, nucleofection, microinjection, short-lived ribonucleoproteins and electroportation [23]. Some of these methods, including microinjection and electroportation, can damage cells and are not possible in vivo [23]. In electroportation, an electric field increases permeability of cell membrane, allowing entry of the CRISPR/Cas system into the cell [23]. Nucleofection, nanoparticles and lipofectamine are less commonly used by researchers due to the tendency for low cell penetrance using these methods [23]. Viral vectors are the most common and effective delivery systems. AAV (adeno-associated viral vector) is the most commonly studied vector [26, 27]. The limiting factors in the use of viral vectors are low cargo capacity, immunogenicity and tissue specificity [26]. AAV is a small virus that does not cause disease, just a very mild immune response [26]. It attaches and infiltrates the host cell. The virus transfers DNA into the nucleus, leading to sustained gene expression [26]. There is ~ 4.7 kb AAV vector packaging limit [26]. Therefore, when using this delivery system with spCas9, two AAVs are required: one to package spCas9 and the other to package the sgRNAs [26]. With smaller Cas types, such as saCas9, only one AAV is required [26]. AAV is specific to muscle, liver, brain and eye tissue [27]. Immune response (mainly humoral, due to prior

infection with AAV) was found in 96% of patients in one study [27]. These patients demonstrated antibodies for AAV [26, 27]. Other delivery systems use other viruses, such as adenovirus and lentivirus [26]. Short-lived ribonucleoproteins (RNP) are proteins that shuttle the CRISPR-Cas system into a cell [27]. Since they are short-lived, the action of the CRISPR-Cas system tends to be local to where the RNPs are injected [27]. These decrease off-target effects and are less immunogenic than viral vectors [23].

3.4 Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are synthetic nucleic acid sequences that bind RNA to modulate gene expression [28]. ASOs can restore protein function by splice modification, decrease aberrant protein function by silencing, modify protein function or reduce toxicity of an aberrant protein [28]. ASOs penetrate their target with the help of ribose alteration, avoid degradation by nucleases and avoid immune response by alterations in their phosphate group, ribose and nucleosides [28]. Two ASOs were FDA approved in 2016: eteplirsen for Duchenne muscular dystrophy and nusinersen for spinal muscular atrophy [28]. Proteinuria has been a common side effect with the ASOs, although it has been mostly benign [28].

About 14% of patients who have Duchenne muscular dystrophy contain the mutation at exon 51, where eteplirsen takes action [29]. It works by mRNA knock-down via activation of RNA-H, which breaks down the RNA-DNA complex before translation [29]. It aims to skip the mutated exon to convert the frame shift mutation back into the reading frame [29].

Nusinersen works via alteration of gene splice site [30]. It targets intronic splicing silencer N1 (ISSN1), causing inclusion of exon 7 in SMN2 pre-mRNA [30]. This results in SMN2 protein translation that looks identical to SMN1 protein [30].

CRISPR-Cas systems have some benefit over ASOs, such as being less cytotoxic and requiring less number of treatments [23].

3.5 Other methods of genetic manipulation

Meganucleases are large endonucleases, able to cut out large, 14–40 base pair (BP) long, DNA sequences [31]. They were discovered in the 1980s [31]. Although these are specific, they are costly to create, requiring expertise and more time than CRISPR, which makes it inefficient [31]. Meganucleases have been studied in Duchenne muscular dystrophy [31]. One group designed a meganuclease that cuts upstream of the deletion 'hot spot' of intron 44 of the dystrophin gene [31]. It was delivered via a lentiviral vector [31]. After administration, expression of a fully corrected dystrophin gene was observed via western blot [31].

Zinc finger nucleases recognize short sequences (3 BP) of DNA, but can be combined with several other zinc fingers to accommodate longer sequences [32]. They are less specific, but are expensive to make as they require expertise, time and effort to create [32]. They are cytotoxic to cells, so currently, they are mainly in use for modifying stem cells and immune cells [32]. Ousterout et al. used this technology in myoblast cell cultures to yield dystrophin expression [32].

Transcription activator-like effector nuclease (TALEN) are artificial restriction enzymes, fused to a nuclease and designed to recognize specific DNA sequences of 33 or 34 amino acid repeats [33]. They are able to perform DNA repair, replacement, insertion or deletion [33]. This is a precise method that is easy to make and is not costly [33]. TALEN has been used successfully in human cell cultures (myoblasts and dermal fibroblasts) with Duchenne muscular dystrophy, as well as to treat Golden Retrievers with muscular dystrophy [33].

RNA interference (RNAi) is used in the cell to control gene expression [34]. Two types of RNAs are known to perform this function: small interfering RNAs (siRNA) and microRNAs (miRNA) [34]. After RNA polymerase II produces mRNA, the mRNA travels to the cytoplasm for transcription, unless it is intercepted by RNA interference [34]. siRNA or miRNA binds to enzymes that break down mRNAs that match or closely match a sequence in them [34]. miRNAs are about 21 nucleotides long and bind to dicer, an enzyme that cleaves mRNA that matches the single-stranded microRNA [34]. After cleavage, the mRNA is degraded [34]. Argonaut is another enzyme that performs the same function [34]. Once bound to miRNA or siRNA, the complex is called RISC (RNA-induced silencing complex) [34]. siRNAs differ from miRNAs in that they are double stranded [34]. RNAi requires multiple treatments and can be cytotoxic [34].

4. Use of genetic treatments in treating motor neuron disease

4.1 CRISPR treatment of ALS

Treatment of ALS has been limited by the limited understanding of the mechanism of disease [2]. Some of the use of CRISPR/Cas9 research done in ALS is to identify the mechanism by which the various genes cause toxicity, discovering modifiers and RNA-processing pathways [35].

Researchers did a proof of concept study, which demonstrated that using CRISPR/Cas9 in an AAV delivery system in G93A-SOD1 mice targeting the SOD1 mutation has delayed onset, increased survivability of motor neurons, decreased motor atrophy, increased motor function and prolonged lifespan, compared to control mice [36]. G93A-SOD1 transgenic mice were infused at birth or first day of life with the delivery system [36]. Typically, the mice develop symptoms at 90 days of life [36]. A single peptide is changed in the SOD1 mutation in this model [36]. The amount of mutant SOD1 protein in the spinal cord was reduced by the infusion, compared with control mice [36]. This delayed the onset of disease by a range of 2–36 days, but did not slow disease progression once the onset came [36]. However, the delay in onset prolonged survival in test mice by 25%, compared to diseased controls [36].

Another group was able to produce gene-corrected fibroblast stem cells using a CRISPR/Cas9 system from ALS patients with SOD1 and FUS mutations [37]. They first collected and cultured FUS and SOD1-mutated fibroblasts and confirmed their mutations [37]. Then, they used a CRISPR/Cas system with electroportation to target the FUS mutation for correction with single-stranded oligodeoxynucleotide as a repair template [37].

In 2017, researchers demonstrated that they could use genetically modified mesenchymal stem cells to express neurotrophic factors [38]. Neurotrophic factors are peptides that promote growth, survival and differentiation of neurons [38]. This paper proposes that CRISPR/Cas in an AAV delivery system is a good way to genetically modify mesenchymal stem cells to express these factors, which are neuroprotective [38].

Other researchers are looking at using CRISPR/Cas13 to target aberrant mRNAs in C9ORF72 ALS patients [39]. CRISPR/Cas13 cuts RNA and this group modified it to be more specific toward the toxic mRNAs, which do not leave the nucleus and lead to R-loops in DNA [39]. Their mouse models have shown improvement in motor symptoms [39].

This group [40] was able to eliminate toxic microsatellite repeat expansion RNAs with an RNA-targeting Cas9 in myotonic dystrophy cell cultures. They developed

a programmable CRISPR/Cas9 system to visualize and eliminate repetitive RNAs retained and aggregating in the nucleus [40]. Although these experiments were conducted in myotonic dystrophy cell cultures, not cell cultures expressing C9ORF72, they are theoretically applicable [40].

In 2018, a group did a genome-wide survey, looking for suppressors and enhancers of C9ORF72 dipeptide repeat toxicity in human cells [41]. These were validated using primary mouse neurons with CRISPR/Cas9 screening [41]. They discovered several modifiers, but one in particular, called TMX2, modulated the endoplasmic reticular stress caused by C9ORF72 dipeptide repeats, increasing survival to 100% (from 10%) in their mouse models [41].

Additional researchers used a SaCas9 endonuclease to disrupt HERV-K env, a retroviral gene, human mouse mammary tumor virus-like 2, related to prostate cancer motor neuron disease [42]. They found this inhibited molecules involved in amyotrophic lateral sclerosis, including epidermal growth factor receptor (EGF-R), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), SF2/ASF and TDP-43 [42]. These molecules are important for RNA-binding and alternative splicing [42].

4.2 ASO editing of ALS

Tofersen is an antisense oligonucleotide that binds to the mRNA from the SOD1 gene [43]. This drug is being studied in patients with ALS caused by the SOD1 mutation [43]. In the phase 1/2 trial, treatment with tofersen 100 mg in 10 patients with SOD1 fALS over a three-month period resulted in a statistically significant lowering of SOD1 protein levels in the cerebrospinal fluid and a slowed decline in the ALS Functional Rating Scale-Revised (ALSFRS-R) compared to 12 patients receiving a placebo [44]. They also noted slowed decline in muscle strength and vital capacity in the study group [44].

5. Conclusion

Amyotrophic lateral sclerosis is a disease with no cure; however, current research is promising for a cure in the near future. Technologies in genetic editing show particular promise in the field of neurodegeneration. Molecular mechanisms of genetic diseases, even those with known mechanisms, are oftentimes much more complex than initially thought. Discoveries regarding transcription modulators have proven particularly useful in research to find treatments for ALS. Given the recent advances in these areas, the future appears brighter for patients with ALS.

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Conflict of interest

The author declares no conflict of interest.

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