T.C.
ÜSKÜDAR UNIVERSITY
INSTITUTE OF SCIENCE

INSTITUTE OF SCIENCE DIVISION
MOLECULAR BIOLOGY MASTER'S PROGRAM

MASTER THESIS

SYNTHETIC BIOLOGY APPROACHES IN THE TREATMENT OF SPINAL MUSCULAR ATROPHY: *IN VITRO* INVESTIGATION OF THE EFFECTS OF COMBINED XENO-NUCLEIC ACID/ DNA ANTISENSE OLIGONUCLEOTIDES (XNA/DNA-ASO) ON *SMN2* GENE EXPRESSION AND CELL VIABILITY

CEMRE CAN İNCİ

Thesis Advisor
Assit. Prof. Dr. Cihan TAŞTAN

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ÖZET

SPİNAL MUSKÜLER ATROFİ TEDAVİSİNDE SENTETİK BİYOLOJİ YAKLAŞIMLARI: KOMBİNE XENO-NÜKLEİK ASİT/DNA ANTİSENS OLİGONÜKLEOTİDLERİNİN (XNA/DNA-ASO) *SMN2* GEN EKSPRESYONU VE HÜCRE CANLILIĞI *ÜZERİNDEKİ* ETKİLERİNİN *IN VİTRO* ARAŞTIRILMASI

Spinal Musküler Atrofi (SMA), Survival Motor Neuron (SMN) proteininin yetersiz üretimi nedeniyle motor nöronların kaybı ile karakterize ciddi bir nörodejeneratif hastalıktır. Bu eksiklik temel olarak *SMN1* genindeki mutasyonlardan kaynaklanır ve *SMN2* geninin 7. ekzonunun dahil edilmesiyle kısmi olarak telafi edilir. Nusinersen (ticari adı SPINRAZA®) gibi geleneksel antisens oligonükleotidler (ASO'lar), ekzon 7'nin dahil edilmesini teşvik etmek ve SMN protein seviyelerini artırmak için *SMN2* 'yi hedef almıştır. Ancak son çalışmalar, yeni ASO tasarımlarının, özellikle de Xeno Nükleik Asit/DNA-ASO (XNA/DNA-ASO) yapılarının daha fazla etkinlik ve stabilite sunabileceğini göstermektedir. Bu tez, SMA Tip II fibroblast modellerinde *SMN2* ekzon 7 inklüzyonunu yukarı regüle etmede, SMN protein ekspresyonunu artırmada ve hücre canlılığını iyileştirmede XNA/DNA-ASO'ların ve RNA-ASO'ların karşılaştırmalı etkinliğini araştırmaktadır. Spesifik olarak, yeni tasarlanan iki XNA/DNA-ASO bileşiği, MERLiNA ve GENLiNA, SMN protein seviyeleri, hücre canlılığı (MTT testi) ve apoptoz (Annexin V/PI boyama) gibi temel parametrelere odaklanarak Nusinersen ile birlikte değerlendirilmiştir. Bulgular, XNA/DNA-ASO'ların, özellikle de 100 nM konsantrasyonundaki MERLiNA'nın, SMN ifadesini önemli ölçüde artırırken, Nusinersen'e kıyasla daha düşük apoptoz seviyelerini ve daha yüksek hücre canlılığını koruduğunu ortaya koymuştur. Bu sonuçlar, XNA/DNA-ASO teknolojilerinin SMA tedavilerini iyileştirmek için umut verici bir terapötik yolu temsil ettiğini ve mevcut RNA tabanlı ASO tedavilerine göre stabilite ve etkinlik açısından potansiyel avantajlara sahip olduğunu göstermektedir. Bu çalışma, bu yeni bileşiklerin terapötik potansiyeline ilişkin daha ileri preklinik ve klinik araştırmaların önünü açmaktadır.

Anahtar Kelimeler: Antisens Oligonükleotid (ASO), Spinal Müsküler Atrofi (SMA), *SMN2*, XNA/DNA-ASO.

ABSTRACT

SYNTHETIC BIOLOGY APPROACHES IN THE TREATMENT OF SPINAL MUSCULAR ATROPHY: *IN VITRO* INVESTIGATION OF THE EFFECTS OF COMBINED XENO-NUCLEIC ACID /DNA ANTISENSE OLIGONUCLEOTIDES (XNA/DNA-ASO) ON *SMN2* GENE EXPRESSION AND CELL VIABILITY

Spinal Muscular Atrophy (SMA) is a severe neurodegenerative disease characterized by the loss of motor neurons due to insufficient production of Survival Motor Neuron (SMN) protein. This deficiency primarily arises from mutations in the *SMN1* gene, with the *SMN2* gene providing partial compensation through the inclusion of exon 7. Traditional antisense oligonucleotides (ASOs), such as Nusinersen (trade name SPINRAZA®), have targeted *SMN2* to promote exon 7 inclusion and increase SMN protein levels. However, recent studies suggest that novel ASO designs, particularly Xeno Nucleic Acid/DNA-ASO (XNA/DNA-ASO) constructs, may offer enhanced efficacy and stability. This thesis investigates the comparative efficacy of XNA/DNA-ASOs and RNA-ASOs in upregulating *SMN2* exon 7 inclusion, increasing SMN protein expression, and improving cell viability in SMA Type II fibroblast models. Specifically, two newly designed XNA/DNA-ASO compounds, MERLiNA and GENLiNA, were evaluated alongside Nusinersen, focusing on key parameters such as SMN protein levels, cell viability (MTT assay), and apoptosis (Annexin V/PI staining). The findings revealed that XNA/DNA-ASOs, particularly MERLiNA at 100 nM concentration, significantly enhanced SMN expression while maintaining lower apoptosis levels and higher cell viability compared to Nusinersen. These results suggest that XNA/DNA-ASO technologies represent a promising therapeutic avenue for improving SMA treatments, with potential advantages over existing RNA-based ASO therapies in terms of stability and efficacy. This work paves the way for further preclinical and clinical investigations into the therapeutic potential of these novel compounds.

Keywords: Antisense Oligonucleotide (ASO), Spinal Muscular Atrophy (SMA), *SMN2*, XNA/DNA-ASO.

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Cemre Can İNCİ

DECLARATION FORM

I hereby declare that I have obtained all the information and documents in this study within the framework of academic rules, that I have presented all visual, audio and written information and results in accordance with the rules of scientific ethics, that I have not falsified any of the data I have used, that I have cited the sources I have used in accordance with scientific norms, that my thesis is original except where references are cited, that it has been produced by me and that it has been written according to Üsküdar University Institute of Health Sciences Thesis Writing Guide

30.09.2024

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INDEX OF SYMBOLS AND ABBREVIATIONS

ASO: Antisense Oligonucleotide

BD: Becton Dickinson

BSA: Bovine Serum Albumin

CMV: Cytomegalovirus

cDNA: Complementary Deoxyribonucleic Acid

DMD: Duchenne Muscular Dystrophy

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

FBS: Fetal Bovine Serum

FITC: Fluorescein Isothiocyanate

hnRNP: Heterogeneous Nuclear Ribonucleoproteins

HPRT1: Hypoxanthine-Guanine Phosphoribosyltransferase 1

ISS-N1: Intronic Splicing Silencer N1

LNA: Locked Nucleic Acid

MFI: Mean Fluorescence Intensity

MLPA: Multiplex Ligation-dependent Probe Amplification

mRNA: Messenger Ribonucleic Acid

NGS: Next-Generation Sequencing

PBS: Phosphate-Buffered Saline

PEI: Polyethylenimine

PI: Propidium Iodide

PS: Phosphorothioate

qPCR: Quantitative Polymerase Chain Reaction

RNA: Ribonucleic Acid

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SMA: Spinal Muscular Atrophy

SMN: Survival Motor Neuron

snRNP: Small Nuclear Ribonucleoproteins

TALEN: Transcription Activator-Like Effector Nuclease

XNA: Xeno Nucleic Acid

ZFN: Zinc Finger Nuclease

1. INTRODUCTION

Rare diseases are chronic and progressive diseases that affect a small percentage of the population, usually of genetic origin. It is estimated that 300 million people worldwide are living with a rare disease and approximately 6000 to 8000 rare diseases have been identified (Nguengang Wakap et al., 2020). Many of these diseases are caused by genetic mutations that result in impaired cellular and molecular function and often have incurable or even life-threatening consequences. Although the diagnosis and treatment of these genetically driven diseases is challenging, advances in genetic engineering and molecular biology have led to the development of promising therapeutic approaches for some rare diseases (Boycott et al., 2019).

Molecular biotechnological methods such as antisense oligonucleotide (ASO) and gene therapy have come to the forefront in the treatment of genetically based rare diseases. These methods offer therapeutic effects by targeting incorrect protein production or deficiency caused by genetic mutations. Antisense oligonucleotides bind to target mRNA, regulate gene expression and promote the production of proteins required for the disease. Among these approaches, ASO-based therapies developed for neuromuscular diseases such as Spinal Muscular Atrophy (SMA) stand out (Rinaldi & Wood, 2018).

Spinal Muscular Atrophy (SMA) is a serious genetic disease characterized by progressive degeneration of motor neurons, leading to muscle atrophy and, in many cases, early death. The disease is caused by a homozygous deletion or mutation of the Survival Motor Neuron 1 (*SMN1*) gene, resulting in a deficiency of the SMN protein, which is essential for the survival and function of motor neurons. The parallel *SMN2* gene also produces SMN protein but usually produces a transcript that lacks exon 7 and is therefore non-functional. In this context, increasing the inclusion of exon 7 in the *SMN2* transcript has become a critical therapeutic target in the treatment of SMA (Finkel et al., 2017).

Antisense oligonucleotides (ASOs) have emerged as a promising therapeutic approach aimed at increasing the production of full-length SMN protein by regulating the

exonization process of *SMN2* mRNA. Among these, RNA-based ASOs (RNA-ASOs) have shown efficacy in increasing the incorporation of exon 7, and this efficacy has been demonstrated with the FDA-approved drug Nusinersen. However, challenges such as limited cellular uptake, sensitivity to nuclease degradation and potential off-target effects have led to the development of more advanced ASO technologies (Watts & Corey, 2012).

Xeno Nucleic Acids (XNA), specifically Locked Nucleic Acids (LNA), represent a new generation of ASOs designed to overcome these limitations. XNAs are synthetic analogs of natural nucleic acids and, thanks to their modified backbone, provide enhanced stability, increased binding affinity to target RNA and resistance to enzymatic degradation (Braasch & Corey, 2001). LNA-modified DNA-ASOs (XNA/DNA-ASOs) have been shown to improve exonylation modulation with greater potency and specificity compared to conventional RNA-ASOs (Petersen & Wengel, 2003).

This study aims to evaluate the potential of intron 7-targeted XNA/DNA-ASOs to increase *SMN2* gene expression and improve cell viability in SMA models by investigating their *in vitro* efficacy. By comparing the performance of XNA/DNA-ASOs with RNA-ASOs, the research aims to evaluate the potential of XNA/DNA-based therapies as a superior alternative for the treatment of SMA. Understanding these mechanisms and the relative advantages of combined XNA/DNA-ASOs may provide critical information for the development of more effective and durable SMA therapies.

To achieve this aim, SMA Type II fibroblast cells will be cultured and treated with different concentrations of XNA/DNA-ASOs under controlled conditions. After treatment, SMN protein levels between the different treatment groups will be evaluated using immunocytochemistry with flow cytometry. Cells will be analyzed by loading into a Beckman Coulter CytoFLEX flow cytometry device and the expression of SMN protein will be measured by fluorescence intensity in the cells. In this way, the effect of ASOs on SMN protein production will be quantitatively evaluated. Furthermore, RNA will be isolated and *SMN2* mRNA levels quantified, which will provide information on the exonization efficiency of ASOs. Cell viability as a critical indicator of treatment efficacy will be assessed by MTT assay, which will provide information on viability and cytotoxicity by measuring cellular metabolic activity.

Apoptosis and necrosis rates will be assessed using flow cytometry with Annexin V and Propidium Iodide (PI) staining, respectively. Annexin V positive/PI negative cells indicate early apoptosis, while Annexin V positive/PI positive cells indicate late apoptosis or necrosis stage. These assays will play an important role in understanding the mechanisms of cell death induced by ASO therapies.

Finally, cellular morphology will be monitored over time using fluorescence microscope and experimental results will be visually validated. Through these comprehensive experimental approaches, the study will elucidate the potential of XNA/DNA-ASOs as a superior therapeutic strategy for the treatment of SMA and contribute to a broader understanding of antisense oligonucleotide therapies in genetic disorders.

2. GENERAL INFORMATION

2.1. Rare Diseases

Rare diseases are defined as diseases that affect less than 1% of the general population, are usually genetic in origin and have a chronic course (Eurordis, 2017). Approximately 300 million people worldwide are living with a rare disease and estimates suggest that there are between 6000 and 8000 rare diseases (Nguengang Wakap et al., 2020). These diseases occur predominantly in early childhood and are often associated with a severe clinical course. Most rare diseases are of genetic origin and can show autosomal dominant, autosomal recessive or X-linked inheritance patterns. Non-genetic rare diseases may be associated with infections, autoimmune diseases, poisoning or environmental factors (Luzzatto et al., 2015). Loss of function or incorrect protein expression due to genetic mutations can lead to damage in many organs and systems, which significantly reduces the quality of life of patients. Furthermore, as rare diseases are often difficult to diagnose and require lengthy processes, they may be undiagnosed or misdiagnosed. This can lead to delayed treatment and disease progression (Boycott et al., 2019).

One of the most important challenges in the diagnosis of rare diseases is their genetic origin and genetic heterogeneity. Many rare diseases can present with different clinical symptoms, which can be confused with common diseases. Although the development and introduction of genetic testing has greatly improved the diagnosis of rare diseases, many rare diseases remain undiagnosed or misdiagnosed (Wright et al., 2018). Next-generation sequencing (NGS) technologies, in particular, enable the simultaneous screening of multiple diseases with large gene panels and accelerate the diagnostic process (Splinter et al., 2018). These delays in diagnosis often lead to delayed initiation of treatment and patients' quality of life decreases as the disease progresses. Current treatment options for most rare diseases are limited, and treatments are often aimed at alleviating symptoms rather than curing the disease completely (Austin et al., 2018). Therefore, increasing research and clinical trials in the field of rare diseases is critical for the development of new treatment options.

One of the most important advances in the treatment of rare diseases is the development of gene therapy and oligonucleotide-based therapeutic approaches. Gene therapy is a therapeutic strategy that aims to correct a faulty gene or synthesize a missing

protein. These treatment modalities hold great promise, especially in rare diseases associated with genetic mutations (High & Roncarolo, 2019). In recent years, gene therapies approved by the FDA have been an important milestone in the field of rare diseases. For example, onasemnogene abeparvovec (trade name Zolgensma®), used in the treatment of Spinal Muscular Atrophy (SMA), was developed as a gene therapy aimed at correcting the mutation in the *SMN1* gene and has proven its success in this field (Mendell et al., 2017). In addition, antisense oligonucleotide (ASO)-based therapeutic approaches are another important treatment modality used in rare diseases. ASOs aim to alter the expression of disease-causing genes by targeting them at the mRNA level. This method has been successfully applied in diseases such as Duchenne Muscular Dystrophy (DMD) and SMA (Aartsma-Rus & Krieg, 2017). Especially in SMA, nusinersen (commercially known as SPINRAZA®®) regulates the splicing of the *SMN2* gene, increasing the production of functional SMN protein and slowing the progression of the disease (Finkel et al., 2017).

Clinical trials and research in the field of rare diseases have made great advances in understanding the pathophysiology of disease. However, due to the nature of rare diseases, funding and resources to treat them are limited. Many pharmaceutical companies avoid investing in research to treat rare diseases because patient populations with these diseases are often small. Despite this, governments and international organizations have launched several programs to raise awareness of rare diseases and support the development of treatments. For example, the Orphan Drug Act (1983) in the US is an important law that encourages the development of drugs for rare diseases (Kesselheim et al., 2019). As the number of research and clinical trials on rare diseases increases, treatment options are evolving. In particular, gene therapy, cellular therapies and oligonucleotide-based therapies are opening new horizons in the treatment of these diseases. Advances in genetic engineering and biotechnology offer hope for many rare diseases that cannot be cured.

Among rare diseases, Spinal Muscular Atrophy (SMA) has made great progress in terms of treatment options in recent years. SMA is a serious genetic disease characterized by the degeneration of motor neurons and occurs in infancy. SMA is caused by mutations in the *SMN1* gene, resulting in a deficiency of the SMN protein. This protein is critical for the healthy functioning of motor neurons (Kolb & Kissel, 2015). In the treatment of SMA, gene therapies and ASO-based approaches have shown highly

effective results. Gene therapies such as Onasemnogene Abeparvovec aim to directly correct the mutation in the *SMN1* gene, while ASO-based therapies such as Nusinersen increase the production of functional SMN protein by regulating the splicing process of the *SMN2* gene (Mendell et al., 2017). These therapeutic approaches have significantly improved the survival and quality of life of SMA patients. Rare diseases are now better understood and treated thanks to advances in genetic sciences and biotechnology. Examples such as SMA show great promise in the treatment of rare diseases and demonstrate the potential of new treatment modalities such as gene therapy and oligonucleotide-based approaches (Mercuri & Finkel, 2018).

2.2. Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by progressive degeneration of motor neurons. Located in the central nervous system, motor neurons are cells that control muscle movements and initiate muscle contractions. In SMA, the production of SMN protein in motor neurons is deficient due to mutations or deletions in the Survival Motor Neuron 1 (*SMN1*) gene. SMN protein is vital for motor neuron survival, maintenance of function and regulation of cellular processes, particularly RNA processing and snRNP biogenesis (Burghes & Beattie, 2009). The *SMN1* gene is localized on the long arm (5q13.2) of chromosome 5 of the human genome. The *SMN2* gene, located in the same chromosomal region, is a close homolog of the *SMN1* gene and the main difference between these two genes is that the *SMN2* gene carries a mutation that produces a mostly non-functional protein isoform. Although the *SMN2* gene has the capacity to produce SMN protein, it produces an mRNA that is largely missing exon 7, resulting in a non-functional form of SMN protein (Lorson et al., 1999). The copy number of the *SMN2* gene often determines the severity of SMA disease; more copies of *SMN2* may contribute to a milder course of the disease (Prior, 2008).

This figure shows the localization of the *SMN1* and *SMN2* genes, which form the genetic basis of Spinal Muscular Atrophy (SMA), on the long arm of chromosome 5 (5q13) in the human genome. The *SMN1* gene is located in the telomeric region, while the *SMN2* gene is located in the centromeric region. The pathogenesis of SMA is usually caused by mutations or deletions in the *SMN1* gene, whereas the *SMN2* gene produces a

limited functional SMN protein. The copy number of the *SMN2* gene is an important factor influencing the severity of SMA disease (Lunn & Wang, 2008).

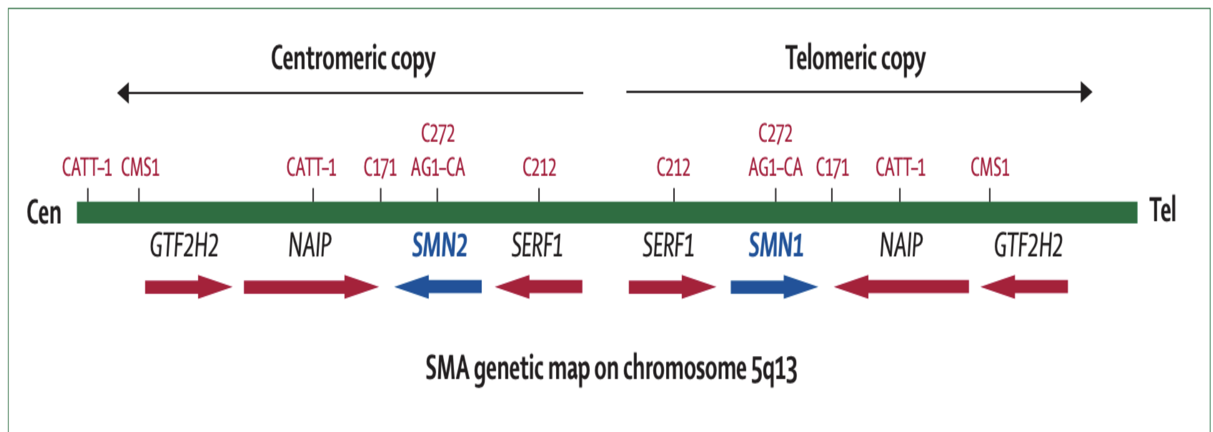

Figure 1: Localization of *SMN1* and *SMN2* Genes on Chromosome 5q13 (Lunn & Wang, 2008).

Functional loss of the *SMN1* gene leads to both impaired cellular function and weakened synaptic connections in motor neurons. These impairments result in clinical symptoms such as muscle weakness, atrophy and, in severe cases, respiratory failure (Lefebvre et al., 1995). The localization of the *SMN1* and *SMN2* genes on the genetic map of SMA is critical for understanding the molecular basis of the disease and this map plays an important role in the diagnosis of SMA and the development of treatment strategies.

The clinical manifestations of SMA and the severity of the disease differ according to the age of onset and loss of motor function. There are four clinical types of SMA and these types are classified according to age of onset, severity of symptoms and disease progression. Each type of SMA is associated with different genetic and molecular changes.

2.2.1. SMA Type 1 (Werdnig-Hoffmann Disease)

SMA Type 1 is the most severe form of SMA and occurs shortly after birth. Babies with type 1 usually cannot lift their heads, sit up or have difficulty swallowing. Type 1 SMA patients often have homozygous deletions or severe mutations in the *SMN1* gene. These mutations lead to an almost complete loss of production of the functional SMN protein (Lefebvre et al., 1995; Burghes & Beattie, 2009). SMN protein has a critical role in snRNP (small nuclear ribonucleoprotein) biogenesis in the cell nucleus. SMN protein deficiency leads to defects in RNA processing and splicing in motor neurons, resulting in motor neuron death and neuronal degeneration (Lorson et al., 1999). In these patients, the

SMN2 gene is a homologous gene similar to *SMN1*, but the majority of the protein produced from *SMN2* are partial proteins that do not include exon 7. This results in the production of non-functional SMN protein isoforms (Monani et al., 2000). One of the most important factors in SMA Type 1 is the splicing error of exon 7. SMA Type 1 patients usually have very few copies of *SMN2* and this severely limits functional SMN protein production (Mailman et al., 2002). Treatment options include antisense oligonucleotides (ASO) such as nusinersen, which promotes the production of full-length mRNA containing exon 7 of *SMN2*. Nusinersen enables the inclusion of exon 7 through splicing editing of the *SMN2* gene so that more functional SMN proteins are produced (Finkel et al., 2017).

2.2.2 SMA Type 2 (Moderate SMA)

SMA Type 2 is usually diagnosed in infancy, between 6-18 months. It has a milder course than Type 1. SMA Type 2 patients can sit up, but usually cannot walk and experience secondary complications such as scoliosis or breathing problems. SMA Type 2 patients usually have homozygous mutations or deletions in the *SMN1* gene, but they have more copies of *SMN2* than Type 1 patients (Wirth et al., 2006). These extra copies allow the production of more functional SMN protein and lead to a milder phenotype of the disease. SMA Type 2 patients are less deficient in SMN protein because they produce a greater amount of functional SMN protein due to the increased *SMN2* copy number (Coovert et al., 1997). However, the amount of SMN protein produced is still far below normal levels, which prevents motor neurons from functioning properly and leads to a progressive loss of motor skills (Lorson et al., 1999). As a treatment, nusinersen is also used for Type 2 SMA patients. This drug slows motor neuron loss by enabling the *SMN2* gene to produce a full-length functional SMN protein through the inclusion of exon 7 (Finkel et al., 2017). New gene therapies and oral splicing modulators, such as risdiplam, are also being developed for Type 2 patients. Risdiplam alters the splicing process of *SMN2* mRNA, increasing the inclusion of exon 7 and raising levels of functional SMN protein (Ratni et al., 2018).

2.2.3 SMA Type 3 (Kugelberg-Welander Disease)

SMA Type 3 is a milder form of SMA and is usually diagnosed during childhood or adolescence. Type 3 SMA patients can walk, but may lose the ability to walk in later

years. These patients have homozygous mutations in the *SMN1* gene, but have many more copies of *SMN2* compared to other types of SMA, resulting in the production of more functional SMN proteins (Lefebvre et al., 1995). At the molecular level, Type 3 SMA patients generally have greater efficiency in SMN protein production, but this production does not reach normal levels. In these patients, motor neuron loss progresses more slowly, which allows motor skills to be preserved for longer periods of time (Wirth, 2000). Generally, respiratory and swallowing functions are preserved in Type 3 SMA patients, but walking ability may be affected. The increased copy number of *SMN2* in type 3 SMA patients is the main reason for the milder course of the disease. The amount of functional SMN protein produced in these patients may be sufficient for the survival of motor neurons, but this level is not sufficient for complete preservation of motor functions (Wirth et al., 2006). In terms of treatment, SMA Type 3 patients may also benefit from splicing modulators such as nusinersen.

2.2.4 SMA Type 4 (Adult Onset SMA)

SMA Type 4 is the mildest form of SMA and is usually diagnosed in adulthood. This form progresses slowly and patients can usually move independently. The level of SMN protein is higher in Type 4 SMA patients because they have a much higher number of copies of *SMN2* (Lefebvre et al., 1995). SMA Type 4 patients produce sufficient amounts of SMN protein to maintain the function of motor neurons. However, in the later stages of the disease, motor neurons may start to lose their function. Symptoms in type 4 SMA patients are usually mild and limited to mild muscle weakness (Burghes & Beattie, 2009). Type 4 patients produce a full-length SMN protein containing exon 7, explaining the mild phenotype of the disease. For these patients, treatments such as nusinersen and risdiplam can be used (Ratni et al., 2018).

Each of the types of Spinal Muscular Atrophy (SMA) is based on a lack of functional SMN protein resulting from mutations or deletions in the *SMN1* gene. The main difference between these types is related to the *SMN2* gene copy number; patients with more copies of *SMN2* have a milder disease course. For example, SMA Type 1 usually has very few copies of *SMN2*, while Type 3 and Type 4 patients have more copies of *SMN2*, increasing the amount of functional SMN protein produced. However, most of the SMN protein produced from *SMN2* is not sufficient as it does not contain exon 7 and

therefore the disease phenotype cannot be fully prevented (Lefebvre et al., 1995; Mailman et al., 2002).

Clinical differences between SMA types are also directly related to the rate of motor neuron death and the severity of muscle atrophy. In SMA Type 1, motor neuron death is rapid and aggressive, whereas in Type 4 the process is much slower. This determines how long patients experience symptoms and how long they can maintain their motor skills. Treatment strategies are also shaped according to these differences; while early intervention is vital to preserve motor function in Type 1 patients, treatment in Type 3 and Type 4 patients is more focused on improving quality of life (Finkel et al., 2017).

Finally, modern approaches such as nusinersen, risdiplam and gene therapies are being used to treat SMA types by increasing SMN protein production and slowing disease progression. By targeting genetic mutations, these therapies directly affect the biological process in motor neurons lacking the SMN protein, allowing specific strategies to be developed for each SMA type (Ratni et al., 2018).

This table summarizes the clinical features of the different types of Spinal Muscular Atrophy (SMA). SMA is classified according to criteria such as age of onset of the disease, peak level of motor development, life expectancy, proportion of the patient population and *SMN2* gene copy number (Chaytow et al., 2021).

Table 1: Classification of Spinal Muscular Atrohpy (Chaytow et al., 2021).

	Age of Onset	Highest Achieved Motor Milestone	Life Expectancy*	Proportion of Patients With SMA	*SMN2* Copies
Type 0	Birth	Never sitting	<6 months	<1%	1
Type I	<6 months	Never sitting	8-24 months	50%-60%	2-3
Type II	6-18 months	Sitting	20s-30s	30%	2-4
Type III	18 months-30 years	Walking	Normal	10%	3-5
Type IV	>30 years	Walking	Normal	5%	3-5

*Without disease-modifying treatment or mechanical ventilation.

2.2.5 Importance and Methods of Genetic Tests in SMA Diagnosis

Spinal Muscular Atrophy (SMA) is a serious neuromuscular disease affecting motor neurons and is of genetic origin. The diagnosis of SMA is based on the detection of deletions or pathogenic mutations in the *SMN1* (Survival Motor Neuron 1) gene. These diagnostic methods are vital for correctly classifying the disease and directing the patient to the appropriate treatment. The genetic makeup of SMA also influences the clinical diagnostic processes, so accurately analyzing *SMN1* and *SMN2* gene copies plays an important role in predicting disease severity and response to treatment. The main genetic tests used to diagnose SMA include Multiplex Ligation-dependent Probe Amplification (MLPA), PCR-based tests and DNA sequencing. These tests are considered the gold standard, especially in the detection of mutations and deletions in the *SMN1* gene.

MLPA (Multiplex Ligation-dependent Probe Amplification) is one of the most widely used methods for the diagnosis of SMA and is usually performed to determine *SMN1* and *SMN2* gene copy numbers. The vast majority of SMA patients have homozygous deletions in the *SMN1* gene or other mutations that cause a lack of functional SMN protein. MLPA is a highly sensitive method used to detect deletions and copy number differences in these genes (Prior, 2008). The MLPA test is considered the gold standard for diagnosing SMA because it provides high accuracy and sensitivity. In this method, DNA sequences are targeted using specific probes for each gene region. Probes designed specifically for the *SMN1* and *SMN2* genes detect the copy number of these genes. With MLPA, both homozygous deletions in the *SMN1* gene and *SMN2* gene copy

number can be determined simultaneously. Determination of *SMN2* copy number is critical in predicting the clinical course of the disease, as patients with more *SMN2* copies usually have a milder clinical picture (Chen et al., 2010). MLPA is widely used not only in the diagnosis of SMA, but also in carrier testing and prenatal diagnosis. In carrier individuals, deletions or mutations in the *SMN1* gene can be easily detected by MLPA. In prenatal diagnosis, MLPA is used to analyze fetal DNA samples obtained by chorionic villus biopsy or amniocentesis. In this way, it is possible to detect the disease before birth in pregnancies at risk of SMA.

Polymerase Chain Reaction (PCR) is another important molecular method used in the diagnosis of SMA. PCR enables the amplification of specific DNA segments and is applied to detect specific mutations or deletions in the *SMN1* gene. Homozygous deletions, which are common in SMA patients, can be detected quickly and reliably by PCR. In this method, DNA sequences are specifically targeted and the amplified DNA fragments are analyzed to detect mutations or deletions (Lunn & Wang, 2008). PCR-based tests are also used to detect small nucleotide differences between the *SMN1* and *SMN2* genes. For example, the C-thymine change in exon 7 in the *SMN1* gene is an important genetic marker in the diagnosis of SMA. PCR can be customized to distinguish these small mutations. Variations such as allele-specific PCR, where specific alleles are amplified, are commonly used to detect such small genetic differences. In addition to SMA diagnosis, PCR-based tests are often preferred for the detection of carrier individuals. In individuals who are carriers of SMA, one copy of the *SMN1* gene is deletion, which can be easily detected by PCR (Wirth et al., 2006). PCR-based methods are very important in prenatal diagnosis. In individuals with a family history of SMA, PCR tests can be performed on fetal DNA samples obtained by amniocentesis or chorionic villus biopsy. These tests allow the detection of homozygous deletions in fetal DNA and thus prenatal diagnosis of SMA can be made (Lunn & Wang, 2008).

DNA sequencing is used to detect rare mutations in SMA patients. Sequencing allows for detailed analysis of all coding regions of the genetic material, allowing for direct examination of rare mutations in the *SMN1* gene. Although homozygous deletions in the *SMN1* gene are the most common genetic cause of SMA, some patients may also have pathogenic point mutations. Traditional sequencing methods such as Sanger sequencing can be used to detect these mutations, but methods such as Next-Generation

Sequencing (NGS) may be preferred for more comprehensive analysis (Chen et al., 2010). Sanger sequencing is particularly effective in detecting mutations in exon 7 and other coding regions of the *SMN1* gene. This method is widely used due to its low cost and reliability. However, next-generation sequencing methods such as NGS provide a more comprehensive analysis and make it possible to sequence many genes simultaneously. This is particularly useful for detecting rare mutations in genetic diseases such as SMA. NGS can be used both for prenatal diagnosis and for the detection of carrier individuals and provides fast results. Since SMA is an autosomal recessive disease, there is a 25% chance that a child will develop the disease if both parents are carriers. Therefore, prenatal genetic testing is of great importance, especially for individuals with a family history of SMA. Prenatal diagnosis is made by examining fetal DNA samples obtained through amniocentesis or chorionic villus biopsy. Using methods such as MLPA, PCR and sequencing, deletions or pathogenic mutations in the *SMN1* gene are detected on these samples. Prenatal diagnosis allows early detection of SMA and provides information to the family about the disease and helps to determine possible treatment options (Prior, 2008). Carriage tests are performed to detect deletions or mutations in the *SMN1* gene in individuals who may be carriers of SMA. Carriage tests can be performed by both MLPA and PCR-based methods. These tests are vital for family planning, especially in individuals at risk of SMA. MLPA is the most commonly used method to detect *SMN1* gene deletions in carrier individuals. In addition, results obtained during prenatal diagnosis can also confirm carrier status (Chen et al., 2010).

Spinal Muscular Atrophy (SMA) is a common genetic disorder worldwide and has different prevalence rates in many countries. The prevalence of SMA varies depending on genetic differences between populations and carrier rates. The worldwide prevalence of SMA is better understood, especially with the widespread availability of genetic testing, and studies on the prevalence of SMA have shown that SMA is more common in some regions than others.

2.2.6 SMA Prevalence

The worldwide prevalence of SMA varies according to regional distribution. Europe has the highest prevalence of SMA, accounting for approximately 65.75% of the global SMA prevalence. In addition, the prevalence of SMA in North America is around

18.16%, indicating that the disease is also highly prevalent in this region (TREAT-NMD, 2020). In Central and South America, the prevalence of SMA is lower, accounting for about 5.17% of the total prevalence. In regions such as Australia and New Zealand, the prevalence of SMA is much lower, only around 1.11%. Finally, other regions of the world (e.g. Asia and Africa) are included in the so-called "Rest of the world" group, which accounts for 9.81% of the total SMA prevalence (TREAT-NMD, 2020). Looking at the worldwide distribution of SMA, it is noteworthy that the disease is most common in Europe and North America, while it is less common in other regions. This may be due to the greater availability of SMA screening and genetic testing in Europe and North America. In addition, genetic counseling and prenatal diagnosis practices are more common in these regions, allowing for early diagnosis and recording of SMA (Prior, 2008).

Turkey is a country with a high prevalence of genetic diseases and SMA has an important place among these genetic diseases. The prevalence of SMA in Turkey is similar to the averages in Europe. According to studies, the carrier rate of SMA in Turkey is around 1/40, which means that one in every 40 people is a carrier of SMA (Topaloglu et al., 2018). Studies on the prevalence of SMA in Turkey have revealed that the disease is more common in regions where consanguineous marriages are common. Consanguineous marriages may cause diseases with autosomal recessive inheritance to occur more frequently. In Turkey, SMA patients are usually diagnosed with genetic tests such as MLPA and PCR. With the expansion of SMA screening programs and the increase in genetic counseling, early diagnosis and treatment planning of the disease are carried out more effectively (Topaloglu et al., 2018). Awareness-raising studies and genetic screening programs for SMA in Turkey aim to better understand the prevalence of SMA and to obtain more comprehensive data.

SMA poses a significant public health problem as a genetic disease worldwide. While Europe and North America are among the regions where SMA is most prevalent, early diagnosis of the disease is becoming possible in countries such as Turkey through genetic screenings and carrier tests. Studies on the global distribution and prevalence of SMA are helping to better understand the genetic basis of the disease and to develop strategies for treatment. The global distribution of SMA varies under the influence of many factors such as genetic factors, carrier rates and the availability of genetic tests.

This figure shows the global distribution of a particular dataset, where Europe accounts for the largest proportion, comprising 65.75% of the total data. North America follows with 18.16%, while the rest of the world makes up 9.81%. The map highlights the significant concentration of data or activity in Europe, suggesting a potential focus of interest or research in that region. Smaller contributions come from Central & South America (5.17%) and Australia & New Zealand (1.11%), indicating lower participation or data presence from these regions.

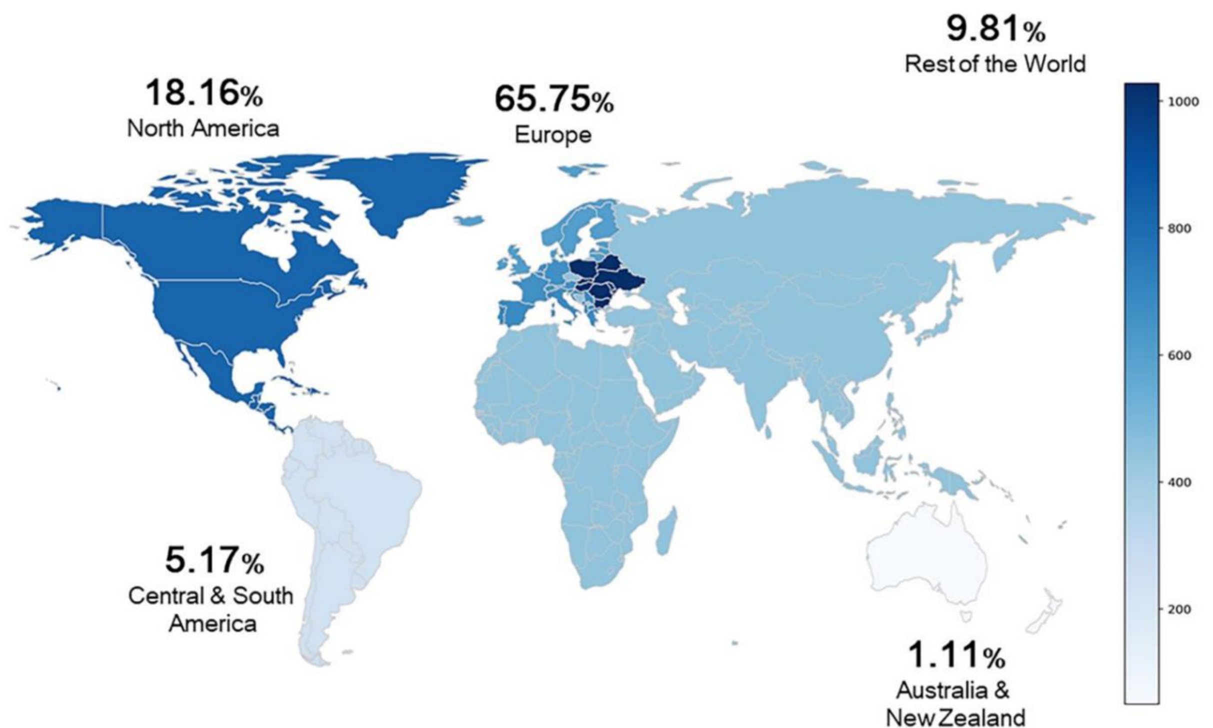

Figure 2: Worldwide distribution of SMA patients registered under TREAT-NMD. (Chong et al., 2021)

2.2.7 Genetic Basis and Pathophysiology of SMA

SMA is mainly caused by mutations or deletions in the Survival Motor Neuron 1 (*SMN1*) gene. The *SMN1* gene is responsible for the production of the SMN (Survival Motor Neuron) protein, which is vital for the survival and function of motor neurons (Lefebvre et al., 1995). SMN protein is required for the biogenesis of small nucleated ribonucleoprotein (snRNP) complexes and spliceosome functions in motor neuron cells. This protein ensures the correct processing of pre-mRNAs and regulation of alternative exonylation pathways (Burghes & Beattie, 2009). There is a second gene in the human genome, *SMN2*, which has the capacity to produce SMN protein. However, the mRNA produced by the *SMN2* gene is processed through alternative splicing, which largely

excludes exon 7. This leads to the production of a non-functional SMN protein isoform. In SMA patients, the ability of the *SMN2* gene to produce full-length mRNA containing exon 7 is limited, resulting in insufficient levels of SMN protein. Generally, an increased copy number of the *SMN2* gene is associated with a milder clinical picture in the phenotype of the disease; more copies of *SMN2* may enable the production of more functional SMN proteins and slow the course of the disease (Lorson et al., 1999).

This figure compares the splicing processes of the *SMN1* and *SMN2* genes between healthy individuals and SMA patients. (a) In a healthy individual, the *SMN1* gene performs correct splicing to produce full-length SMN protein. The *SMN2* gene, on the other hand, produces an incomplete (truncated) SMN protein, with exon 7 usually omitted. However, the *SMN2* gene also produces a small amount of full-length SMN protein. These processes ensure healthy motor neuron function with sufficient levels of SMN protein in the individual's motor neurons (Singh et al., 2021). (b) In the SMA patient, the *SMN1* gene is completely lost or dysfunctional, so no functional full-length SMN protein is produced. The missing SMN protein produced from the *SMN2* gene is insufficient for motor neurons to maintain their function. This results in motor neuron degeneration, which leads to the clinical manifestations of SMA (Chong et al., 2021).

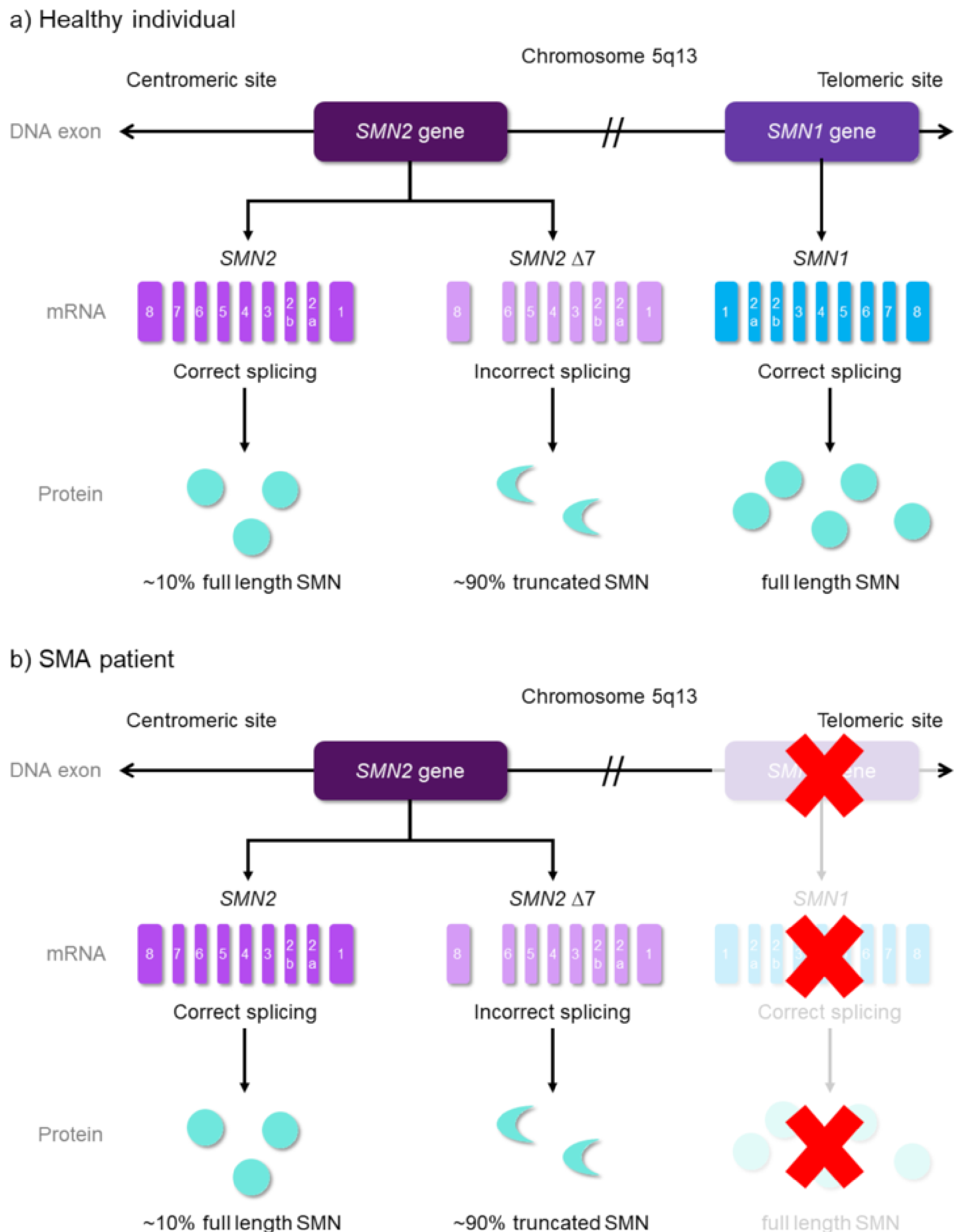

Figure 3: Molecular Mechanisms of *SMN1* and *SMN2* Gene Expression in Healthy Individuals and SMA Patients (Chong et al., 2021).

SMN protein deficiency impairs the synaptic function of motor neurons and the formation and maintenance of synapses at the neuromuscular junction. This leads to degeneration of motor neurons and results in loss of synaptic connections. Impaired nerve-muscle communication leads to muscle weakness and atrophy. Furthermore, the

degeneration of motor neurons leads to irreversible damage to the spinal cord, reinforcing the progressive nature of the disease (Burghes & Beattie, 2009).

The pathophysiology of SMA focuses specifically on the cellular functions of the SMN protein. The SMN protein interacts with Gemin proteins, a protein complex known for its RNA binding capacity. This complex is involved in the assembly of snRNPs in the nucleus and in the biogenesis of spliceosomal small nuclear RNAs (snRNAs). Deficiency of the SMN protein disrupts these critical cellular processes, resulting in loss of function and eventual death of motor neurons (Lefebvre et al., 1995).

Motor neurons are critical cells in the central nervous system that transmit movement commands to the muscles and initiate muscle contractions. These neurons function properly with a balance of excitatory and inhibitory signals. At the nerve-muscle junction (neuromuscular junction), neurotransmitters released from the axon terminals of motor neurons bind to receptors on muscle cells, causing them to contract. This process is regulated by a complex series of events, including the transport of synaptic vesicles, neurotransmitter release and stimulation of muscle cells. Healthy motor neurons produce high levels of SMN (Survival Motor Neuron) protein, which is essential for the proper functioning of these processes. In particular, SMN protein plays a critical role in the biogenesis of small nucleated ribonucleoproteins (snRNP), RNA processing and maintenance of synaptic structure (Burghes & Beattie, 2009). Adequate levels of SMN protein are essential for motor neurons to maintain synaptic plasticity and synaptic integrity (Lefebvre et al., 1995).

In SMA patients, production of SMN protein is deficient due to mutations or deletions in the *SMN1* gene. This deficiency leads to a range of morphological and functional defects in motor neurons. SMN protein deficiency causes critical impairments in synaptic vesicle transport and maintenance of synaptic structures (Lorson et al., 1999). As a consequence, synaptic connections between motor neurons and muscle cells are weakened and synapses are lost. The lack of synaptic vesicles reduces neurotransmitter release and leads to insufficient stimulation of muscle cells (Burghes & Beattie, 2009). Motor neurons undergo degeneration in this process and other neurons in the vicinity may also be affected and develop hyper-excitability (Fletcher et al., 2017). This synaptic dysfunction is one of the main mechanisms underlying muscle weakness and atrophy seen in SMA (Lefebvre et al., 1995).

Deficiency of the SMN protein also produces various autonomous and non-autonomous effects within motor neurons themselves. Autonomous effects affect the intrinsic processes of motor neurons (e.g. RNA processing and synaptic vesicle transport), while non-autonomous effects disrupt interactions between motor neurons and relationships with peripheral support cells (Burghes & Beattie, 2009). Loss of synapses, reduced excitability and ultimately death of motor neurons are characteristic features of SMA. These degenerative processes caused by SMN protein deficiency are key factors explaining the progressive nature of the disease and motor neuron loss (Lefebvre et al., 1995).

This figure shows normal motor neuron function versus synaptic dysfunction and motor neuron degeneration due to SMN protein deficiency. Under normal conditions, motor neurons establish healthy synaptic connections and efficiently stimulate muscles. However, in SMA, SMN deficiency leads to impaired synaptic connections of motor neurons, synapse loss and cellular degeneration. These processes are the main mechanisms underlying the muscle weakness and atrophy associated with motor neuron loss in SMA.

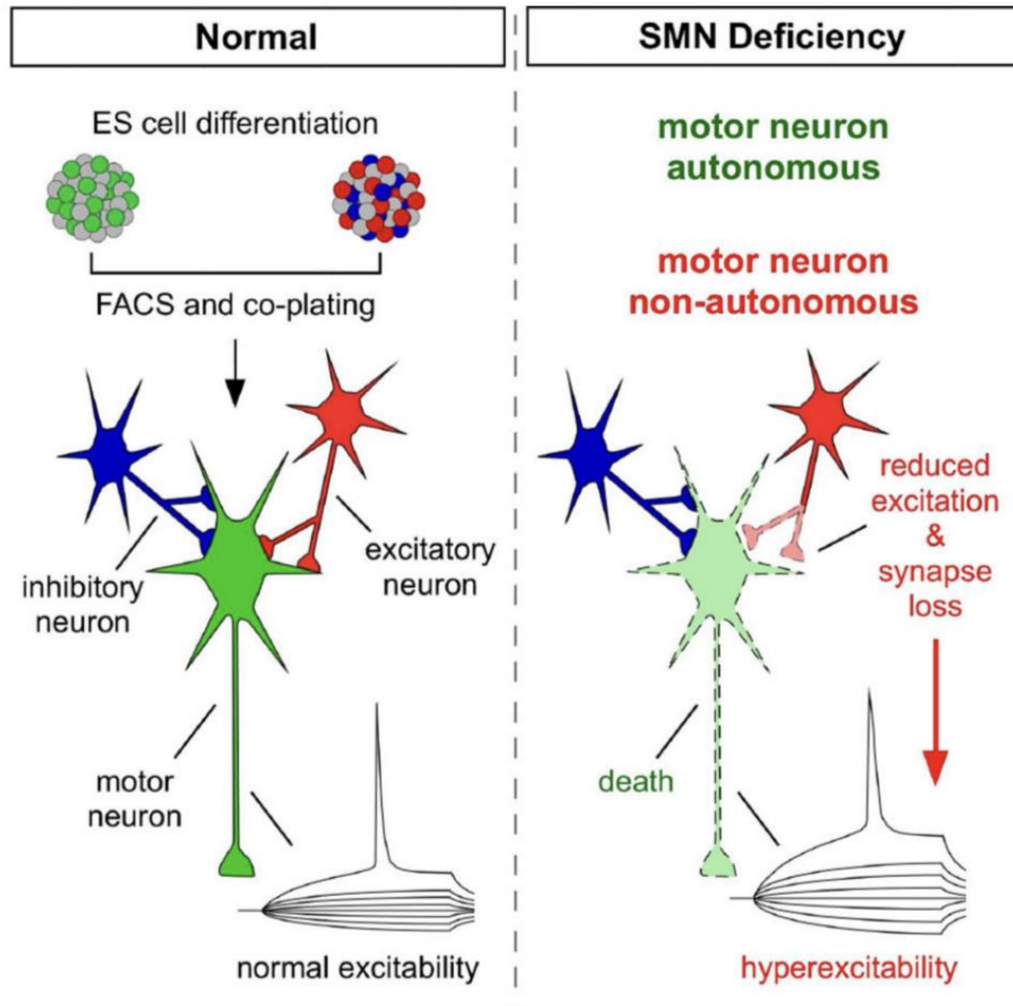

Figure 4: Effects of SMN Deficiency on Motor Neurons in SMA Disease (Simon et al., 2016)

2.2.8 Spinal Muscular Atrophy (SMA) History: From Genetic Discoveries to Therapeutic Advances

Spinal Muscular Atrophy (SMA) has been identified and studied since the late 19th century as a serious genetic disorder affecting motor neurons. The scientific history of SMA ranges from clinical observations to genetic discoveries and modern treatment methods. The discovery of the genetic origins of SMA and the treatments developed based on this knowledge have been an important milestone in the management of this disease.

The first clinical description of SMA was made by Guido Werdnig and Johann Hoffmann in 1891. Both scientists studied muscle weakness and motor neuron degeneration in infants and young children and named this condition "Werdnig-

Hoffmann Disease" (Pearn, 1978). Werdnig and Hoffmann described this disease, characterized by muscle weakness and atrophy, as a clinical entity that would later be known as SMA. At that time, SMA was often confused with other neuromuscular diseases such as muscular dystrophy. SMA was included in the group of neuromuscular diseases because the genetic makeup of the disease was not yet fully known at that time. However, clinical observations revealed the innate characteristics of SMA and gave clues that the disease has an autosomal recessive inheritance pattern (Lefebvre et al., 1995).

Until the 1950s, studies were limited to observational studies to better understand the clinical course of SMA. The discovery of genetic origins was not yet possible at this time; therefore, the clinical diagnosis of diseases such as SMA was often a slow process and there was no definitive treatment. Nevertheless, Werdnig and Hoffmann's clinical observations clearly demonstrated that the disease was a condition affecting motor neurons and laid the foundation for later genetic research (Pearn, 1978).

Since the 1950s, advances in genetic science have contributed greatly to the understanding of the genetic structure of SMA. The discovery that autosomal recessive inheritance pattern plays a role in SMA led to a better understanding of the genetic transmission mechanism of the disease. Carrier frequency studies conducted during this period showed that SMA is linked to cases in family history. In particular, studies conducted in the families of patients with SMA revealed that this disease can be transmitted through recessive genes (Wirth et al., 2006).

Genetic linkage analysis has been an important step in getting to the root of SMA. In the early 1990s, DNA analysis and genetic linkage studies revealed that SMA is associated with a gene on chromosome 5. Scientists discovered that SMA is caused by mutations in the *SMN1* (Survival Motor Neuron 1) gene, which plays a critical role in the survival of motor neurons (Lefebvre et al., 1995). Genetic research during this period is considered a major step towards understanding the molecular genetics of SMA. In addition, these discoveries opened the door to the development of genetic-based therapies.

In 1995, the molecular mechanism of SMA became clearer and scientists identified mutations in the *SMN1* gene as the main cause behind SMA. This important discovery was a breakthrough in understanding the biological basis of the disease. The

SMN1 gene is responsible for the production of the SMN protein, which is vital for the survival of motor neurons. Deficiency of this protein leads to loss of motor neuron functionality and symptoms such as muscle weakness and atrophy (Lorson et al., 1999). Furthermore, besides mutations in the *SMN1* gene, the *SMN2* gene also has an important role in the course of the disease. However, *SMN2* fails to produce a full-length functional SMN protein because exon 7 is often left out during the splicing process of this gene (Mailman et al., 2002).

The discovery of the *SMN1* and *SMN2* genes has led to a focus on molecular-based treatments for SMA and opened the door to the development of gene therapy and antisense oligonucleotide (ASO)-based therapies. These discoveries have strengthened the idea that SMA is a treatable disease. In addition, the widespread use of genetic testing and studies to determine the carrier status of SMA also gained momentum during this period (Chen et al., 2010).

The first major advance in the treatment of SMA was achieved with Nusinersen, which was approved by the FDA in 2016. Nusinersen is an antisense oligonucleotide therapy that enables proper splicing of the *SMN2* gene to include exon 7 and produce functional SMN protein. This treatment reduces motor neuron loss and preserves motor skills in SMA patients. Nusinersen's clinical successes have raised hopes that SMA is a treatable disease (Finkel et al., 2017).

Another important step in the treatment of SMA was taken with the development of the gene therapy Onasemnogene Abeparvovec. Onasemnogene Abeparvovec aims to restore the *SMN1* gene to patients by directly targeting the genetic cause of SMA. This treatment has the potential to improve motor function, especially when administered in infancy, and is considered a revolutionary approach to SMA treatment. Onasemnogene Abeparvovec is administered to patients as a single dose via adeno-associated viral vector (AAV9) and this treatment allows the *SMN1* gene to become active again (Day et al., 2019).

Risdiplam (trade name Evrysdi®) is another SMA treatment approved in 2020 and is an orally available splicing modulator. Risdiplam increases the production of SMN protein by promoting the splicing of exon 7 in the *SMN2* gene. This treatment option has

the potential to improve motor skills, especially in infant and pediatric SMA patients (Ratni et al., 2018).

Advances in the treatment of SMA will continue in the future with developments based on innovative treatment methods such as genetic engineering and stem cell research. Gene editing technologies such as CRISPR/Cas9 can provide a permanent cure for the disease by directly correcting mutations in the *SMN1* gene. By precisely intervening in specific regions of DNA, this technique corrects genetic mutations and offers an effective treatment that addresses the root cause of the disease. The potential for CRISPR/Cas9 technology to be used in the treatment of SMA is promising, especially as it directly targets mutations and can treat the disease at the genetic level. Research suggests that such gene editing strategies could be used to treat SMA in the near future (Schuster et al., 2019).

In addition, stem cell therapies hold significant promise in the treatment of SMA. Stem cells can be used to regenerate motor neurons and repair damage to the nervous system. This approach allows the production of healthy cells to replace damaged motor neurons. Stem cell therapy can compensate for motor neuron loss and restore muscle function in advanced stages of SMA (Ramos et al., 2020). However, this treatment modality is still being investigated in the early stages and further studies are required for clinical use.

Another strategy among future treatment approaches is the personalization of pharmacological treatments and the development of targeted treatment options at different stages of the disease. Combination therapies, gene therapies, antisense oligonucleotide (ASO) therapies and stem cell therapies will be used together to manage the disease more effectively. These strategies offer various ways to protect motor neurons and maintain their functionality. Moreover, customizing treatment options according to the individual genetic makeup of patients may improve the efficacy of treatment (Finkel et al., 2017).

Early detection of SMA carriers and preventive treatment strategies will also be critical to reduce the prevalence of the disease in the future. Genetic screening and prenatal diagnostics will allow for early detection of the disease and thus the implementation of appropriate treatment strategies. Implementation of large-scale

screening programs, especially for SMA carriers, can prevent the spread of the disease in the community and contribute to the protection of future generations (Prior, 2008).

As a result, future SMA treatment approaches will be built on innovative methods such as genetic engineering, stem cell therapies, personalized medicine and combination therapies. These treatments will aim to improve symptoms and halt the progression of SMA by intervening in the genetic basis of the disease. Increasing access to treatment and developing safer, more effective methods will contribute to SMA being recognized as a treatable disease.

2.2.9 Potential Genetic Therapies for the Treatment of SMA: Methods and Molecular Mechanisms

Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by degeneration of motor neurons due to mutations in the *SMN1* gene. SMA is one of the most common fatal genetic diseases in children and great advances have been made in the treatment of the disease. In recent years, genetic therapies have played an important role in the treatment of SMA, and several approaches have been developed that aim to halt or slow disease progression by increasing SMN protein levels. In this chapter, the methods of genetic therapies used and potentially used in the treatment of SMA are reviewed in detail.

RNA-based therapeutics are molecular tools that revolutionize the treatment of genetic diseases and directly modulate gene expression. These therapeutics include antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), micro RNA (miRNA) modulators and RNA aptamers. ASOs bind to specific mRNA molecules and disrupt the function of these mRNAs or regulate splicing processes, thereby stopping or increasing the production of specific proteins (Finkel et al, 2017). siRNAs target and degrade mRNA by RNA interference (RNAi) mechanism, which in turn inhibits the synthesis of target proteins (Adams et al., 2018). miRNA modulators regulate gene expression at the post-transcriptional level by inhibiting mRNA translation or promoting mRNA degradation (Janssen et al., 2013). RNA aptamers bind to specific proteins or small molecules with high affinity and inhibit their biological functions (Ng et al., 2006). These mechanisms enable RNA-based therapeutics to target disease-causing processes by directly interfering with genetic material in the treatment of genetic diseases.

However, RNA-based therapeutics also have several limitations and challenges. The efficacy of these therapeutics largely depends on factors such as access to target cells, intracellular bioavailability and stability (Crooke et al., 2018). By their very nature, RNA molecules can be rapidly degraded by nucleases, which shortens their intracellular lifespan (Adams et al., 2018). Furthermore, off-target effects and immunogenic responses are among the major issues limiting the safety and efficacy of these therapeutics (Janssen et al., 2013). Carrier systems and chemical modifications have been developed to improve the stability and delivery of RNA therapeutics to target cells, but these solutions are not always effective (Ng et al., 2006). As a result, the development of RNA-based therapeutics is a complex process that requires continuous improvements and research to increase efficacy and minimize side effects (Crooke et al., 2018).

Summarized how RNA-based therapeutics function and modulate gene expression at the mRNA level. Each strategy is used in the treatment of diseases, offering a different mechanism of intervention directed against specific genetic targets. The efficacy of such therapeutics depends on their ability to bind to the target mRNA, their efficiency of cellular uptake and their specificity (Zogg et al., 2023).

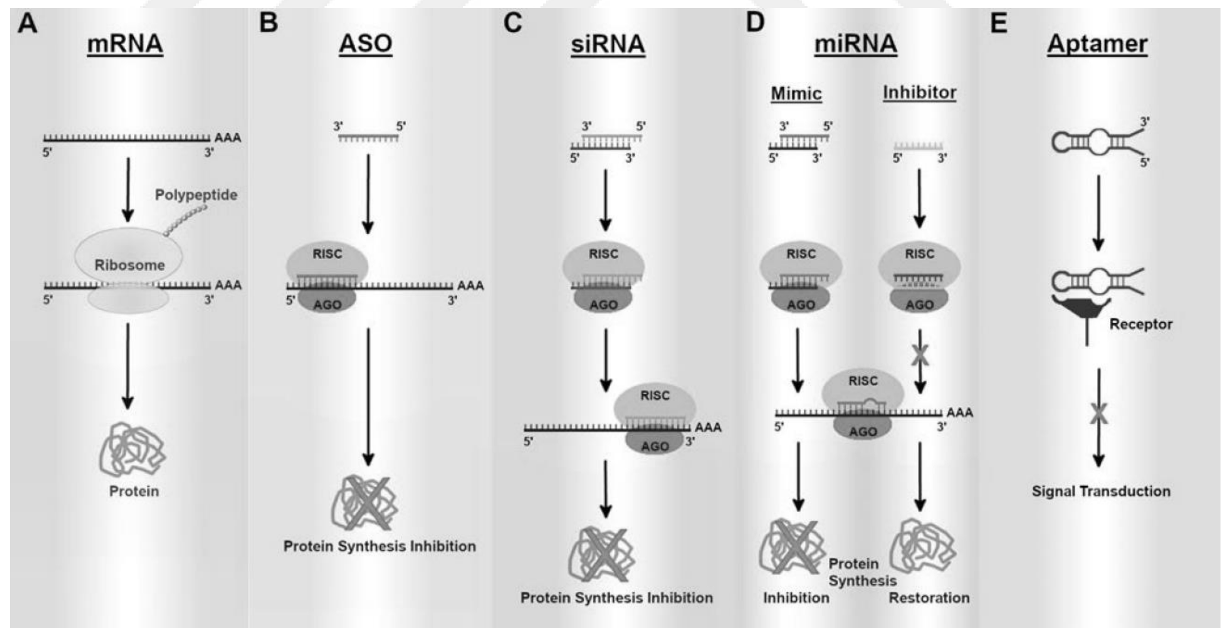

Figure 5: Molecular Mechanisms of RNA-Based Therapeutics: mRNA, ASO, siRNA, miRNA and Aptamer Strategies (Zogg et al., 2023).

Viral vector-based gene transfer is a method of genetic therapy in which viruses are used as carriers (vectors) to correct a missing, faulty or mutated gene or to carry a functional gene copy into target cells. Viruses have the natural capacity to carry genetic

material into cells, making them an ideal tool for gene therapy. The most common viral vectors used in this method include adeno-associated viruses (AAV), retroviruses and lentiviruses.

Viral vectors are viruses that have usually had their pathogenic genes removed and replaced with therapeutic genes. When these vectors enter cells, they carry genetic material into the nucleus of the target cell. This genetic material can be integrated into the cell's genome (as in retroviruses and lentiviruses) or remain as an extrachromosomal circle (as in AAV vectors). In the cell nucleus, the gene carried by the vector is transcribed and converted into mRNA, which is then translated by ribosomes and the therapeutic protein is synthesized. Thus, the function of the missing or defective gene is restored or a new function is introduced (Naso et al., 2017).

Viral vectors offer great advantages with their capacity to efficiently deliver genetic material to target cells and provide long-term expression. AAV vectors are often preferred due to their low immunogenic profile and high tropism capacity for various cell types. Retroviruses and lentiviruses, on the other hand, provide continuous expression thanks to their ability to integrate genetic material into the host cell genome. However, viral vector-based gene transfer also involves risks, such as potential immune responses and uncontrollability of genetic integration. In particular, genome integration of retroviruses and lentiviruses carries the risk of insertional mutagenesis, which can lead to side effects such as cancer (Naldini, 2015). Other limitations include limited carrying capacity for large genes and high production costs.

The vector is equipped with mutated AAV2 ITR regions and controls SMN gene expression with the CMV enhancer, CB promoter and SV40 intron. The AAV9 vector binds to the cell surface and is taken into the cell by endocytosis. In the cytoplasm, the outer sheath of the vector is opened, releasing the genetic material. This material then reaches the nucleus, where it participates in episomal formation, leading to the production of SMN protein (Nishio et al., 2023).

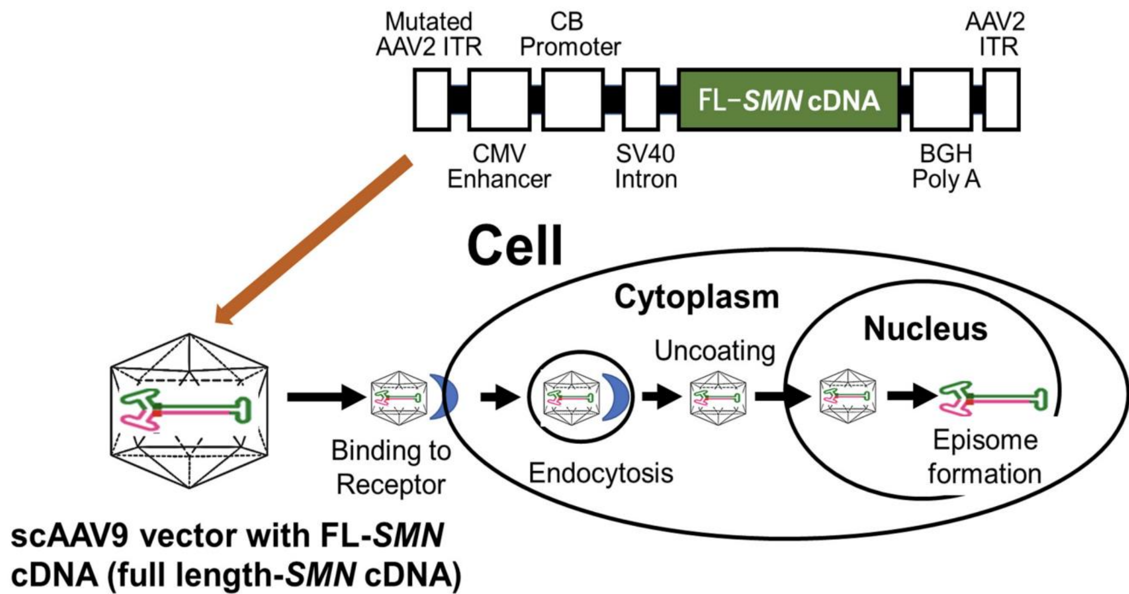

Figure 6: Intracellular mechanism of gene therapy with scAAV9 vector carrying full-length SMN cDNA (FL-SMN) for the treatment of Spinal Muscular Atrophy (Nishio et al., 2023).

Gene editing-based therapies are innovative treatment methods that offer the ability to modify, correct or silence specific DNA sequences by directly targeting genetic material. The most prominent of these technologies are CRISPR/Cas9, TALEN (Transcription Activator-Like Effector Nucleases) and ZFN (Zinc Finger Nucleases). These methods have the potential to correct genetic disorders or silence disease-causing genes by interfering with targeted gene sequences with high specificity.

The CRISPR/Cas9 system recognizes the target DNA sequence through a guide RNA (gRNA) and cuts it by the Cas9 endonuclease. The resulting double-strand breaks (DSB) are repaired by the cell's natural DNA repair mechanisms (non-homologous end joining, NHEJ or homology-directed repair, HDR). NHEJ often leads to gene silencing by making faulty repairs, whereas HDR makes targeted edits using a repair template (Doudna & Charpentier, 2014).

TALEN consists of proteins that can bind to and cut specific DNA sequences. Each TALEN protein contains DNA binding sites designed specifically for the target DNA sequence. After binding to DNA, these proteins create double-stranded DNA breaks, which are regulated by the cell's repair mechanisms. TALENs may have more specific targeting capacity compared to CRISPR/Cas9, but require a more complex and time-consuming design process (Boch & Bonas, 2010).

Zinc Finger Nucleases are proteins consisting of zinc finger motifs that bind specifically to DNA. These proteins bind to the target DNA sequence and create double-

strand breaks. The breaks are repaired by the cell and in the process the target DNA sequence is modified or edited. ZFNs are an older technology and are not as widely used as more modern methods such as CRISPR and TALEN, but are still preferred in some gene editing applications (Urnov et al., 2010).

Gene editing-based therapies have the potential to revolutionize the treatment of genetic diseases with the capacity to make targeted changes directly to DNA. Tools such as CRISPR/Cas9, TALEN and ZFN operate with high specificity and efficiency. CRISPR/Cas9's flexibility and relative ease of design have made it the most popular method of gene editing. However, gene editing technologies also carry some important limitations, such as off-target effects, risk of insertional mutagenesis and immune response. Off-target effects of systems such as CRISPR/Cas9 can lead to unwanted genetic alterations, which may jeopardize the safety of the treatment (Hsu et al., 2014). Furthermore, more safety and efficacy studies are needed before these technologies can be widely used in clinical practice.

Genetic therapies used in the treatment of SMA include a wide range of molecular methods aimed at correcting genetic defects underlying the disease or regulating protein production. RNA-based therapeutics, viral vector-based gene transfer and gene editing technologies are all methods with great potential in the treatment of SMA. Each of these therapies, with their own advantages and limitations, can be used at different stages in the treatment of SMA. In the future, more research is needed to improve the efficacy of these methods and to reach a wider group of patients. Furthermore, reducing the costs of these therapies and ensuring their long-term safety are critical for the success of genetic therapies in the treatment of SMA.

This figure includes three FDA-approved drugs (nusinersen, onasemnogene abeparvovec and risdiplam) and other drugs in clinical trials (branaplam, olesoxime, reldesemtiv and SRK-015). Nusinersen acts as an antisense oligonucleotide (ASO) that facilitates correct splicing of *SMN2* transcripts. Onasemnogene abeparvovec is a gene therapy method that replaces the *SMN1* gene with the AAV9 vector. Risdiplam and branaplam are small molecules that similarly regulate *SMN2* splicing. Olesoxime is a neuroprotective compound, while reldesemtiv and SRK-015 are agents targeted at enhancing muscle contractions. (Chong et al., 2021)

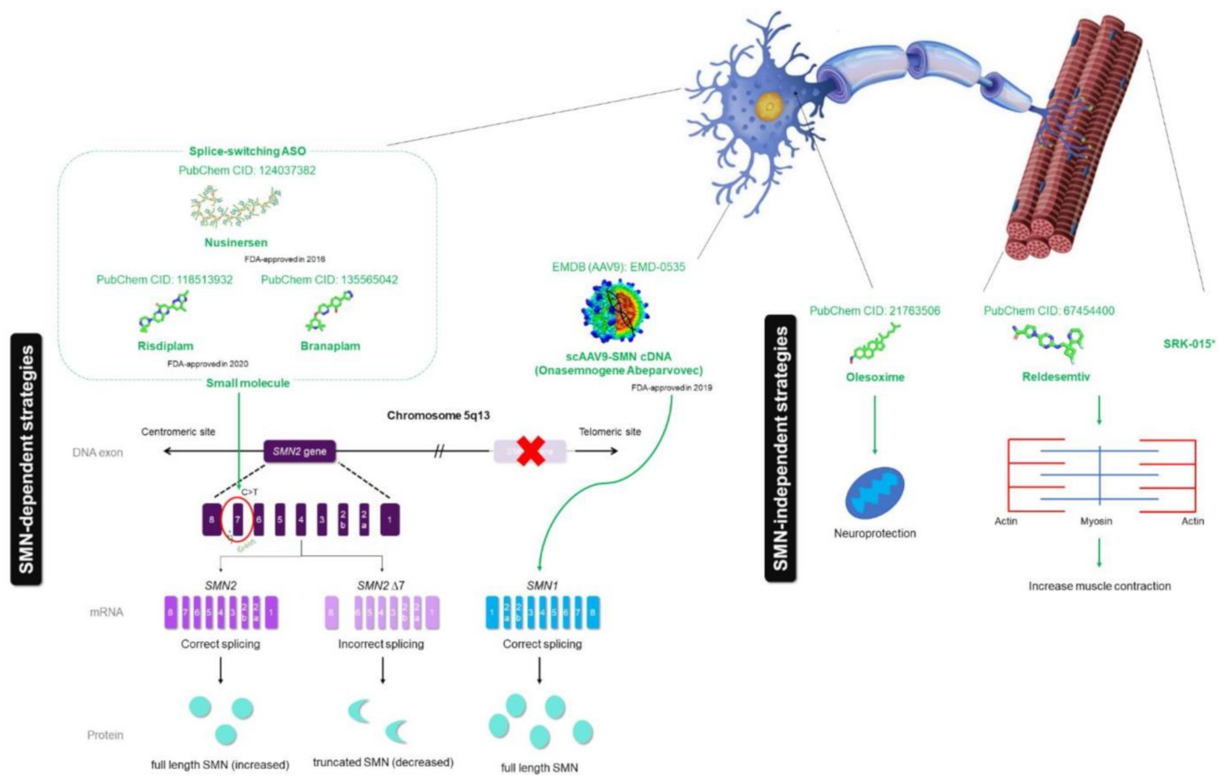

Figure 7: Scheme showing the therapeutic mechanisms of drugs used in the treatment of SMA (Chong et al., 2021).

2.2.10 Current Gene Therapies for the Treatment of SMA: Mechanisms, Shortcomings and Costs

Spinal Muscular Atrophy (SMA) is a neuromuscular disease characterized by progressive degeneration of motor neurons caused by inadequate production of SMN protein due to mutations in the *SMN1* gene. In the treatment of SMA, gene therapies aim to increase SMN protein levels and ensure the survival of motor neurons. Currently, there are three main gene therapies approved for the treatment of SMA: Nusinersen (trade name SPINRAZA®), Onasemnogene Abeparvovec (trade name Zolgensma®) and Risdiplam (trade name Evrysdi®). The molecular mechanisms, efficacy, shortcomings and costs of these treatments are reviewed in detail below.

Nusinersen is the first approved drug for the treatment of SMA and is classified as an antisense oligonucleotide (ASO). At the molecular level, Nusinersen acts on the pre-mRNA of the *SMN2* gene. Normally, the mRNA produced by the *SMN2* gene largely lacks exon 7, resulting in the production of non-functional SMN protein isoforms. Nusinersen binds to *SMN2* mRNA and ensures that exon 7 is included during splicing, thereby increasing the production of functional SMN protein (Finkel et al., 2017). This

mechanism promotes motor neuron survival by compensating for the lack of SMN protein. One of the major shortcomings of Nusinersen is the method of administration. The drug is delivered by direct intrathecal injection into the spinal fluid. This invasive procedure is particularly challenging for young children and infants and needs to be repeated at regular intervals. Furthermore, Nusinersen requires lifelong treatment, which creates an ongoing treatment burden for patients (Mercuri et al., 2018). The cost of Nusinersen treatment is high, approximately USD 750,000 for the first year and approximately USD 375,000 per year for subsequent years (Coriell et al., 2018). This cost can severely limit access to treatment.

Onasemnogene Abeparvovec is defined as a gene therapy that transfers the *SMN1* gene into motor neurons using an adeno-associated viral vector (AAV9). This treatment moves a healthy *SMN1* gene into motor neurons to replace a missing or mutated *SMN1* gene. This gene transfer allows motor neurons to restart production of the missing SMN protein (Mendell et al., 2017). Onasemnogene Abeparvovec is administered intravenously as a one-time treatment and is usually used in patients under 2 years of age. One of the main challenges of Onasemnogene Abeparvovec is that the treatment is only effective at a very early age, usually in children under 2 years of age. In addition, immune responses that may develop against the AAV9 vector may limit the effectiveness of the treatment. These immune responses may result in possible complications after treatment and reduced efficacy (Al-Zaidy et al., 2019). Furthermore, it is not yet fully known how sustainable the effects of Onasemnogene Abeparvovec are in the long term. Onasemnogene Abeparvovovec is considered one of the most expensive medicines in the world. The one-time treatment cost is approximately 2.1 million USD (Mendell et al., 2017). This high cost is one of the most important factors limiting the widespread availability of the treatment.

Risdiplam is one of the newest drugs for the treatment of SMA and is an orally administered small molecule compound. Risdiplam modulates the splicing of *SMN2* pre-mRNA, allowing the inclusion of exon 7, thereby increasing the production of functional SMN protein (Ratni et al., 2018). The major advantage of Risdiplam is that it can be taken orally, thus avoiding the need for invasive procedures such as intrathecal injection. This feature significantly improves patients' adherence to treatment and quality of life. Potential shortcomings of Risdiplam include limited data on the long-term efficacy and safety of the drug. As with other gene therapies, Risdiplam requires continuous treatment,

which creates a long-term treatment burden for patients (Mercuri et al., 2020). It also requires careful management of the drug's bioavailability and dosing as it is taken orally. The annual cost of Risdiplam has been estimated at approximately 340,000 USD (Ratni et al., 2018). While this cost is relatively low compared to other SMA treatments, it still represents a significant economic burden.

These drugs aim to slow or stop the progression of the disease by increasing SMN protein levels in SMA treatment and act through different molecular mechanisms (Nishio et al., 2023).

Table 2: Gene Therapies for SMA Disease: Nusinersen, Onasemnogene Abeparvovec and Risdiplam (Nishio et al., 2023).

	Nusinersen	**Onasemnogene Abeparvovec**	**Risdiplam**
Drug type	Antisense oligonucleotide	Adeno-associated viral vector	Small molecular compound
Mechanism of action	Modification of *SMN2* pre-mRNA Splicing	*SMN* gene transfer	Modification of *SMN2* pre-mRNA Splicing
Administration	Intrathecal	Intravenous	Oral
FDA approved year	2016	2019	2020
Patient age	All	<2 years	>2 months *
Clinical trials involving symptomatic patients	ENDEAR CHERISH	START	FIREFISH SUNFISH
Clinical trials involving pre-symptomatic patients	NURTURE	SPR1NT	RAINBOWFISH

* Risdiplam was approved in the US for pediatric and adult patients with SMA of all ages in 2022.

Each treatment strategy focuses on the effect on exon 7 of the *SMN2* and *SMN1* genes. While similar results were obtained between these treatments in terms of the distribution and function of the SMN protein, each treatment acts by a different mechanism. The figure highlights the effects of each treatment option on mRNA editing and SMN protein production (BMC Pediatrics, 2022).

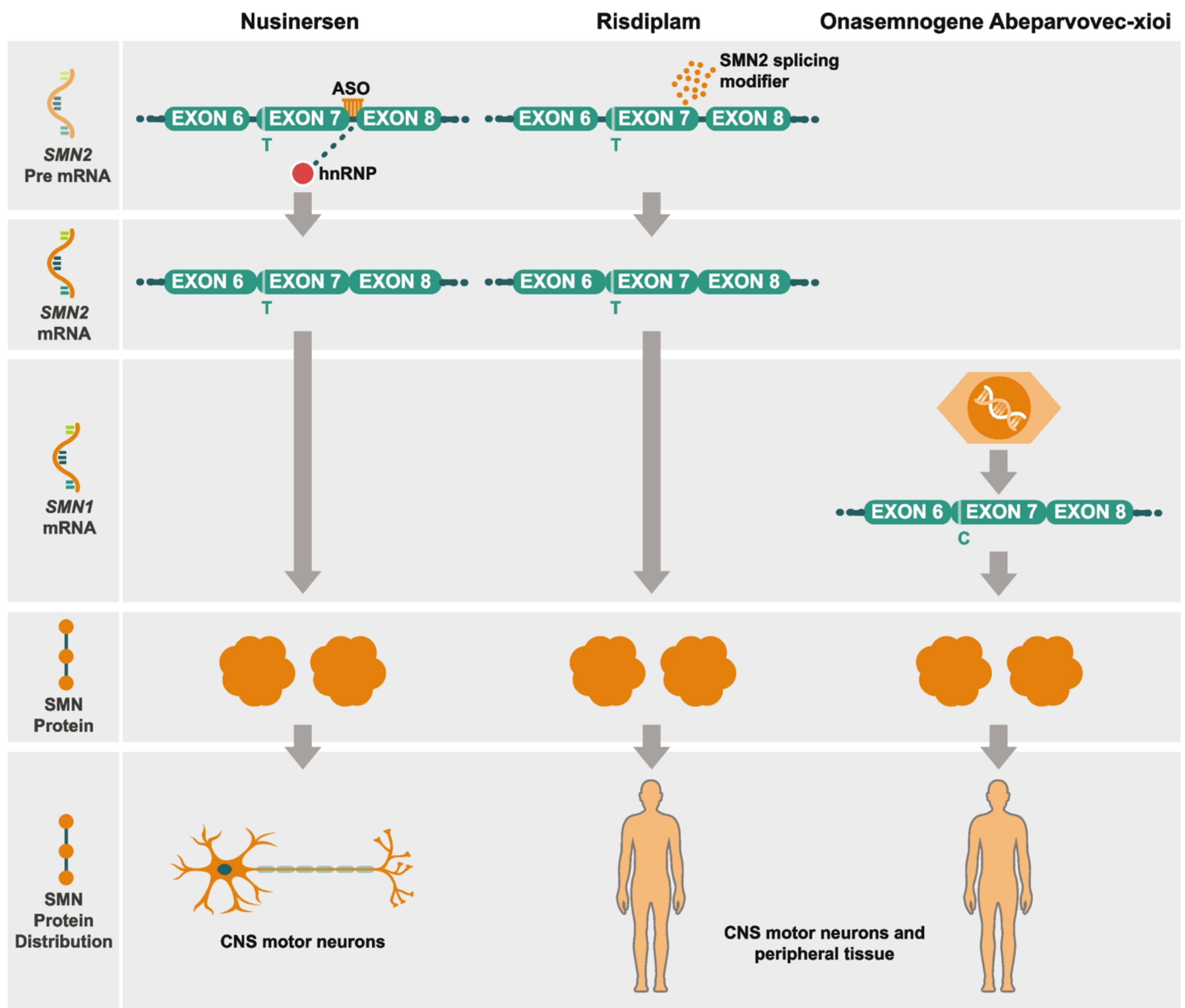

Figure 8: This figure compares three different treatment approaches used to treat Spinal Muscular Atrophy (SMA): Nusinersen, Risdiplam and Onasemnogene Abeparvovec-xioi (BMC Pediatrics, 2022).

While these three gene therapies have made significant strides in the treatment of SMA, each of these therapies faces various shortcomings and challenges. The high cost of treatment is one of the biggest factors limiting access to these therapies. In addition, more research is needed on the long-term efficacy and safety of the treatments. Future research should focus on improving the efficacy, reducing side effects and lowering the costs of these therapies. At the same time, research into other potential therapeutic targets other than the SMN protein is also ongoing, which could allow SMA treatment to develop further.

2.3. RNA-Based Therapeutics and Molecular Mechanisms

RNA-based therapeutics are innovative treatment strategies that modulate gene expression, interfering with the genetic origin of diseases. These therapeutics provide treatment by blocking the translation of mRNA, suppressing the function of specific genes or reducing the production of disease-causing proteins. There is a growing role for RNA-based therapeutics in the treatment of genetic diseases such as SMA, as well as cancer, infectious diseases and neurodegenerative diseases. Below, the main types of RNA-based therapeutics and their molecular mechanisms are discussed in more detail.

Antisense oligonucleotides (ASOs) are short, single-stranded DNA or RNA molecules that bind specifically to mRNA molecules within the cell, altering the function of these mRNAs. ASOs modulate the expression of the target gene by stopping the translation of a specific mRNA or regulating alternative splicing processes. This can lead to reduced or complete cessation of protein synthesis. ASOs trigger RNA-DNA hybrid formation by binding to the target mRNA. This binding causes degradation of the target mRNA, usually by the enzyme RNAse H, thus inhibiting translation of the mRNA and reducing protein production. Furthermore, some ASOs interfere with the splicing mechanism, allowing exons to be included or skipped. This process is particularly seen with Nusinersen in the treatment of SMA; Nusinersen promotes the inclusion of exon 7 by binding to *SMN2* pre-mRNA, which increases the production of functional SMN protein (Finkel et al., 2017).

The efficacy of ASOs depends on their fit to the sequence of the target mRNA, their capacity to enter the cell and their ability to remain stable inside the cell. Chemical modifications of ASOs (e.g. phosphorothioate backbone) improve these factors, thus increasing bioavailability and strengthening their resistance to nucleases. However, the specificity of binding with the target mRNA is critical to minimize off-target effects.

Small interfering RNAs (siRNAs) are double-stranded RNA molecules that use the RNA interference (RNAi) mechanism to target specific mRNAs and inhibit protein production by causing their degradation. siRNAs trigger the degradation of specific mRNAs using the cell's natural defense mechanisms and thus exert a gene silencing effect.

When siRNAs enter the cell, they combine with Argonaute (AGO) proteins to form a complex known as the RNA-induced silencing complex (RISC). The RISC recognizes and binds to the target mRNA with the siRNA's antisense strand (guide strand). This binding leads to the cutting and degradation of the mRNA by RISC. The cut mRNA cannot be translated by ribosomes, which stops the production of the target protein (Adams et al., 2018). The efficiency of siRNAs depends on their efficiency of entry into the cell, their capacity to integrate into the RISC complex and their binding specificity to the target mRNA. Chemical modifications and carrier systems can improve the entry and biostability of siRNAs into the cell. In addition, off-target effects of siRNAs are also minimized, as these effects determine the side effect profile of siRNA therapies.

Micro RNAs (miRNAs) are endogenously produced small RNA molecules that regulate gene expression at the post-transcriptional level. miRNAs inhibit translation or trigger degradation of mRNA by binding to target mRNA. miRNA modulators are used to treat various diseases by altering genetic regulation.

miRNAs bind to the 3' UTR (untranslated region) of specific mRNA molecules. This binding inhibits the translation of the mRNA or triggers the degradation of the mRNA. miRNA modulators are used either to enhance the effect of endogenous miRNA (miRNA mimetics) or to block the function of specific miRNAs (antagomiRs). For example, a miRNA mimetic can restore normal gene regulation by replacing the missing miRNA (Janssen et al., 2013). The efficacy of miRNAs depends on their binding specificity to the target mRNA and their bioavailability inside the cell. In addition, the duration of miRNA stability in the cell and its interactions with other miRNAs or RNA-binding proteins also determine efficacy. Off-target effects and side effects are carefully scrutinized during the development of miRNA-based therapies.

RNA aptamers are short RNA molecules that can bind with high affinity to specific target molecules. Aptamers usually bind to proteins, small molecules or cellular surface receptors, inhibiting the function of these targets. The binding abilities of aptamers are similar to those of monoclonal antibodies, but aptamers are smaller and less immunogenic. RNA aptamers bind specifically to the target protein or molecule, inhibiting its biological activity. Aptamers can block the catalytic activity of enzymes or ligand-receptor interactions by binding to the active site of the target molecule. This mechanism can block specific cellular signaling pathways or stop disease-causing biochemical reactions (Ng et al., 2006).

The efficacy of RNA aptamers depends on their binding specificity to the target molecule, thermodynamic stability and bioavailability. The design of aptamers is usually done by the SELEX (Systematic Evolution of Ligands by Exponential enrichment) method, which involves the selection of aptamers that provide high affinity and specific binding with the target molecule. The small size and low immunogenic profile of aptamers are among their advantages, but plasma stability and *in vivo* bioavailability are important factors to consider in clinical applications.

RNA-based therapeutics have revolutionized modern medicine, offering promising strategies for the treatment of many diseases. Various RNA technologies, such as ASOs, siRNAs, miRNA modulators and RNA aptamers, are used in many fields, from genetic diseases to infectious diseases and cancer. The development of these therapeutics has been made possible by rapid advances in molecular biology and genetic engineering, but research in these fields is still ongoing. In the future, more work needs to be done to improve the efficacy of these therapies and to develop new RNA-based therapeutics.

2.4. Antisense Oligonucleotides (ASOs)

Antisense oligonucleotides (ASOs) are short, artificial nucleic acid sequences used to regulate genetic information. These small oligonucleotides, which are complexed to DNA or RNA, are designed to bind to target mRNA to repress, alter or modify gene expression. The basic principle of this technology is to interfere with the translation process of mRNA and target protein production. These promising molecules for the treatment of genetic diseases, cancer and viral infections have gained great importance in the field of gene therapy and drug development (Crooke, 2004).

2.4.1 Molecular Mechanism of Antisense Oligonucleotides

The main function of antisense oligonucleotides is to bind to genetic material and disrupt the function of the targeted mRNA, thereby inhibiting protein synthesis. When ASOs bind to mRNA, this binding either leads to direct degradation of the mRNA or prevents the mRNA from being recognized by ribosomes and participating in the translation process. Two basic mechanisms control this process:

1. **RNase H Activation:** This mechanism starts with the binding of DNA-based antisense oligonucleotides to the target mRNA. After hybridization

between mRNA and ASO, an enzyme called RNase H recognizes this double helix and cleaves and destroys the mRNA. This process effectively stops protein synthesis and prevents the production of disease-causing proteins (Bennett & Swayze, 2010).

2. **Translation Inhibition:** ASOs can also prevent mRNA from being recognized by ribosomes during translation. In this case, ASOs bind to the mRNA but do not allow the mRNA to be cleaved by RNase H. Instead, the reading of the mRNA by the ribosome and thus protein synthesis is blocked (Dias & Stein, 2002).

In addition, ASOs can also modify pre-mRNA splicing events. This mechanism controls alternative splicing by altering the processing of intron and exon sections of mRNA. For example, nusinersen, used in the treatment of SMA, interferes with the splicing mechanism of the *SMN2* gene, resulting in an mRNA isoform that produces functional SMN protein (Finkel et al., 2017).

This figure shows how antisense oligonucleotides (ASOs) modulate gene expression by binding to mRNA. (A) Binding of ASOs to mRNA results in the formation of an RNA-DNA hybrid, which is recognized by the enzyme RNase H and leads to the degradation of mRNA. This process reduces the protein level of the target gene. (B) ASOs can also regulate the pre-mRNA splicing process. The inclusion (exon inclusion) or skipping (exon skipping) of exons depends on the specific binding sites of ASOs. An ASO that binds to a specific exon can promote its inclusion or cause its skipping during splicing. These mechanisms play an important role in the treatment of diseases by regulating gene expression.

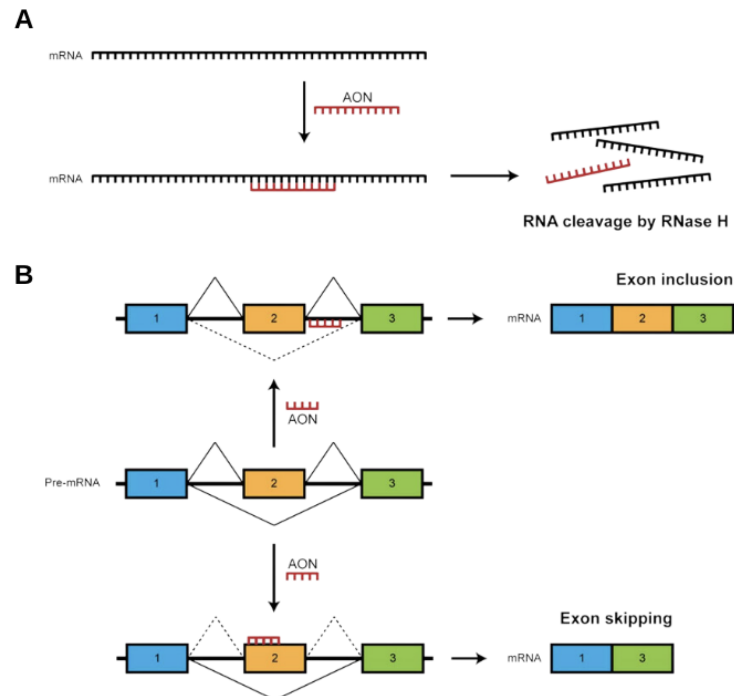

Figure 9: Antisense Oligonucleotides (ASO) Bind to mRNA and Control Gene Expression through Splicing Regulation and RNase H Activation (Front. Neurosci., 2024).

ASOs have a wide range of uses. Used in many diseases such as genetic diseases, neurological disorders, cancer and viral infections, ASOs are particularly effective in the treatment of genetically based diseases. Some of the important disease areas where these molecules are used are as follows:

SMA is a genetic disease affecting motor neurons and is characterized by the inability to produce sufficient amounts of SMN protein due to mutations in the *SMN1* gene. Nusinersen, used in the treatment of SMA, interferes with the splicing mechanism of the *SMN2* gene, resulting in the production of more functional SMN proteins. This treatment helps SMA patients maintain motor skills and slow disease progression (Ratni et al., 2018). Nusinersen promotes the preservation of muscle function by promoting the production of an mRNA containing exon 7 of *SMN2*.

The *SMN1* gene produces full-length FL-*SMN1* mRNA that includes exon 7 (C7), while the *SMN2* gene usually excludes exon 7 and produces Δ7-*SMN2* mRNA that is missing. However, in some cases FL-*SMN2* mRNA can also occur. The Δ7-*SMN2* mRNA lacking exon 7 fails to form the proper oligomer structure of the SMN protein, leading to loss of function. In contrast, FL-*SMN1* and FL-*SMN2* mRNAs can form functional oligomers, allowing proper SMN protein production (Nishio et al., 2023).

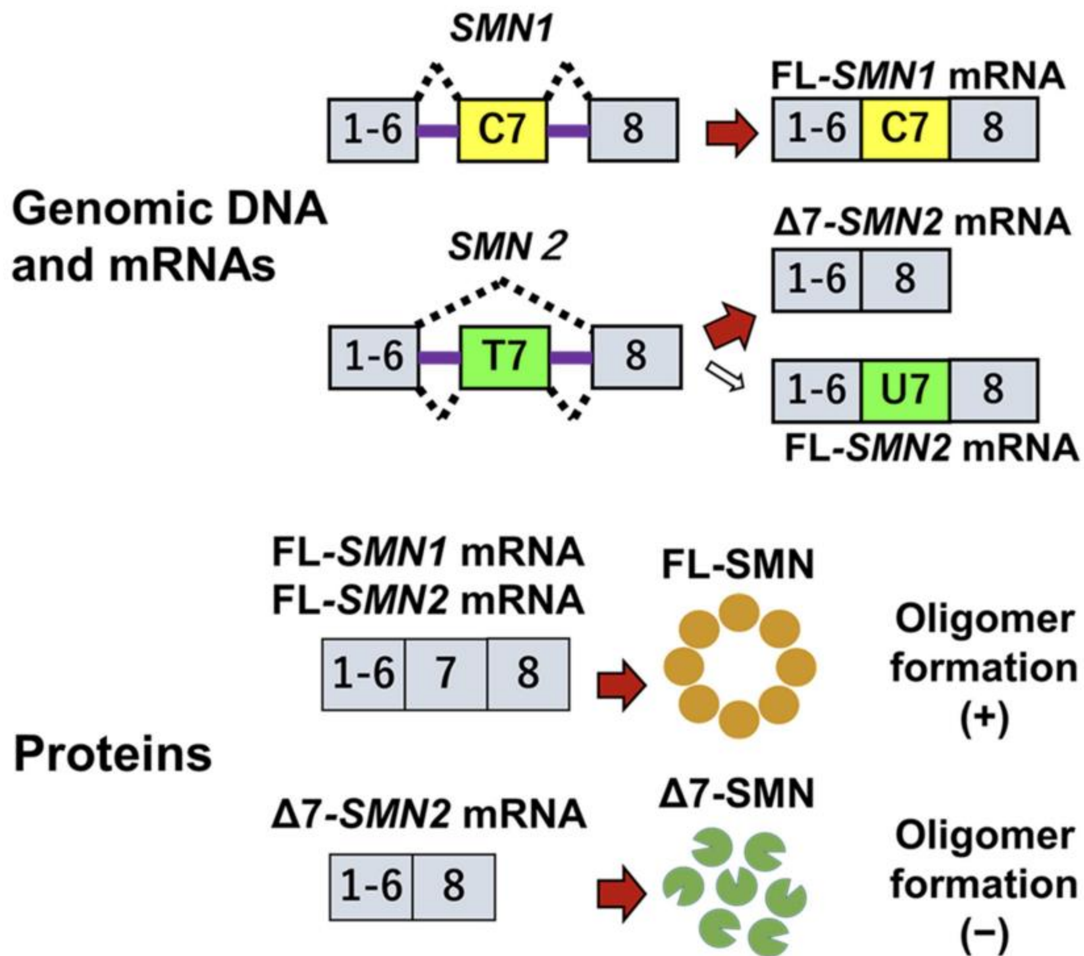

Figure 10: *SMN1* and *SMN2* genes function differently on exon 7 (Nishio et al., 2023).

Duchenne Muscular Dystrophy occurs when the gene responsible for the production of the protein dystrophin is mutated. ASOs can correct faulty protein production by "circumventing" certain exons of this gene. Eteplirsen, used in the treatment of DMD, aims to preserve muscle function in patients by using this mechanism. This drug partially restores dystrophin production by skipping exon 51 (Kole & Krieg, 2015).

ASOs also have great potential in cancer treatment. Used against oncogenes that are overexpressed in cancer cells, ASOs suppress these genes. ASOs target genetic material and degrade mRNA to stop cancer cells from proliferating. An example of ASO use in cancer is oligonucleotides targeting the BCL-2 gene. The BCL-2 protein causes cells to resist apoptosis. By suppressing this gene, the susceptibility of cancer cells to apoptosis can be increased (Crooke, 2017).

Antisense oligonucleotides can stop viruses from replicating by targeting viral RNA. This method is particularly promising in the treatment of RNA viruses such as HIV and hepatitis C. ASOs bind to specific regions of the viral genome, preventing the RNA from replicating and thus stopping the virus from spreading. For example, ASOs developed against hepatitis C virus prevent the spread of infection by suppressing virus replication (Stein & Castanotto, 2017).

The development and use of ASOs has revolutionized genetic-based therapies, but further refinements and modifications are needed. Tissue-specific ASO delivery, specific targeting and chemical modifications can enhance the therapeutic effects of ASOs. Especially in combination with gene editing technologies such as CRISPR/Cas9, ASOs could offer more effective and safe treatment options. This would lead to significant advances in the treatment of both genetic diseases and complex diseases such as cancer (Schuster et al., 2019).

Drugs are categorized according to their chemical structure, mechanism of action, target organ or mRNA, indications, route of administration, year of approval and orphan drug status. The Chemistry column shows the different chemical modifications (e.g. 2'-O-MOE, PS, PMO) used in ASOs. The Mechanism of Action column explains that ASOs work by methods such as RNase H1 activation for mRNA degradation, siRNA-mediated gene silencing or exon skipping. The table highlights the diversity in target organs and mRNAs and highlights the therapeutic effects of drugs for genetic diseases such as Spinal Muscular Atrophy (SMA), Duchenne Muscular Dystrophy (DMD) and Hereditary Transthyretin Amyloidosis (hATTR). It also notes that many drugs have orphan drug status, indicating that the diseases being treated are rare. (Nucleic Acids Research, 2018)

Table 3: This table provides a comprehensive overview of Antisense Oligonucleotide (ASO)-based drugs approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

Name (market name)Company	Chemistry	Mechanism of action	Target/organ	Indication	Route/Dosing	Year of approval	Designation
Fomivirsen (Vitravene™), Ionis Pharma, Novartis	21 mer PS DNA	RNase H1	CMV IE-2 mRNA/eye	CMV retinitis	IVT/300 μg every 4 weeks	FDA (1998) EMA (1999)	-
Mipomersen (Kynamro™), Ionis Pharma, Genzyme, Kastle Tx	20 mer, 2′-O-MOE, PS, 5-methyl cytosine	RNase H1	Apo-B-100 mRNA/liver	HoFH	SC/200 mg once weekly	FDA (2013)	Orphan
Eteplirsen (Exondys 51®), Sarepta Tx	30 mer PMO	Exon skipping	DMD pre-mRNA exon 51/Skeletal muscle	DMD	I.V. infusion/30 mg/kg once weekly	FDA (2016)	Orphan
Nusinersen (Spinraza®), Ionis Pharma, Biogen	18 mer PS, 2′-O-MOE, 5-methyl cytosine	Exon inclusion	SMN2 pre-mRNA exon 7/CNS	SMA	ITH/12 mg once every 4 months	FDA (2016) EMA (2017)	Orphan
Patisiran (Onpattro®), Alnylam	2′-O-Me, 2′F, PS siRNA	AGO2	TTR mRNA/liver	hATTR	I.V. infusion 0.3 mg/kg once every 3 weeks	FDA (2018) EMA (2018)	Orphan
Inotersen (Tegsedi®), Ionis Pharma, Akcea Pharma	20 mer 2′-O-MOE, PS	RNase H1	TTR mRNA/liver	hATTR	SC/300 mg once weekly	FDA (2018) EMA (2018)	Orphan
Vutrisiran/ALN-TTRSC02	2′-O-Me, 2′-F,PS	RNase H1	TTR mRNA/liver	hATTR	SC/25 mg every 3 months	FDA (2022)	Orphan
Milasen Boston Children's Hospital	22 mer 2′-O-MOE, PS, 5-methyl cytosine	Splicing modulation	Intron 6 spice acceptor cryptic site/CNS	CLN7	ITH/42 mg once every 3 months	FDA (2018)	Orphan
Volanesorsen (Waylivra®), Ionis Pharma, Akcea Pharma	20 mer, PS,2′-O-MOE	RNAse H	mRNA APOCIII/liver	FCS	SC/285 mg once weekly	EMA (2019)	Orphan
Givosiran (Givlaari®), Alnylam	PS - SiRNA GalNAc	AGO2	ALAS1 mRNA/liver	AHP	SC/2,5 mg/kg once every months	FDA (2019), EMA (2020)	Orphan
Golodirsen (Vyondys 53™), Sarepta Tx	25 mer PMO	Exon-Skipping	DMD pre-mRNA/muscle	DMD	I.V/30 mg/kg once weekly	FDA (2019)	Orphan
Viltolanersen (Viltepso™), NS Pharma	PMO	Exon-skipping	DMD pre-mRNA/muscle	DMD	IV/80 mg/kg once weekly	FDA (2020)	Orphan
Lumasiran (Oxlumo™), Alnylam	siRNA	AGO2	HA01 mRNA/liver	PH1	SC/dose and frequency depend on the patient's weight	FDA (2020) EMA (2020)	Orphan
Inclisiran (Leqvio®), The Medicines Company, Novartis	2′F, 2′-O-Me, PS siRNA- GalNAc	AGO2	mRNA PCSK9/liver	FH	SC/300 mg once every 6 months	EMA (2020) FDA (2021)	-
Casimersen (Amondys 45 ™), Sarepta	22 mer PMO	Exon-Skipping	DMD pre-mRNA/muscle	DMD	30 mg/kg once weekly	FDA (2021)	Orphan
Tofersen	2′-O-MOE-PS	RNase H1	SOD1 mRNA/brain	ALS	100mg/15 mL (6.7 mg/mL) single-dose vial	FDA (2023)	

Antisense medicines approved by the Food and Drug Administration (FDA) or European Medicines Agency (EMA). 2′- F— 2′-Fluoro, 2′-O- MOE—2′-O-methoxyethyl, AHP—acute hepatic porphyria, ALAS—Aminolevulinate synthase, Apo—Apolipoprotein, CLN7— Neuronal Ceroid Lipofuscinosis, CMV—cytomegalovirus, CNS—central nervous system, DMD—Duchenne muscular dystrophy, FCS—Familial chylomicronemia syndrome, FH—Familial hypercholesterolemia, GAlNAc—N-acetylgalactosamine, HAO1— Hydroxyacid oxidase 1, hATTR—hereditary transthyretin amyloidosis, HoFH—omozygous familial hypercolesterolaemia, ITH—Intrathecal, IV—Intravenous, IVT—intravitreal, PCSK9—Proprotein convertase subtilisin/kexin type 9, PH1 — Hyperoxaluria type 1, PMO, Phosphorodiamidate morpholino; PS, Phosphorothioate, SC—Subcutaneous, siRNA—Small interfering RNA, SMA—spinal muscular atrophy, SMN—survival of motor neurons, TTR—Transthyretin.

As can be seen, the number of drugs approved by both regulatory agencies has increased over the years. It is noteworthy that FDA approvals increased faster than EMA approvals, especially in 2016 and onwards. By 2020, there was a peak in the number of drugs approved by the FDA, while EMA approvals showed a more steady increase. This figure highlights the developments in the regulatory approval process for ASO therapies and the upward trend over time. (Nucleic Acids Research, 2018).

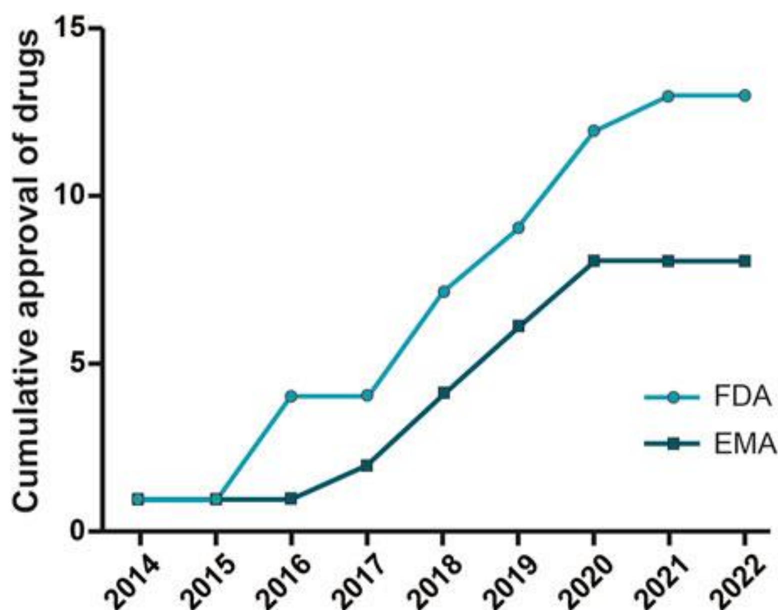

Figure 11: Shows the distribution of antisense oligonucleotide (ASO)-based drugs approved cumulatively by the FDA (US Food and Drug Administration) and EMA (European Medicines Agency) between 2014 and 2022.

2.4.2 ASO Design and Production

Antisense oligonucleotides (ASOs) are powerful tools that modulate gene expression by targeting mRNA with sequences specific to genetic material. Proper design and manufacturing strategies are crucial to increase the efficacy of ASOs, which are widely used in the treatment of genetic diseases. The design of ASOs requires an optimized structure for specific regions of the target mRNA so that both high-affinity binding of the oligonucleotide to the target and bioavailability can be achieved (Bennett et al., 2017).

The critical first step in ASO design is the accurate selection of the targeted mRNA sequence. The selection of the target mRNA is usually performed with the help of bioinformatics tools. These tools analyze the structure, accessible regions and gene expression levels of the target mRNA to determine the most effective binding site for the ASO (Schoch & Miller, 2017). The open regions of the target mRNA should be selected taking into account ribosomal protected domains, as these regions enable high affinity binding of ASO and trigger degradation of the mRNA by RNase H.

Another important factor used in the design of ASO is the oligonucleotide length. Generally, sequences of 15 to 25 nucleotides in length are preferred. This length maximizes the ASO's ability to bind specifically to the target mRNA while minimizing

the risk of nonspecific binding (Bennett et al., 2017). In addition, chemical modifications are applied to ASOs to increase their stability and keep them active inside the cell. These modifications provide resistance against cellular nucleases and increase therapeutic efficacy.

In ASO design, chemical modifications of the native nucleotide chain are commonly used to both increase the stability of the ASO and optimize its binding strength to the target. Phosphorothioate (PS) modification is one of the most widely used backbone modifications. This modification prevents ASO from being degraded by nucleases and facilitates its crossing of the cell membrane (Crooke, 2017). Furthermore, modifications such as 2'-O-methoxy ethyl (MOE) and locked nucleic acid (LNA) increase the binding strength of ASO to mRNA through chemical changes to the ribose ring and optimize biological utilization (Watts & Corey, 2012). The correct use of chemical modifications directly affects the therapeutic efficacy of ASO. For example, the PS backbone keeps ASO stable, while base modifications such as MOE and LNA strengthen the specific binding of ASO. Newer modifications, such as Xeno Nucleic Acid (XNA), offer much higher stability and binding affinity than native nucleotide sequences, making ASOs effective for longer periods of time in the treatment of genetic diseases (Braasch & Corey, 2001).

The ASO production process is largely carried out by solid phase synthesis. This process starts by chemically assembling each nucleotide on a polymer support, respectively. Phosphoramidite chemistry is the most widely used method for ASO production; this technique allows for rapid and highly efficient oligonucleotide synthesis (Beaucage & Iyer, 1992). At each nucleotide addition step, the nucleotide bases are activated with phosphoramidite derivatives, thereby forming the oligonucleotide chain. In the production step, after each nucleotide is added, the protective groups are removed and the chain continues to grow. After the production is complete, the purification phase comes. Techniques such as HPLC (High-Performance Liquid Chromatography) ensure that pure oligonucleotides are obtained. Purification maximizes the efficacy of the therapeutic oligonucleotide and minimizes the risk of side effects (Bennett et al., 2017).

Quality control during the ASO production process is critical to ensure the therapeutic efficacy of oligonucleotides and to prevent faulty production. Each ASO produced is subjected to quality control testing after purification. Mass spectrometry and capillary electrophoresis are commonly used techniques to check the molecular weight

and accuracy of ASO. These tests confirm that the oligonucleotide sequence is correct and free of unwanted modifications (Schoch & Miller, 2017).

Quality control processes are of great importance, especially for ASOs developed for clinical use. Incorrect sequences or impure products can reduce the efficiency of binding to the target mRNA and lead to side effects. Therefore, rigorous control at every stage of the manufacturing process ensures safe and effective ASOs.

Technological advances in ASO production are enabling the development of faster and more efficient synthesis methods. Highly efficient production techniques and more sophisticated quality control methods will make the clinical use of ASOs more reliable. Furthermore, new modification strategies and more specific targeting techniques will continue to increase the potential of ASOs in the treatment of genetic diseases (Watts & Corey, 2012). The design and production of ASOs plays a critical role in the treatment of genetic diseases. Modifications used to ensure high specificity binding to target mRNA, optimize bioavailability and increase intracellular stability maximize the efficacy of ASO. Solid phase synthesis, phosphoramidite chemistry and advanced quality control methods enable the production of reliable and effective ASOs. In the future, studies on ASO production and design will open the door to more innovative approaches in the treatment of genetic diseases.

2.4.3 Basic Structure and Modifications of Antisense Oligonucleotides

ASOs are short oligonucleotides containing the basic building blocks of classical nucleic acids. Structurally, ASOs consist of a phosphodiester backbone, nucleotide bases and ribose or deoxyribose sugars. Although this structure is similar to natural DNA or RNA sequences, these oligonucleotides require modifications in order to remain stable in the biological environment and to be more easily taken into the cell. In particular, structural modifications to ensure that oligonucleotides remain stable in the cell improve their pharmacokinetic and pharmacodynamic properties. In addition, the ability to cross the cell membrane and effectively reach intracellular target sites is among the most important factors that increase the therapeutic efficacy of ASOs (Stein & Castanotto, 2017). When we consider the effects of ASOs (Antisense Oligonucleotides) on gene expression and their therapeutic potential, the importance of modifications made to increase the efficiency of these molecules emerges. The ability of ASOs to modulate gene expression by interacting with target mRNA is highly dependent on their chemical

structure and the modifications made to this structure (Corey, 2020). Therefore, understanding the mechanism of ASOs and their effects on gene expression is essential for understanding how these molecules are optimized (Kurreck, 2003). Antisense oligonucleotides (ASOs) are short DNA or RNA sequences used to regulate gene expression by specifically binding to target mRNA sequences. These oligonucleotides have been developed as powerful tools for genetic diseases and various therapeutic approaches. However, certain chemical modifications are required for oligonucleotides to remain stable in the biological environment, to effectively cross the cell membrane, to bind to the targeted mRNA with high affinity, and not to induce immune responses. The chemical structures of ASOs and the modifications made to these structures directly affect the bioavailability, intracellular stability and binding strength of ASOs to target mRNA. Therefore, chemical modifications of ASOs are critical to optimize their clinical efficacy and safety profile (Crooke, 2004).

2.4.4 Chemical Modifications: Structural Strength and Functionality

The activity of antisense oligonucleotides is largely based on chemical modifications. These modifications can be made to the oligonucleotide's backbone, sugar ring or nucleobases. The aim of each modification is to increase the bioavailability of ASO, reduce immunogenicity and improve target binding affinity.

The phosphodiester backbone consists of nucleotides linked together by phosphorus bonds, which is the natural structure of nucleic acids. However, this structure is susceptible to rapid degradation by nucleases in the biological environment. Therefore, chemical modifications to phosphodiester bonds increase the stability of ASOs. One of the most common modifications is phosphorothiolate (PS) modification. PS modification is achieved by replacing an oxygen atom in the phosphodiester bond with sulfur. This modification makes ASO more difficult to be degraded by nucleases, thus allowing it to remain active in the cell for longer (Bennett & Swayze, 2010). The phosphorothiolate backbone also increases the ability of ASO to bind to plasma proteins, which further improves its bioavailability (Stein et al., 2010). However, the PS modification also has some disadvantages. This modification can negatively affect the binding affinity of the oligonucleotide to the target. In particular, in ASOs targeting mRNA degradation through RNase H, it has been observed that PS modification can reduce binding efficiency (Stein et al., 2017).

The sugar ring plays an important role in the stability and binding strength of ASO's nucleotide sequence. Chemical modifications to the ribose sugar can enable ASO to bind more strongly to mRNA. One of the most important modifications developed for this purpose is Locked Nucleic Acid (LNA) technology. LNA "locks" the sugar ring by forming a methylene bridge in the ribose ring, making the structure more rigid. This rigid structure significantly increases the binding strength of the nucleic acid to the target mRNA. LNA modification is widely used in oligonucleotides used to interfere with the splicing events of ASOs. For example, nusinersen, an ASO used in the treatment of SMA, has been modified with LNA technology and aims to correct splicing errors in the *SMN2* gene (Elmehed et al., 2020).

Another important sugar ring modification is the 2'-O-methoxy (2'-O-Me) and 2'-O-methyl (2'-O-Met) groups. These modifications are achieved by replacing the hydroxyl group at the 2' position of ribose with methoxy or methyl groups. 2'-O-modifications make ASOs more resistant to nucleases and increase their binding affinity to mRNA. These modifications also increase the biological safety of ASOs by reducing immunological responses (Wengel, 2000).

Modifications to the nucleotide bases can increase ASO specificity to the target mRNA and improve binding affinity. ASOs usually contain classical nucleotide bases such as adenosine (A), cytosine (C), guanosine (G) and thymine (T); however, chemical modifications can be used to alter the structure of these bases to provide a stronger interaction with the mRNA. For example, 5-methylcytosine modification increases base pairing stability by adding a methyl group to the structure of the cytosine base. Such modifications allow the oligonucleotide to bind more specifically and strongly to the target mRNA (Nielsen, 2001).

In addition, base modifications, such as peptide nucleic acid (PNA), create structures that differ from those of natural nucleic acids. PNAs offer a structure consisting of peptide bonds instead of an oligonucleotide backbone, which increases the binding affinity to the target. In addition to being more stable than conventional nucleic acid sequences, PNAs show low immunogenicity in biological systems, making them ideal for therapeutic uses (Nielsen, 2001).

These modifications can be applied to different regions of the oligonucleotide to increase the therapeutic efficacy of ASOs. Modifications such as 2′-OMe, 2′-MOE, 2′-Fluoro and Tc-DNA are made on the ribose sugar, while phosphorothioate (PS)

modification on the backbone provides resistance to nuclease degradation. Furthermore, nucleobase modifications (e.g. 5-methylcytosine, 5-methyluridine) increase the binding affinity of the oligonucleotide to mRNA, while alternative chemistries such as PMO and PNA are used to create more stable oligonucleotides (Haque & Yokota, 2023).

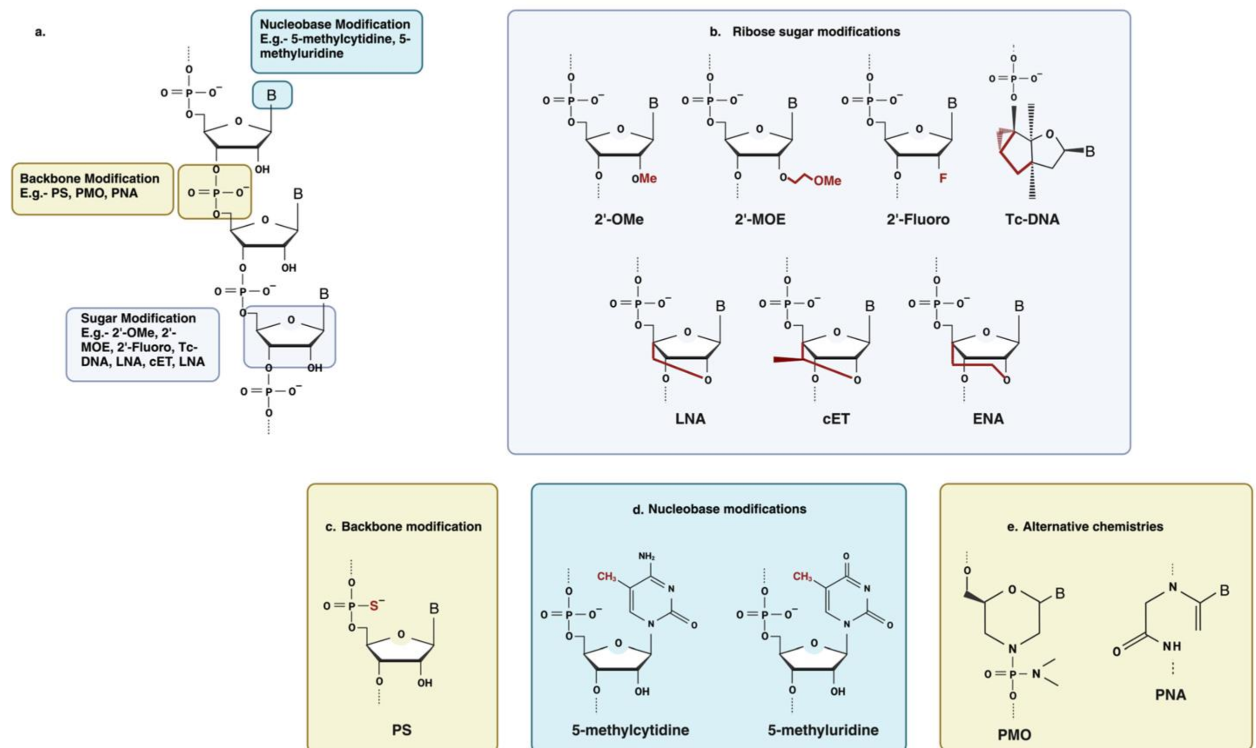

Figure 12: This figure shows common modifications used in the chemical structure of antisense oligonucleotides (Haque & Yokota, 2023).

2.4.5 Targeting and Intracellular Delivery: Lipid Nanoparticles and Delivery Systems

Antisense oligonucleotides (ASOs) are a powerful therapeutic approach that holds potential promise in the treatment of genetic diseases. However, the clinical efficacy of ASOs is highly dependent on their safe and efficient delivery to targeted cells and tissues. The efficient delivery of intracellular ASOs to the cytoplasm or nucleus plays a critical role in therapeutic efficacy. Since the ability of ASOs to cross the cell membrane is limited, various methods and modifications have been developed to optimize intracellular delivery (Juliano, 2016).

The most common mechanism by which ASOs are taken into the cell is endocytosis. Endocytosis allows large molecules from the cell membrane to be taken into the cell in vesicles. Specific receptors on the cell surface recognize ASOs, which are then transported into the cell in vesicles called endosomes. However, in this process, most ASOs can be transported from endosomes to lysosomes and degraded (Seth et al., 2012). In order for ASOs to exert their therapeutic effects, they need to ensure endosome escape. Research shows that a large proportion of ASOs accumulate in endosomes and undergo lysosomal degradation, leading to limited intracellular activity (Crooke, 2017). Therefore, strategies have been developed to increase the escape of ASOs from endosomes to the cytosol or nucleus.

Various chemical modifications have been made to facilitate the passage of ASOs across the cell membrane and to ensure their stability inside the cell. Phosphorothioate (PS) modification is a common modification of the backbone of ASO to prevent its degradation by nucleases. PS modification helps ASO to more efficiently pass through the cell membrane and increase its bioavailability (Bennett et al., 2017). In addition, chemical modifications such as 2'-O-methoxy ethyl (MOE) increase the biostability of ASO and optimize its binding strength to target mRNA. MOE modifications increase cellular efficiency by protecting ASOs from lysosomal degradation after they are transported into the cell via endocytosis (Watts & Corey, 2012). Other modifications include locked nucleic acid (LNA) and peptide nucleic acid (PNA). These modifications allow ASO to bind more strongly to mRNA and remain stable in the cell for longer (Petersen & Wengel, 2003).

Another common way to increase intracellular uptake is to use lipid-based carriers or nanoparticle systems. Lipid nanocarriers form a protective layer that allows ASO to more easily cross the cell membrane. Lipid carriers help to increase endosome escape while transporting ASO within the vesicle. These transporters also facilitate the specific delivery of ASO to the target tissue (Seth et al., 2012). In addition to lipid carriers, polymer-based nanoparticles are also used for the safe delivery of ASOs. These systems have been designed to optimize the intracellular uptake of ASOs and have been successfully used in many studies to improve therapeutic efficacy. Nanoparticles offer a versatile platform that can be used to enhance the bioavailability of ASO and ensure its specific delivery to target cells (Juliano, 2016).

To optimize the therapeutic effects of ASOs, specific delivery to the target cell type is required. This strategy has been developed to ensure that ASO only reaches target cells and does not cause side effects in other cells. For example, GalNAc (N-acetylgalactosamine) ligands used as specific ASO carriers to hepatocytes have been widely used to regulate gene expression in liver diseases (Prakash et al., 2014).

In neurological diseases, ASO needs to cross the blood-brain barrier (BBB). Crossing the BBB is a major challenge for neurological targeting and ASO is usually delivered directly to the central nervous system via intrathecal injections (Crooke, 2017). This strategy has been applied in particular to drugs such as Nusinersen, which is used to treat Spinal Muscular Atrophy (SMA). Nusinersen is injected directly into the spinal fluid, targeting motor neurons and increasing SMN protein levels, making it effective in treating SMA (Finkel et al., 2017).

Once injected systemically, ASOs accumulate in various tissues before reaching target tissues. The brain resists the passage of ASO due to the difficulty of crossing the blood-brain barrier. The liver is one of the organs with the highest accumulation of ASO (40-60%), which may limit the therapeutic efficacy of ASO. ASO accumulation is also observed in organs such as the kidney and spleen, with 18-40% of ASO accumulating in the kidneys and being eliminated through renal clearance. The lower part of the figure shows the mechanism of ASO uptake into the cell. ASOs are taken into the cell by endocytosis and pass into the cytoplasm by escape from endosomes. In this process, ASOs that fail to escape from endosomes undergo lysosomal degradation and lose their therapeutic efficacy. Successful ASOs are transported to the nucleus and modulate target gene expression.

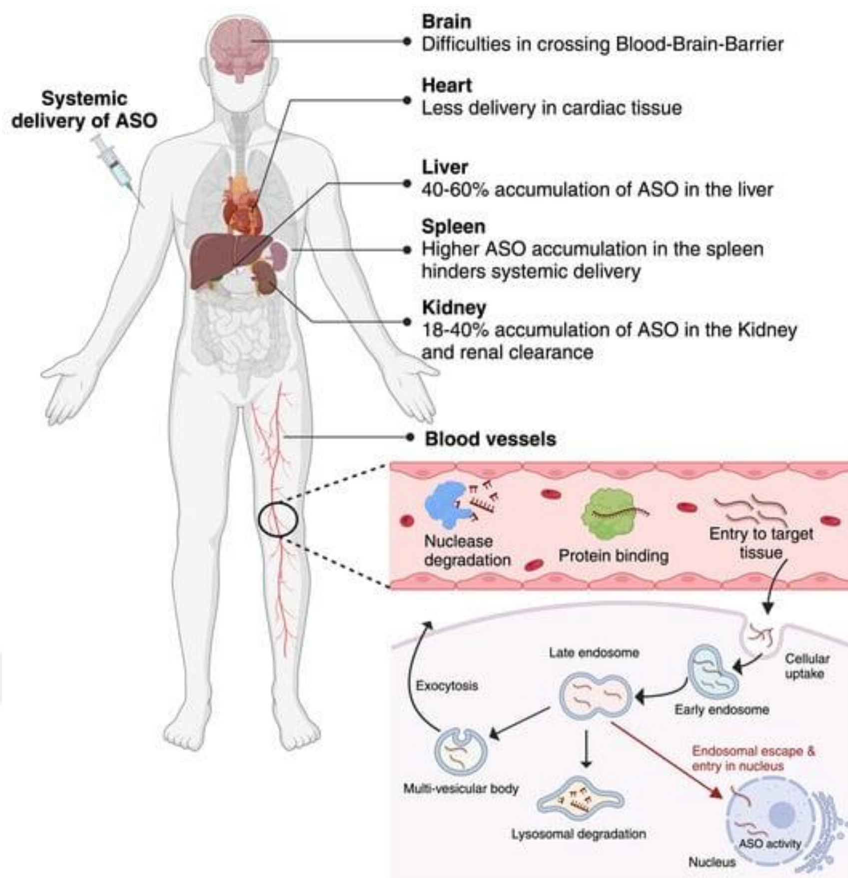

Figure 13: This figure illustrates the distribution and intracellular transport barriers that antisense oligonucleotides (ASOs) face in the body after systemic delivery (Haque & Yokota, 2023).

Work is ongoing on novel delivery systems and modifications to further enhance the therapeutic efficacy of ASOs. Future delivery systems may incorporate bioengineering techniques to optimize ASO delivery to target tissues. For example, pH-sensitive carriers or peptide-based delivery systems could make endosome escape more efficient, allowing ASO to be more bioavailable inside the cell (Juliano, 2016). Nanoparticle technology also has great potential to enhance intracellular uptake. Nanoparticles, especially those developed using biodegradable polymers, enable safe delivery of ASO to target tissues while minimizing toxicity. Future research will include further bioengineering modifications to improve the clinical efficacy of these systems (Crooke, 2017).

The efficient delivery of antisense oligonucleotides into the cell is one of the most important elements of their therapeutic success. Approaches such as uptake into the cell via endocytosis, chemical modifications and nanoparticle-based carriers enable ASOs to cross the cell membrane and exert activity in the cytoplasm or nucleus. In the future, the

development of delivery systems to optimize the bioavailability and specific delivery of ASOs to the target tissue will further expand the clinical use of these therapies.

Gapmers, a specific subclass of antisense oligonucleotides, target mRNA degradation by mechanisms such as chemical modification and RNase H activation. Gapmers are oligonucleotides with end regions composed of modified nucleotides and a "gap" region in the middle. The modified ends allow for stronger binding to mRNA, while the gap region is recognized by RNase H and degraded. This design increases the binding strength of ASOs to mRNA while facilitating the degradation of the target mRNA (Crooke, 2017).

Gapmers have modifications that both increase extracellular stability and allow RNase H to efficiently cleave the target mRNA. This technology is recognized as a potential tool for the treatment of many diseases such as cancer, genetic diseases and neurodegenerative disorders (Stein & Castanotto, 2017).

2.4.6 Combination Modifications

Antisense oligonucleotides (ASOs) have significant potential in the treatment of genetic diseases. The efficacy of ASOs largely depends on their high-affinity binding to mRNA, intracellular stability and bioavailability. However, due to their natural structure, ASOs are at risk of degradation in the cellular environment. Therefore, chemical modifications have been developed to improve therapeutic efficacy. Furthermore, combination modifications are strategies where multiple modifications are used together to increase stability while ensuring high affinity binding of ASOs to the target (Bennett et al., 2017).

Phosphorothioate (PS) backbone modification makes ASOs more durable than native DNA or RNA structures. This modification prevents degradation of ASO by intracellular nucleases and increases its biological usefulness (Watts & Corey, 2012). The PS backbone preserves the therapeutic effect of ASO, enabling its long-term use and is widely preferred in clinical applications. Besides PS modification, base modifications such as Locked Nucleic Acid (LNA) are also one of the strategies that increase the efficacy of ASOs. LNA is a modification of the ribose ring that increases the binding affinity of ASO to mRNA. ASOs with LNA modifications provide a stronger binding strength and higher targeting specificity (Petersen & Wengel, 2003). The combination of LNA and PS modifications optimizes both the stability and binding strength of ASOs,

making them particularly effective in the treatment of genetic diseases such as Spinal Muscular Atrophy (SMA) (Rinaldi & Wood, 2018). To further enhance the efficacy of ASOs, a combination of different oligonucleotide modifications such as Xeno Nucleic Acid (XNA) and DNA-ASO are used. XNAs are synthetic analogs of natural nucleic acids and provide increased stability and binding affinity due to their modified backbone structure. XNAs are more resistant to enzymatic degradation and enhance their therapeutic effects by strong binding to target RNA (Braasch & Corey, 2001). In particular, XNA-modified ASOs offer significant advantages in the treatment of Spinal Muscular Atrophy (SMA) and other genetic diseases, as they provide higher bioavailability and long-term stability (Rinaldi & Wood, 2018). XNA/DNA-ASO combinations increase *SMN2* gene expression in diseases such as SMA, leading to more efficient production of SMN protein, which in turn prevents motor neuron degeneration (Finkel et al., 2017). Peptide Nucleic Acid (PNA) is another synthetic nucleic acid analog used in modifications of ASOs. PNA has replaced the backbone of nucleotides with a protein-based structure. This structure allows PNAs to bind very strongly to the target mRNA and increases the resistance of oligonucleotides to cellular degradation (Nielsen et al., 1991). PNAs are seen as a promising tool for the treatment of genetic diseases due to their biostability and specific binding properties. Gapmer technology is a strategy developed using chemical modifications at specific sites of oligonucleotides. Gapmers combine both PS backbone and LNA or XNA base modifications, providing both stability and mRNA degradation. This strategy is widely used in genetic diseases such as SMA as it enables ASO to efficiently degrade the target mRNA and remain stable in the cell for longer (Bennett et al., 2017).

Combination modifications are an important way to optimize the therapeutic effect of ASOs. The PS-LNA combination has shown successful results in the treatment of genetic diseases by increasing the intracellular stability and binding affinity of ASO to mRNA (Petersen & Wengel, 2003). Likewise, the XNA-DNA ASO combination increases the bioavailability of ASO, providing longer effects over the course of treatment and alleviating disease symptoms by increasing gene expression in diseases such as SMA (Finkel et al., 2017). Combination modifications are also used to improve the systemic distribution and cellular uptake of ASOs. In particular, nanoparticle carriers or lipid-based systems enable modified ASOs to reach the target tissue more efficiently and increase therapeutic efficacy (Seth et al., 2012).

Combination modifications used to enhance the therapeutic efficacy of antisense oligonucleotides offer a revolutionary advance in the treatment of genetic diseases. The combination of modifications such as PS, LNA, XNA and DNA-ASO allows ASO to bind to mRNA with high specificity while increasing its bioavailability. In particular, XNA-DNA ASO combinations provide effective results in the treatment of genetic diseases such as SMA and increase the clinical potential of ASOs. In the future, ASO modification strategies will find wider use in the treatment of genetic disorders.

2.4.7 Current ASOs Used in SMA Treatment and Their Efficacy

One of the strategies used to compensate for mutations of the *SMN1* gene is to increase the production of functional SMN protein by introducing exon 7 of the *SMN2* gene. Nusinersen is the first SMA treatment approved by the FDA and is in the Antisense Oligonucleotide (ASO) class. Nusinersen targets the splicing mechanism of the *SMN2* gene, allowing the inclusion of exon 7, thereby increasing the production of functional SMN protein (Finkel et al., 2017). The effect of Nusinersen is realized by binding to the intronic splicing silencer N1 (ISS-N1) site in the *SMN2* pre-mRNA, preventing the binding of splicing factors. This ensures the incorporation of exon 7 into the mRNA and promotes the production of full-length functional SMN protein. This process ensures the survival of motor neurons and maintenance of muscle function (Hua et al., 2010).

The efficacy of Nusinersen has been proven by several clinical trials. In the ENDEAR study, it was reported that motor function improved in early SMA patients, with improvements such as being able to hold their head and sit without support. Treated patients had higher motor function scores compared to the placebo group. The earlier treatment was started, the more favorable the clinical outcomes (Finkel et al., 2017). Furthermore, the NURTURE study showed that the progression of the disease can be largely halted by the administration of Nusinersen in infants where symptoms have not yet appeared. The results of this study showed that starting treatment early is critical (De Vivo et al., 2019).

In recent years, other ASOs developed as an alternative to Nusinersen have also attracted attention in the treatment of SMA. Molecules such as Risdiplam are preferred in the treatment of SMA due to their ease of oral administration. Risdiplam promotes the production of more functional SMN proteins by changing the way *SMN2* mRNA is

processed. In clinical trials, Risdiplam has shown significant improvements in motor function and quality of life in SMA patients (Ratni et al., 2018).

ASOs are considered an important tool for specific targeting in the treatment of genetic diseases. ASOs such as nusinersen increase the production of functional SMN protein by altering the alternative splicing mechanism of the *SMN2* gene in SMA patients. Although ASOs are effective in the central nervous system, they must be administered intrathecally. This can pose delivery challenges. In contrast, small molecules (Risdiplam) are effective in the treatment of SMA through different mechanisms. Treatments such as Nusinersen, Onasemnogene Abeparvovec and Risdiplam used in the treatment of SMA aim to slow the progression of the disease and improve motor functions through different mechanisms. Nusinersen, an ASO that regulates the splicing mechanism of the *SMN2* gene, increases the production of functional SMN protein. Risdiplam is used as a small molecule that promotes the production of functional SMN protein by regulating the splicing process of *SMN2* mRNA. While each treatment has its own advantages and limitations, these advances in SMA treatment have led to significant improvements in patients' quality of life. In the future, it is aimed to develop more effective treatment strategies with the combination of these therapies or genetic engineering techniques.

2.4.8 XNA Features and Advantages

Antisense oligonucleotides (ASOs) are short nucleotide sequences that have the potential to revolutionize the treatment of diseases by regulating gene expression. However, various chemical modifications are required for ASOs to remain stable in biological systems, bind efficiently to specific targets and reduce immunogenicity. These modifications are made in the form of Xeno Nucleic Acids (XNAs), synthetic nucleic acids that provide higher stability and efficiency compared to classical DNA and RNA structures. XNAs are characterized by resistance to biological degradation, high binding affinity and specific targeting capacity. In this article, the role, advantages and molecular-level effects of XNAs and especially Locked Nucleic Acid (LNA) technology in ASO therapies will be discussed. Xeno Nucleic Acids (XNAs) are synthetic analogs obtained by adding various modifications to the chemical structures of natural nucleic acids. The main feature of XNAs is that their biological stability is increased by chemical modifications to the nucleic acid backbone or sugar ring. Some important advantages of XNAs compared to natural DNA and RNA; Nuclease Resistance: XNAs are resistant to

degradation by exonucleases and endonucleases present in the biological environment. This allows XNAs to remain stable in the cell for longer periods and increases their therapeutic efficacy (Nielsen, 2001). High Binding Affinity: XNAs have the capacity to bind with high affinity to target mRNA or DNA sequences. This allows oligonucleotides to regulate gene expression by binding more efficiently and specifically to their targets (Stein & Castanotto, 2017). Low Immunogenicity: XNAs, unlike natural nucleic acids, significantly reduce the potential to induce immune responses. This provides a great advantage in terms of minimizing side effects, especially in systemic therapies (Crooke, 2004).

2.4.8.1 Xeno Nucleic Acids Types

XNAs can contain various chemical modifications and structural variations. These modifications can be customized according to therapeutic targets, resulting in different types of XNAs. There are various types of XNAs such as Peptide Nucleic Acids (PNA), Locked Nucleic Acid (LNA), Hydroxy Nucleic Acids (HNA), Glyco Nucleic Acids (GNA). These species differ according to their chemical structure and therapeutic effects. PNA tends to be more stable thanks to its backbone consisting of peptide bonds; however, LNA is the most researched and used type of XNA in terms of therapeutic potential (Nielsen, 2001).

Locked Nucleic Acid (LNA) is a type of XNA that modifies the chemical structure of oligonucleotides to increase intracellular stability and target binding affinity. LNA is characterized by "locking" the sugar ring by forming a methylene bridge between the 2' oxygen atom and the 4' carbon atoms in the ribose sugar ring. This "locked" structure allows the LNA to acquire a more rigid and flat structure, which increases the double helix stability of nucleic acids. LNAs are used in ASO therapies specifically to regulate splicing events and significantly increase the binding strength to the target mRNA (Wengel, 2000).

LNAs hybridize with target nucleic acid sequences with high stability. This enables stronger binding of LNA oligonucleotides to the target by significantly increasing

the thermal denaturation temperature (Tm). This property is particularly critical for therapeutic approaches that require high efficacy even at low concentrations (Kauppinen et al., 2005). LNA modification enables oligonucleotides to bind to specific target sequences with high affinity and reliability. This high specificity increases the safety of treatment by minimizing off-target effects. This high binding specificity of LNAs is one of the most important factors that increase the applicability of ASO therapies in genetic diseases (Elmehed et al., 2020). LNAs are much more resistant to nucleases compared to natural oligonucleotides. This property allows LNA-modified ASOs to remain stable in biological systems for longer periods of time, which improves treatment efficacy (Stein et al., 2010).

LNAs are a type of ASO modification used to alter alternative splicing mechanisms or target RNA. LNA-modified ASOs could potentially be used to correct splicing errors of the *SMN2* gene in the treatment of diseases such as SMA. LNA technology provides a higher affinity binding to mRNA, which offers a great advantage in strategies to regulate gene expression (Elmehed et al., 2020). LNA modifications not only increase biological stability but also improve the pharmacokinetic properties of ASOs. ASOs containing LNAs can have longer half-lives and larger volumes of distribution. This provides therapeutic efficacy at lower dosages and improves patient compliance. For example, the higher bioavailability of LNA-based oligonucleotides allows for reduced dosing frequency and lower treatment costs (Bennett & Swayze, 2010).

LNAs offer higher thermal stability and binding affinity compared to other XNAs. Therefore, ASOs containing LNA modifications are particularly favored in therapeutic applications where gene expression needs to be modified specifically and with high accuracy. Moreover, the reduced off-target effects of LNA make this modification a reliable option for the treatment of genetic diseases (Wengel, 2000). Xeno Nucleic Acids (XNAs) and especially Locked Nucleic Acid (LNA) technology are revolutionizing the regulation of gene expression in antisense oligonucleotide therapies. LNAs are being used as an important tool in the treatment of genetic-based diseases, thanks to both their structural modifications that increase biological stability and their high binding affinity. LNA-containing ASOs are used to correct gene splicing errors, particularly in diseases

such as SMA, and these modifications have the potential to reduce side effects while increasing therapeutic efficacy. In the future, the use of LNA and other XNA modifications in a wider spectrum of diseases will allow the development of more effective and safe approaches to the treatment of genetically based diseases.

This figure shows the chemical structures of DNA and various Xeno Nucleic Acids (XNAs). XNAs are modified versions of natural nucleic acids, often used in oligonucleotide therapies. In addition to DNA, the figure shows XNA types such as PNA, LNA, CeNA, HNA, ANA, TNA and GNA. These structural modifications increase the biological stability of XNAs, conferring resistance to nucleases and enabling stronger target binding in therapeutic applications (Eid et al., 2018).

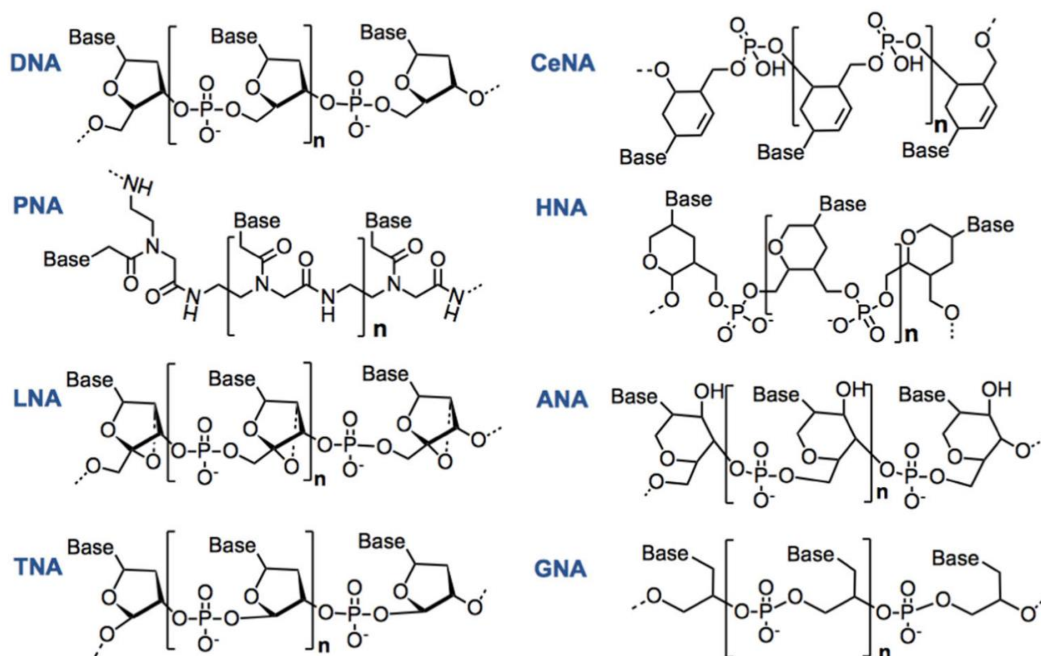

Figure 14: Structural Comparison of DNA and Xeno Nucleic Acids (Eid et al., 2018).

Antisense oligonucleotides (ASOs) have emerged as an important tool in the treatment of genetic diseases. By binding to target mRNA, ASOs can regulate protein production, induce RNA degradation or alter splicing processes. ASOs historically started with DNA-based oligonucleotides and have evolved into more durable and effective therapeutic molecules over time. RNA-ASOs and Xeno Nucleic Acid (XNA) and DNA-ASO combinations (XNA/DNA-ASO) are two different types of this technology. There

are structural and functional differences between these two types of ASOs, leading to different results in their clinical efficacy.

RNA-ASOs are oligonucleotides, usually based on ribonucleotides. These oligonucleotides have an RNA-specific ribose sugar and, when bound to mRNA, either degrade the target RNA or modify alternative splicing. RNA-ASOs are used to regulate functional protein production and in this context target various biological processes such as splicing correction or mRNA degradation. Nusinersen is one of the successful examples of RNA-ASOs in the clinic and is used in the treatment of SMA (Hua et al., 2010). XNA/DNA-ASOs are structurally more stable and modified oligonucleotides. Xeno Nucleic Acids (XNAs) are structurally modified synthetic analogs of natural nucleotides. XNAs are chemically modified to be more robust in biological systems where natural nucleotides are weak. These structures have higher stability and resistance to nuclease degradation compared to RNA or DNA oligonucleotides (Watts & Corey, 2012). XNA/DNA-ASOs contain modifications to the ribose sugar structure such as 2'-O-methoxy ethyl (2'-MOE) or Locked Nucleic Acid (LNA) to increase the stability of DNA-ASOs, which significantly increases their therapeutic efficacy.

Although RNA-ASOs have the capacity to bind to target mRNA sequences with high specificity, they suffer from intracellular stability and distribution problems. RNA-based oligonucleotides can be rapidly degraded by nucleases inside the cell. Therefore, the long-term stability and therapeutic effect of RNA-ASOs in biological systems may be limited (Bennett & Swayze, 2010). The activity of RNA-ASOs largely depends on their modifications. For example, phosphorothioate (PS) backbone modification and ribose sugar modifications allow the RNA-ASO to bind more tightly to the target mRNA and increase its resistance to biological degradation (Crooke, 2017). XNA/DNA-ASOs provide more efficient targeting compared to RNA-ASOs. XNAs bind to target mRNA with higher affinities than natural RNA or DNA oligonucleotides. This higher binding affinity allows XNA/DNA-ASOs to produce fewer off-target effects. Experimental results show that XNA/DNA-ASOs show higher stability and specificity compared to RNA-ASOs. Especially in gene silencing and splicing regulation, XNA/DNA-ASOs perform better than RNA-ASOs (Watts & Corey, 2012).

RNA-ASOs are unstable molecules in biological systems without chemical modifications. They have a high propensity to be degraded by the enzyme nuclease within the cell. Therefore, the biological utility of RNA-ASOs may be limited and frequent dosing may be required to maintain the therapeutic effect (Geary et al., 2015). This limited stability of RNA-ASOs complicates the treatment process in some clinical applications. XNA/DNA-ASOs have a superior profile in this regard. Since XNAs are structurally modified synthetic analogs of nucleic acids, they are much more durable in biological systems. Modifications to the ribose or deoxyribose sugar of XNA provide high resistance to nuclease degradation. This allows XNA/DNA-ASOs to exert long-lasting therapeutic effects and to be effective with less frequent doses (Braasch & Corey, 2001). In terms of bioavailability, XNA/DNA-ASOs have an advantage over RNA-ASOs.

RNA-ASOs are generally well tolerated in clinical use, but can produce immunogenic side effects at high doses or with prolonged use. Off-target effects and difficulties in crossing the cell membrane are also among the factors limiting the clinical efficacy of RNA-ASOs. Therefore, clinical applications of RNA-ASOs may remain limited despite stability-enhancing modifications. In contrast, XNA/DNA-ASOs offer lower toxicity and higher efficacy. In experimental studies, XNA/DNA-ASOs have been observed to be safer and more effective than RNA-ASOs. Thanks to the stability and specificity of XNA/DNA-ASOs, higher therapeutic efficacy can be achieved at lower doses. Low off-target effects make these molecules more reliable in the treatment of genetic diseases (Petersen & Wengel, 2003).

In conclusion, RNA-ASOs have an important role in the treatment of genetic diseases and are widely used in clinical applications. However, their limitations in intracellular stability, distribution and targeting may negatively affect the clinical efficacy of RNA-ASOs. XNA/DNA-ASOs are modified oligonucleotides that overcome these limitations and provide higher stability, specificity and therapeutic efficacy. In experimental and clinical studies, XNA/DNA-ASOs have shown superior performance compared to RNA-ASOs. Therefore, XNA/DNA-ASOs may be more widely used in the treatment of genetic diseases in the future and may contribute to making therapeutic strategies more effective.

3. MATERIALS AND METHODS

3.1 Cell Culture

In this study, we used the GM03813 cell line (Coriell Institute for Medical Research) derived from patients with Spinal Muscular Atrophy (SMA) Type 2. The GM03813 cell line consists of human fibroblast cells that are frequently used to study the genetic and molecular mechanisms of SMA. This cell line was derived from SMA patients carrying a homozygous deletion in the *SMN1* gene and is a suitable *in vitro* model to understand how SMA progresses at the molecular level.

SMA Type 2 Fibroblast cells were cultured using Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich). EMEM is an enriched formulation containing essential amino acids, vitamins and salts required for cell growth. This medium was prepared by adding 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher), 1% Penicillin-Streptomycin (Pen-Strep) (Gibco), and 1% L-glutamine. FBS provides growth factors necessary for cell growth and division, while Pen-Strep provides protection against possible bacterial and fungal contamination. L-glutamine is an amino acid that plays an important role in cell metabolism and is essential for cell proliferation. Cells were cultured in a humid incubator at 37°C in a 5% CO_2 atmosphere. 5% CO_2 provides optimal growth conditions for the cells by keeping the pH level constant. These standard cell culture conditions guarantee healthy growth and division of fibroblasts.

The SMA Type 2 Fibroblast cell line was passaged when the confluency level of the cells reached 80-90%. Cells were first washed with phosphate-buffered saline (PBS) to remove old medium and then trypsinized using Trypsin-EDTA (Gibco, Thermo Fisher) to prevent cells from adhering to the surface. During trypsinization, cells were incubated at 37°C for approximately 3 minutes. After this time, trypsin was inactivated with EMEM, 10% FBS, 1% Pen-Strep, 1% L-glutamine and cells were centrifuged. After centrifugation, the cells were divided 1:3, added to new medium and re-cultured in growth medium. This was done when the cells had reached the required confluence to ensure healthy growth in culture for a long period of time.

Cell viability and cell count were measured by Trypan Blue staining method using Bio-Rad TC20 Cell Counter device. Trypan Blue Exclusion Assay is a method in which live cells do not absorb the dye, but dead cells absorb the dye. In this way, the viability rate of the cells was calculated. When counting live cells, the TC20 Cell Counter automatically counts each cell and accurately determines the viability rate. At each stage of the experiments, cell density and viability were monitored using this method and cells were ensured to have a viability of greater than 80%. This is critical to increase the reliability of the experiments and to guarantee that the cells used in the experiment are healthy.

To establish cell stock, the SMA Type 2 Fibroblast cell line was frozen after the cells had grown. For freezing, cells were suspended in a cryoprotectant solution containing 90% Cell Medium (EMEM, 10% FBS, 1% Pen-Strep, 1% L-glutamine) and 10% dimethyl sulfoxide (DMSO). DMSO protects the cells from the damaging effects of ice crystals that may form during freezing. Cells were first placed in a -80°C cabinet and transferred 24 hours later to a liquid nitrogen tank for long-term storage. During the recultivation of the frozen cells, the cells were thawed rapidly in a 37°C water bath and immediately transferred to 15 ml falcon tubes. Cell medium was added slowly to avoid cell shock, and then the cells were centrifuged at 400 g for 5 minutes at 4°C. After centrifugation, the supernatant was removed and the cells were resuspended with fresh medium and transferred to appropriate flasks. Cells were incubated and cultured in a 37°C oven containing 5% CO2.

3.2 Antisense Oligonucleotide (ASO) Applications

The splicing suppressor region in exon 7 of the *SMN2* gene (ISS-N1) has been selected as a critical site for antisense oligonucleotide targeting. ISS-N1 is a negative splicing regulator that regulates the exclusion of exon 7 from splicing. ASO design for this region was done with the aim to increase the incorporation of exon 7 into *SMN2* mRNA. The ISS-N1 target site was optimized based on RNA structural analysis and experimental data. A 15-20 nucleotide long ASO was designed to bind to the ISS-N1 target site. The designed XNA/DNA-ASO and the positive control Nursinersen were obtained from Qiagen Inc. This ASO was intended to bind to the target site and prevent the binding of hnRNP proteins, thus enabling exon 7 to be incorporated into mRNA by

splicing. The designed oligonucleotides were optimized using bioinformatics tools and RNA structure analysis. Phosphorothioate (PS) modification was performed on the backbone of ASO to increase its biological stability and improve its therapeutic efficacy. It was also combined with a modification of the XNA variant, LNA, at the ribose sugar to enhance its intracellular utilization and stronger binding to the target. This combined modification of XNA/DNA-ASO promotes the production of functional SMN protein by ensuring that oligonucleotides remain stable in the cell and exon 7 is incorporated by correct splicing. (Zhang et al., 2022; Bennett et al., 2017)

The table represents the modified antisense oligonucleotide sequences used in the study. MERLiNA and GENLiNA are XNA/DNA-ASO hybrid sequences designed for therapeutic application, utilizing phosphorothioated XNA and DNA bases for enhanced stability. The positive control, Nusinersen, an RNA-based antisense oligonucleotide, is included as a benchmark, showcasing typical modifications for improved hybridization and efficacy. The sequences highlight the structural differences aimed at optimizing therapeutic performance.

Table 4: Modified XNA/DNA-ASO and RNA-ASO Sequences

Antisense Oligonucleotide Modified Sequence Names	XNA-DNA mixmer sequence Phosphorothioated XNA* bases/ Phosphorothioated DNA* bases
XNA/DNA-ASO (MERLiNA)	+T*C*+A*C*+T*+T*+T*C*+A*+T*+A*+A*+T*G*C*+T*G*G
XNA/DNA-ASO GENLiNA	T*+C*A*+C*T*T*T+C*A*T*A*A*T*+G*+C*T*+G*+G
Positive Control RNA-ASO (Nusinersen)	mU*mC*A*mC*mU*mU*mC**A*mU*A*A*mU*G*mC*mU*G*G

This study will evaluate the efficacy of oligonucleotide therapies in Spinal Muscular Atrophy (SMA). Two different Xeno Nucleic Acid Antisense Oligonucleotides (XNA/DNA-ASO), GenLiNA and MerLiNA, and an RNA-based Antisense Oligonucleotide (RNA-ASO) Positive control, Nusinersen, were used in the experiment.

3.2.1 Control Groups:

- DNA Transfected Control: Cells were transfected with GFP plasmid to monitor transfection efficiency.
- PEI Control (Polyethyleneimine): PEI control group was created to see the effects of PEI used as a transfection agent on the cells.
- Negative Control (NC): Cell culture only (no oligonucleotide treatment).

3.2.2 Treatment Groups:

- Nusinersen (RNA-ASO): An ASO used clinically in SMA patients, it allows the inclusion of exon 7 in *SMN2* mRNA.
- GenLiNA (XNA-ASO): A Xeno Nucleic Acid-based ASO developed for the treatment of SMA.
- MerLiNA (XNA-ASO): A Xeno Nucleic Acid-based ASO developed for the treatment of SMA

Experiments were performed with ASO transfection at concentrations of 500 nM, 200 nM, 100 nM, and 10 nM, 1 nM. Cell density was adjusted to 2×10^5 cells per well. Cells were transfected using 12-well plates.

This figure outlines the experimental workflow used to treat SMA Type II fibroblast cells with XNA/DNA-ASO (GENLiNA and MERLiNA) and RNA-ASO (Nusinersen). First, 2 × 10^5 fibroblast cells were seeded into a 12-well plate and exposed to various concentrations (1 nM, 10 nM, 100 nM, 200 nM, and 500 nM) of the respective ASO treatments. Control conditions included both PEI and DNA-transfected cells, along with a negative control group. At 48 hours post-treatment, SMN staining was performed to analyze SMN protein expression through flow cytometry. In parallel, RNA isolation was conducted to assess SMN gene expression levels. Cell viability was measured using the MTT assay, while apoptosis and necrosis rates were determined through Annexin V and Propidium Iodide (PI) staining. Finally, cell morphology was observed at both 24 and 48 hours using fluorescent and phase-contrast microscopy, allowing for a comprehensive evaluation of the treatment's impact on cell survival, SMN expression, and apoptosis.

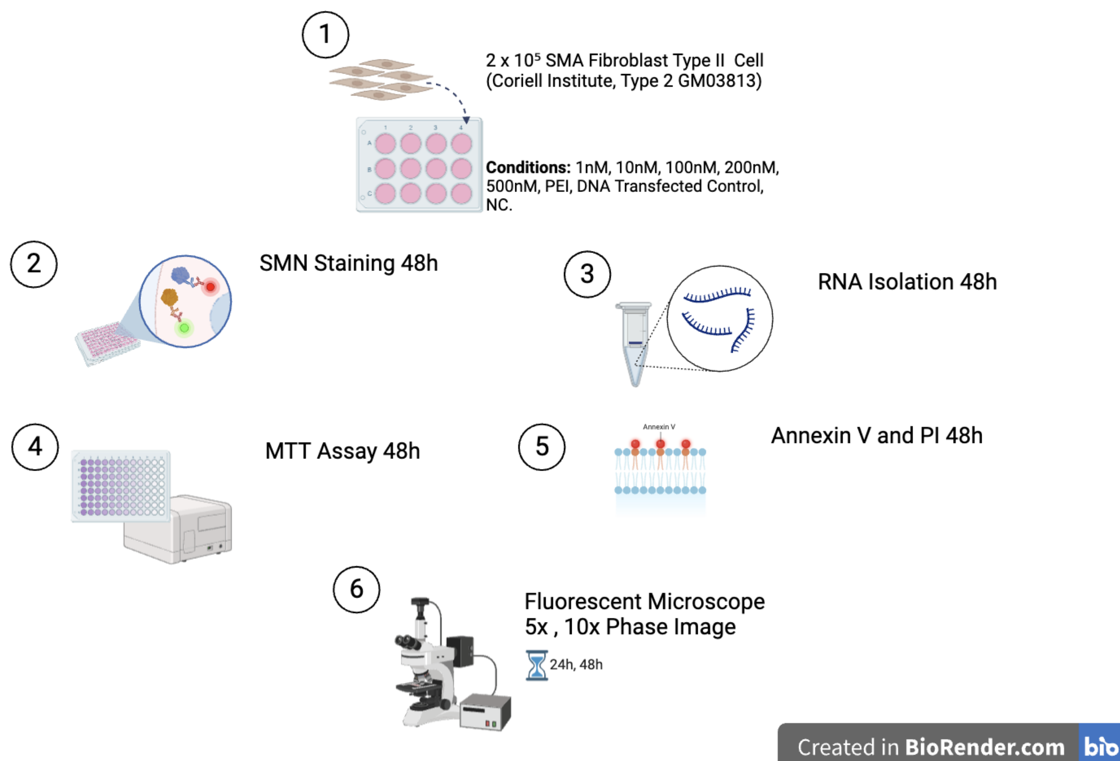

Figure 15: Workflow of Experimental Procedures for SMA Type II Fibroblast Treatment with XNA/DNA-ASO (GENLiNA and MERLiNA) and RNA-ASO (Nusinersen).

3.3 Transfection Protocol in Target Cell Lines

Cells were subjected to transfection when they reached 80% confluency before the start of the experiment in accordance with the culture conditions. Polyethyleneimine (PEI) was used for transfection. PEI is a transfection agent that allows both XNA/DNA-ASOs and the positive control RNA-ASOs to enter the cell efficiently. Cells were transfected with GFP plasmid and different concentrations of ASOs and no oligonucleotide was added in the control groups.

Transfection was performed by a method using Polyethyleneimine (PEI). In this study, 2×10^5 SMA fibroblast type 2 cells were treated with Antisense Oligonucleotide (ASO) at concentrations of 1 nM, 10 nM, 100 nM, 200 nM and 500 nM. The different concentrations were chosen to examine the dose-dependent effects of ASO.

Cells were seeded in a 12-well plate and approximately 2×10^5 cells were added to each well. Cells were grown in an incubator at 37°C in 5% CO_2 at 37°C until they reached 80-90% confluency.

ASO (Antisense Oligonucleotide) solution was prepared at specific concentrations in serum-free medium (Opti-MEM). Different concentrations of ASO (1

nM, 10 nM, 100 nM, 200 nM and 500 nM) were used to examine the dose-dependent effects of ASO in cells. PEI (Polyethyleneimine) solution was prepared in serum-free medium and PEI ratio was determined as 5:3.5. The PEI/ASO mixture was incubated at room temperature for 10 minutes to increase the passage of ASO through the cell membrane. Afterwards, the old medium was discarded from the cell culture medium and PEI/ASO mixture at concentrations of 1 nM, 10 nM, 100 nM, 200 nM and 500 nM was added to each well in appropriate amounts.

Cells were incubated with the transfection mixture for 48 hours in a 5% CO_2 incubator at 37°C. During this time, PEI transported ASOs into the cells and allowed the cells to take up the oligonucleotides. At the end of 48 hours, the cells were subjected to validation procedures.

3.4 Intracellular SMN Staining and Analysis by Flow Cytometry

In the study, SMN protein expression in cells was analyzed using intracellular staining and flow cytometry. SMA Type II fibroblast cells were stained using anti-SMN-AlexaFluor 647 antibody and the results were evaluated by flow cytometry. The protocol used is described step by step below:

SMA Type II fibroblast cells were cultured under appropriate conditions. After the cells were harvested, the cell suspension was centrifuged at 400g for 5 minutes at +4°C to obtain pellets. Cells were subjected to fixation and permeabilization using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit. 200 µl of Fixation/Permeabilization solution was added to the cell pellet and cells were incubated at +4°C for 20 minutes. After fixation, the cells were washed twice with BD Perm/Wash™ Buffer. The washes were performed carefully to preserve the permeabilized nature of the cells. Cells were stained with anti-SMN-AlexaFluor 647 antibody (Santa Cruz Biotechnology, Anti-SMN Antibody (2B1) Alexa Fluor® 647, sc-32313 AF647) at a dilution ratio of 1:50. During the staining process, the samples were vortexed and then incubated at +4°C for 30 minutes. After staining, the cells were washed twice with BD Perm/Wash™ Buffer and centrifuged at 300g for 5 minutes. They were then suspended in 300 µl of BD Perm/Wash™ Buffer. Analysis by Flow Cytometry: Stained cells were loaded into a Beckman Coulter CytoFlex flow cytometry device and analyzed. SMN protein expression levels were assessed by both Mean Fluorescence Intensity (MFI) and percentage distribution within the cell population.

The flow cytometry method used in this protocol is critical to quantitatively measure SMN protein expression levels of cells. As a result of the analysis, changes in SMN protein levels in different ASO treatment groups were determined and compared.

3.5 Determination of Cell Viability by MTT Assay

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] test was applied to evaluate cell viability and possible toxicity. MTT assay is a colorimetric method that measures the metabolic activity of cells. With this method, living cells reduce the MTT agent to purple colored formazan salt and the amount of these salts indicates the mitochondrial activity of the cell. In the experiment, 1x10^4 cells were seeded in each well.

After the cells reached the appropriate morphology, 10 µl of MTT agent was added to each well and the cells were incubated at 37°C for 4 hours. At the end of this time, the medium with MTT agent was removed and 100 µl DMSO was added to each well to dissolve the formazan salts on the cells. The addition of DMSO dissolved the purple formazan crystals and the absorbance values corresponding to cell viability were measured at wavelengths of 570 nm and 655 nm. Measurements were performed using an ELISA (FLUOstar, OMEGA) device.

This assay was used to determine the cytotoxic effects of XNA/DNA-ASO and RNA-ASO treatments on cells and to assess cell viability. Cell viability revealed the differences between the experimental groups and provided an opportunity to compare the toxic effects of ASOs on cells.

3.6 Determination of Apoptosis Rates in Target Cell Lines by Flow Cytometry

In this study, toxicity and apoptosis rates in target cell lines were analyzed by flow cytometry using Annexin V-FITC and Propidium Iodide (PI). Apoptosis was determined by staining cells with Annexin V and PI. Annexin V marks the migration of phosphatidylserine to the outer surface of the cell membrane, while PI stains cells in which the cell membrane has become permeable so that necrotic cells can be identified.

The experimental protocol used to determine toxicity and apoptosis rates in target cell lines was based on Annexin V-FITC and Propidium Iodide (PI) staining methods. Cells were diluted with 1X Annexin binding buffer to 1x10^5 cells in each test tube. To these cell suspensions, 5 µL Annexin V-FITC and 1 µL PI (100 µg/mL) working solution

were added per tube. The staining process is used to determine the apoptotic state of the cells and the staining mixture was incubated for 15 minutes at room temperature. After incubation, the cells were resuspended by adding 400 µL of 1X Annexin binding buffer to each tube. The prepared cell suspension was analyzed by Beckman Coulter CytoFlex flow cytometry device. This analysis determined the early and late apoptosis rates of the cells according to Annexin V and PI staining results.

This method was used to measure the rates of apoptosis induced in cells by XNA/DNA-ASO and RNA-ASO therapies and to compare the toxic effects of the treatments.

3.7 Total RNA Isolation

Total RNA isolation from cells was performed using PureLink RNA Mini Kit (#12183018A) according to the manufacturer's instructions. Following the isolation process, absorbance measurements were performed with a Microplate Reader (OmegaStar, ELISA) to assess the purity and concentration of the isolated RNA. Measurements were performed at 230 nm, 260 nm and 280 nm wavelengths and the A260/A280 ratio was taken into account for RNA purity. Isolated total RNA was stored at -20°C to be used for cDNA synthesis in subsequent experiments. At this stage, cDNA synthesis was not performed and cDNA synthesis is planned to be performed in the future using the High-Capacity cDNA Reverse Transcription Kit (The Applied Biosystems™, #4368814) according to the manufacturer's instructions.

3.8 Statistical Analysis

Each experiment was performed in three technical replicates and experimental results are reported as mean ± standard deviation (SD). Data were manually entered into GraphPad Prism software in the appropriate format according to cell groups. Data obtained in apoptosis and viability analyses were reported as Percentage Distribution.

The statistical significance threshold was accepted as $p<0.05$. P-values lower than this value indicated that the differences between the groups were significant.

The results of statistical analyses are reported in graphs and tables, and significant differences are indicated with an asterisk (*) (e.g. *p<0.05, **p<0.01, **p<0.001).

Results are presented as the mean ± standard deviation (SD) of each experimental group. Each graph includes individual data points, means and SD for each group. The results of the statistical analysis are presented in graphs and tables generated with GraphPad Prism.

4. FINDINGS

The results of this study provide significant insights into the comparison between XNA/DNA-ASO treatments (GENLiNA and MERLiNA) and RNA-ASO (Nusinersen) in the context of spinal muscular atrophy (SMA) therapy. The investigation primarily focuses on key cellular outcomes, such as cell viability, SMN protein expression, apoptosis, and mitochondrial activity. Through various experiments, it is observed that XNA/DNA-ASO treatments demonstrate notable efficacy in enhancing SMN protein expression and maintaining cell survival, while also effectively reducing apoptosis rates compared to Nusinersen. The ability of XNA/DNA-ASO to sustain high levels of SMN expression while minimizing apoptotic markers underlines its therapeutic potential, particularly in SMA Type II fibroblasts. These findings suggest that XNA/DNA-ASO could offer superior stability and efficacy over conventional RNA-ASO therapies, making it a promising candidate for further clinical development.

The study's analysis of early and late apoptosis rates also reveals that XNA/DNA-ASO treatments cause lower levels of cellular toxicity, as indicated by the reduced percentage of late apoptotic cells. This aspect is crucial for minimizing off-target effects and improving long-term therapeutic outcomes. Meanwhile, Nusinersen demonstrates considerable performance in enhancing SMN expression, but its efficacy appears limited compared to the superior results obtained with XNA/DNA-ASO.

Mitochondrial activity assays (MTT) further confirm the advantageous effects of XNA/DNA-ASO, which shows greater maintenance of mitochondrial function and cellular energy levels. The combination of these findings establishes a foundation for considering XNA/DNA-ASO as a next-generation therapeutic approach for SMA, highlighting the need for future studies to explore its long-term efficacy, safety, and applicability in clinical settings. Through this comprehensive comparison, the study contributes valuable knowledge to the growing body of research focused on developing advanced therapies for genetic disorders like SMA.

This figure shows the effects of different doses of Nusinersen treatment on cell viability (left), SMN protein expression (middle), apoptosis rates (right, Annexin V/PI staining) and cell morphology in SMA Type 2 fibroblast cell line. Flow cytometry

analysis shows that Nusinersen at a dose of 100 nM increased SMN expression by up to 60.06%, while apoptosis rates remained at lower levels (17.82%) at this dose. Cell viability and morphology suggest that Nusinersen maintains cell health with low toxicity. In SMA Type 2 fibroblasts, these findings confirm that Nusinersen is an effective treatment option. (**Figure 16**)

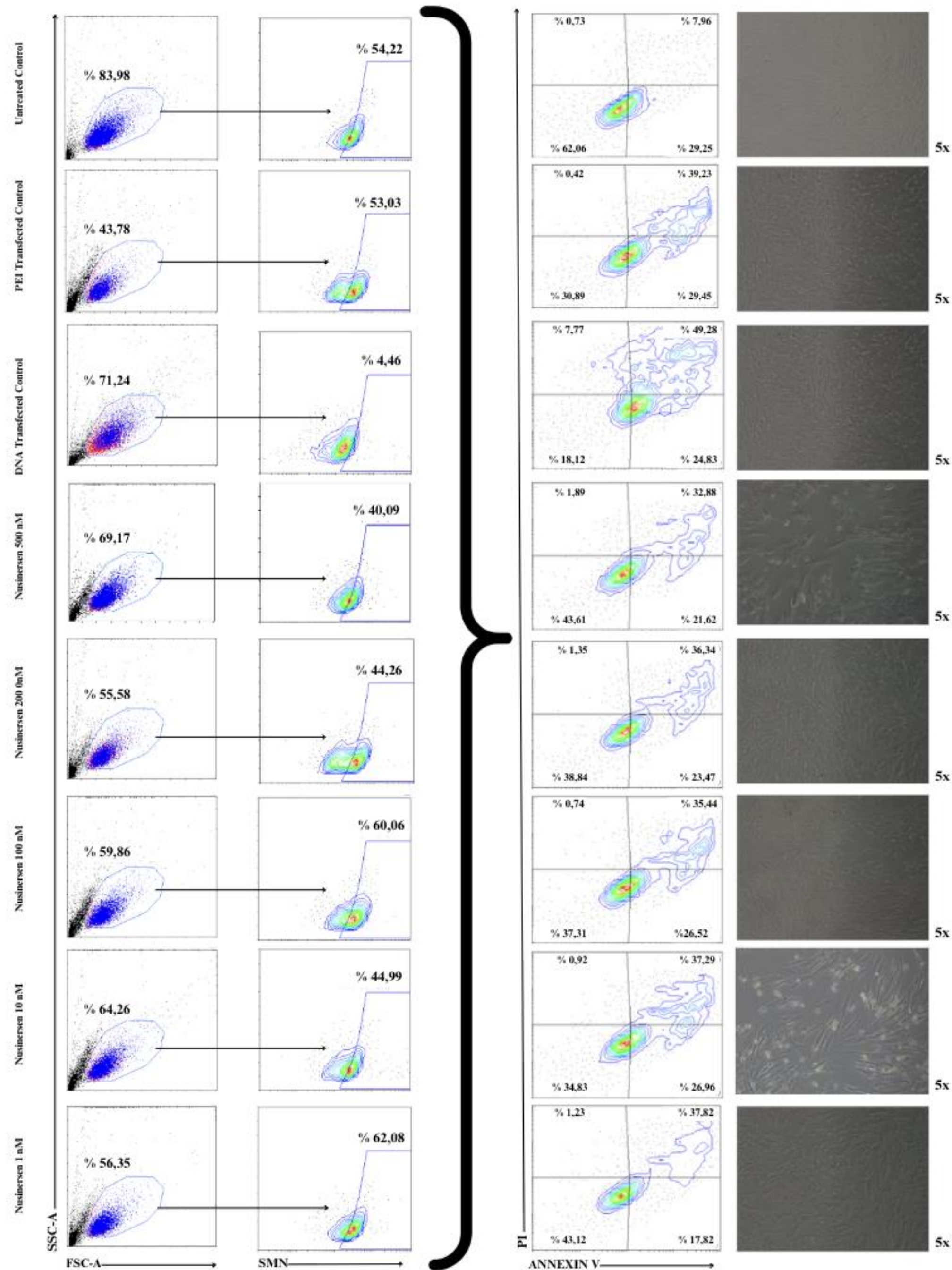

Figure 16: Effects of Different Nusinersen Doses on Cell Viability, SMN Expression, Apoptosis and Cell Morphology in SMA Type 2 Fibroblast Cells

This figure shows the results of treatment in SMA Type 2 fibroblast cell line using XNA/DNA-ASO (GENLİNA). Cell viability (left), SMN protein expression (middle), apoptosis rates (right, Annexin V/PI staining) and cell morphology were assessed by flow cytometry analysis. GENLİNA 100 nM dose achieved the highest SMN expression rate (72.34%). At the same dose, apoptosis rates remained low and cell viability was maintained. Cell morphologies also indicate that the treatment has low toxicity. These results indicate that GENLİNA may be an effective alternative in the treatment of SMA. (**Figure 17**)

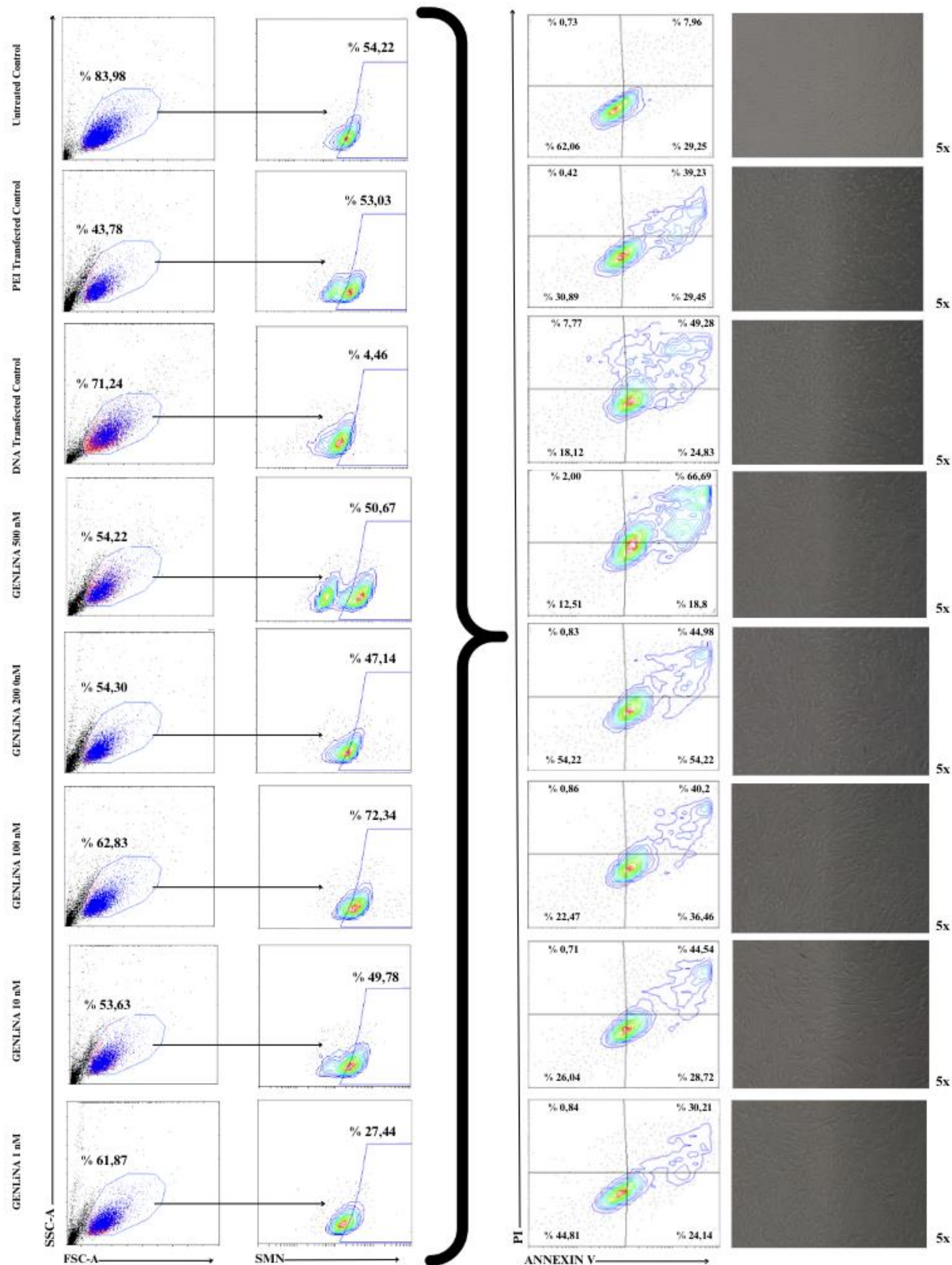

Figure 17: Effects of Different GENLiNA Doses on Cell Viability, SMN Expression, Apoptosis and Cell Morphology in SMA Type 2 Fibroblast Cells

This figure shows the effects of XNA/DNA-ASO (MERLiNA) treatment on the SMA Type 2 fibroblast cell line. On the left, the effect of different doses of MERLiNA on cell viability is shown; up to 85% viability was observed at a dose of 100 nM. The centre graph shows the increase in SMN protein expression by MERLiNA; at a dose of

100 nM, SMN expression increased up to 60.06%. On the right side, apoptosis rates determined by Annexin V/PI staining are presented; at a dose of 100 nM, the apoptosis rate was 37.61%. Furthermore, morphological analyses showed that cell health was preserved at low doses (1 nM and 10 nM). These data suggest that MERLiNA may have the potential to increase cell health and SMN expression in the treatment of SMA. (**Figure 18**).

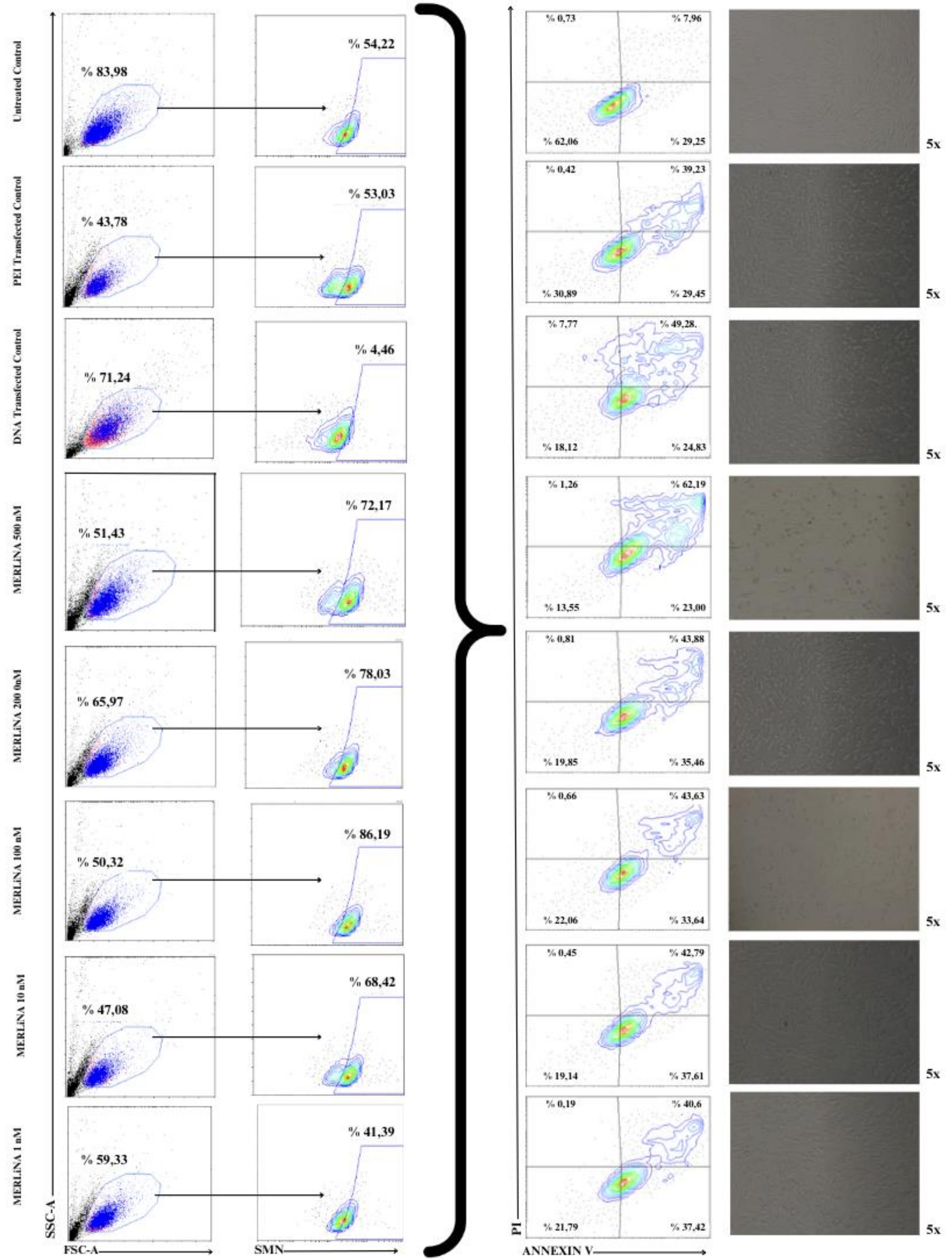

Figure 18: Effects of Different MERLİNA Doses on Cell Viability, SMN Expression, Apoptosis and Cell Morphology in SMA Type 2 Fibroblast Cells

Nusinersen was included as a comparative control to benchmark against the novel XNA/DNA-ASO therapies being evaluated. The data indicate that Nusinersen achieved an SMN expression increase of 60.06% at a dose of 100 nM, with a relatively low

apoptosis rate of 17.82%. The high cell viability and well-maintained cell morphology suggest that Nusinersen has minimal cytotoxic effects and preserves cellular integrity. However, despite these positive outcomes, Nusinersen's ability to significantly elevate SMN protein levels remains limited compared to the more innovative XNA/DNA-ASO treatments. This limitation underscores the necessity for exploring alternative therapies with enhanced efficacy in SMN expression while maintaining a similar safety profile.

In contrast, the XNA/DNA-ASO GENLiNA demonstrated superior results in SMN protein expression, reaching 72.34% at a dose of 100 nM, significantly outperforming Nusinersen. However, the apoptosis rate at this dose was measured at 36.46%, indicating that higher doses of GENLiNA may induce more apoptotic activity, requiring careful dose optimization to avoid excessive cell death. Lower doses of GENLiNA (1 nM and 10 nM) maintained cell viability while preserving cell morphology, though SMN expression levels were not as elevated as at the higher doses. This dose-dependent behavior suggests that while GENLiNA has the potential to be an effective treatment, it necessitates precise titration to balance efficacy and safety, making it a viable alternative for further exploration.

The XNA/DNA-ASO MERLiNA emerged as the most promising candidate from this series of tests, achieving an SMN expression of 86.19% at 100 nM, the highest among the tested therapies. This remarkable increase in SMN expression illustrates MERLiNA's strong therapeutic potential in addressing SMN protein deficits in SMA. However, the apoptosis rate of 43.63% at the same dose indicates that cytotoxic effects must be monitored carefully, especially at higher doses. Nonetheless, MERLiNA maintained robust cell viability at lower doses (1 nM and 10 nM) with significantly reduced apoptosis rates, indicating a favorable safety profile at these levels. Morphological analysis showed that while high doses of MERLiNA caused some cellular degeneration, low doses effectively preserved cell structure and health, further supporting its candidacy as a viable SMA treatment.

In summary, the data strongly support the advancement of XNA/DNA-ASO therapies as a more potent alternative to traditional RNA-ASOs like Nusinersen. Both GENLiNA and MERLiNA displayed a significantly greater capacity for increasing SMN protein expression, with MERLiNA showing the most remarkable results in terms of efficacy.

However, both XNA/DNA-ASO treatments present dose-dependent challenges, particularly regarding apoptosis rates and cytotoxicity at higher doses, underscoring the need for careful dose optimization. Morphological assessments reinforce the therapeutic potential of these XNA/DNA-ASOs, with lower doses maintaining cell viability and structural integrity. Thus, MERLiNA and GENLiNA present as strong candidates for further development, potentially offering more effective, targeted, and customizable treatment solutions for SMA compared to current RNA-ASO therapies.

In cell viability measurements, the effects of Nusinersen (RNA-ASO), MERLiNA (XNA/DNA-ASO), GENLiNA (XNA/DNA-ASO) and control groups at different concentrations were examined. The results show that cell viability was generally maintained between 1 nM and 500 nM in all ASO groups. While about 60% viability was maintained at MERLiNA 100 nM dose, Nusinersen showed a viability rate close to 40% at 100 nM. Especially in PEI transfected control and GFP control groups, the viability rate exceeded 60%, while NC group showed the highest viability rate. Similar viability rates were observed at high concentrations (above 50%) in all groups, reflecting the dose-dependent effect of ASO treatment (**Figure 19**).

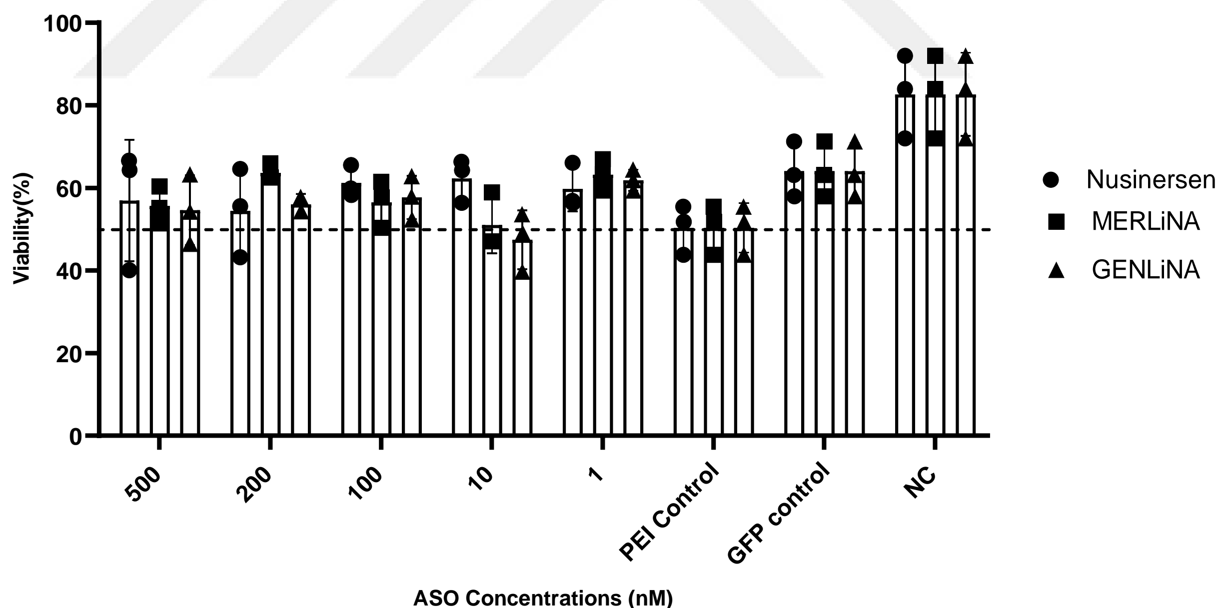

Figure 19: Cell Viability Analysis

According to MTT results, mitochondrial activity was compared at different ASO concentrations. In the range from 500 nM to 1 nM, significant differences were observed between Nusinersen and MERLiNA groups. Nusinersen group showed a slight decrease in mitochondrial activity at low doses (1-10 nM), while MERLiNA and GENLiNA

groups showed a more stable activity at these doses. Especially at a dose of 100 nM, MERLiNA exhibited an activity above 0.7%, indicating that the mitochondrial activity of the cells was highly preserved. Higher mitochondrial activity was observed in GFP and PEI groups. In general, mitochondrial activity was preserved in a dose-dependent manner between ASO groups (**Figure 20**).

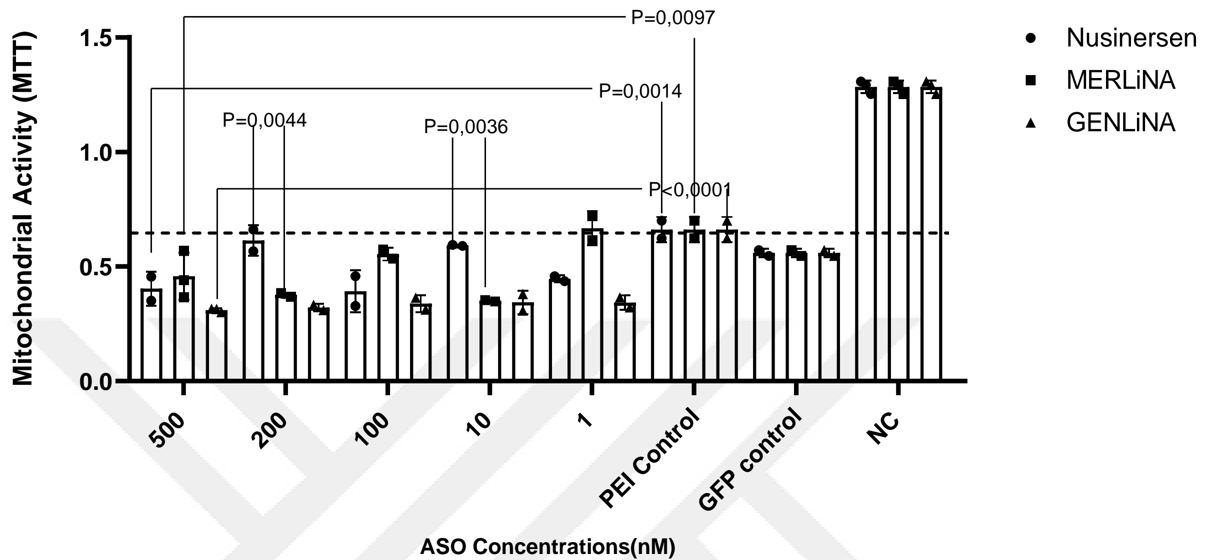

Figure 20: MTT Analysis (P < 0.05 is significant NS= non-Significant.)

The effects of Nusinersen, MERLiNA and GENLiNA groups at different concentrations were examined in terms of SMN protein expression. While the Nusinersen group generally achieved approximately 60% SMN expression at 500 nM, this rate exceeded 70% in the MERLiNA and GENLiNA groups. At lower concentrations (1-10 nM), the SMN expression rates of MERLiNA and GENLiNA groups were higher, especially at 100 nM, with an expression rate close to 72%. GFP and PEI control groups showed the highest level of SMN expression. These results indicate that XNA/DNA-ASO is more effective in SMN expression compared to RNA-ASO (**Figure 21).**

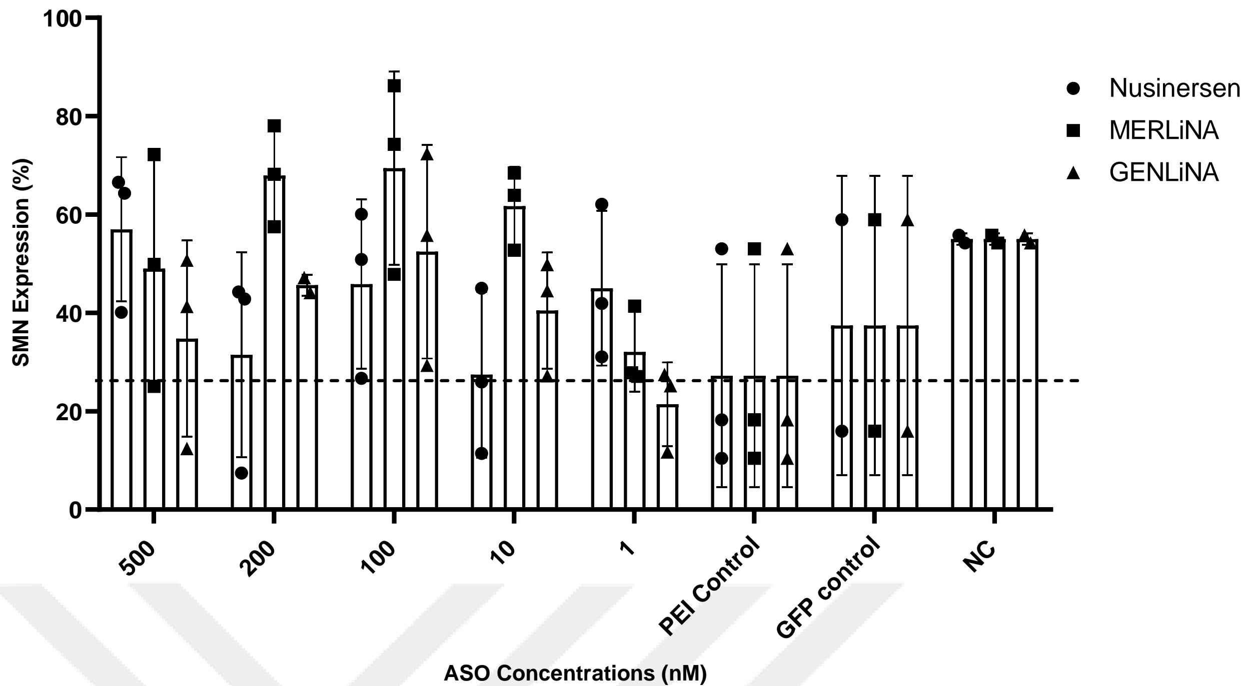

Figure 21: SMN Expression Analysis.

Survival apoptosis data compared the effects of Nusinersen (RNA-ASO), MERLiNA (XNA/DNA-ASO), GENLiNA (XNA/DNA-ASO) and control groups at different concentrations. Survival rates indicate whether apoptosis mechanisms are suppressed or not. At high concentrations (500 nM), a survival rate of around 40% was observed in the Nusinersen and MERLiNA groups, while the survival rate in the GENLiNA group was slightly below 30%. At lower concentrations (1-10 nM), MERLiNA suppressed apoptosis more effectively, increasing the survival rate above 40%. Especially at a dose of 10 nM, MERLiNA provided a survival rate of around 35% and these results were generally more favorable compared to the control groups. Survival analysis showed that MERLiNA had a better apoptosis suppressing effect at lower concentrations and maintained the cell survival rate more than the other groups. This suggests that MERLiNA may be more effective than RNA-ASO in reducing cell death in SMA treatment. (**Figure 22**)

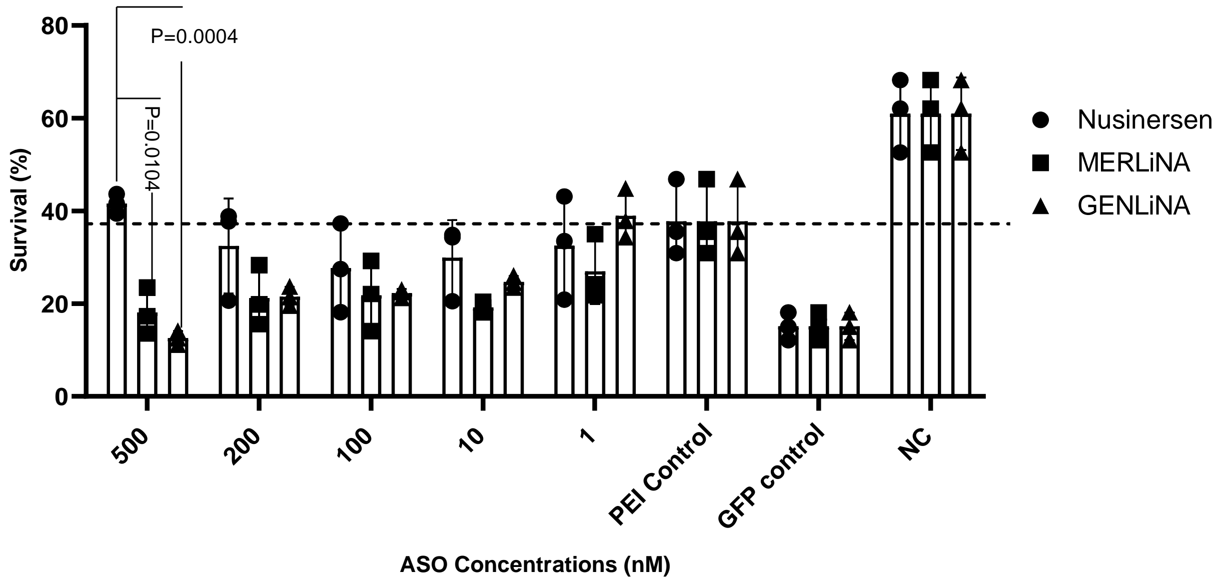

Figure 22: Survival Apoptosis Analysis (P < 0.05 is significant NS= non-Significant.)

In late apoptosis assays, the effects of Nusinersen, MERLiNA and GENLiNA groups on apoptosis were compared. At 500 nM concentration, approximately 60% late apoptosis rate was observed in the Nusinersen group, while this rate remained in the range of 40-50% in the MERLiNA and GENLiNA groups. Especially at low concentrations (1-10 nM), the apoptosis rate in the MERLiNA group decreased significantly and remained below 30%. In GFP and PEI control groups, apoptosis rates were higher compared to theother groups. These results suggest that MERLiNA and GENLiNA are effective in reducing the apoptosis rate. (**Figure23)**

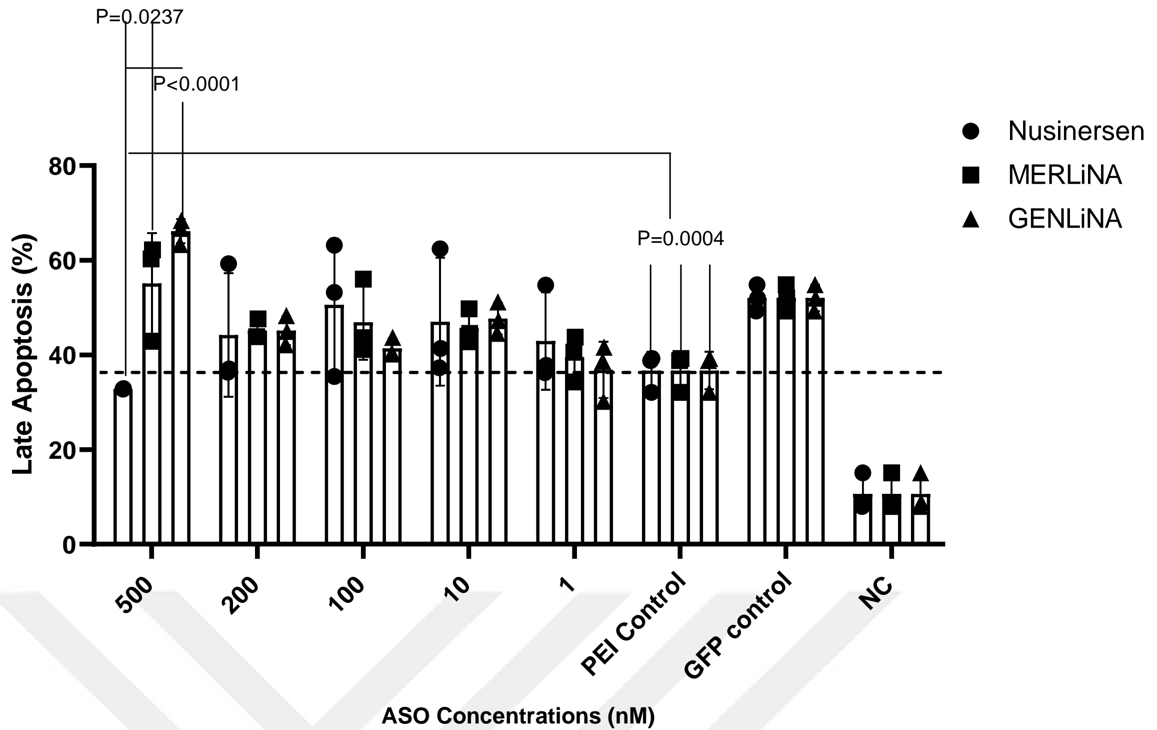

Figure 23: Late Apoptosis Analysis (P < 0.05 is significant NS= non-Significant.)

Early apoptosis results showed similar trends in Nusinersen, MERLiNA and GENLiNA groups. At 500 nM concentration, Nusinersen showed an early apoptosis rate of approximately 30%, while this rate was close to 20% in MERLiNA and GENLiNA groups. At lower doses (1-10 nM), especially MERLiNA reduced the early apoptosis rate below 20% and apoptosis was largely suppressed. GFP and PEI control groups had the highest early apoptosis rates. These results suggest that MERLiNA and GENLiNA are more effective in suppressing apoptosis than RNA-ASO. (**Figure 24)**

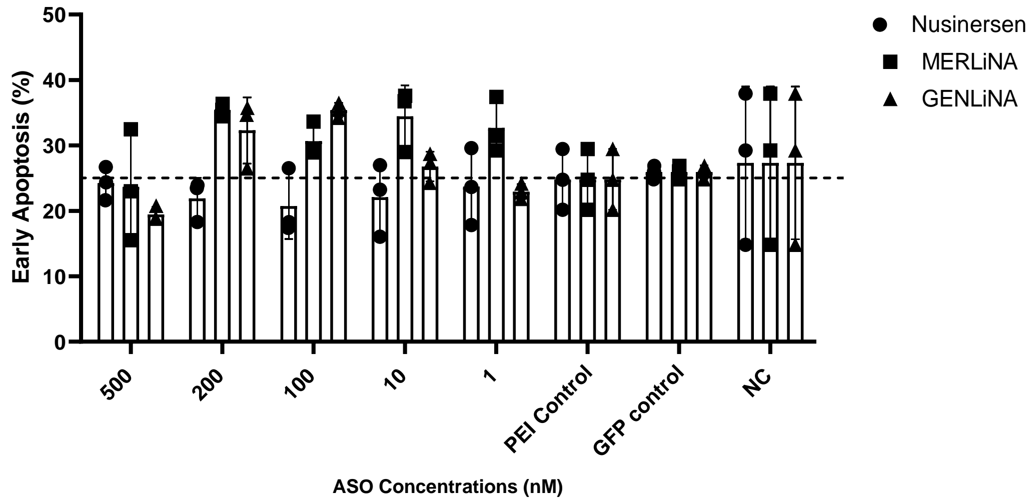

Figure 24: Early Apoptosis Analysis.

This figure illustrates the geometric mean (GeoMean) values of SMN protein expression in treated fibroblast cells under different experimental conditions. The graph presents comparative GeoMean values for cells treated with XNA/DNA-ASO (GENLiNA and MERLiNA), RNA-ASO (Nusinersen), and control groups. The data indicate a significant increase in SMN protein expression, especially in cells treated with XNA/DNA-ASO formulations, highlighting the potential of XNA/DNA-ASO therapies in enhancing SMN expression more effectively than RNA-ASO in this model system **(Figure 25).**

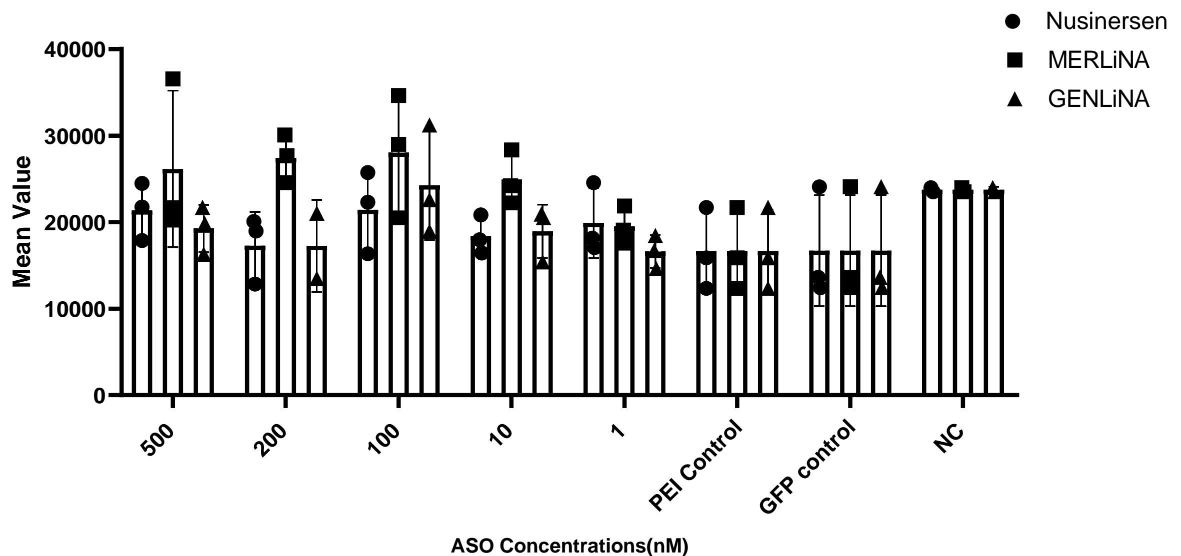

Figure 25: GeoMean Analysis of SMN Protein Expression in Treated Cells.

5. DISCUSSION

In this study, the effects of MERLiNA and GENLiNA XNA/DNA-ASOs in SMA Type 2 fibroblast cells were evaluated in comparison with Nusinersen (trade name Nusinersen, RNA-ASO). Although the results are generally consistent with the findings in the literature, some unique findings increase the importance of the study. In particular, XNA/DNA-ASOs seem to stand out as potential therapeutic options in the treatment of SMA. The findings contribute to a deeper understanding of the effects of ASOs used for SMA treatment on cell viability, SMN expression and apoptosis.

When the MTT assay results were analyzed, a decrease in cell viability was observed at doses of 500 nM and above, while viability was preserved at low doses (100 nM and below). In particular, MERLiNA maintained cell viability at low concentrations (10 nM and 1 nM), indicating that the toxicity levels of this ASO are low. It is noteworthy that compared to nusinersen, MERLiNA presented similar or lower toxicity levels. This finding is consistent with reports in some studies in the literature that XNA modifications improve the therapeutic efficacy of ASO by increasing intracellular stability (Stein & Castanotto, 2017). Nusinersen, on the other hand, showed overall more stable results in terms of cell viability, which supports previous studies in the literature confirming the efficacy of Nusinersen in the treatment of SMA (Chiriboga et al., 2016; Finkel et al., 2017). However, in this study, MERLiNA showed efficacy close to Nusinersen at low doses, indicating that XNA/DNA-ASOs may also be a potential alternative in the treatment of SMA. Moreover, the increased toxicity observed at high doses (500 nM) is in line with findings in the literature that excessive doses of ASOs can lead to intracellular stress and toxic effects (Crooke et al., 2017).

SMN expression analyses revealed that MERLiNA and GENLiNA produced a significant increase in SMN levels, especially at doses of 100 nM and 10 nM. This increase is a very favorable finding compared to other ASOs used in the treatment of SMA. In the literature, Nusinersen has been reported to improve motor neuron function and slow disease progression by increasing SMN expression (Hua et al., 2010). The potential of MERLiNA to increase SMN expression offers promising results in terms of XNA/DNA-ASOs, especially affecting the splicing mechanism of *SMN2* mRNA, enabling the incorporation of exon 7. Furthermore, the capacity of GENLiNA to increase SMN expression at low doses suggests that XNA-based modifications may be more potent and stable compared to RNA-based therapies. It has been reported in the literature

that XNA modifications enable stronger binding of ASOs to the target RNA and thus can alter splicing mechanisms more effectively (Bennett & Swayze, 2010). This shows that the results obtained in our study are consistent with previous findings in the literature.

Apoptosis assays showed that early apoptosis rates decreased in MERLiNA and GENLiNA groups at low doses, whereas apoptosis increased significantly at high doses (500 nM). Especially at doses of 100 nM and 10 nM, MERLiNA decreased apoptosis, indicating that this ASO can suppress intracellular stress mechanisms. Compared to nusinersen, MERLiNA induced lower apoptosis rates at lower doses, suggesting that XNA/DNA-ASOs may potentially have lower intracellular toxic effects. Increased apoptosis rates at higher doses, however, suggest that doses of XNA/DNA-ASOs for the treatment of SMA should be carefully optimized. The findings in the literature that excessive ASO doses may cause intracellular toxicity and apoptosis are in agreement with the high dose toxicity observed in our study (Corey, 2017).

In terms of cell morphology, cells were healthy and adherent in MERLiNA and Nusinersen groups at low doses. However, at high doses (500 nM), signs of deterioration in cell morphology were noted. The rounding of the cells suggests that morphological changes can be considered as an indicator of intracellular stress. The ability of cells to maintain their healthy morphology is critical for determining safe and effective dose ranges of ASOs used in the treatment of SMA. These findings are consistent with reports in the literature that high doses of ASOs can lead to cytoskeletal disruptions (Crooke et al., 2017).

It was observed that cell viability also decreased in groups with high apoptosis rates. Especially in the MERLiNA and GENLiNA groups at 500 nM dose, apoptosis rates increased significantly, while viability rates decreased. This coincides with studies in the literature suggesting that high doses of ASO can lead to cell death and apoptosis (Bennett & Swayze, 2010). The prominence of apoptosis as an important finding in this study emphasizes that ASOs used in SMA treatment should be designed to not only increase SMN expression but also minimize cell death rates.

The findings of this study suggest that XNA/DNA-ASOs such as MERLiNA and GENLiNA are potential therapeutic agents for the treatment of SMA. Compared to nusinersen, XNA/DNA-ASOs showed similar efficacy, especially at low doses, but increased intracellular stress and apoptosis was observed at high doses. This suggests that XNA/DNA-ASOs should be carefully optimized during the treatment process. Although

the success of Nusinersen in SMA treatment has been proven in the literature, this study emphasizes that XNA/DNA-ASOs may be promising alternatives for SMA treatment. In the future, larger scale *in vivo* studies and clinical trials are needed to utilize these ASOs in clinical applications. In particular, it is thought that XNA modifications may provide longer-term effects than RNA-based therapies by increasing intracellular stability (Stein & Castanotto, 2017).

6. CONCLUSIONS AND RECOMMENDATIONS

The results obtained in this study demonstrate that XNA/DNA-ASO therapies, specifically MERLiNA and GENLiNA, offer notable advantages over traditional RNA-based ASO treatments, such as Nusinersen, in treating Spinal Muscular Atrophy (SMA). At doses as low as 100 nM, both MERLiNA and GENLiNA exhibited superior SMN protein expression and cell viability when compared to Nusinersen. This underscores the potential of XNA/DNA-ASOs to provide higher efficacy and stability, particularly at lower doses. The chemical modifications inherent to XNA/DNA-ASOs significantly enhance their intracellular stability optimizing therapeutic effects.

In the MERLiNA group, SMN expression was significantly higher than in Nusinersen-treated cells, particularly in SMA Type 2 fibroblasts, suggesting a more efficient regulation of SMN2 exon 7 splicing. This increased efficacy points to the therapeutic potential of XNA/DNA-ASOs in genetic disorders, offering a new avenue to improve SMN protein production and potentially halt disease progression.

Apoptosis assays further highlight the favorable toxicity profiles of XNA/DNA-ASOs. While Nusinersen showed low apoptosis rates, the MERLiNA and GENLiNA groups also exhibited low apoptosis, especially at optimized doses, suggesting that these treatments can preserve cell viability and function. Morphological analyses confirm that these treatments have limited negative effects on cell structure, emphasizing their safety and potential as therapeutic agents.

In mitochondrial viability assays (MTT), MERLiNA and GENLiNA outperformed Nusinersen, demonstrating more stable mitochondrial activity, a critical indicator of improved cellular metabolism and overall cell health. This, combined with the increased intracellular stability of XNA/DNA-ASOs, highlights their superiority over RNA-ASOs like Nusinersen, which can be more prone to degradation and less effective over time.

The results indicate that XNA/DNA-ASOs, such as MERLiNA and GENLiNA, could represent a more potent, stable, and reliable therapeutic option for SMA treatment. Their chemical modifications offer enhanced efficacy in promoting SMN protein expression and maintaining cell health with fewer apoptotic effects. These findings suggest that with further research, particularly in optimizing dosage and long-term studies, XNA/DNA-ASOs may set a new standard for treating genetic diseases like SMA, offering greater long-term benefits in both survival rates and quality of life for patients.

Combining SMN protein increased by XNA/DNA-ASO treatment with myostatin inhibitor is a promising strategy for muscle regeneration and improvement of motor neuron function. In neuromuscular diseases such as Spinal Muscular Atrophy (SMA), SMN protein deficiency causes degeneration of motor neurons and consequent muscle atrophy. This leads to severe loss of motor function and significantly reduces the quality of life of patients. Increasing SMN protein through XNA/DNA-ASO ensures the survival of motor neurons, while myostatin inhibitors promote muscle development and regeneration, and the effect of the treatment process can be increased by combining these two mechanisms.

Myostatin inhibitors are known to specifically enhance the development and function of muscle cells by blocking mechanisms that inhibit muscle growth. Myostatin inhibitors, such as apitegromab, may work synergistically with the protective effect of SMN protein on motor neurons, thereby preserving motor function and muscle mass in SMA patients. In particular, increasing the level of SMN protein enables motor neurons to transmit signals to muscle cells more efficiently and helps muscle cells to develop resistance to atrophy.

Furthermore, suppression of myostatin activity in muscle tissue by combining XNA/DNA-ASO and myostatin inhibitors may improve the communication between motor neurons and muscle cells and increase their positive effects on muscle strength. The SMN protein plays a key role in maintaining motor neuron function and is essential for the healthy development of muscle tissue. Myostatin inhibition may contribute to this process, accelerating muscle regeneration and allowing patients to gain more muscle mass. This may alleviate symptoms such as muscle weakness and weakness in patients.

Future studies may investigate this synergy between SMN protein and myostatin inhibition in more depth and evaluate the results of clinical applications combining these two therapeutic approaches in the treatment of SMA. For example, suppression of myostatin activity in muscle tissue in combination with increasing SMN levels may increase muscle mass while minimising motor neuron damage. This combination could both slow the loss of motor function and preserve muscle mass in progressive muscle diseases such as SMA. Thus, the quality of life of patients can be improved and a more functional musculature can be gained.

In conclusion, SMN protein increased by XNA/DNA-ASO treatment may be more effective when combined with myostatin inhibitors due to its protective effect on

motor neurons and its contribution to muscle regeneration. This combination, which increases muscle growth and strength, may offer an effective solution against muscle weakness while maintaining the health of motor neurons in diseases such as SMA. This new treatment strategy has the potential to improve patients' quality of life by improving muscle regeneration and motor neuron function.

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