T.C.
ÜSKÜDAR UNIVERSITY
INSTITUTE OF SCIENCE

MOLECULAR BIOLOGY DEPARTMENT
MOLECULAR BIOLOGY MASTER'S PROGRAM

MASTER'S THESIS

COMPARISON OF INTRON 7 TARGETED NEXT GENERATION ANTISENSE XENO NUCLEIC ACID (XNA-ASO) APPROACHES TO INCREASE *SMN2* GENE EXPRESSION AND CELL VIABILITY IN SPINAL MUSCULAR ATROPHY (SMA) DISEASE IN SMA FIBROBLAST TYPE 2 AND *SMN1* KNOCKOUT CELLS

Özüm KILIÇ

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İSTANBUL-2024

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Özüm KILIÇ

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İSTANBUL-2024

ÖZET

SMA Fibroblast Tip 2 ve *SMN1* Knockout Hücrelerinde Spinal Musküler Atrofi (SMA) Hastalığında *SMN2* Gen Ekspresyonunu ve Hücre Canlılığını Artırmak İçin Intron 7 Hedefli Yeni Nesil Antisense Kseno Nükleik Asit (XNA-ASO) Yaklaşımlarının Karşılaştırılması

Spinal Musküler Atrofi (SMA), omurilikteki motor nöronların dejenerasyonu ile karakterize edilen ve ilerleyici kas zayıflığı ve atrofisine yol açan genetik bir hastalıktır. SMA, SMN proteinini üretmekten sorumlu olan *SMN1* (Survival Motor Neuron 1) genindeki mutasyonlar veya delesyonlar nedeniyle oluşur. Bu protein, motor nöron fonksiyonu için kritik öneme sahiptir ve eksikliği SMA semptomlarına yol açar. Hastalık öncelikle bebekleri ve küçük çocukları etkiler ancak *SMN2* geninin şiddetine ve kopya sayısına bağlı olarak daha sonraki aşamalarda da ortaya çıkabilir. *SMN2* geninin, sınırlı miktarda işlevsel SMN proteini üreten bir yedek gendir. Nusinersen (Spinraza) gibi ASO terapileri, *SMN2* geninin ekleme sürecinde ekson 7'nin dahil edilmesini artırarak işlevsel SMN proteininin üretimini güçlendirerek çalışır. SMA araştırmaları bu tedavilerle önemli ölçüde ilerlemiş olsa da, hastalığın hücrelerde modellenmesi, daha fazla anlayış ve yeni terapötik yaklaşımlar geliştirmek için de önemlidir. U87 hücreleri, nörolojik çalışmalarda yaygın olarak kullanılan bir insan glioblastoma hücre hattıdır. U87 *SMN1* nakavt hücreleri, SMA araştırmaları için modifiye edilmiş hücre hattı görevi görmüştür. Bu hücrelerdeki *SMN1* genini nakavt ederek, araştırmacılar SMA'da görülen hücresel ortamı taklit edebilirler. *SMN1* nakavt U87 hücreleri, azalmış SMN protein seviyeleri, bozulmuş RNA eklemesi ve snRNP'de (küçük nükleer ribonükleoproteinler) kusurlar gibi SMA'nın temel moleküler özelliklerini sergiler - mRNA eklemesi için temel bir işlemdir. Bu nakavt modelleri, bilim insanlarının SMA'nın moleküler patolojisini kontrollü bir laboratuvar ortamında incelemelerine olanak tanır ve SMN protein üretimini geri kazandırmayı ve hastalığın etkilerini hafifletmeyi amaçlayan Xeno Nükleik Asit (XNA) tabanlı antisens oligonükleotidler (ASO'lar) gibi terapileri test etmek için değerli araçlar olarak hizmet eder. Özetle, *SMN1* nakavt hücreleri ve ASO'lar gibi hedefli tedavileri kullanan SMA modellerinin birleşimi, SMA'nın moleküler temeline ilişkin anlayışımızı ilerletme ve tedavi sonuçlarını iyileştirme potansiyeline sahiptir.

Anahtar Kelimeler: SMA, CRISPR, ASO, XNA/DNA-ASO, KNOCKOUT

ABSTRACT

Comparison of Intron 7 Targeted Next Generation Antisense Xeno Nucleic Acid (XNA-ASO) Approaches to Increase *SMN2* Gene Expression and Cell Viability in Spinal Muscular Atrophy (SMA) Disease in SMA Fibroblast Type 2 and *SMN1* Knockout Cells

Spinal Muscular Atrophy (SMA) is a genetic disorder characterized by the degeneration of motor neurons in the spinal cord, leading to progressive muscle weakness and atrophy. SMA is caused by mutations or deletions in the *SMN1* (Survival Motor Neuron 1) gene, which is responsible for producing the SMN protein. This protein is critical for motor neuron function, and its deficiency leads to the symptoms of SMA. The disease primarily affects infants and young children but can also manifest in later stages depending on the severity and the number of copies of the *SMN2* gene, a backup gene that produces limited amounts of functional SMN protein. ASO therapies like Nusinersen (Spinraza) work by increasing the inclusion of exon 7 in the *SMN2* gene's splicing process, enhancing the production of functional SMN protein. While SMA research has significantly advanced with these treatments, modeling the disease in cells is also crucial for further understanding and developing new therapeutic approaches. U87 *SMN1* knockout cells provide a vital platform for SMA research. U87 cells are a human glioblastoma cell line widely used in neurological studies. By knocking out the *SMN1* gene in these cells, researchers can mimic the cellular environment seen in SMA. The *SMN1* knockout U87 cells exhibit key molecular characteristics of SMA, such as reduced SMN protein levels, impaired RNA splicing, and defects in snRNP (small nuclear ribonucleoproteins) assembly—an essential process for mRNA splicing. These knockout models allow scientists to study the molecular pathology of SMA in a controlled laboratory setting and serve as valuable tools for testing therapies like Xeno Nucleic Acid (XNA)-based antisense oligonucleotides (ASOs), which aim to restore SMN protein production and mitigate the effects of the disease. In summary, the combination of SMA models using *SMN1* knockout cells and targeted therapies like ASOs has the potential to advance our understanding of SMA's molecular basis and improve treatment outcomes.

Keywords: SMA, CRISPR, ASO, XNA/DNA-ASO, KNOCKOUT

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DECLARATION FORM

I hereby declare that all the information and documents used in this study have been obtained in accordance with academic rules, and that all visual, auditory, and written data and results have been presented in compliance with scientific ethical principles. I affirm that no falsification has been made in the data I used, that proper attribution has been provided to the sources in accordance with scientific standards, and that, except where cited, the thesis is original, produced by myself, and written in accordance with the Thesis Writing Guide of the Institute of Health Sciences at Üsküdar University.

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

Spinal Muscular Atrophy (SMA) is a neuromuscular disorder characterized by the loss of alpha motor neurons and muscle atrophy (Bowerman et al., 2017). The disease manifests through motor neuron degeneration along with additional defects at the neuromuscular junction. The incidence of SMA varies across Europe, America, and Asia, with differences observed among ethnic groups. Carrier frequency ranges from 1:40 to 1:60, while the incidence in live births varies between 1:6000 and 1:10000 (Bozorg Qomi et al., 2019).

SMA typically follows an autosomal recessive inheritance pattern and is also referred to as proximal SMA (Bowerman et al., 2017). Two main genes associated with the disease, *SMN1* and *SMN2*, encode the survival motor neuron (SMN) protein. Deletion of exon 7/8 in the *SMN1* gene is found in approximately 95% of patients, significantly reducing SMN protein expression. The SMN protein produced from the *SMN2* gene is approximately 90% truncated and unstable, referred to as non-functional SMNΔ7 protein.

Although the *SMN2* gene is highly similar to *SMN1*, a single nucleotide difference in exon 7 results in the production of a shorter, less stable SMN protein (Ross & Kwon, 2019b; Schorling et al., 2020). In SMA patients, the *SMN2* copy number is correlated with disease severity; a lower *SMN2* copy number leads to more severe types of SMA (Feldkötter et al., 2002).

The CRISPR-Cas9 system has opened a new chapter in genome editing technologies and has been instrumental in studying the mechanisms and regulation of various genetic diseases. In the context of SMA, genetic models have garnered significant attention, particularly in advancing the understanding of molecular pathogenesis and the development of novel therapeutic approaches. *In Vitro* models can be created by inactivating the *SMN1* gene, providing a valuable tool for studying SMA pathogenesis.

The CRISPR-Cas9 system comprises the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequences and the Cas9 endonuclease associated with the CRISPR system, which acts as a prokaryotic immune mechanism. This system can be adapted to target and cleave the *SMN1* gene for SMA modeling. The inactivation of *SMN1* provides an invaluable cellular model to investigate the molecular and biological basis of SMA. These models facilitate the analysis of biological processes such as the impact on

motor neurons and protein-protein interactions in cellular signaling pathways. Moreover, they are essential for evaluating new drugs developed to treat SMA. CRISPR-Cas9-generated *SMN1*-inactivated cell models offer an optimal platform for studying the disease etiology and screening potential therapeutic strategies.

To date, three drugs have received FDA approval for the treatment of SMA: Biogen's Nusinersen (Spinraza®), Novartis' Onasemnogene Abeparvovec-xioi (Zolgensma®), and Risdiplam (Evrysdi™). Despite the significant therapeutic outcomes shown by these drugs, their high cost remains a significant barrier. While various therapeutic approaches are being developed, no significant improvements have been made in terms of cost reduction. SMA is classified into five subtypes based on phenotypic severity and *SMN2* copy number: SMA Type 0, SMA Type 1, SMA Type 2, SMA Type 3, and SMA Type 4 (Oskoui & Kaufmann, 2008; Verhaart et al., 2017). The primary goal of current treatment strategies is to increase SMN protein expression, utilizing small molecules, antisense oligonucleotide (ASO) technology, and gene therapy. The side effects associated with FDA-approved SMA drugs highlight the need for alternative therapies. Current treatments are linked to common side effects such as infections and allergic reactions, as well as severe outcomes, including cardiac arrhythmia, pulmonary hypertension, neurological complications, and even death (ZOLGENSMA® [onasemnogene abeparvovec-xioi], n.d.; Alhamadani et al., 2022; Masson et al., 2022).

The use of antisense oligonucleotides (ASOs) has emerged as a unique therapeutic approach for SMA. ASOs are short synthetic DNA or RNA sequences designed to bind specific gene regions and modify gene expression. For SMA treatment, ASOs aim to enhance the production of partially functional SMN protein from *SMN2*-derived transcripts. *SMN1* produces a truncated, unstable protein, whereas ASOs manipulate the alternative splicing of *SMN2* pre-mRNA to increase the production of full-length, functional SMN protein (Hua et al., 2010).

Nusinersen (Spinraza®) became the first ASO shown to be effective in treating SMA. Approved by the FDA in 2016 as the first therapy for SMA, Nusinersen increases SMN levels by manipulating the alternative splicing of *SMN2* to produce full-length SMN protein. However, challenges associated with side effects, the requirement for multiple doses, and high costs limit its practicality. Thus, research continues into developing ASOs with better safety profiles and longer-lasting effects.

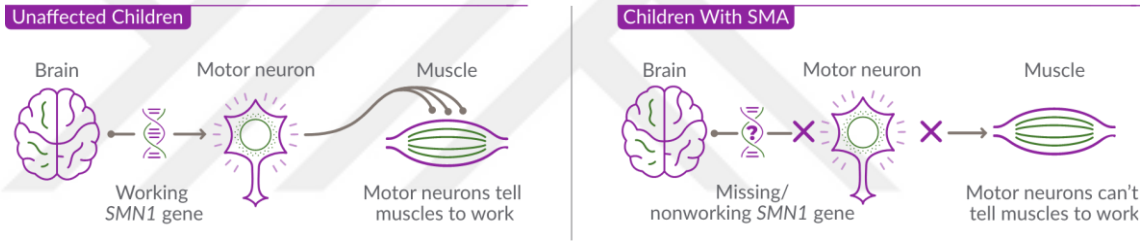
In this context, targeting the ISS-N1 region within intron 7 of *SMN2* with a novel class of Xeno Nucleic Acid (XNA)-based ASOs (XNA-DNA-ASOs) has shown promise in terms of higher stability and lower toxicity. By achieving sufficient production of SMN protein, this approach offers new hope for SMA treatment.

This thesis aims to investigate the efficacy of XNA-based ASO therapy, a next-generation genetic approach for treating SMA. The study focuses on the impact of XNA-DNA-ASOs on SMN protein production in both SMA Fibroblast Type 2 and *SMN1* Knockout cell models. The primary objective is to explore the potential of XNA-DNA-ASO therapy in enhancing SMN protein levels by regulating *SMN2* splicing. The study will also examine the effects of XNA-DNA-ASOs on cell viability, functionality, and protein expression in greater detail. These experiments will be conducted on cellular models to assess the clinical practicality of XNA-DNA-ASOs and their potential benefits in the treatment of SMA. Another goal of this thesis is to compare the advantages of XNA-DNA-ASOs with traditional ASO therapies and to highlight how this novel approach could revolutionize SMA treatment. This work responds to the growing demand for unconventional solutions to rare genetic diseases and aims to demonstrate the benefits and practical applications of this innovative therapy.

2. GENERAL INFORMATION

2.1. Definition of Disease

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease. The incidence of SMA is 1 in 10,000 live births, with a carrier frequency of approximately 1 in 50 adults across all racial backgrounds (Pern 1980). SMA, one of the most common genetic causes of infant mortality, can be fatal in the absence of disease-modifying treatments. SMA symptoms result from degeneration of motor neurons in the spinal cord and cranial motor nuclei, leading to muscle weakness and atrophy **(Figure 1)**. The most common form of SMA results from homozygous mutations in the *SMN1* gene located on chromosome 5q13, resulting in deficient levels of SMN (Survival Motor Neuron) protein production in motor neurons (Farrar et al., 2009).



HYPERLINK

Figure 1. SMN1 protein in healthy and diseased individuals

2.2. History of SMA

Spinal muscular atrophy was described for the first time in 2 infant siblings by Guido Werdnig in 1891 and in 7 additional cases by Johan Hoffmann between 1893 and 1900 (Kolb et al., 2011). Although the name Werdnig-Hoffmann disease was eventually added to the severe infantile SMA form, the cases were actually of moderate severity; the first descriptions of severe infantile SMA were made by Sylvestre in 1899 and Beevor in 1903 **(Figure 2)**. Whereas a milder form of SMA, in which patients retained the ability to stand and walk and achieved long-term survival was not described in more detail until the 1950s by Wohlfart, Fez, and Eliasson and later by Kugelberg and Welander (Dubowitz et al., 2009). After these clinical findings, the gene responsible for SMA was found in the Meki laboratory in the year 1995. It has been identified that 95% of the cases

of SMA, irrespective of their type, are because of a homozygous deletion in the *SMN1* gene at chromosome 5q13.3. In humans, each allele has two forms of the SMN gene: the telomeric form is known as *SMN1* and the centromeric form as *SMN2*.

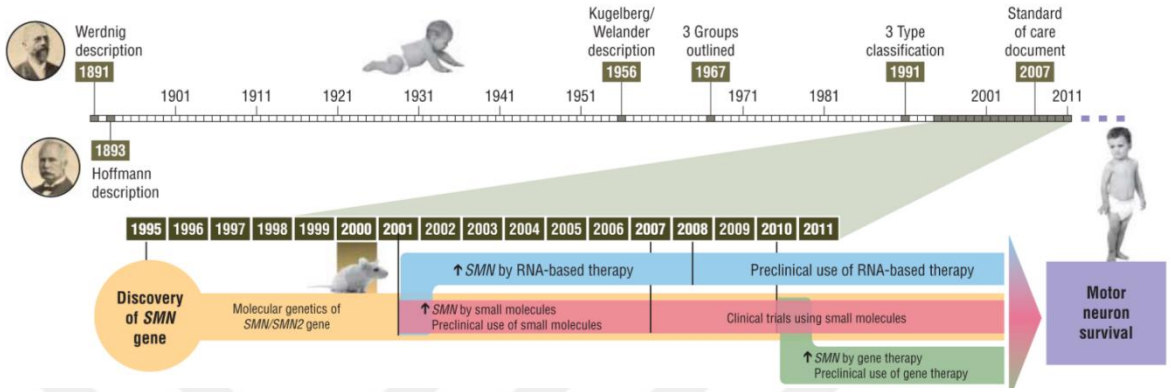


Figure 2. Spinal muscular atrophy (SMA) timeline (Kolb et al., 2011).

In the twenty-first century, advances in gene therapy and gene editing technology have further expanded treatment options for SMA. In 2016, gene therapy Onasemnogene abeparvovec (Zolgensma) offered an important therapeutic alternative, addressing the genetic basis of SMA (Simmons et al., 2019). These historical advances complete the future research and treatment approaches as a continuous process of development in the management and treatment of SMA **(Table 1).**

Table 1. History of the disease from past to present

Year	Milestone
1891	Initial description by Werdnig
1893	Hoffmann description
1956	Kugelberg/Welander description
1967	3 Groups outlined
1991	Consensus classification of 3 types of SMA
1995	Discovery of SMN gene
2001	First demonstration of increased SMN expression resulting from a potential small molecule therapy in cell culture
2001	First demonstration of increased SMN expression resulting from a potential antisense oligonucleotide therapy in cell culture
2001	First preclinical study using small molecule therapy
2007	First clinical trial using small molecule therapy
2007	Consensus statement for Standard of Care established
2008	First preclinical studies using RNA-based therapy
2010	First preclinical studies using SMN1 gene therapy

2.3 Molecular Genetics of SMA

Spinal Muscular Atrophy is a neurodegenerative disease characterized by progressive loss of motor neurons that consequently affects muscle functions. SMA is genetically caused by mutations or deletions in the *SMN1* gene on chromosome 5q13.2. It encodes for the SMN protein, important in the survival and functioning of motor neurons. Most (~95%) SMA cases have been attributed to homozygous deletions or mutations within the *SMN1* gene, leaving 4-5% of the patients to present compound heterozygous mutations-a situation where one allele has a deletion and another has a point mutation. Of the features of SMA, by far the most interesting involves a gene termed *SMN2*, which is remarkably similar to *SMN1*-but for one critical exception: it contains a C-to-T transition in exon 7 of its pre-mRNA.

This single change leads to splicing of exon 7 from the majority of its transcripts to produce a truncated, unstable isoform of the SMN protein called SMNΔ7. This results in the rapid degradation of the isoform and only a small percentage of full-length

functional SMN protein. Though the *SMN2* gene may also produce functional protein, this is often too low in percentage to compensate for the complete loss of the function of *SMN1* in more severe forms of SMA disease. One of the most important modulating factors in the degree of severity in the disease manifestations is the copy number of *SMN2*. Thus, the patients with a higher number of this gene copies usually have milder forms of SMA.

It was due to an increase in the production of full-length functional SMN protein. Thus, this straightforward relationship between the copy number variations in the *SMN2* gene and the disease phenotypic variation points to the very important role this gene plays in modifying the clinical course of the disease. On the molecular level, the pathogenesis of SMA is directly related to the processes of alternative splicing, which regulate the inclusion or exclusion of specific exons during pre-mRNA maturation. This process requires the spliceosome-a complex assembly whose key components are small nuclear ribonucleoprotein complexes, or snRNPs. The SMN protein is an essential determinant in snRNP biogenesis **(Figure 3).**

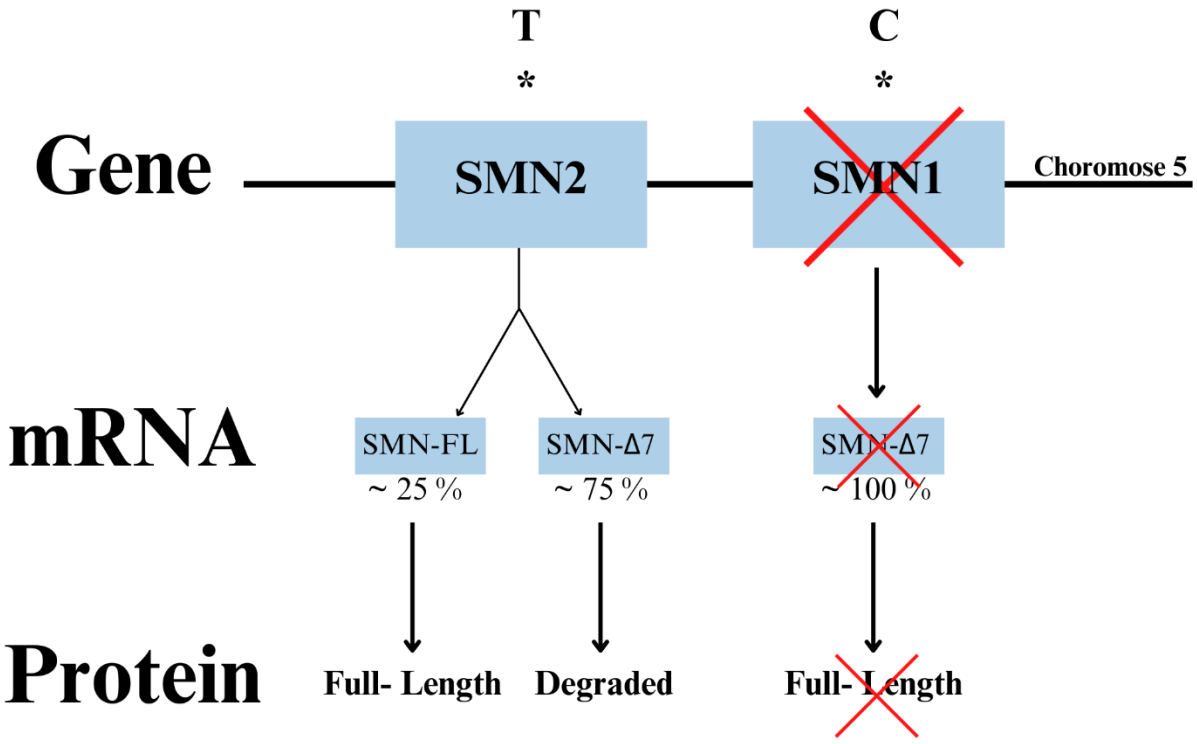


Figure 3. SMN1 and SMN2 proteins level

Its deficiency hinders normal spliceosomal function. This defect in RNA processing leads to incorrect expression of proteins, contributing finally to motor neuron dysfunction and degeneration in the case of *SMN1* Singh & Howell, 2017. This transition within the *SMN2* gene results in the disruption of an ESE within exon 7. This transition created an ESS promoting the binding of splicing suppressor proteins to aggravate the skipping of exon 7. Another mutation involves the substitution of A to G in intron 7 and activates an intronic splicing silencer called ISS-N1, which binds to hnRNPA1, further inhibiting the inclusion of exon 7. These two alternative splicing events mentioned above restrict the levels at which functional SMN protein is produced by *SMN2* with critical consequence. Current approaches to the treatment of SMA aim at targeting these splicing mechanisms.

Such antisense oligonucleotides, for example, are designed in order to target the ISS-N1 region with the specific aim of promoting the inclusion of exon 7 in the *SMN2* transcripts so that maximum full-length SMN protein is produced. This method has limited scope for mitigating symptoms in SMA by compensation for the loss of function of *SMN1*. Rao et al., 2018; Shorrock et al., 2018. In summary, the molecular genetics of SMA have been a rather complex interaction between mutations of the *SMN1* gene, copy number variation of *SMN2*, and regulation of splicing mechanisms. Such an understanding of interactions has not only been great in identifying the disease but also in developing therapies toward modulation of levels of SMN protein with an aim of reducing clinical manifestation of SMA.

2.4 Molecular Cell Mechanism of SMA

SMA is a severe neurodegenerative disease characterized by progressive loss of motor neurons, leading to muscle weakness and atrophy. The molecular basis of this disease includes mutations or deletions in the gene *SMN1* (Survival Motor Neuron 1) located on chromosome 5q13.2, encoding the SMN protein important for proper functioning and survival of motor neurons. Important cellular processes that SMN participate in include snRNP assembly and maturation. Such complexes are responsible for the splicing of pre-mRNA-transcript RNA editing, whereby introns are removed and exons joined to yield mature mRNA, a prerequisite for protein synthesis (Shorrock et al., 2018; Schorling et al., 2019).

2.4.1 SMN Protein and Its Role in Ribonucleoprotein Complexes

The Sudanese Crocodile team may modestly propose, argue, or postulate on this matter. The SMN protein is ubiquitously expressed throughout the body, both chronically and acutely, with a well-established molecular weight of 38kDa; it is also highly conserved across species. This suggests that the SMN protein has a very fundamental role in basic cellular functions, especially regulating ribonucleoprotein (RNP) complexes. RNP complexes comprised of RNA molecules and their bound proteins vary in function, and in classic ways support different aspects of RNA metabolism including transport, stability, and splicing. Of the various RNP complexes, the snRNPs are core components of the splicing machinery, wherein they contribute to a large matter and RNA-protein complex called a spliceosome which accurately removes introns from the pre-mRNA. See: younger et al., 2023; Singh and Howell, 2017. Furthermore, these SMN complexes also work in an assembly for the small nuclear RNAs with Sm proteins by interacting with other proteins Gemins 2-8, and unrip. This is a critical step in successful assembly of snRNP in the cytoplasm. Newly developed snRNP complexes move to the nucleus, mature Cajal bodies, cellular compartments which are enriched in various transcriptional and RNA processing factors . Once matured two of the snRNP complexes integrate into active spliceosome's in the nuclear speckles to regulate splicing events that ultimately creates functional mRNA. Beyond snRNP assembly, the SMN participates in the assembly of other important RNP complexes like U1snoRNPs responsible for modifying non-coding RNAs, and mRNA-proteins in support of the mRNA transport. When levels of SMN is depleted, as is the case for SMA, it breaks or dysregulates these important functions in our cells and literally takes a toll across multiple systems and total cellular function, and more severely at long-axoned motor neurons that are reliant upon efficient RNA processing and RNA transport.

2.5. Cellular Pathways Disrupted by SMN Deficiency

In Spinal Muscular Atrophy (SMA), we know SMN-deficiency effects at a cellular level go beyond the effects of RNA processing. One of the cellular pathways that is impacted is the dynamics of actin, the cytoskeletal structure which support cell shape, cell movement and more. In motor neurons, SMN actually interacts with a protein called profilin that is important for actin polymerization. When SMN is depleted, profilin is

impaired in its function/control of actin. This would ultimately lead to abnormal signaling through the RhoA pathway that impairs proper cytoskeletal reorganization to inhibit axon growth. The axonal growth of motor neurons is essential for forming connections with muscles well as synaptic function and this is potentially one of the most important ways this contributes towards the motor symptoms associated with SMA (Schorling et al., 2019).

Mitochondrial dysfunction is another important component of the pathogenesis of SMA. The motor neurons require substantial amounts of energy for functions such as axonal transport. Mitochondria are the powerhouses of energy in the cell and reduced expression of mitochondrial proteins in SMA, i.e. phosphoglycerate kinase 1 (PGK1) will affect mitochondrial function and energy demands of the cell. Consequently, dysfunctional mitochondria contribute to reduced motor neuron survival and function (Rao et al., 2018).

Lastly, we know that there is disruption towards cellular ubiquitin homeostasis, which relates to the impaired assignment SMN has with E1 ubiquitin-activating enzyme, UBA1. Ubiquination tags proteins for degradation, therefore proper functionality of this workflow is necessary for the capacity to maintain cellular protein balance. This breakdown in `degradation' will build up misfolded or damaged proteins which are potentially just as toxic toward motor neurons (Shorrock et al., 2018).

2.5.1 Protein Homeostasis and Local Translation

Furthermore, SMN has a role in the local translation of proteins within motor neurons, namely axonal growth cones.Growth cones are specialized structures at the termini of axons. They are critical in controlling axonal directionality throughout growth and synaptogenesis. In order for some mRNAs to be transported into growth cones, such as ß-actin mRNA, local assembly of the actin cytoskeleton, which is facilitated by SMN, drives axonal outgrowth. Motor neurons from SMA have decreased levels of SMN protein and therefore have shorter axons with smaller growth cones, which have significantly less ß-actin mRNA and protein. Disruption of mRNA transport and local translation impacted axonal growth, and contributed to motor neuron degeneration in SMA (Singh & Howell, 2017).

2.5.2 Splicing Mechanisms and SMA Pathogenesis

The pathogenesis of SMA has been prominently linked to malfunction of the *SMN2* paralog of *SMN1*. *SMN2* is practically identical with *SMN1*; however, a C-to-T transition in exon 7 introduces other factors that blocks the inclusion of this exon during splicing. This ultimately leads to the recognition of a ambiguous, highly unstable truncated protein the SMNΔ7 form. *SMN2* gives rise only to very small quantities of the functional SMN protein and cannot compensate for the loss of function imparted by *SMN1*. Antisense oligonucleotide therapies correct the splicing defect directly by targeting the SCN2 pre-mRNA, including the ISS-N1 region, enhancing the ability to splice in exon 7 and allowing increased synthesis of functional SMN protein.

The pathogenesis of spinal muscular atrophy (SMA) reflects the complexity of all of the disrupted cellular processes resulting from low levels of functional SMN protein, including snRNP assembly, RNA processing, axon transport, mitochondrial function, and local protein synthesis, which ultimately results in the progressive degeneration of motor neurons, which are particularly vulnerable based on the length of their axons and their high metabolic demand. The future of targeted therapies, including ASO treatment, appears promising not only by restoring levels of SMN protein, but also in the slowing of disease progression in SMA.

2.6. Epidemiology

The birth prevalence rate of Spinal Muscular Atrophy (SMA), a genetic disorder, is more precisely the number of newborns with SMA. Different studies have focused on calculating the infant birth prevalence rate of SMA. Almost consequently, a study published in 2017 details the average number of SMA cases per 100,000 live births worldwide is 5 to 24 (Verhaart et al 2017). These discrepancies are for several reasons. Studies have been carried out in different countries and the prevalence of SMA varies between regions. For example, if a country experiences higher rates of consanguinity among its parents then it may influence the prevalence. Then there is the fact that many studies have been done within small areas of specific countries, which means the population samples are smaller.

The rarity of SMA, combined with the small numbers of people affected by it, can make prevalence estimates vary wildly. This means it will be harder to pick out a definitive figure for how many people have SMA. Differences in the time periods of these studies, the diagnostic technologies available and clinical classifications used for diagnosis all have direct bearing on the level of medical help which can be given to people with SMA as well as on its incidence. Also, the state of health care systems in different countries can all affect how well we can grasp what types of cases are diagnosed as Sma Wesley And thus the estimates themselves will be distorted accordingly (Verhaart et al. 2017). An analysis of death certificates and hospital admissions in England 2008-2016 found that on average 6.2 children were born with the disease per 100,000 live births (See 'Spinal Muscular Atrophy Type 1: NCARDRS data briefing.gov.uk', 2021). Moreover, a study focusing on data from European countries between 2011 and 2015 found a median incidence of 11.9 cases per 100,000 live births (Verhaart et al. 2017).

This research derived its estimates in part from the number of genetically confirmed SMA cases in laboratories from Europe, where the incidence rate ranged between 6.3 to 26.7 per 100,000 live births depending on the country (Verhaart et al. 2017). In America as well, studies have been conducted regarding patient population studies, including studies regarding the SMA incidence in particular. In a study conducted in 2012 whose focus was on northern California and some parts of the United States, it was noted that one of the incidence of SMA could reach one in 11,000 live births (approximately 9.1 per 100,000). Newborn screening data from New York State collected over the 12 months starting in October 2018 indicate that of 225,093 newborns screened for SMA, 8 were diagnosed with the condition, with an estimated prevalence of 3.6 per 100,000 (Kay et al., 2020; 2022). These statistics came out to be much lower than previously estimated **(Figure 4)**.

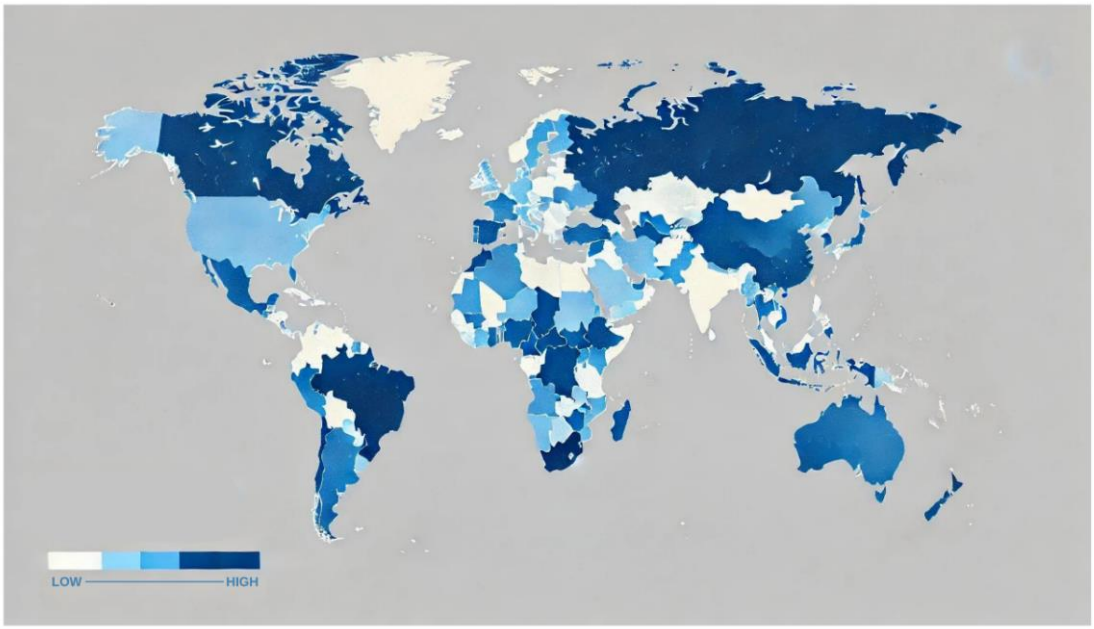


Figure 4. SMA low to high display on World Map

The authors suggested that these differences were due to the fact that CIM studies were undertaken in small population-based studies in Europe, cervical cytology was performed for diagnosis without considering genetics, the evolution of classification systems and diagnostic criteria for SMA and its types, and possible sampling errors (Kay et al., 2020; 2022). Citing sufficiency of literature, as of January 2024, all of the three countries in North America have adopted newborn screening for SMA, including Hawaii. Since more states in the U.S. are starting to make use of universal newborn screening, a better usage of the aforementioned estimation methods should be seen with less over-exaggeration of prevalence when the focus is on specific regions. Other countries also recently offered some more initial findings which headed to the newborn screening and might be important for understanding the control over the spread of SMA. For instance, Taiwan reported 7 cases of SMA where in the study 120267 newborns were screened (5.8 for 100,000), in Australia 9 cases out of 103903 (8.7 for 100,000), whereas in Germany 22 cases were present among 165525 born infants (~13.3 for 100,000) (Vill et al., 2019).

2.7. Clinical Classification and Types of SMA

Spinal Muscular Atrophy (SMA) is a neuromuscular disorder. It starts with symmetrical muscle weakness and atrophy but changes as the roots of its pathology are prenatal (Figs 9-11) and this condition progresses to proximal compression by axial, intercostal, and bulbar muscles In 1991, development was described in three forms according to standard of SMA motor performance and onsets. These descriptions were later changed by subdividing type 3 according to lifespan, adding adult-onset type 4, and prenatal onset type 0. In spite of this, the scheme just goes on growing even in the genetic era when it comes to furnishing clinical and prognostic information **(Table 2)** (Kolb and Kissel, 2015).

Table 2. Types of SMA

Type	Age at Onset	Development	Lifespan	SMN2 Copy Number
0	Prenatal	Respiratory support	< 1 month	1
1	0-6 months	Never sit	< 2 years	2
2	< 18 months	Never stand	> 2 years	3-4
3	> 18 months	Stand unassisted	Adult	3-4
4	> 21 years	Stand unassisted	Adult	4-8

Type 0 SMA is especially seen in births defined by a history of decreased fetal movements. It is also a severe phenotype that starts before birth. This condition is typically accompanied by severe muscle weakness and marked hypotonia at birth. In the examinations performed on infants with type 0 SMA, there are many findings such as areflexia, facial diplegia, atrial septal defects and additional contractures. Arthrogryposis multiple congenita may also occur as part of this phenotype. For infants suffering this type of life-threatening difficulty in the early part of life, respiratory support can start right from birth. In terms of quality of life, availability and comfort; for most babies who get SMA type 0 at birth, it will last only 6 months or less (Kolb and Kissel, 2015; Farrar et al., 2009).

SMA Type 1, also known as Werdnig-Hoffmann disease, is a serious neuromuscular disease that occurs in the first six months of life. Their hypotonicity, weak head control and decreased tendon reflexes are often noticeable by parents when these infants have a routine check-up. They will note that most of the symptoms listed here are similar to those of a number diseases caused by protein misfolding (protein conformational change), but that none them include only respiratory failure and therefore death. Some infants appear perfectly normal at first but quickly become weak. For them the episodes in which J-alternances occur all the time seem particularly long: in a typical night's sleep, 20–30 minutes only will pass without such episodes. They usually manage to achieve a 'frog-legged' posture as they cannot hold their own heads up. Tongue fasciculations, bad sucking reflexes and a collapse in the normal protective mechanisms of the airways are other typical features. As the muscles concerned with swallowing and of the tongue weaken, feeding gets harder aspiration results. However, the child's thinking abilities are often intact and they seem bright and lively. (Kolb and Kissel, 2015; Farrar et al., 2009).

SMA Type 2 is a moderately severe neuromuscular disease that usually manifest before 18 months of age. Infants with type II may be able to sit up unsupported, but cannot stand or walk independently. The condition is defined by progressive weakening from proximal muscles in the legs, sometimes accompanied by hypotonia, areflexia, and tremors on the hands. With a weakening of the muscles, orthopedic problems frequently occur. This may include an evolving scoliosis, limb contractures, and problems in the jaw joint (posterior morbidity). Scoliosis and intercostal muscles are key weaknesses that can bring their own problems, for example limiting the body's lung function. The children are not cognitively impaired and most of them live into adult life, although they will experience considerable morbidity during that period with diminished functionality(Kolb and Kissel, 2015; Farrar et al., 2009)

It was widely accepted to regard SMA Type 3 (Kugelberg-Welander disease) as beginning symptoms "after 18 months." These patients can walk on their own, but their motor functions may fluctuate over time. Some individuals may require a wheelchair during childhood whereas others are still able to walk in adulthood. In this disorder, progressive proximal weakness is most pronounced in the legs. Scoliosis and respiratory muscle weakness are uncommon for this type of disease. Furthermore, in a few instances one can observe pseudohypertrophy of the calf muscles and weakly elevated creatine

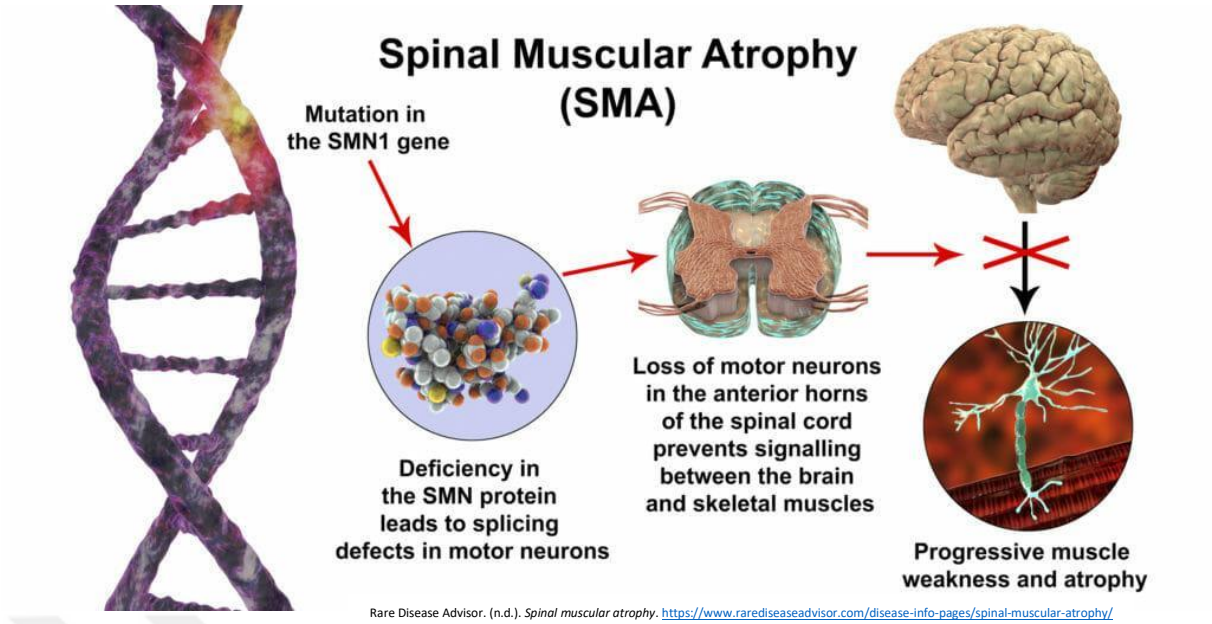
kinase levels. People with SMA Type 3 tend to have the same lifespan as the general population, and there is no effect of this on their mental abilities (Kolb and Kissel, 2015; Farrar et al., 2009).

SMA Type 4 represents the mildest form of the disease less hen 5 percent all this SMA cases. Most commonly seen in adults, it generally occurs in adulthood at age 30 or later age-groups. Pneumonia is more resistant to these individuals than in the case of 2, in fact all seconda SMA Type 4 is genetically heterogeneous; not all cases are due to a deletion of the *SMN1* gene autosomal dominant inheritance may also occur. This phenotype has a full life expectancy, and patients characteristically live well up into old age.

2.8. Pathophysiology

SMA is an autosomal recessive neuromuscular genetic disorder characterized by motor neuron degeneration and muscle weakness. Generally, most of these cases have been due to either mutations or deletions occurring in the *SMN1* gene, since the gene codes for a protein known as Survival Motor Neuron 1, important for the survival of motor neurons.

This thus leads to the disruption of two central cellular processes involving splicing of mRNA and assembly of small nuclear ribonucleoprotein complexes in the motor neurons, which thereby causes degeneration of the motor neurons. While the *SMN2* gene also produces some functional SMN protein, the amount is inadequate to meet the needs of the motor neurons (Figure 2).



Rare Disease Advisor. (n.d.). *Spinal muscular atrophy*. https://www.rarediseaseadvisor.com/disease-info-pages/spinal-muscular-atrophy/

Figure 5. SMA Disease Pathophysiology

This, in fact, constitutes the basic cause of progressive muscle weakness and atrophy. The clinical severity in SMA is modified by the copy number of the modifying gene *SMN2* and, consequently, by the levels of functional SMN protein (Rossoll et al., 2003).

In the past few years, treatments have developed to increase the level of SMN protein. For example, Nusinersen modulates the splicing mechanism of the *SMN2* gene, therefore allowing for more functional protein to be produced. Gene therapy approaches are targeted at the rescue function of the *SMN1* gene itself.

2.9. Signs and Symptoms

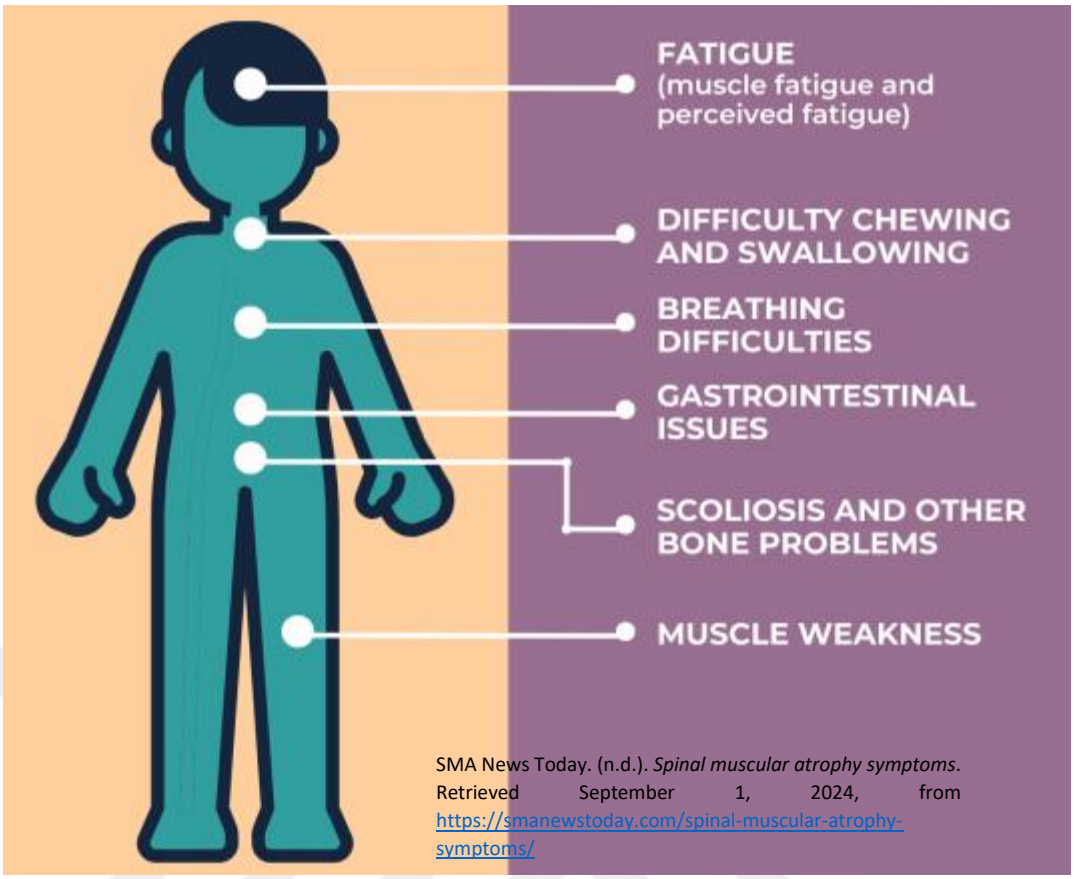
Spinal Muscular Atrophy is a neuromuscular genetic disorder that is characterized by muscle weakness and degeneration of the motor neuron. Symptoms of the disease highly differ according to its SMA type and severity. Symptoms most generally associated with SMA include muscle weakness, hypotonia, or low muscle tone, and delays in motor development. Especially in the early-onset types, as in SMA Type 1, previously termed Werdnig-Hoffmann Disease, symptoms typically manifest within the first six months of life, and patients do not attain motor developmental milestones such

as holding their heads, sitting, or walking. According to Mercuri et al. (2012), patients with SMA Type 1 develop serious respiratory complications due to the weakening of the respiratory muscles. Such patients generally show paradoxical respiratory movements due to intercostal muscle weakness and diaphragmatic breathing. Respiratory muscle weakness may lead to recurring respiratory infections and aspiration risk. These patients usually die within the first two years due to respiratory failure.

In SMA Type 2 and Type 3, the symptoms are later in appearance and milder. These patients are able to acquire the motor skills of sitting and walking. However, these motor skills have been noted to progressively decline with an increase in muscle weakness. While patients with Type 2 usually never gain walking skills, in the case of Type 3, patients may walk in early childhood years only to lose this capacity in later years of their life.

Another characteristic symptom of SMA is muscle atrophy. Generally, muscle atrophy in muscles close to the body, i.e. proximal muscle groups, occurs. This leads to difficulty moving arms and legs and further causes skeletal deformities such as sclerotic bone structure, scoliosis and joint contractures in patients (Mercuri et al., 2012). The loss of motor neurons further leads to a decrease or loss of reflexes in patients (Kolb et al., 2011).

Lastly, difficulties in feeding and retardation in growth are also common symptoms in SMA patients. Those who experience choking while eating or drinking and difficulty swallowing food due to the weakening of the muscles are prone to malnutrition; this may further lead to growth and developmental delays in most patients, especially those with SMA Type 1 and Type 2 (Finkel et al., 2015) (Figure 6).



SMA News Today. (n.d.). *Spinal muscular atrophy symptoms*. Retrieved September 1, 2024, from https://smanewstoday.com/spinal-muscular-atrophy-symptoms/

Figure 6. Most Common SMA Symptoms

These symptoms differ in their severity and rate of onset in view of the genetic background of the disease-particularly, the number of copies of *SMN2*. Patients with more copies of *SMN2* have milder symptoms and slower disease progression.

2.10. Diagnostic Methods

SMA is a neuromuscular genetic disease characterized by the degeneration of motor neurons, and early diagnosis is important. In general, diagnosis can be made by clinical symptomatology in combination with genetic tests. Diagnosis of SMA thus played a critical role in estimating the severity and course of the disease, and early diagnosis had provided an edge in treatment planning. Accurate diagnosis of SMA was made using various procedures which included clinical evaluation, genetic tests, electrophysiological tests, and prenatal screening. These methods help to define the

presence of SMA and the type of it. Such methods have been in use since 2015 by Finkel et al. and Mercuri et al. in 2012.

The basic diagnosis mode in the diagnosis of SMA includes:

- Clinical Assessment: Symptoms specific to SMA, such as developmental delay of motor development, weakness, and hypotonia, are diagnosed clinically (Finkel et al., 2015).
- Genetic Tests: Blood testing for the detection of mutation or deletion of the *SMN1* gene. This is the most frequently applied method of confirmation of diagnosis for SMA (Lefebvre et al., 1995).
- Electromyography (EMG): A test used in measuring the electrical activity of muscles is done in order to test the function of motor neurons. Mercuri et al., 2012.
- Muscle Biopsy: It is performed rarely by taking a sample of muscle tissue to see into the condition of the muscles. It is employed when genetic testing is poor. Dubowitz, 1995.
- Carrier Screening: Genetic testing done in those with a family history of SMA to see if they are carriers of the disease (Prior, 2008).
- Prenatal Testing: Amniocentesis or chorionic villus sampling (CVS) is conducted to diagnose SMA in the fetus during pregnancy (Rodeck & Whittle, 2009).

2.11. Treatment Methods

For long decades, Spinal Muscular Atrophy has been considered to be a disease managed only with supportive treatments. During this time, symptomatic alleviation was done using methods such as physiotherapy, respiratory support, and nutritional support. However, recent advances in genetics have formed a way for revolutionary developments in the treatment of SMA. Nusinersen, approved in 2016, was the first treatment for the genetic basis of SMA. Spinraza (under the trade name) entered into clinical use as an antisense oligonucleotide, modulating the splicing mechanism of the *SMN2* gene to permit more functional SMN protein production. In 2019, this was followed by the approval of Onasemnogene abeparvovec and was named Zolgensma. Zolgensma (under the trade name) acts against SMA at the genetic level by providing the missing gene to

the patient with the aid of a viral vector that contains the *SMN1* gene. Two years later, in 2020, oral Risdiplam was approved in this respect as a different treatment modulating the splicing mechanism of the *SMN2* gene and, thus, further expanding the treatment options. Additionally, stem cell therapies and other gene therapy modalities also continue to be investigated and are promising for future innovations in the treatment of SMA. In addition, other gene therapy modalities and stem cell therapies are under active investigation and hold promise for future innovations in the treatment of SMA (Lunn & Wang, 2008; Darras & Monani, 2019). Due to such treatment modalities, SMA has been recognized as a manageable and possibly curable disease **(Table 3)**.

Table 3. Development of SMA treatment over the years.

Treatment Method	Start Year	Description	Reference
Supportive Therapies	1950s	Symptom management through physical therapy, respiratory support, nutritional support, etc.	Kolb et al., 2015
Antisense Oligonucleotide Therapy (Nusinersen - Spinraza)	2016	Modulation of SMN2 gene splicing to increase functional SMN protein production.	Finkel et al., 2017
Gene Therapy (Onasemnogene abeparvovec - Zolgensma)	2019	Delivery of a functional SMN1 gene using a viral vector.	Mendell et al., 2017
Oral SMN2 Splicing Modifier (Risdiplam - Evrysdi)	2020	An oral drug that modulates SMN2 gene splicing.	Baranello et al., 2021
Stem Cell Therapies	Under Research	Aiming to convert stem cells into motor neurons, not yet in clinical use.	Lunn & Wang, 2008
Preclinical Research (Gene Therapy, Antisense Oligonucleotide Modifications)	Under Research	Ongoing preclinical studies on gene therapy and antisense oligonucleotide modifications.	Darras & Monani, 2019

2.12 CRISPR Gen Editing System

Clustered Regularly Interspaced Short Palindromic Repeats is a gene editing technique that, within the last few years, has emerged as a revolutionary innovation in genetic engineering and biotechnology **(Figure 7)**. Applications have run the gamut from the treatment of genetic diseases to agricultural genetic modifications that have made it possible to make targeted changes within certain regions of the genome. CRISPR-Cas9 is taken from a natural defense mechanism that bacteria and archaea have developed

against viral infection, which allows these hosts to recognize and destroy the DNA of the viruses that infect them.

CRISPR technology is a revolutionary technique for editing genetic material. Compared to other methods of gene editing, the system of CRISPR-Cas9 stands as by far much faster, cheaper, more sensitive, and versatile. While introducing double-strand breaks in the region of DNA of interest, this system introduces cellular mechanisms of repair in such a fashion that desired genetic modifications are actually realized along the process. This makes CRISPR-Cas9 powerful and of widespread use because it is able to recognize target DNA sequences in a highly specific way through the use of short RNA molecules, known as guide RNA or gRNA.

This technology expands the bounds of genetic engineering in treating a lot of genetic diseases, enhancing plant and animal genetics, and even furthering the understanding of complex diseases like cancer. The arrival of CRISPR-Cas9 is considered a paradigm shift in the scientific world, with this technology bound to revolutionize everything from personalized medicine to agriculture in the years to come.

The CRISPR gene-editing technique has brought revolutionary technology in the field of genetic research and biotechnology. It cuts or edits any desired specific target region in the genome of cells. CRISPR has been inspired by the natural defense mechanism developed in bacteria upon viral infections and may find wide application not only for genetic engineering but also for medical treatment.

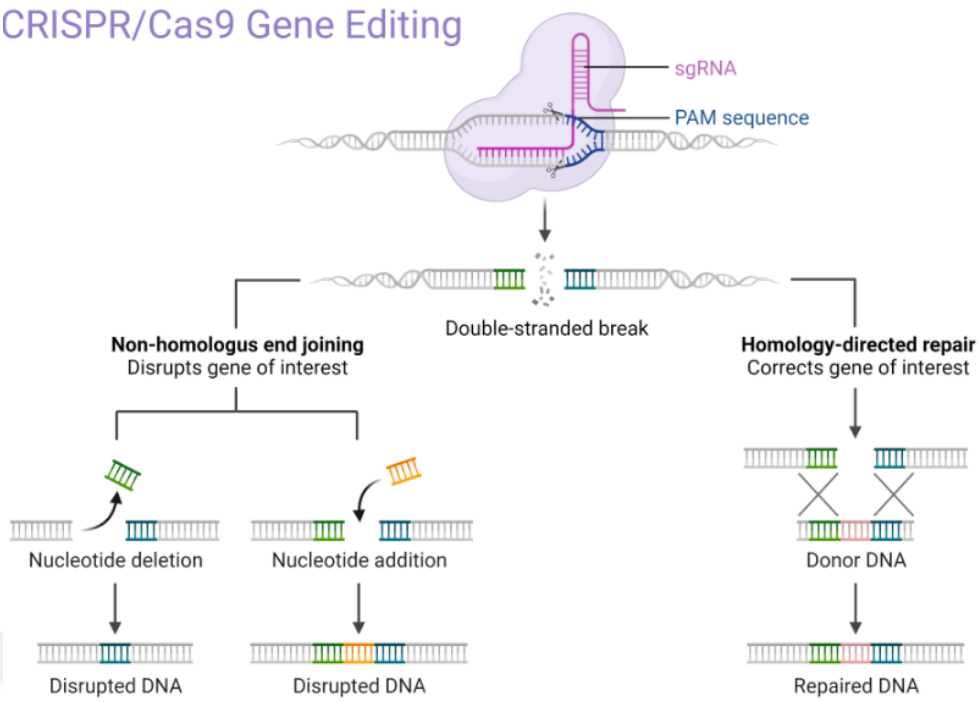


Figure 7. Mechanism of CRISPR/Cas9

The CRISPR System: Basic Components and Principles of Action

1. Guide RNA (gRNA): gRNA stands for guide RNA, a small piece of RNA designed to recognize the DNA target sequence with which it will interact in the process of gene editing. Thus, the RNA sequence specifically binds with the targeted genetic region and guides the cutting enzyme to this region. Sensitivity of the CRISPR system depends on the specificity of the guide RNA. The guide RNA is taken from the CRISPR sequences of bacteria; this allows the recognition of viral DNA fragments that the bacteria have 'seen' before.

2. Cas EnzymeCas9: Cas9 is the most commonly used endonuclease in the CRISPR system. Cas9 performs a double-strand cut in the DNA sequence guided by the guide RNA. This would, therefore, trigger the DNA repair machinery of the cell and allow for the desired genetic alterations to be made.

3. Target DNA cutting and repair mechanisms:

❖ Non-homologous End Joining (NHEJ): This technique rapidly seals double-stranded breaks in DNA by the cell. The NHEJ is mainly error-prone and minor insertions or deletions of DNA are possible for this technique. It can make the gene non-functional or modify its functionality completely.

❖ Homology-Directed Repair (HDR): When the cell is provided with a template DNA for the repair mechanism, the HDR mechanism is activated. In this process, it ensures that the DNA cut region is correctly repaired using template DNA. Target changes in the genetic engineering of organisms are possible by HDR. An example could be a compensatory action involving a mutation that causes a certain disease or addition of new genetic information using HDR.

Genetic Editing of CRISPR Technology

1. Target DNA Region Identification: Essentially, the first step is to identify the genetic region that needs to be modified. This targeting of the DNA region is mediated by guide RNA; therefore, genetic engineers program the CRISPR system to recognize a particular DNA sequence they want to target.
2. Design of Guide RNA: The genetic engineers have to design the guide RNA in such a way that the guide RNA targets a particular DNA sequence. The specificity of the guide RNA is highly essential to be Hlavcompatible with the CRISPR system and to minimize the off-target effects.
3. Cas Enzyme Activation: The guide RNA binds to the endonuclease enzyme such as Cas9, and this enzyme has to make a double-stranded cut in the DNA sequence targeted. This triggers the DNA repair mechanisms inside the cell.
4. DNA Repair Process: The cell uses one of the NHEJ or HDR mechanisms in order to repair the cut DNA. During this, the genetic engineers would introduce desired genetic changes by using a DNA template given to the cell from outside. HDR is a sensitive repair process, mostly used for treating genetic diseases.
5. Verification of Genetic Alteration: After the process of editing of genes has been performed, the cells are checked to see how appropriate and effective the made alterations are. It is done by genetic analysis and with the aid of techniques from molecular biology.

CRISPR technology can be applied to a wide range of areas, from genetic disease treatment to biotechnology, expanding the boundaries of genetic engineering. The technology's precision and efficiency play a critical role in genetically correcting diseases, improving plant and animal genetics, and even developing cancer treatments.

2.13. CRISPR in SMA

CRISPR-Cas9 technology was a great revolution in genetics. The application range goes from therapeutic approaches in SMA research to model generation. Among many good uses, CRISPR-Cas9 mediates the generation of *SMN1* knockout models simulating genetic conditions of SMA in healthy cells and enabling their thorough study of molecular pathophysiology and drug discovery. This is achieved through CRISPR-Cas9-mediated knockout of the healthy copy of the *SMN1* gene, which could induce SMA molecular hallmarks in otherwise healthy cells without the requirement for patient-derived cells.

2.13.1 CRISPR-Cas9 Mediated *SMN1* Knockout for the Generation of SMA Models

The CRISPR-Cas9 is one such technique that gives a license to edit targeted DNA sequences with precision in order to alter it. The major player in relation to SMA research is CRISPR-Cas9, which will deliver knockouts to *SMN1* through the creation of DSBs at its assembled locus upon cuts by Cas9 protein. This it does by creating mistakes mediated by NHEJ, such as indels. Whatever the mutations, these would introduce frameshifts leading to a stop codon and thus terminate the translation, by this comfortable mean allowing *SMN1* knockout modelizing at the molecular level of the disease.

2.13.2 Detailed Process of *SMN1* Knockout

1. To target the initiation of knockout in *SMN1*, a specific exon should be targeted, e.g., 7 or 8, because these exons are required to provide functionality to it. — Design and Delivery of sgRNA This knockout will model the genetic defect in SMA disorder (Lefebvre et al., 1995; Lorson & Androphy Neural Dev.). Next, guide RNA is delivered to the cells in complex with Cas9 protein, which carries it. These sgRNAs can enter cells by lentivirus transduction and electroporation, among others, or could be packaged as lipid nanoparticles.

2. Once the Cas9 protein/sgRNA is inside a cell, it will cut genomic DNA at the site specified by the sgRNA. The DNA cleavage would result in double strand breaks

on the *SMN1* locus. This event provokes the cellular DNA repair machinery to act and repair these DSBs, its most direct way of doing so, preferably in an error-prone manner by NHEJ that creates random indels, transforming which *SMN1* reading frame was disrupted or leads to a defective protein product.

3. Testing of Knockout Cells After the editing has taken place, there is a need to test for knockout. Thus, one can screen for indels in the *SMN1* gene by using PCR/gel or no protein SMNs from western blots among others. In this case, at least your knockout cells need to be expanded/clonal expansion, or you will not expand the cell population to ensure that only cells completely lacking in *SMN1* move to the next experiment.

4. The established phenotype that the investigators will verify in this research is largely reproduced in cells with null mutations in the *SMN1* gene, as evidenced by low levels of the relevant SMN protein, reduced splicing competence especially with respect to snRNP biogenesis, and cell phenotypes pertinent to the SMA disease phenotype.

2.13.3 Application of *SMN1* Knockout Models in SMA Research

1. The SMA Pathogenesis Model: The cell line with knocked-out *SMN1* represents a good model for the investigation of the pathogenetic mechanism of SMA, a disease mainly caused by mutations that lower the level of SMN protein. The deletion of the copy of the mutant gene derived from their ample healthy cells/lines performs two general pathogenic aspects common to this disease. It also allows for the research into what, in general, the loss of SMN impacts within cells, for example, RNA splicing, axon guidance, and synapse formation. For example, see Thelen & Kye/Workman et al. 2006, Ottensen 2017

2. Spliceosomal Defects: It is known that the SMN protein functions within a spliceosome-a complex of snRNPs-and hence the splicing machinery upstream of other pathogenic downstream events, including pre-mRNA splicing. In contrast, defective snRNP assembly has proved to be one of the culprits of abnormal pre-mRNA splicing resulting from the loss of the *SMN1* gene. This is particularly problematic to the motor neurons since splicing requires bringing together both a sufficient number and different proteins to be synthesized and needed in high amounts at the synapse. These require reflecting a cloudy spectrum necessary to maintain/regulate homeostasis-motor neuron connections.

3. Axonal Transport: SMN insufficiency disrupts axonal transport of mRNA, especially that coding β-actin. This mRNA is required for the maintenance of cytoskeleton at the growth cone. This deficit in axonal transport results in degeneration of axons-a feature seen in almost all the pathological processes of SMA (Liu et al., 2020).

4. Drug Screening: The aberrant functionality of *SMN2*, a paralog of *SMN1*, is central to the pathology of SMA. While highly homologous to the gene *SMN1*, the nucleotide change from C to T in exon 7 allows other factors to remove it from being productive in splicing, resulting in the production of a truncated protein form-the highly degraded SMNΔ7. While *SMN2* does produce reasonable amounts of the functional SMN protein, it generally does not produce enough to replace that lost to absence of *SMN1*. Antisense oligonucleotide treatments target splicing defects directly by binding to a region in SCN2 pre-mRNA, known as ISS-N1, allowing or facilitating the use of the splicing factors for the inclusion of exon 7 that helps increase the levels of the SMN protein.

5. Mechanistic Insights and Novel Therapeutic Targets: While in SMA, dysfunction of *SMN2*-a paralog of *SMN1*-is an important pathological factor, structurally the *SMN2* gene is highly similar to the *SMN1* gene. However, a functionality C to T transition present in exon 7 directs additional factors to skip the exon through splicing. This yields a poorly functional product termed SMNΔ7-an unstable product present in splicing. The *SMN2* gene expresses low levels of functional SMN protein; this however is usually not enough to compensate for the loss of *SMN1* protein levels. Antisense oligonucleotide interventions target areas in the *SMN2* pre-mRNA and hence effectively address the splicing defect. For example, therapies that are targeted at ISS-N1 inclusively on the *SMN2* pre-RNA increase the expression of exon 7 and, hence, more functional forms of the SMN protein (Rao et al, 2018).

The use of CRISPR-Cas9 technology in the development of *SMN1* knockout cell lines realizes an extremely promising pathway of study with regard to SMA. Cell models are accordingly contributing to an understanding of the molecular mechanisms of SMA, particularly concerning RNA splicing, axon transport, and synaptic function. These cellular models will allow compound screening and validation of therapeutic targets, bringing us a step closer to a treatment for SMA-or, if one looks at it in a broader manner, finding strategies that improve SMA. As CRISPR-based technologies continue to

improve, so will our generations of *SMN1* knockout models, further facilitating investigation into this devastating disease.

2.14. Antisense Oligonucleotides

At present, antisense oligonucleotides have now clearly emerged as an important tool in both gene editing and gene therapy. These are short synthetic DNA or RNA molecules designed to bind with specific RNA sequences with a view to regulate gene expression, correct disease-associated genetic errors, or inhibit RNA function. Antisense oligonucleotides have been attracting increasing interest in the treatment of genetic diseases and in the management of neurological disorders.

Antisense oligonucleotides (ASOs) were discovered to influence RNA processing and modulate protein expression; however, progress in translating these agents into the clinic has been hampered by insufficient target engagement, biological activity, and off-target toxicities. Over time, various chemical modifications of ASOs have been used to overcome these difficulties. These changes, together with a better understanding of the mechanism of action of ASOs and improved clinical trial design, have given momentum to the translation of ASO-based tactics into treatments. Many neurological illnesses have no effective therapy; nevertheless, as research continues to untangle the pathogenic underpinnings of these diseases, they present a great platform for testing ASO-based approaches. This continuous development has reached a zenith in recent years with the approval of ASOs for the treatment of spinal muscular atrophy, which constitute watershed moments in a field where disease-modifying medicines were almost non - existent. With the rapid development of enhanced next-generation ASOs for clinical use, this technique has the potential to significantly impact the treatment of numerous neurological disorders in the near future.

ASOs consist of small, synthetic chains of nucleotides that interact directly with target RNA to modulate its function. Gene expression modulation by ASOs takes place through an interaction with target RNA because of translation inhibition of the target RNA or because of induction of its degradation. In the treatment of genetic diseases, ASOs have the potential to correct the genetic processes leading to disease by interfering at the RNA level.

Antisense oligonucleotides are synthetic polymers usually consisting of 15-21 nucleotides, engineered in such a way as to attach to some sequences of nucleic acids, mRNA, or its precursor pre-mRNA by complementary base-pairing (Vegeto et al., 2019). ASOs have been divided into several generations according to their structures. They can also be single-stranded DNA ASOs or RNA nucleotide sequences, which are often complementary to endogenous microRNA sequences (Bajan and Hutvagner, 2020). Other classes of nucleic acid-based molecules, known as short interfering RNA, exist in double-stranded complexes (Bajan and Hutvagner, 2020).

ASOs are chemically designed to bond only with their target nucleic acid sequence-usually with the purpose of regulating gene expression. The stability of ASOs is an important issue because the single-stranded molecules, per se, need to survive for a sufficient amount of time to reach their target intact and resist degradation by the circulating nucleases (Levin, 2019). In contrast, siRNAs are more stable within biological settings owing to their duplex structure, which protects them from nucleases in both the bloodstream and within cells (Bajan and Hutvagner, 2020). However, systemic delivery through siRNAs is difficult because of their higher molecular weight and negative charge, which complicates interactions with cellular membranes, including rapid renal excretion (Meister et al., 2004).

Once bound to their cognate RNA target, ASOs can trigger multiple molecular mechanisms. Those are generally divided into two categories: (a) those promoting RNA cleavage through recruitment of endogenous RNA-degrading enzymes such as RNase H, and (b) those interfering with mRNA translation or splicing without degrading the target RNA (Ward et al., 2014).

Although ASOs and siRNA are structurally different and their mechanism also differs, both kinds of molecules prove to be useful tools in the modulation of gene expression. Both hold immense therapeutic promise for specific genetic disorders by reshaping the course of the disease with precision and specificity. In the continued effort to develop the therapeutic potential of ASOs and siRNAs, the unique advantages and disadvantages of these molecules will be weighed, and their combined contribution to nucleic acid-based therapeutics will be recognized.

Antisense oligonucleotides represent highly specific ways of acting on gene expression. Because of their unique capability, antisense oligonucleotides can bind to the

complementary RNA sequence and thus be capable of degrading or modulating the function of the target RNA; hence, they find application in a wide array of genetic disorders. Backbone and chemical modifications of ASOs crucially depend on the selection for stability, specificity, and therapeutic efficacy. Chemical modification overcomes critical intrinsic limitations of nucleic acids, including instability in biological milieus, as well as susceptibility to enzymatic degradation (Figure 8).

One of the simpler chemical modifications within ASOs is the PS backbone. In native nucleic acids, there is a phosphate backbone that links nucleotides together. However, in phosphorothioate-modified ASOs, one of the oxygen atoms in the phosphate group is replaced by a sulfur atom. These subtle but different changes confer a number of advantages on the ASOs, which are rendered more resistant to nucleases, enzymes that otherwise would degrade RNA or DNA in biological systems. Consequently, with a phosphorothioate backbone, ASOs possessing this feature have the ability to circulate longer in the bloodstream-a trait that assures stability and prolongs activity. Modification, in this way, improves cellular uptake, hence allowing the effective targeting of the site inside the cell. A very important role played by the PS backbone in therapeutic applications is that drugs, such as Spinraza (Nusinersen) for the treatment of SMA, require drug stability within CSF after an intrathecal injection.

Other modifications that further enhance the stability and binding affinity of ASOs include 2'-O-Methyl (2'-OMe) and 2'-O-Methoxyethyl (2'-MOE). The common feature of these modifications includes the substitution of the 2′ hydroxyl group of the ribose sugar by either a methyl or methoxyethyl group. This substitution further enhances RNA-binding affinity and provides greater protection of the ASO against enzymatic degradation; hence, its half-life in circulation is improved. For instance, modifying 2'-MOE exerts a twofold positive effect in not only stabilizing the ASO but also reducing its immunogenicity-that is, it is less likely to induce an immune response-so it is safer for long-term use. These modifications enhance the stability of ASOs, making the modified ASOs active to target pre-mRNA splicing-a particularly useful strategy under conditions like SMA, where splicing errors have to be corrected for the rescue of functional protein production.

Of the significant development done in ASO design, the most important has been the creation of LNAs beyond simple backbone and sugar group stabilization. In LNAs,

the ribose sugar is "locked" into a rigid conformation with a methylene bridge between the 2' oxygen and 4' carbon. This structural rigidity greatly increases the thermal stability of the ASO and significantly enhances the specificity and strength of RNA binding. LNAs will be of particular use in applications where high-affinity binding is needed, such as applications against oncogenes in cancer therapy. Because of their higher binding affinity, LNAs will bind to their target RNA sequence in a very specific and strong manner, thus becoming a powerful tool for both gene silencing and splicing modulation. (Lundin et al., 2013).

Peptide Nucleic Acids Another innovative advance in the field of ASO chemistry is peptide nucleic acids. Unlike other 'classical' ASOs, which rely on the sugar-phosphate backbone, PNAs possess peptidomimetic backbones composed of repeating N-(2-aminoethyl) glycine units. This provides a backbone that is very resistant to enzymatic degradation and thus able to bind to complementary RNA or DNA sequences with high affinity. With a neutral backbone, however, PNAs are somewhat at a disadvantage in cell membrane penetration and usually require delivery vectors, such as liposomes or nanoparticles, to access intracellular targets. Nevertheless, PNAs hold great promise for applications in gene-editing technologies and diagnostics, provided efficient delivery methods are developed (Nielsen, 2001).

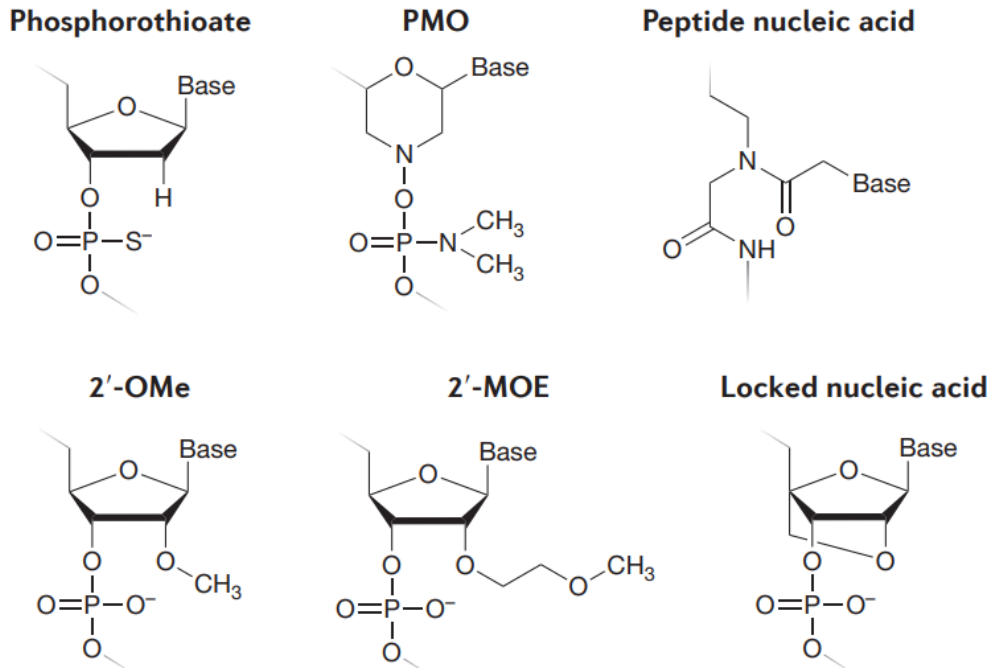


Figure 8. Chemical modifications of the ASO backbone

In the past few decades a number of chemical modifications to the phosphodiester backbone have been made to improve antisense oligonucleotide (ASO) pharmacokinetic properties, tolerability profile, and target binding affinity. Phosphorothioate DNA, phosphorodiamidate morpholino (PMO), and peptide nucleic acid designs all confer resistance to nucleases and enhanced uptake in cells, resulting in increased potency of the ASO. Ribose substitutions, including 2′-O-methyl (2′-OMe), 2′-O-methoxyethyl (2′-MOE), and locked nucleic acid, are frequently used in combination to further increase stability, enhance target binding, and generally confer less toxicity than unmodified designs.

Another class of ASO, less general but not less important, is represented by the morpholino oligonucleotides. Unlike conventional ASOs, morpholinos utilize a special backbone made up of morpholine rings linked by phosphorodiamidate groups. Because of this special structure, they show very high resistance against enzymatic digestion and chemical decomposition in biological systems. With very good stability and low toxicity, morpholino oligonucleotides find wide applications in research and therapeutic settings in gene knockdown experiments at early developmental stages or in models of Duchenne Muscular Dystrophy (Summerton & Weller, 1997). However, because they do not bear any charge, the big barrier of the cell membrane hinders them, and hence particular modes of delivery like electroporation or microinjection techniques have to be employed for their effectiveness.

A subclass of ASOs, known as gapmers, is of special value, since they are able to degrade target RNAs via RNase H activation. Gapmers consist of a DNA part flanked by modified RNA nucleotides in the center. This central DNA gap hybridizes with the target RNA, and the latter type of RNA-DNA duplex recruits RNase H, which cleaves the RNA strand specifically, thus degrading that strand. This is a very potent approach toward the knockdown of gene expression, especially in those diseases where one overproduced mRNA may drive the pathology of the disease, such as in certain cancers and metabolic disorders. Gapmers effect specific and potent gene silencing via RNase H-mediated cleavage.

Overall, such chemical modifications of ASOs allow their wide therapeutic application, in particular in the treatment of genetic disorders like SMA, DMD, and various forms of cancer. For example, Spinraza (under the trade name) corrects splicing of the *SMN2* gene toward restoration of the expression of full-length functional SMN protein in patients with SMA (Finkel et al., 2017). Spinraza (under the trade name) forms a very stable and potent complex in the cerebrospinal fluid for very long periods by incorporating phosphorothioate and 2'-MOE modifications and can, therefore, be given infrequently with consistent therapeutic benefit. LNAs and gapmers have also shown great promise in cancer research as a way of effectively silencing oncogenes or other genetic drivers of the disease, making a new paradigm for targeted therapies that avoid many of the liabilities of cytotoxic chemotherapy.

Advanced chemical changes have significantly increased the stability and specificity of ASOs. From phosphorothioate backbones that withstand enzymatic degradation to LNAs that increase binding affinity, each change contributes to the development of ASOs as an exceptionally strong tool for gene silencing, splicing manipulation, and gene therapy. Such advancements have broadened the application horizon of ASOs, bringing promise for more effective therapies for a wide spectrum of genetic illnesses. Taken together, the ongoing evolution of ASO chemistry, together with advancements in delivery methods, would imply that these molecules would play an increasingly important role in the future of precision medicine.

2.15. XNAs and Next Generation Therapy

This class of unnatural molecules, the XNAs, carries different structural features from the natural nucleic acid backbone. Their structural function in carrying genetic information remains like that of DNA and RNA; however, chemical alteration in the structures of XNAs has proved them highly resistant in biological milieus. These are the features that make XNAs efficient tools in medicinal, genetic engineering, and other biotechnological applications. These changes in structure from normal DNA and RNA give XNAs longer life by overcoming the enzymatic degradation and stability problems the natural nucleic acids normally go through. Since the sugar-phosphate backbone is modified, enzymatic degradation and problems of stability associated with normal DNA

and RNA are overcome, hence granting them longer life. This also enables interaction with genetic material much more effectively (Nielsen, 2001).

The structural changes make XNAs more resistant to enzymatic degradation within the biological system. Modifications around the backbone of the phosphodiester, especially, make XNAs more stable; hence, they are able to function in biological fluids over longer periods. Because of these changes, structural modifications cause the XNAs to interact stronger with target nucleic acids in a more specific way. Thus, for example, substitution of sulfur atoms for the oxygen atoms of the phosphate groups provides for the phosphorothioate modification. This modification highly reduces the rate at which nucleases degrade XNAs and thus is able to remain active in biological systems over longer periods (Gagnon et al., 2018).

With different natures, various XNAs still have different uses. For example, PNA has a peptidomimetic backbone without the phosphodiester. This structure makes PNA far more robust compared to natural DNA and RNA. PNAs are bound to target gene sequences with high affinity; thus, finding applications in genetic research, molecular diagnostics, and biomolecular interaction studies. Such modifications make the molecules resistant to nucleases; hence, they can stay active for a longer period inside the cell and are ideal tools for applications of gene editing (Nielsen, 2001).

Similarly, LNAs have structure featuring a bridge between the 2' and 4' carbons of the ribose. This feature imparts a capability for strong and specific interaction of these LNAs with target RNA. These are used in antisense therapies for specific genetic alterations. HNA on the other hand is short form for Hexose Nucleic Acids and has a backbone from hexose sugar other than ribose or deoxyribose. Generally, HNA is applied in the gene therapies as well as the synthetic biologies because it is more robust as compared to the other kinds of nucleic acids while applying in the biological systems. FNA stands for Furanosyl-Nucleic Acids; this form of nucleic acid includes a furanosyl ring instead of a ribose ring. In the light of this replacement, the structure of FNA becomes more stable in the biological system. The latter improves long-term usability within the biological system and at the same time the interaction of XNAs with genetic material (Lundin et al., 2015).

That is why, over time, XNAs have gained great importance for therapeutic purposes in the editing of genes and for treatments of genetic diseases. Also, antisense

oligonucleotides, involved in drugs such as Nusinersen, are applied for correction of splicing errors within the *SMN2* gene in SMA patients. Such therapeutic molecules, based on the robust structure of XNAs, might be the ones to bring revolutionary changes in the treatment of genetic diseases (Gagnon et al., 2018).

Besides being genetic carriers, the other important feature of the XNA molecule is that it too can take part in editing and modification processes of genetic material. The XNA molecules are, therefore capable of storing genetic information for longer periods in a stable manner and act as carrier molecules much more than natural nucleic acids. Therefore, XNAs also become one of the power tools for novel use in synthetic biology and bioengineering.

XNA, meaning xeno nucleic acids, refers to chemically altered chemical entities that transcend the limit of natural nucleic acids. They have extensive use in genetic research and therapy these days. More resistant within biological systems, insensitive to degradation by nucleases, and their interaction with genetic targets is stronger; such structures are being considered highly important in genetic engineering and treatment approaches. Further, applications of XNAs to gene editing and synthetic biology hold great promise for the future development of genetic-based treatments.

2.15.1. Lock Nucleic Acids LNA

Locked nucleic acids are synthetic nucleotides based on natural nucleic acids but from a quite different and more advanced class regarding biological and chemical properties. LNAs are structured with a modification that entails a methylene bridge between the 2' and 4' carbons of the ribose sugar. The structural shift highly increases the binding capability and stability of LNAs to DNA or RNA targets. LNAs are formidable tools, not only in molecular biology and genetic engineering but also in general biotechnology, with some remarkable advantages, especially therapeutic ones.

In LNA-modified oligonucleotides, several natural nucleotides are replaced with nucleotide analogs having a modified sugar moiety in which the ribose 2′-Oand 4′-C atoms are linked by a methylene bridge (Grunweller et al., 2007).

As the name suggests, the introduced chemical modification locks this RNA analog in one conformation. This change reduces the entropy of LNA hybridization with

their target DNA/RNA, or in other words, it increases the chance for such a duplex to form (Qassem et al., 2024).

While the natural world contains the architectures of scaffoldings of DNA and RNA, there have been additions to include hydrophobic Xeno-Nucleic Acid polymers with different chemical backbones (Chaput et al., 2019). These XNA molecules carry variations in their sugar moieties maintain nucleobases similar to their DNA and RNA counterparts. One of the most striking features of these XNA sequences, however, is that they have increased affinity for the binding of targeted RNA sequences, with an added increase in resistance against native nuclease enzymes (Kilic et al.,2023).

Harnessing these attributes, XNA-ASO ensembles usher in RNA-based therapeutic strategies, offering a nuanced avenue characterized with lower toxicity, intervention in splicing processes is further made possible because the XNA sequences at both ends of DNA sequences give rise to hybrid XNA-DNA mixmer structures that could affect the mechanics of splicing. Another promising ability involves that of the XNA probe forming stable complexes by binding in a highly specific manner with target DNA or RNA sequences. Taken together, these XNA sequences open up a new frontier for next-generation biopolymers with novel chemical properties that can replace DNA and RNA and open up new avenues in research and therapeutic applications (Kilic et al.,2023).

In this phase of thesis, aiming at a higher expression level of SMN protein by investigating the efficacy of XNA-based antisense oligonucleotides against SMA Type 2 fibroblast and *SMN1* knockout cells. The use of *SMN1* knockout cells is of utmost importance to better understand the genetic basis of SMA and ensure accuracy in disease modeling. These cells model the functional loss of the *SMN1* gene, enabling direct investigation of the cellular and molecular effects of SMA. This new strategy will allow a better understanding of the molecular pathophysiology of SMA and will unravel the potential of XNA-ASOs in genetic therapies, thus enabling the elaboration of new strategies in the treatment of genetic diseases.

3. MATERIALS AND METHODS

3.1. Obtaınıng *SMN1* Knock-Out Cells Wıth CRISPR Cas-9

To inactivate *SMN1* in U87 cell lines and produce an *SMN1*-knockout cell model, BLAST searches were performed on *SMN1* and *SMN2* genes to find acceptable gene sites for the inactivation method, and several areas were discovered. BLAST analysis resulted in gRNA designs targeting distinct areas of intron 5 and intron 7 in the *SMN1* and *SMN2* genes. Following the design process, Addgene provided gRNA designs with particular single point mutations in intron 5 and intron 7 areas, which were then cloned into the lentiCRISPR v2 (Plasmid #52961) plasmid. *SMN1* was then deactivated using the CRISPR/Cas9 technology. Cells with effective gene modification were then picked using Puromycin antibiotic selection.

Table 4. Primers designed for Sanger sequencing

Position	Strand	gRNA Sequence	Pam Sequence (NGG)	On-Target Score	Off-Target Score
SMN1 upstream cut					
1325	-	TCACTTGACCCTGGAGGCGG	AGG	51.5*	63.8
SMN1 downstream cut					
217	+	CAGGAAGGCACCACCATGCG	TGG	61.3*	67.4

3.2. Transformation and Plasmid DNA Isolation

Competent E. coli DH5α strain bacterium (NEB® 5-alpha). Competent E. coli (High Efficiency) (NEB C2987H) were grown in liquid LB medium at 37°C. Bacteria were transformed with plasmid DNAs using the heat shock technique per the manufacturer's specifications. Bacteria were plated on ampicillin-containing LB agar plates and incubated overnight at 37°C. The ZymoPURE II Plasmid Maxiprep Kit (#D4203, ZYMO) was used to isolate plasmids from chosen colonies.

3.3. Agarose Gel Electrophoresis

Plasmid DNA acquired during isolation was visualized using an agarose gel. First, a 1% agarose gel was created. The gel was made using EzView Stain Red Safe Solution (standardized at 5 µL per 100mL), which is comparable to ethidium bromide. The plasmid DNA was measured at 1 µg and put onto the gel. The gathered data were then converted into pictures and analyzed.

3.4. Lentivirus Production and Transfection

The designed Up and Down sequences were cloned into lentiCRISPR v2 (Plasmid #52961) plasmid by Addgene. For lentivirus production, DNAs of *SMN1* upstream and downstream plasmids were separately transfected into host HEK293T cells with pSPAX2 (Addgene #12260) and pVSV-G (Addgene #138479) envelope and packaging plasmid DNAs. All plasmids (2:1 pSPAX2:1 pVSV-G) were treated with P-PEI (Polyethylenimine) transfection reagent (Merten et al., 2016) and lentivirus production was performed using HEK293T host cells. At 72 hours after transfection, HEK293T cell supernatants were collected and packaged recombinant lentiviruses were obtained. The produced lentiviruses were concentrated 100x with Lenti-X Concentrator (#631232, Takara) to increase the virus concentration (Cooper et al., 2011).

3.5. Lentivirus Titration

Jurkat (RPMI medium containing 10% fetal bovine serum, 200 U/ml penicillin/streptomycin antibiotic, 2 mM L-glutamine, 1X MEM vitamin solution and 1X NEAA) cells were inoculated into 96-well petri dishes in 100 µL of medium. Lentiviruses were added to the wells in volumes of 10 µL, 3 µL, 1 µL, 0.3 µL, 0.1 µL and 0.03 µL to contain lentivirus at 100x concentration. Then, the cells were incubated for 72 hours at 37 ^{o}C, 5% CO2. After the cells were transferred to 96-well petri dishes, 2 µg/ml puromycin was applied to Jurkat cells for puromycin selection and incubated for 120 hours. Then, cell viability was tested using BIO-RAD TC20 Automated Cell Counter. Finally, >99% puromycin resistant *SMN1* knockout cells were selected and cultured.

3.6. Cell Culture

Primary SMA Type 1 (#GM00232) and SMA Type 2 (#GM03814) fibroblast cell lines were obtained from the Coriell Institute. SMA Type 1 and SMA Type 2 fibroblast (EMEM medium containing 10% fetal bovine serum, 200 U/ml penicillin/streptomycin antibiotics, 2 mM L-glutamine, and 1X NEAA); HEK293T, HeLa, WT fibroblast, SH-SY5Y (Gibco DMEM with 10% FBS and 1% penicillin/streptomycin, L-glutamine medium); U87, A172 (DMEM medium containing 15% fetal bovine serum, 200 U/ml penicillin/streptomycin antibiotics, 2 mM L-glutamine) cell lines were cultured in the appropriate media. The cells were incubated at 37ºC, 5% CO2. When the cell density reached 90%, the passaging process was performed. After the medium was removed, washing was done with PBS solution. Then, an appropriate amount of EDTA-Trypsin was added and kept at 37ºC for 3 minutes. Medium was added at a ratio of 1:1 and the cells were collected. After centrifugation, the cells were counted and seeded back into the petri dishes. For the freezing stage of the cells, after the cells were removed and settled, the freezing solution was added and the suspension was transferred to a cryo tube and raised to -80. Then, the cells were taken into liquid nitrogen and stored there.

3.7. Puromycin Selection

After obtaining puromycin-HCl (33835.01, Serva), antibiotic selection experiment was established in the cells. Cells were seeded in 96-well petri dishes in 200 µL medium as 10,000/well. Puromycin was applied to the cells in duplicate at concentrations of 0.5, 1, 2, and 4 µg/ml. The cells were incubated at 37ºC and 5% CO2. Then, morphological images were recorded under light microscope at the end of 24-48-72-96 and 120 hours and their viability was analyzed by flow cytometry (Beckman Coulter, CytoFlex). Afterwards, specific antibiotic concentration and hours were determined for each cell line. As a result of virus transduction by flow cytometry, >99% resistant cells containing the puromycin resistance gene were selected and culture was continued. Antibiotics were applied at the specified doses to healthy cells that were not transduced with the virus and were used as a positive control group (**Table 6)**.

Table 5. Day and puromycin concentrations determined in cell lines

Cell Line	Puromycin Concentration	Hour
Jurkat	2 µg /ml	120h
HEK293T	2 µg /ml	120h
U87	2 µg /ml	120h
Wild Type Fibroblast	2 µg /ml	72h
HeLa	2 µg /ml	72h

3.8. Single Cell Cloning (SCC)

In order to determine whether the *SMN1* knockout cell line was successfully created in U87 cell lines containing CRISPR-cas9 system and to create a more homogeneous population, single cell cloning was performed. After the cells were lifted with trypsin, cell counting was performed with TC20 Automated Cell Counter. 50,000 cells were collected in 1 ml of complete medium in Eppendorf. 9990 µL of complete medium was added in a separate falcon and 10 µL of 1 ml of cell mixture was taken and added to the medium in the falcon. It was diluted 1:100 here and 500 cells/ml cells were obtained. After 10 ml of media was added to a separate falcon, 5 ml of 10 ml of cell mixture was taken and added to the medium in the falcon. It was diluted 1:10 here and 50 cells/ml cells were obtained. The total mixture was divided into 96-well petri dishes by pipetting, with 150 µL per well. The aim was to reduce single cells in each well and obtain pure colonies.

3.11. DNA Isolation and Sanger Sequencing

Genomic DNA from U87 CRISPR-cas9 modified SMA Type 1, Type 2 cell lines was isolated with PureLink Genomic DNA Mini Kit (#K182001). Sanger sequencing was performed to verify the effectiveness of the CRISPR-PE system in performing on-target

effect *SMN2* modification in the cell genome. Sanger sequencing was performed at Intergen Genetic Diseases Diagnostic Research and Application Center. *SMN2* gene-specific primers were designed for Sanger sequencing **(Table 7)**.

Table 6. Primers designed for Sanger sequencing

Oligo Name	Base Sequence 5'-3'	Base Pair	Concentration
5forward Sanger	GGG ATT GTA GGC ATG AGC CAC TG	23	200 nmol
5reverse Sanger	GTC TGC TGG TCT GCC TAC TAG TG	23	200 nmol

3.9. Multiplex Ligation-Dependent Probe Amplification (MLPA) Analysis

SALSA® MLPA® Probemix P021-B1 SMA is widely used in the diagnosis and monitoring of SMA (Pane et al., 2018). This technique is used to determine *SMN2* copy numbers, which are very important in assessing the severity of the disease (Müller-Felber et al., n.d.). In addition, the use of this probe mixture allows the determination of homozygous loss of *SMN1* function, which is the distinguishing feature of SMA in patients (Veldhoen et al., 2022). The evaluation of samples with the MLPA technique was carried out at the Intergen Genetic Diseases Diagnostic Research and Application Center with service procurement. SALSA MLPA Probemix P021-B1 SMA contains 32 MLPA probes with amplification products between 175-445 nucleotides. There are 7 SMN exon 7 and 8 probes that can detect the copy number of *SMN1* and *SMN2* genes. In normal individuals carrying two copies of *SMN1* and two copies of *SMN2*, these probes detect a total of four gene copies. In the case of homozygous *SMN1* deletion, these probes are used to determine the number of *SMN2* copies more accurately.

3.10. XNA-DNA ASO mixmer Design and Delivery

Two distinct ASO kinds with various sequence designs were examined to find the best XNA-DNA-ASO sequence. The ISS-N1 motif sequence inside the *SMN2* intron 7 region (10-24) is the target area for the XNA-DNA-ASO mixmer sequences. These XNA DNA mixmers are designed to enhance hydrogen bonding by grouping them individually

based on whether they include A-T or G-C nucleotides. This study used phosphorothioated XNA-DNA mixmers with DNA nucleobases (G *, A *, T *, and C *) as ASO sequences. Using the service procurement technique, Qiagen Biotechnology (Hilden, Germany) provided ASO sequences tailored to create a compact structure with the target mRNA sequence. The planned XNA-ASO arrays were purified at 10 nmol concentration using high-performance liquid chromatography (HPLC). The XNA-DNA-ASO sequences were transmitted to the SMA type 1 fibroblast cell line using PEI transfection reagent figure (Bremer, J. et al, 2022). RNA-ASO analysis of 18-mer 2′-O-(2-metoksietil) (MOE) reveals a 5′-TCACTTTCATAATGCTGG-3′ sequence (Intron 7 position;10-27). Qiagen Biotechnology offers RNA-ASO products that are suitable for this modification and application (**Table 8**).

Table 7. XNA-DNA-ASO mixmer sequences targeted at *SMN2* gene intron 7 region

XNA-DNA-ASO	XNA-DNA mixmer sequence Phosphorothioated XNA* bases/ Phosphorothioated DNA* bases
MERLiNA	+T*C*+A*C*+T*+T*+T*C*+A*+T*+A*+A*+T*G*C*+T*G*G
Positive Control	mU*mC*A*mC*mU*mU*mC**A*mU*A*A*mU*G*mC*mU*G*G

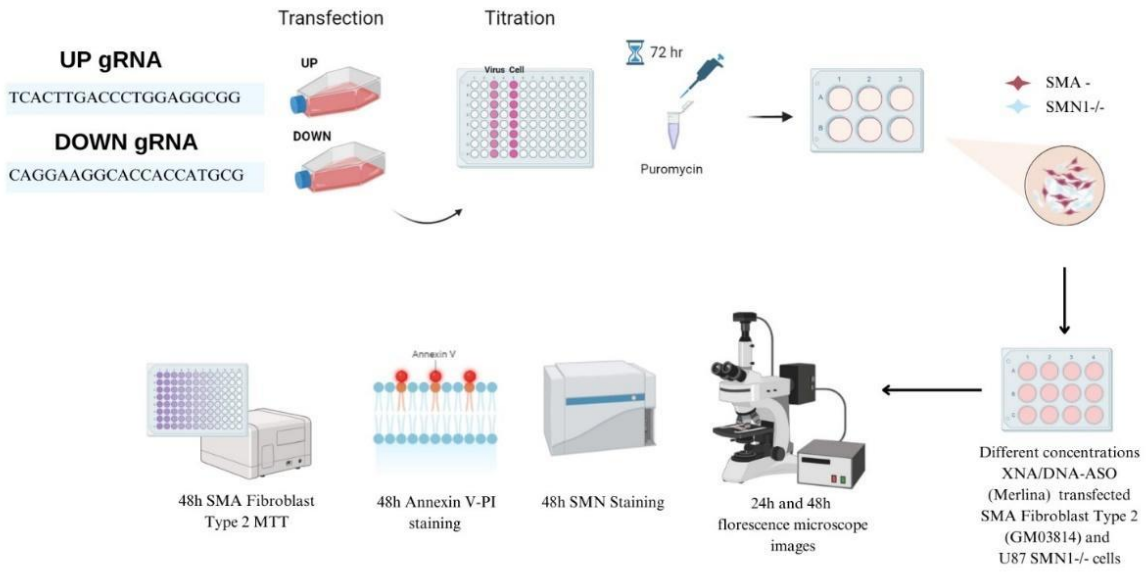


Figure 9. Experimental set-up

3.11. Imaging under a Fluorescence Microscope and Flow Cytometry Analysis

In order to determine the functional SMN protein level, which is targeted to be increased in different cell lines, and to determine its long-term stability, localization within the cell and effects on cell morphology, records were taken under fluorescence microscope. In order to determine the SMN expression levels in SMA Type 1, SMA Type 2 fibroblast, U87 cell lines, intracellular staining was performed with anti-SMN-AlexaFlour 647 antibody (sc-32313, AF647, Santa Cruz Biotechnology) and SMN expressions were analyzed by flow cytometry. Fixation/permeabilization procedures were performed using BDCytofix/Cytoperm™Fixation/Permeabilization Kit (#554714). After the cells were collected to be 100,000 cells, they were centrifuged at 400g for 5 min at +4oC. After removing the supernatant, 200 µl of Fixation/Permeabilization solution (BD) was added to the pellet and incubated at +4ºC for 20 min. After fixation, the cells were washed twice with BD Perm/Wash™ Buffer (BD). Then, anti-SMN-AlexaFlour 647 was added to the cells at a dilution ratio of 1:50, the samples were gently vortexed and incubated at +4ºC for 30 min. The cells were washed twice with BD Perm/Wash™ Buffer (BD) and centrifuged at 300 g for 5 min. After the pellet was dissolved with 200 µl of BD Perm/Wash™ Buffer (BD), it was analyzed by flow cytometry (Beckman Coulter,

CytoFlex) and evaluated according to SMN protein expression percentage and Mean Flouresence Intensity (MFI) value.

3.12. RNA Isolation and cDNA Synthesis from Cell Lines

Total RNA extraction from cells was carried out using the PureLink RNA Mini Kit (#12183018A) RNA purification kit according to the vendor's instructions. Absorbance measurements (230 nm, 260 nm and 280 nm) were taken via Microplate Reader (OmegaStar, ELISA) analysis to determine total RNA concentration. cDNA synthesis was performed using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (#4358814). 10 µl (2 µg) of RNA samples isolated from SMA Type 1, SMA Type 2 and U87 cell lines and 10 µL of 2X RT mix were added to the PCR tube. The reaction was carried out at 25°C for 10 minutes, then at 37°C for 2 hours and finally at 85°C for 5 minutes.

3.13. Quantitative rt-PCR (RT-qPCR) Analysis

rt-qPCR analysis was performed using PowerUp™ SYBR™ Green Master Mix (#A25742). PCR analysis was performed to determine the expression of full-length SMN transcripts (Full length, FL) and exon 7 truncated SMN transcripts (Δ7SMN mRNA). Primers for SMN mRNA and Δ7SMN mRNA were determined (Zheleznyakova et al., 2011) and synthesized by Sentromer. Quantitative analysis was performed using Roche Lightcycler 96. The synthesized primer sequences to determine the onset of FL and Δ7SMN SMN transcription are listed below **(Table 9)** (Zheleznyakova et al., 2011). For the reaction to take place, 5 µl cDNA (20 ng/µl) and 15 µl SYBR Green mix were added to each well so that the final volume was 20 µl. Cycling conditions; The thermal cycler was set to have 2 minutes of polymerase activation at 95°C, 15 seconds of denaturation at 95°C, and 1 minute of annealing/extension at 60°C. The data obtained as a result of the cycle were analyzed.

Table 8. Primers designed for RT-qPCR

Oligo Name	Base Sequence 5'-3'	Base Pair	Concentration
5forward RT-qPCR (FL)	CTG ATG CTT TGG GAA GTA T	19	200 nmol
5reverse RT-qPCR (FL)	GCC AGC ATT TCT CCT TAA	18	200 nmol
5reverse RT-qPCR (Δ7SMN)	GCC AGC ATT TCC ATA TAA TAG	21	200 nmol
5forward RT-qPCR (Δ7SMN)	GTC CAG ATT CTC TTG ATG AT	20	200 nmol

3.14. Annexin V- propidium iodide (PI) Staining

In order to determine the apoptosis stages and viability of cells in cell populations, Annexin V-PI analysis was performed with Annexin V-FITC Apoptosis Detection Assay (Grisp, #GTC21.0100). Cells were collected in Eppendorf as 1×1^5 and centrifuged. Then, cells were dissolved in 500 µL of cold media. After precipitation, they were dissolved with 100 µL of 1x Binding buffer. 2.5 µL of Annexin V-FITC and 5 µL of PI were added and incubated in the dark for 15 min. After incubation, they were precipitated and dissolved in 200 µL of Binding buffer. Finally, flow cytometry analysis of stained cells was performed and apoptosis stages were evaluated.

3.15. Determination of Cell Viability by MTT Assay

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] agent was used to evaluate all possible toxicity that may occur as a result of CRISPR Prime Editing encoding lentivirus transfer and to control cell viability. Cell viability was determined by colorimetric method as a result of MTT agent forming purple colored formazan salt in living cells. Primary SMA Type 1, SMA Type 2 fibroblast, U87 cells were seeded in each well of 96-well petri dish as 10,000 cells. It was waited for 24 hours for the cells to adhere and take morphology. Then, 10 µl of MTT agent was added to each well and the cells were incubated at 37ºC for 4 hours. After the medium containing MTT agent was removed, 100 µl of DMSO was added to each well and kept in the dark for 5 minutes. Finally, after dissolving formazan salts with DMSO, absorbance values were measured

and analyzed at 570nm and 655nm wavelengths in an ELISA (FLUOstar, OMEGA) device.

3.16. Statistical Analysis

The results obtained from all experiments were examined using statistical methods compatible with the number of data using GraphPad Prism 8.0.1 software (GraphPad Software, Inc., San Diego, CA, USA) and presented as graphs/tables. Data were analyzed with two-tailed t-test and the standard deviation of the mean (Standard Error of Means (SEM)) was determined with bar graphs. For all experiments, significance was accepted as $p<0.05$ and non-significant (NS=Non-Significant).

4. RESULTS

4.1.Quality control tests after transformation and isolation of plasmids

After transformation of the plasmids, plasmid DNA isolation was first performed with the ZymoPURE II Plasmid Maxiprep Kit (#D4203, Zymo). The isolated plasmids were visualized on agarose gel as part of quality control (**Figure 10A**). When the gel images were examined, the bands were clearly seen. Bacterial chromosomal DNA contamination and Supercoiled plasmid DNA gel electrophoresis were analyzed with the Biorad Molecular Imager Gel Doc Imaging System. Analysis reports of the gel images are included (**Figure 10B**).

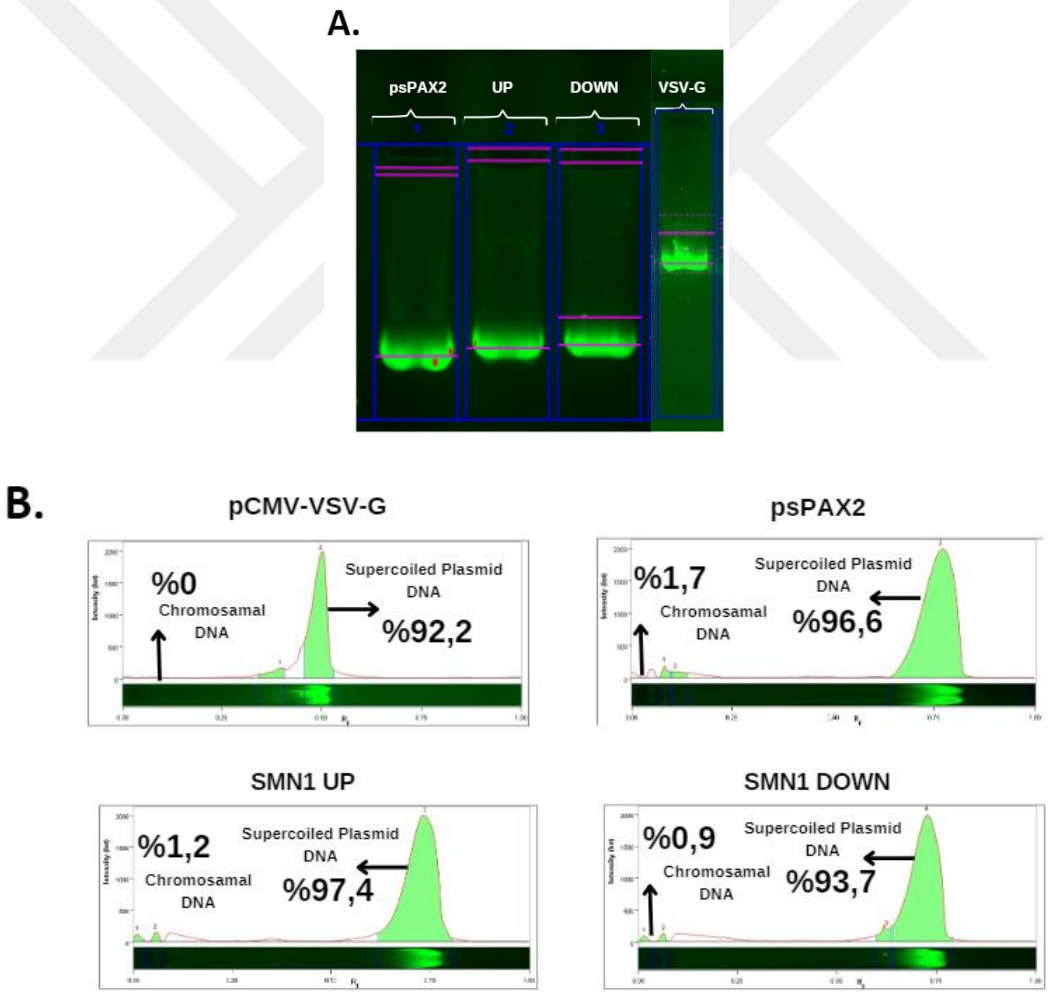


Figure 10. Gel electrophoresis image including quality controls of isolated plasmid DNAs and analysis by Biorad Molecular Imager Gel Doc Imaging System.

Gel electrophoresis image including quality controls of isolated plasmid DNAs and analysis by Biorad Molecular Imager Gel Doc Imaging System. A) pCMV- VSV-G Plasmid (Addgene, #8454), pegRNA1, psPAX2 Plasmid (Addgene #12260), *SMN1*_UP, *SMN1*_DOWN, pLENTİ-PE2-BSD (Addgene, #161514),

pHIV-EGFP (Addgene, #21373) Plasmid Agarose Gel Image. B) Plasmid Chromosomal DNA Contamination and Supercoiled Plasmid DNA Analysis Report.

4.2.Evaluation of isolated plasmid DNAs according to quality control acceptance criteria

The isolated plasmid DNAs were evaluated according to the specified quality control acceptance criteria **(Table 10)** (Taştan et al., 2020). When the A260/A280 values of the isolated plasmids (pCMV-VSV-G, psPAX2, *SMN1*_UP, *SMN1*_DOWN) were examined, it was determined that the values of each plasmid were within the expected range. It was confirmed that the obtained plasmids were isolated purely. Then, the measurement of the isolated plasmid DNA concentrations was determined using the Microplate Reader (FLUOSTAR OMEGA) **(Table 11)**. As a result, it was observed that all plasmids were successful in quality controls and were found suitable for use in future experiments.

Table 9. Plasmid DNA Quality Control Acceptance Criteria (Taştan et al., 2020)

Analysis for plasmids	Methods	Expected value
DNA Concentration	Microplate Reader (FLUOSTAR OMEGA)	>0.1 µg/µl
DNA Purity	Microplate Reader (FLUOSTAR OMEGA)	1.8 < A260/A280 < 2.0
Bacterial Chromosomal DNA Contamination	Gel Electrophoresis and Imaging System	<%1 (w/w)
Supercoiled Plasmid DNA	Gel Electrophoresis and Imaging System	>%80

Table 10. Quality Control Results of Isolated Plasmid DNAs

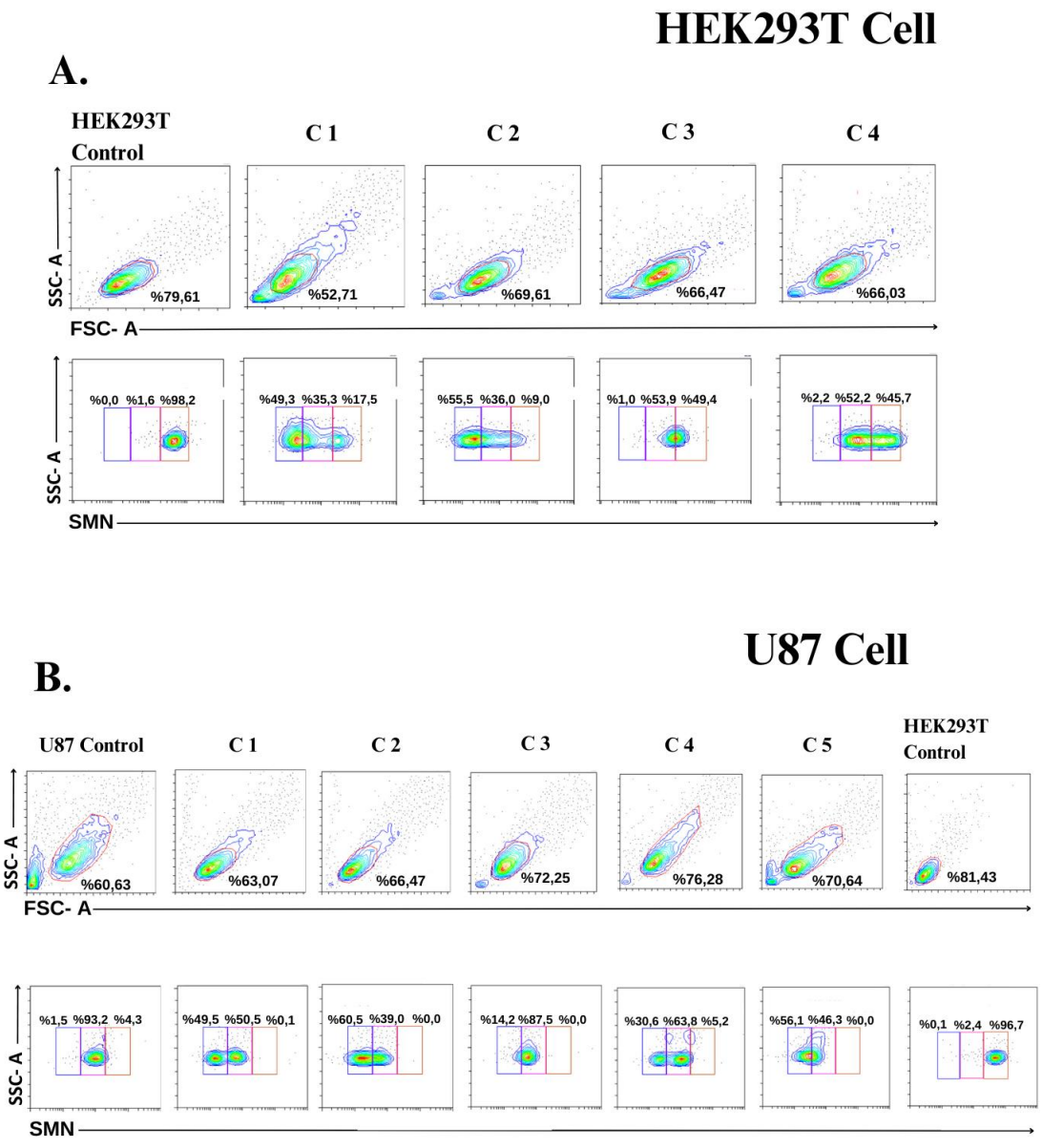
Analysis for plasmids				
	DNA Concentration	DNA Purity	Bacterial Chromosomal DNA Contamination	Supercoiled Plasmid DNA
VSV-G	2,13 µg/µl	1,74	%0	%92,2
PSPAX2	1,77 µg/µl	1,75	%1,7	%96,6
SMN1_UP	1,07 µg/µl	1,75	%1,2	%97,4
SMN1_DOWN	1,05 µg/µl	1,75	%0,9	%93,7

4.3 Evaluation of SMN expression levels after establishment of HEK293T, HeLa, Jurkat and U87 *SMN1* knockout cell lines

Lentiviruses encoding the *SMN1*-targeted CRISPR/cas9 system produced for knockout of the *SMN1* gene were transduced into HEK293T, HeLa and U87 cell lines at an MOI of 3. After 72 hours of transduction, puromycin antibiotic was applied to the cells at appropriate concentrations and the cell population with 99% cell viability was continued to be cultured. The cells were then subjected to SCC to obtain genomically pure clones. After SMN staining, 3 gates representing low-medium-high SMN expression were set for the control and clones. In the SMN staining performed **(Figure 11)**, while the high SMN expression level was detected as 96.73% in the HEK293T cell line with a high SMN profile; in the *SMN1* knockout HEK293T cell clones propagated by SCC, it was detected that SMN levels decreased significantly in independent clones **(Figure 11A)**. The highest SMN expression level in clone C2 decreased to 9%, exhibiting a significant decrease in SMN expression. When the viability of the cells was examined in flow cytometry analysis, it was found that the viability of the clones was on average 80% in the control groups, while it decreased to an average of 66% in the independent clone population **(Figure 11A)**. The flow cytometry result of intracellular SMN staining performed in *SMN1* knockout U87 cell line is given in **Figure 11B**. SMN expression levels, especially in C1-C2 and C5 clones, showed a tendency towards low expression in the independent clone populations. When the medium level SMN expressions were examined in the independent clone populations compared to the control U87 population,

it was 50.49% in clone C1; It was found that it decreased by 38.94% in the C2 clone, 63.75% in the C4 clone and finally 46.32% in the C5 clone. This shows that cells with low SMN expression were successfully produced in the clones in the U87 cell line. When the viability of the cells was examined, it was determined that they progressed with low viability in direct proportion to the low SMN expression level **(Figure 11B)**.

HeLa cell line is a cell line with high SMN expression under normal conditions **(Figure 11)**. The flow cytometry result of intracellular SMN staining performed in *SMN1* knockout HeLa cell line clones is given in **Figure 11C**. It was found that the high SMN expression level gradually decreased in the independent clone populations.



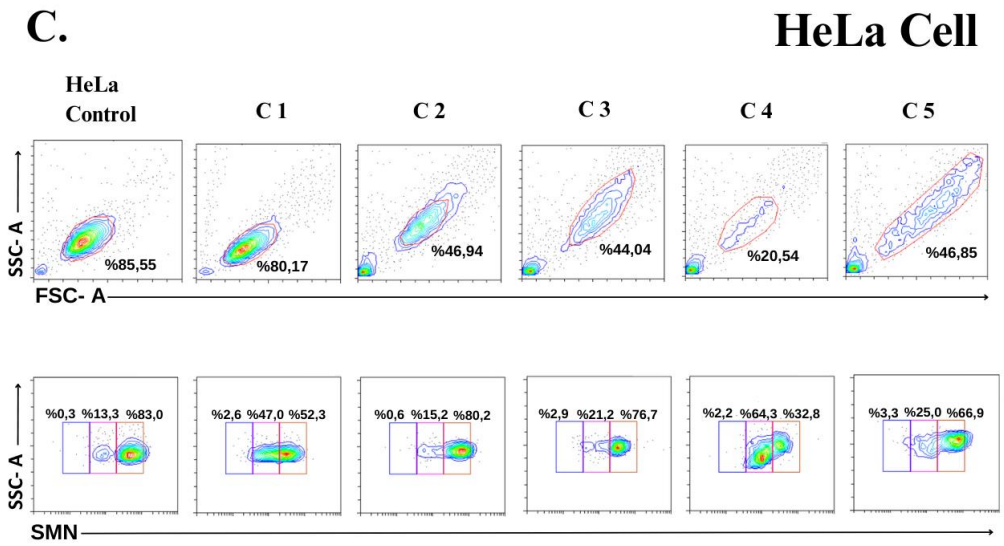


Figure 11. Flow cytometric analysis of staining performed in SMN1 knockout HEK293T, HeLa and U87 cell lines.

A) HEK293T B) U87 C) Flow cytometric analysis of SMN expression in HeLa cell line clones (C1-C2-C3-C4-C5) after intracellular staining with SMN monoclonal antibody.

4.4 Evaluation of low-medium-high SMN expression levels and viabilities in HEK293T, U87 and HeLa *SMN1* knockout cell clones

After obtaining pure clones in *SMN1* knockout HEK293T, HeLa and U87 cell lines, bar graphs were created to compare SMN expressions as low-medium-high and viability levels compared to the control group. Bar graphs created for SMN expressions and viability of HEK293T clones are given in **Figure 12A**. It is seen that high SMN expression level in the control group HEK293T cell line decreases significantly in clone populations. Similarly, medium and low SMN expressions gradually increase compared to the control group, that is, there is a transition from high expression to low expression. Bar graphs created for SMN expressions and viability of *SMN1* knockout HeLa clones are given in **Figure 12B**. When looking at the control group, SMN expression is high. In clone populations, this situation decreases gradually again, and a tendency towards low levels is determined. Bar graphs created for SMN expression and viability of U87 clone populations are shown in **Figure 12C**. It is seen that the control group has more intermediate level SMN expression. It was determined that the intermediate SMN expression level in the clones decreased over time and low SMN expression increased.

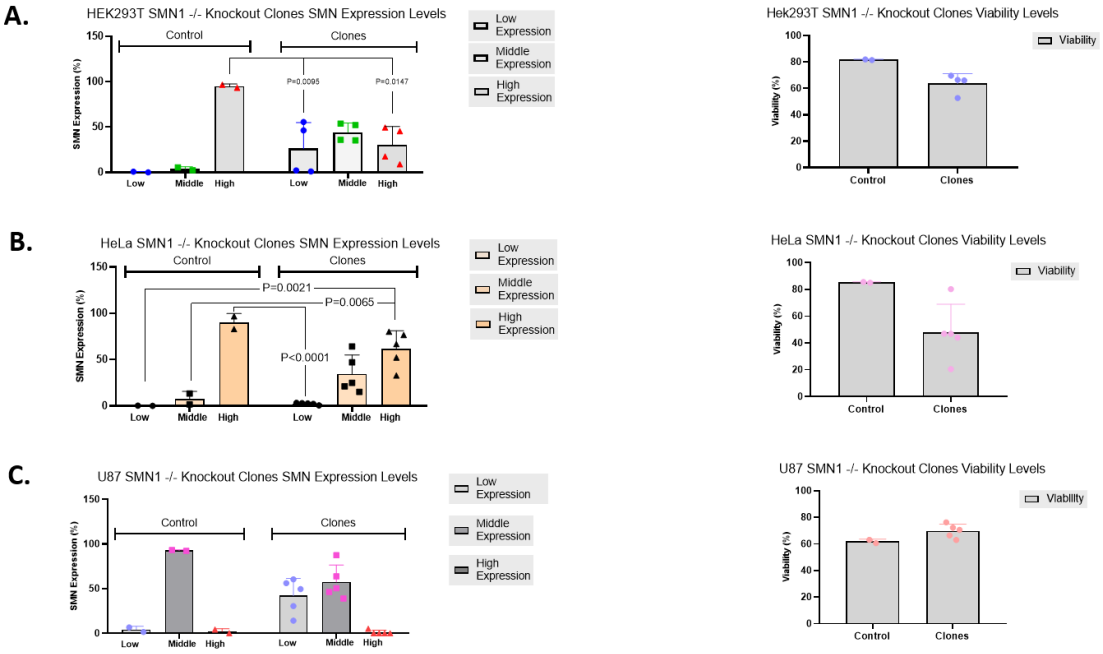


Figure 12. Bar graphs showing SMN expression and viability values in SMN1 knockout HEK293T, HeLa and U87 cell lines. Bar graphs of SMN expression and viaility in SMN1 knockout.

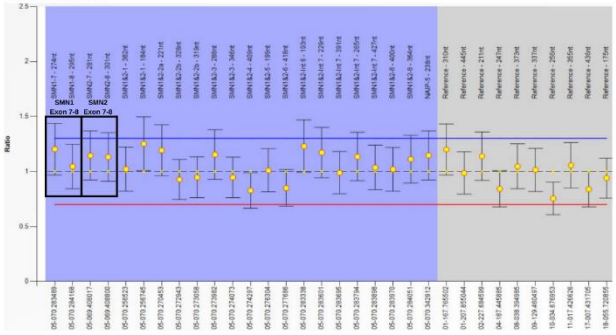
A) HEK293T B) HeLa C) U87 cell line clones. $p<0.05$. NS= Not significant.

4.5 MLPA analysis for *SMN2* copy number in HEK293T *SMN1* knockout cell clones

In order to determine whether the *SMN1* gene was deleted as targeted in the SMA model *SMN1* knockout HEK293T cell line, *SMN1* and *SMN2* copy numbers were determined using the MLPA method with service procurement from Intergen Genetic Diseases Diagnostic Research and Application Center. The MLPA analysis result of the *SMN1* knockout HEK293T cell line clones is given in **Figure 13**. When the analysis results of the control HEK293T cells in **Figure 13A** are examined, it is seen that the *SMN1* exon7-8 and *SMN2* exon7-8 gene regions in the marked boxes are in the same place as other gene regions **(Figure 13A)**. It was determined that the *SMN1* exon7-8 region shifted to negative in the clones in **Figure 13B-C-D and E** and the C2, C3, C4 clone in **Figure 13**.

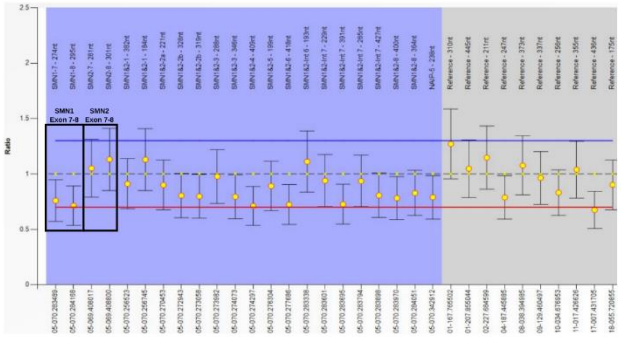
A.

HEK293T Control



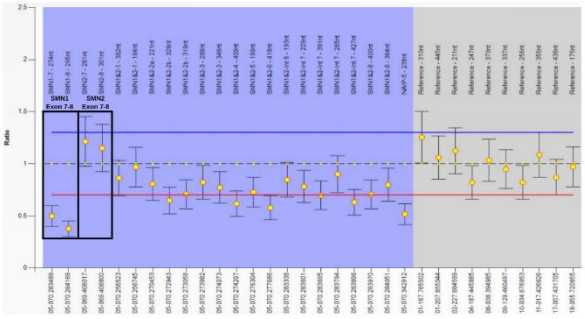
B.

SMN1 Knockout HEK293T Clone C1



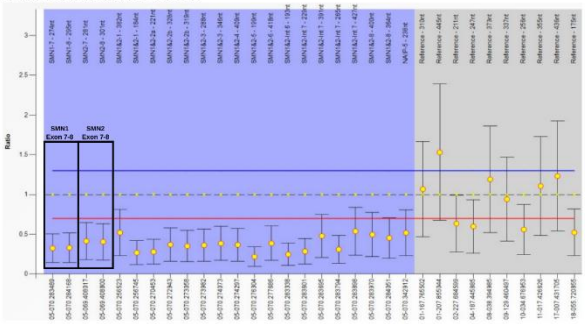
C.

SMN1 Knockout HEK293T Clone C2



D.

SMN1 Knockout HEK293T Clone C3



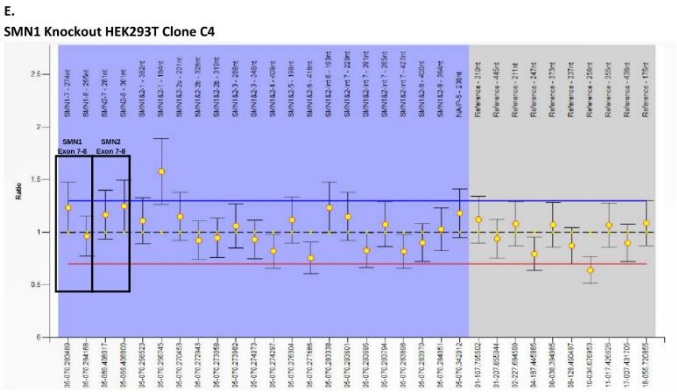


Figure 13. Bar graphs showing SMN expression and viability values in SMN1 knockout HEK293T, HeLa and U87 cell lines. Bar graphs of SMN expression and viability in SMN1 knockout.

A) HEK293T control **B)** C1 clone **C)** C2 clone **D)** C3 clone **E)** Analysis results of C4 clone.

4.7 Flow Cytometry and MTT Analyses in SMA Fibroblast Type 2s with XNA-DNA-ASO

This figure evaluates the effects of treatments developed for SMA (Spinal Muscular Atrophy) disease on cell viability, SMN protein expression and apoptosis under different treatment conditions **(Figure 14).**

In **Figure 14A**, cell viability under different treatment conditions (measured by Forward Scatter-A, FSC-A). In the untreated control, cell viability was determined as 80.38%, while this rate decreased to 63% in PEI transfected cells. While the viability in DNA-ASO treated cells was 54.97%, in XNA-ASO treated cells, the viability was measured as 28% at 200nM concentration, 44.88% at 100nM, and 45.64% at the lowest concentration (1nM).

In Figures **14B and 14C**, SMN protein expression and apoptosis rates were evaluated by flow cytometry. SMN expression showed the best result with a high rate of 92.68% in 100nM XNA-ASO, this concentration was also characterized by the lowest apoptosis rate (7.84%). SMN expression rates and apoptosis levels were also examined in other treatment conditions, SMN expressions of untreated control and PEI transfected control cells were recorded as 53.57% and 53.96%, respectively.

Figure 14D contains morphological images of cells under a microscope. These images show the effects of treatment conditions on cell morphology and reveal that treated cells have a more regular structure.

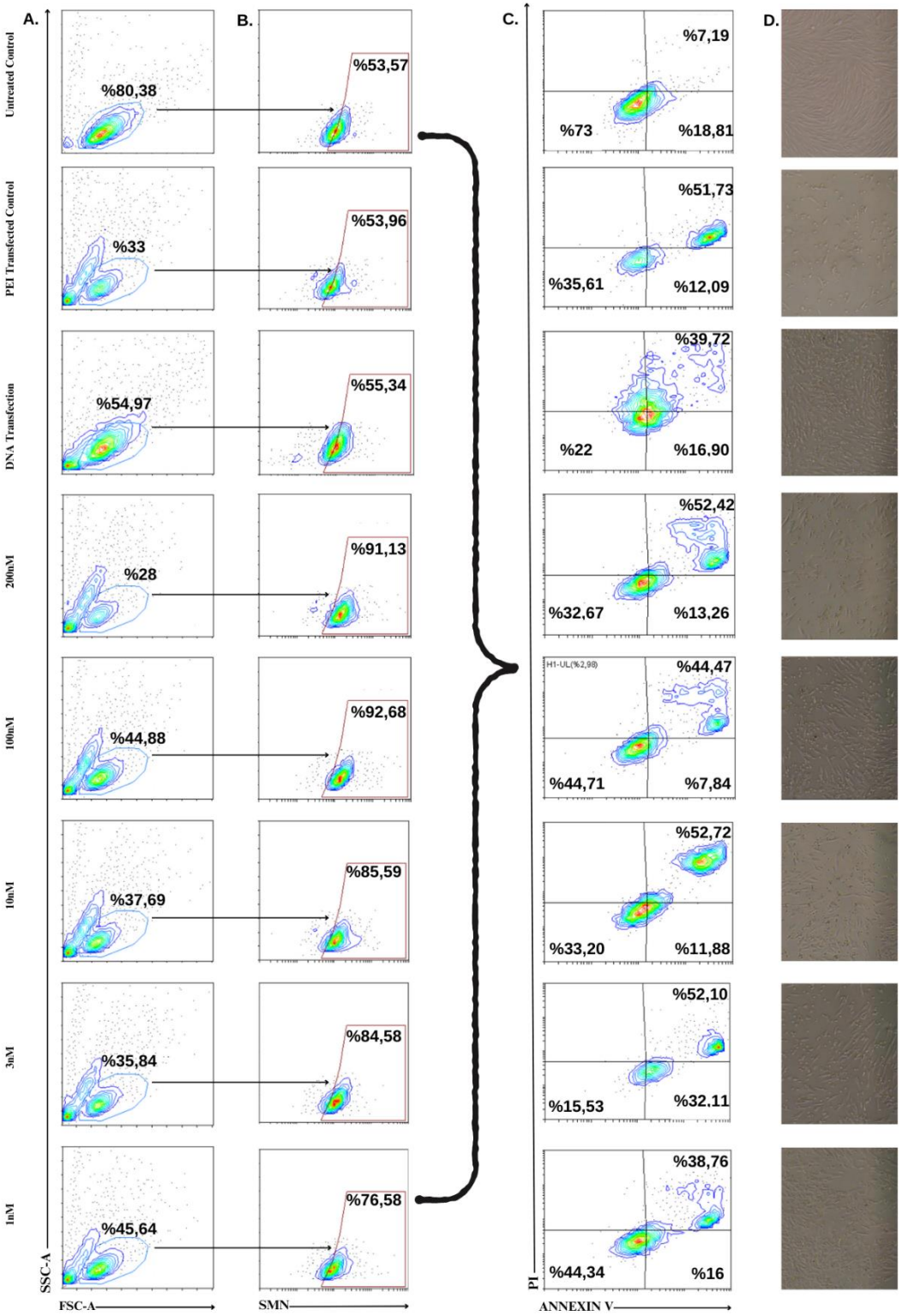


Figure 14. Flow Cytometry Analysis of ASO sequences transfected with PEI.

A. 48th hour analysis of cell viability of SMA Fibroblast cells in flow cytometry treated with untreated control, PEI transfected control, DNA transfection control (GFP DNA), MERLiNA **B**. 48th hour analysis of SMN expression in flow cytometry of SMN antibody staining of SMA fibroblast cells treated with untreated control, PEI transfected control, DNA transfection control (GFP DNA) and MerLiNA doses (200nM, 100nM, 10nM, 3nM, 1nM). **C.** 48th hour flow cytometry analysis of Annexin V-PI antibody staining of SMA fibroblast cells treated with untreated control, PEI transfected control, DNA transfection control (GFP DNA) and MerLiNA doses (200nM, 100nM, 10nM, 3nM, 1nM). **D.** 48th hour 5x phase images of SMA fibroblast cells under fluorescent microscope, treated with untreated control, PEI transfected control, DNA transfection control (GFP DNA) and MerLiNA doses (200nM, 100nM, 10nM, 3nM, 1nM).

These two graphs **(Figure 15)** examine the effects of ASO (antisense oligonucleotide) treatment on SMN expression **(Figure 15A)** and cell survival **(Figure 15B)** in a dose-dependent manner. In the **Figure 15A**, SMN expression is above 80% at 200nM and 100nM ASO concentrations, and is seen at the highest levels. SMN expression is still high at 10nM and 3nM concentrations, approximately 75%. At 1nM concentration, SMN expression decreased slightly to 70%, but it is still significantly higher than the PEI and negative control groups. SMN expression is around 60% and 50% in the PEI and negative control groups, respectively. Cell survival is examined in the **Figure 15B**. Cell survival is at 90% levels at 200nM, 100nM, 10nM and 3nM doses of ASO, and it was observed that these doses did not have a serious negative effect on cell viability. At the 1nM dose, cell survival slightly decreased to around 80%, but this still remains at a healthy level. In the PEI and negative control groups, cell survival was around 60-70%, and ASO treatment appeared to provide better cell survival. These results show that ASO increases SMN expression as the dose increases and significantly protects cell survival.

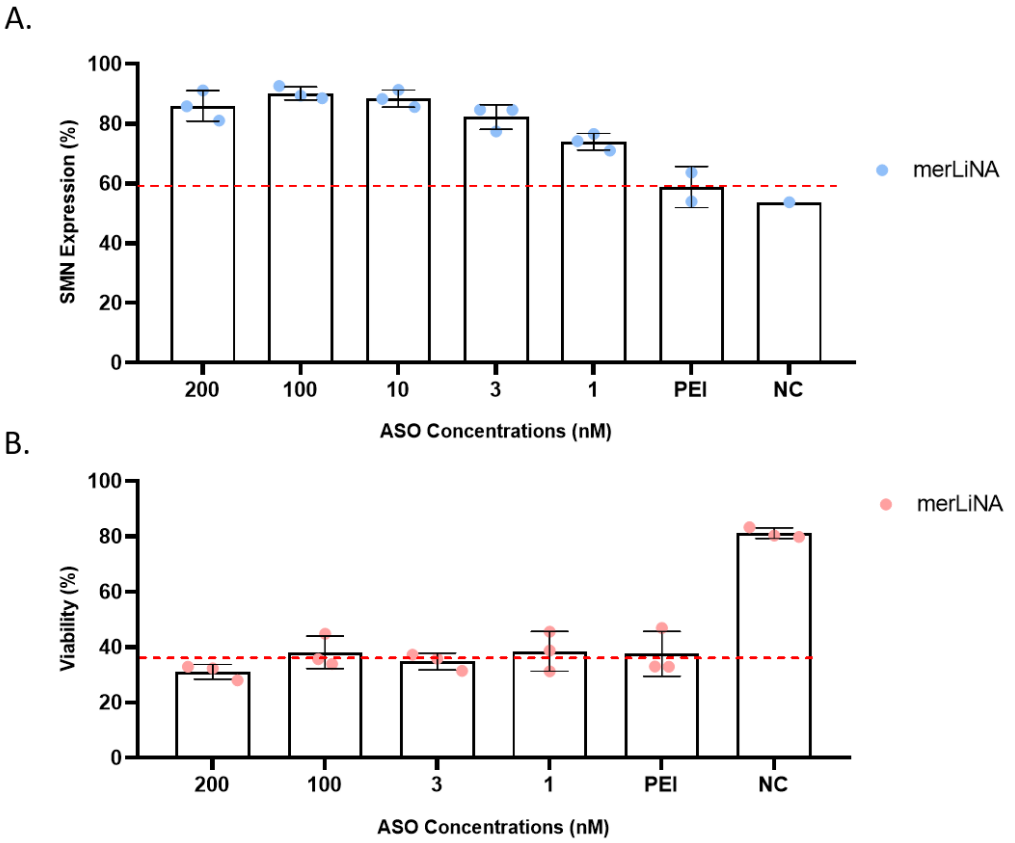


Figure 15. Effects of different ASO (antisense oligonucleotide) concentrations on SMN expression and cell survival.

A. SMN expression was observed at high levels at 200nM, 100nM, 10nM and 3nM concentrations, while this rate decreased slightly at 1nM. SMN expression was lower in PEI and negative control groups. **B.** Cell survival was high at all ASO concentrations, and was preserved at the highest level, especially at 200nM and 100nM concentrations.

Graph **(Figure 16)** shows the effects of different ASO concentrations on SMN protein expression in SMA Type 2 cells. The Y-axis represents the mean expression level of SMN protein (SMN Mean) while the X-axis shows ASO concentrations (in nM) and control groups (PEI and negative control - NC). SMN expression was highest at 100nM and 10nM ASO concentrations, reaching approximately 14,000 units, respectively. At 200nM ASO concentration, this value was slightly lower, around 12,000 units. At lower concentrations (3nM and 1nM), SMN expression decreased significantly and decreased to the lowest levels (around 8,000) in PEI and NC groups. These results show that ASO maximizes SMN expression especially at 100nM and 10nM concentrations, and this effect decreases at lower concentrations.

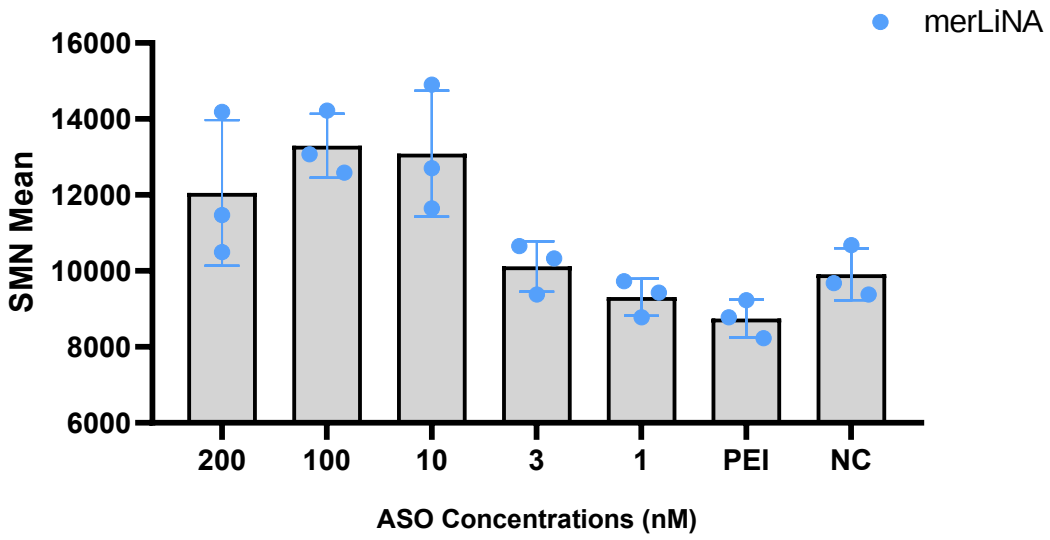


Figure 16. Showing the effects of different ASO concentrations on SMN mean value expression in SMA Type 2 cells.

MTT graph **(Figure 17)** shows the effects of different ASO concentrations on mitochondrial activity in SMA Type 2 cells with the MTT test. At 200nM and 100nM ASO concentrations, mitochondrial activity remained constant at approximately 0.25. At 10nM ASO concentration, activity slightly decreased to around 0.2. At 3nM concentration, a significant increase was observed and activity reached approximately 0.3. The highest mitochondrial activity was observed at 1nM ASO concentration, where activity reached approximately 0.4, indicating that cells were healthier and more viable at this dose. Mitochondrial activity remained low at approximately 0.2 in PEI and DNA transfection groups, and similarly, activity was low in the negative control group. In general, it is observed that low doses of ASO increase mitochondrial activity, while high doses maintain activity.

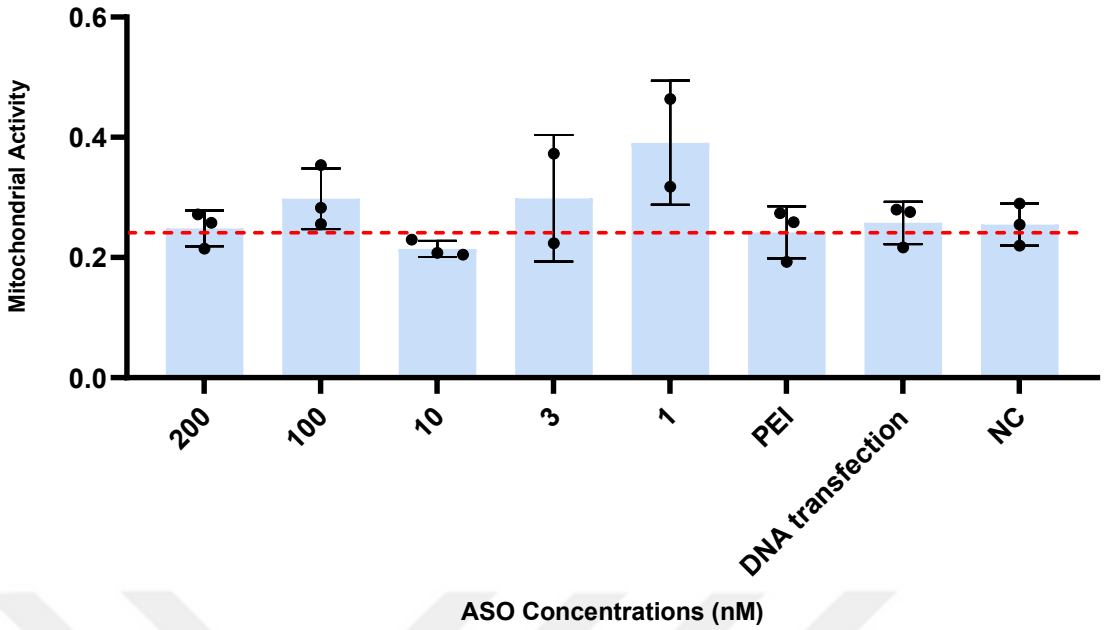


Figure 17. MTT assay results showing the effects of different ASO concentrations on mitochondrial activity in SMA Type 2 cells.

Figure 18 evaluates the effects of different ASO concentrations on cell survival **(Figure 18A)**, early apoptosis **(Figure 18B)** and late apoptosis **(Figure 18C)** in SMA cells, considering the use of PEI (Polyethylenimine) at all ASO doses. In graph **18A** (survival), survival rates were kept around 50% with ASO + PEI combinations, while survival rates were preserved with high concentrations of ASO (200nM and 100nM). This shows that PEI contributes to cell survival at ASO doses. In graph **18B** (early apoptosis), it was observed that the early apoptosis rate increased significantly at 3nM ASO dose, while PEI triggered apoptosis in cells at these doses, but the apoptosis rate was low at high ASO concentrations (200nM and 100nM). In graph **18C** (late apoptosis), it is understood that late apoptosis with ASO + PEI combinations is between 40% and 60% at all doses, and PEI stabilizes late apoptosis rates in cells even at high ASO concentrations. These data suggest that PEI has positive effects on cell survival when used together with ASO, and suppresses apoptosis especially at high ASO doses.

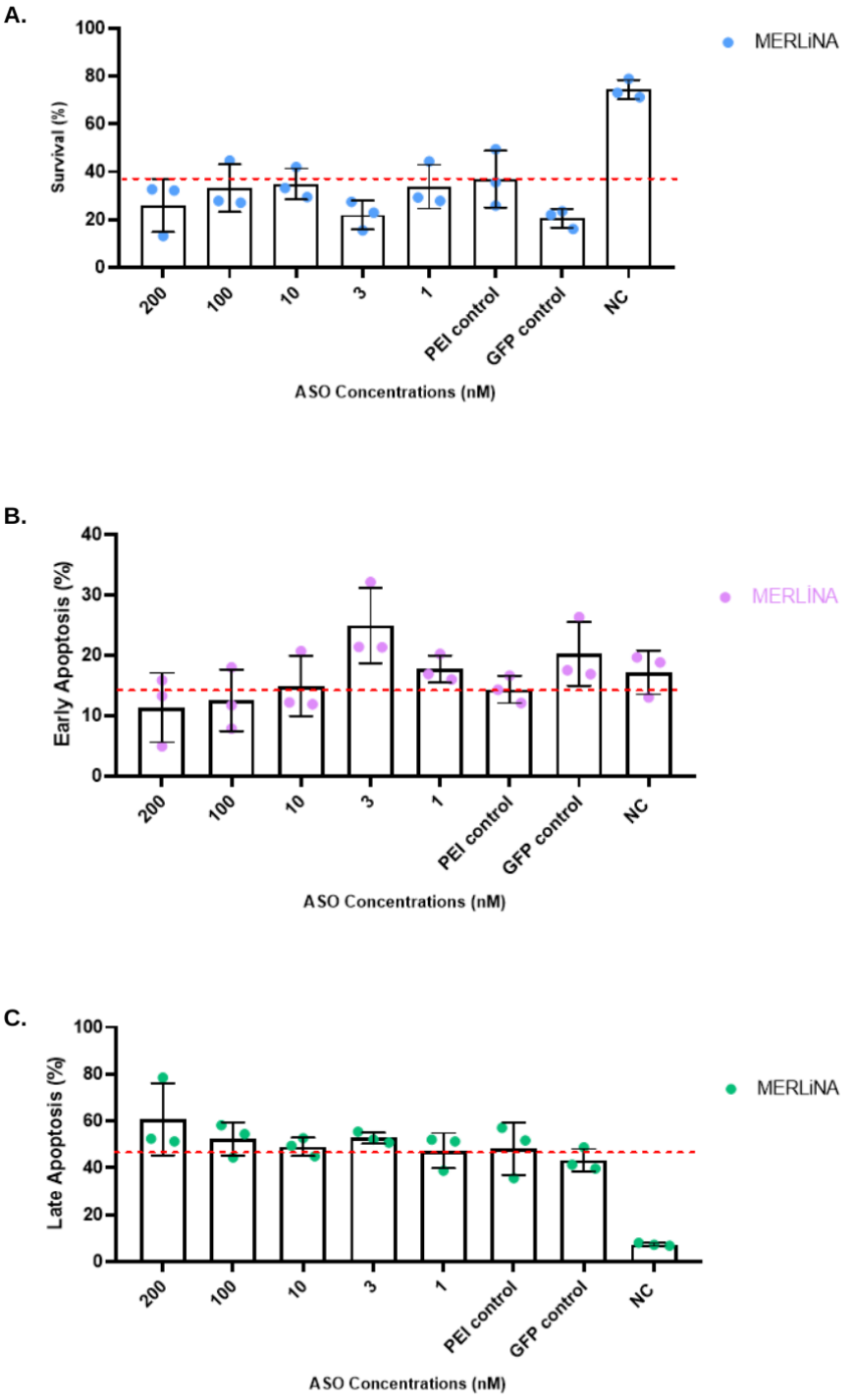


Figure 18. Effects of different ASO concentrations on cell survival

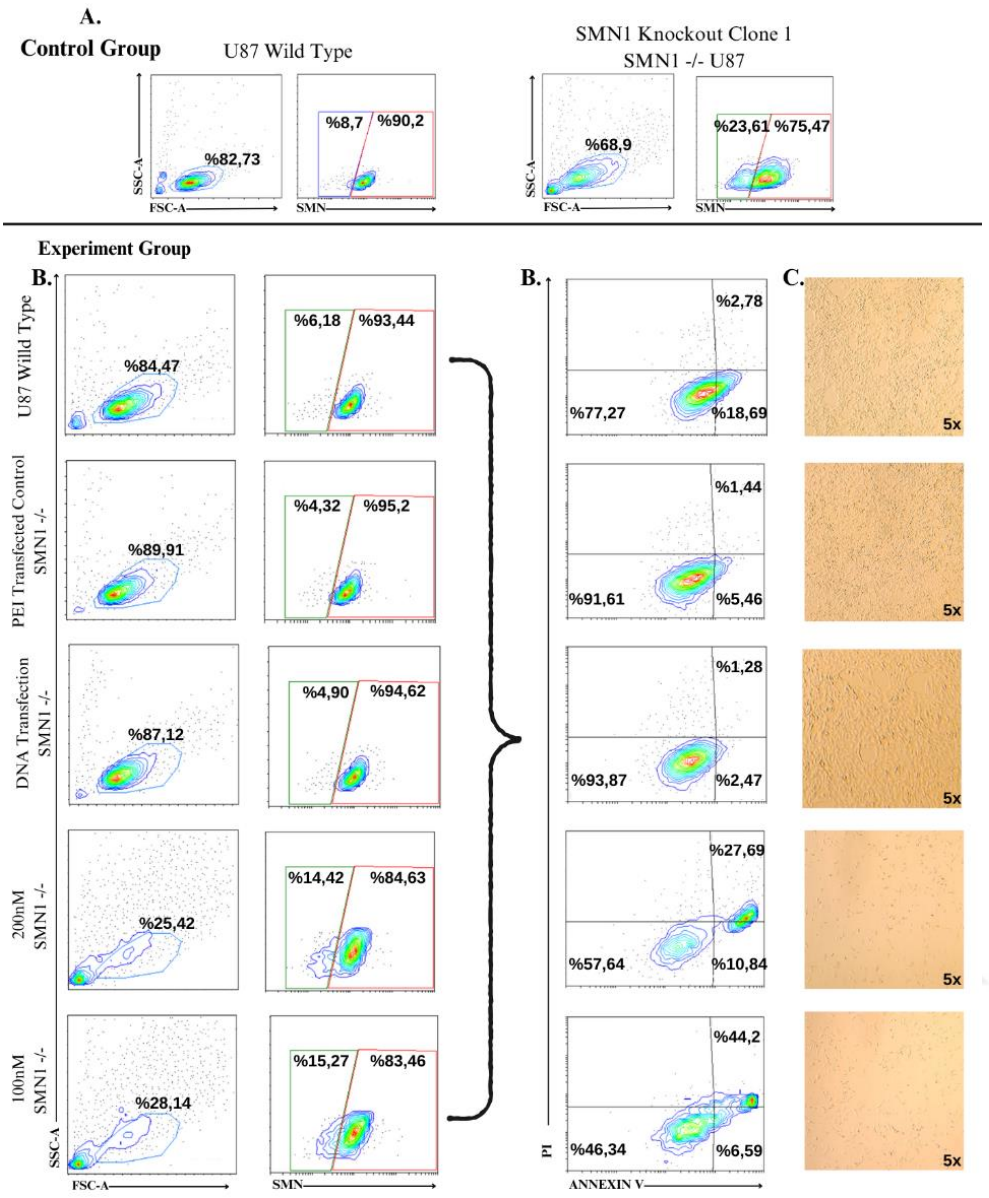
(A), early apoptosis (B), and late apoptosis (C) in SMA cells when used together with PEI.

4.8 Flow Cytometry and Statistic Analyses in U87Cell Line *SMN1* Knockout (*SMN1*-/-) with XNA-DNA-ASO

This experiment examines the effects of XNA-DNA-ASO (MerLiNA) treatment on SMN expression, cell survival, and apoptosis rates in U87 Wild Type and *SMN1* -/- U87 cell lines. The experiment is divided into three main parts **Figure 19A** SMN expression, **Figure 19B** apoptosis rate, and **Figure 19C** cell morphology.

In U87 Wild Type cells, SMN expression was 90.2%, and 82.73% of the cells remained alive. In *SMN1* -/- U87 cells, SMN expression decreased to 75.47%, and the viability rate decreased to 68.9%. These results show that *SMN1* loss negatively affects SMN protein production and cell survival **(Figure 19A)**.

In the experimental group, SMN expression increased to 93.44% in U87 Wild Type cells treated with PEI and DNA transfections. In *SMN1* -/- cells, SMN expression increased up to 95.2% after PEI and DNA transfections. MerLiNA treatment increased SMN expression in *SMN1* -/- cells to 84.63% and 83.46%, especially at 200nM and 100nM concentrations, respectively. At the same time, this treatment kept apoptosis rates at low levels and increased cell survival. When examined in terms of apoptosis rates, apoptosis rates were observed at 66.46% and 61.46% in PEI and DNA transfections, respectively, while these rates decreased to 66.59% at 200nM and 100nM ASO concentrations with MerLiNA treatment. Significant results were also obtained in MerLiNA treatment with low concentrations (10nM, 3nM and 1nM). With 10nM ASO treatment, SMN expression increased to 83.31%, while at 1nM concentration, SMN expression reached 85.92%. Apoptosis rates were kept under control at low doses and cell survival-supporting results were obtained. For example, in cells treated with 1nM MerLiNA, the late apoptosis rate remained at 64.4% **(Figure 19B)**.


A.
Control Group
U87 Wild Type
SMN1 Knockout Clone 1
SMN1 -/- U87
%82,73
%8,7
%90,2
%68,9
%23,61
%75,47
SSC-A
FSC-A
SMN
Experiment Group
B.
C.
U87 Willd Type
PEI Transfected Control SMN1 -/-
DNA Transfection SMN1 -/-
200nM SMN1 -/-
100nM SMN1 -/-
%84,47
%6,18
%93,44
%89,91
%4,32
%95,2
%87,12
%4,90
%94,62
%25,42
%14,42
%84,63
%28,14
%15,27
%83,46
%2,78
%77,27
%18,69
%1,44
%91,61
%5,46
%1,28
%93,87
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%46,34
%6,59
PI
ANNEXIN V
5x

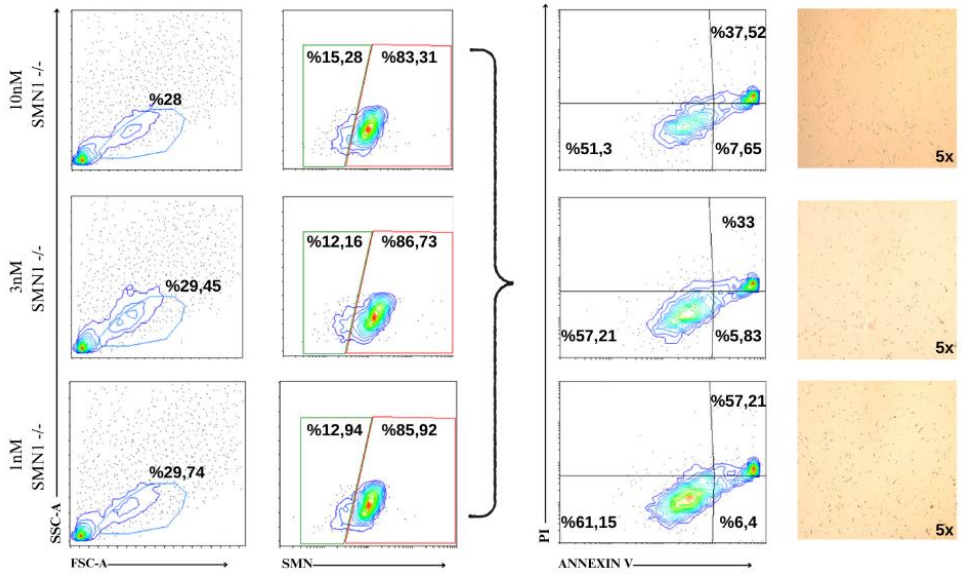

Figure 19. Effects of XNA-DNA-ASO (MerLiNA) treatment on SMN expression, apoptosis and cell survival in U87 Wild Type and SMN1 -/- U87 cells.

A. SMN expression, **B.** apoptosis rate, and **C.** cell morphology

Graph shows **(Figure 20)** the effects of XNA-DNA-ASO (MerLiNA) treatment on SMN expression and cell viability in U87 *SMN1* knockout (*SMN1* -/-) cells. In graph **20A**, the effect of different ASO concentrations on SMN expression is examined. Although SMN expression remained at low levels in general at all ASO concentrations, a slight increase was observed especially at 100nM and 10nM doses. This increase is remarkable compared to PEI and Negative Control (NC) groups because SMN expression was almost zero in these control groups. This shows that MerLiNA treatment can increase SMN expression, albeit low, compared to control groups. In graph **20B**, cell viability was evaluated. While cell viability remained at 20-30% levels in all ASO applied groups, the viability rate was above 80% in PEI and NC groups.

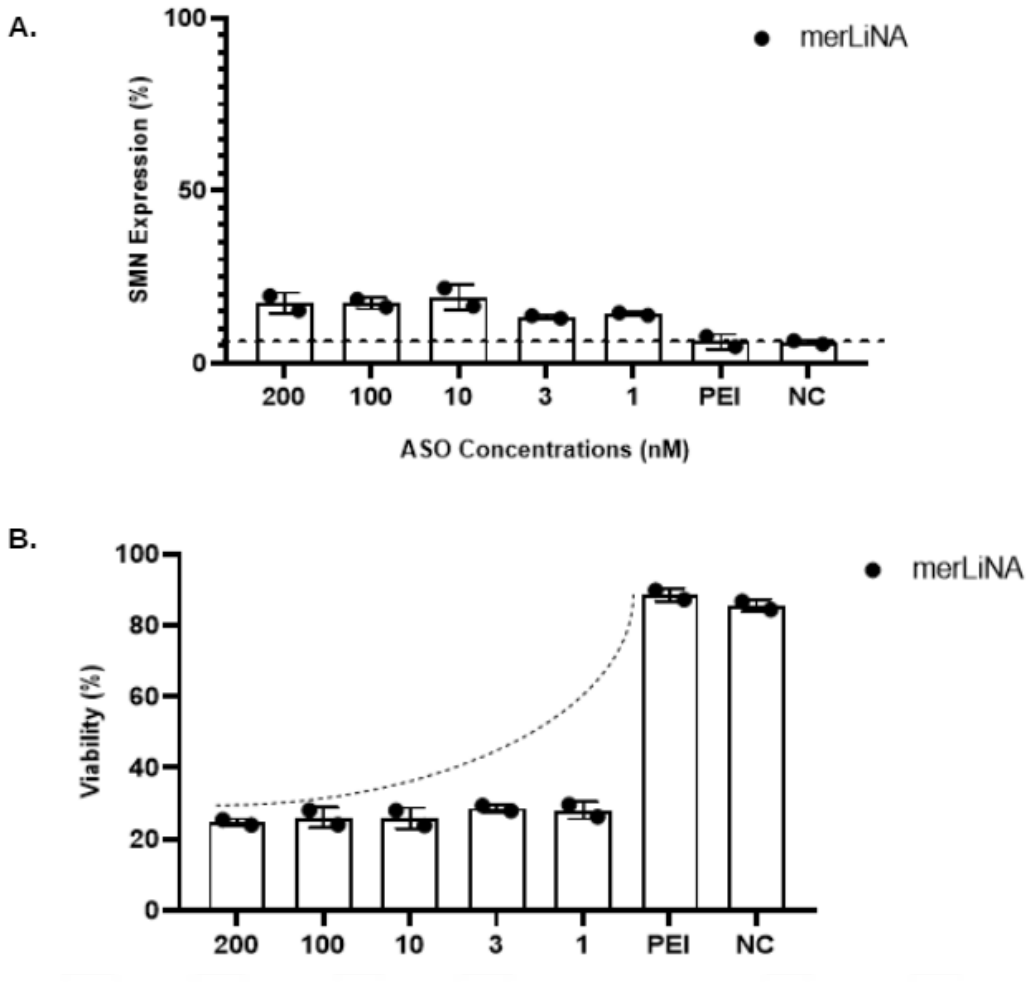

Figure 20. Effects of XNA-DNA-ASO (MerLiNA) treatment on

A. SMN expression and **B.** cell viability in U87 SMN1 −/− cells.

Graph shows **(Figure 21)** the mean values of different ASO concentrations and control groups in U87 cells. High ASO concentrations (200nM and 100nM) kept the mean value at around 270,000, indicating that the treatment was effective. Although this value continued with a slight decrease at 10nM, it remained at an effective level. With low ASO concentrations (3nM and 1nM), the mean values decreased significantly, falling below 250,000. When looking at the control groups, it is seen that the mean value was unexpectedly high in the negative control group, while the mean values remained low in the PEI and GFP control groups. These results show that high ASO doses are more effective, that the treatment efficacy decreases at low doses, and that the abnormally high value in the negative control group should be investigated.

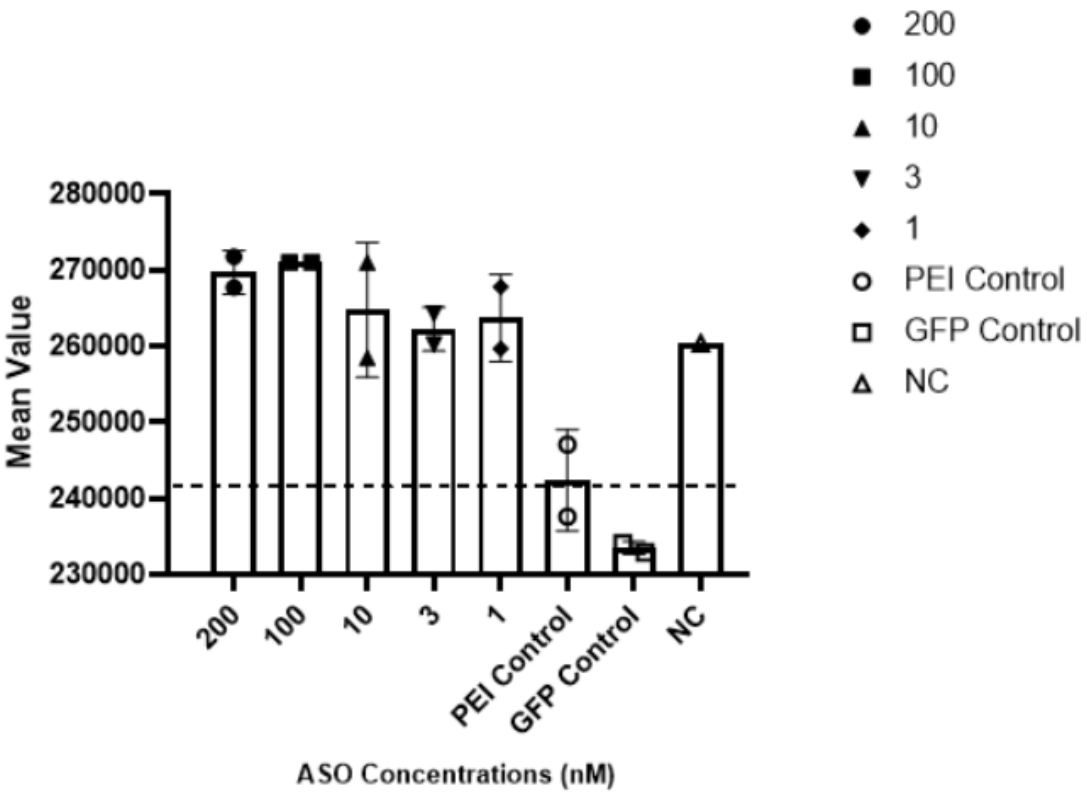

Figure 21. Mean values of different ASO concentrations and control groups in U87 cells.

5.DISCUSSION

In this study, the effects of XNA-DNA-ASO (MerLiNA) treatment on SMN expression, cell viability and apoptosis in *SMN1* knockout (*SMN1* -/-) cells and SMA Type 2 cells were examined. The findings of the study revealed that XNA-DNA-ASOs have a limited but significant effect in increasing SMN expression and this effect varies depending on the applied ASO dose.

First of all, when the effect of XNA-DNA-ASO on SMN expression was examined in *SMN1* knockout cells, a slight but visible increase was observed, especially at high ASO concentrations (200nM and 100nM). As seen in the graphs, SMN expression exhibited a significant increase, albeit low, compared to the PEI and negative control groups. Especially the 100nM and 10nM doses significantly increased SMN expression compared to the control groups. These findings suggest that XNA-DNA-ASOs may provide an improvement in SMN protein production and support gene expression at low levels.

In terms of cell viability, it was observed that the viability rates remained at 20-30% in cells treated with XNA-DNA-ASO, but cell viability reached 80% in PEI and negative control groups. This indicates that ASO treatment is limited in protecting cell survival in *SMN1* knockout cells, but the response to the treatment is not positive when compared to PEI control. Compared to other ASO types, the effect of XNA-DNA-ASOs on cell viability was weak, indicating that XNA-DNA-ASOs need to be made more effective.

In addition, a slight increase in SMN expression was observed in low concentration ASO applications (especially 3nM and 1nM). These increases show that XNA-DNA-ASOs can contribute to gene expression at a certain level even at low doses and that toxicity or side effects on the cell can be kept to a minimum at these doses. However, when looking at cell viability, these effects were limited at low doses and no significant improvement was achieved compared to the PEI and control groups.

The fact that XNA-DNA-ASO generally did not show the expected efficacy in the treatment of *SMN1* knockout cells may be related to the intracellular uptake of the treatment, stability in the cell and binding efficiency to SMN mRNA. Although the chemical modifications used in ASO treatments (such as the combination of XNA and DNA) have the potential to increase efficacy, these modifications need to be optimized to increase bioavailability in the cell. When compared to other types of antisense oligonucleotides (ASOs), such as FDA-approved ASOs such as nusinersen, it has been observed that XNA-DNA-ASOs have lower efficacy. While ASOs such as nusinersen target the alternative splicing regulation of the *SMN2* gene, it appears that further design modifications are needed to increase the efficacy of XNA-DNA-ASOs in *SMN1* knockout cells.

Based on these findings, it was observed that XNA-DNA-ASOs have potential but were not able to provide sufficient SMN expression and cell viability in the treatment of SMA in the current study. It is clear that optimizations should be made in the chemical structure and application protocols of ASOs to increase the treatment efficacy. Comparisons with other ASOs indicate that further research and more effective modifications should be applied to improve the effects of XNA-DNA-ASO on cell survival and SMN expression.

6. CONCLUSION AND RECOMMENDATIONS

The findings in this study, which demonstrate the potential of XNA-DNA-ASOs (MerLiNA) in the treatment of SMA, indicate that some important directions are needed for the future. In particular, the limited efficacy obtained in *SMN1* knockout (*SMN1* -/-) cells indicates that ASO treatments need to be developed and optimized. Several important research areas have been identified for future studies.

First, optimizing the chemical modifications of XNA-DNA-ASOs is of great importance. The low increase in SMN expression can be improved by chemical modifications that will increase the intracellular stability, bioavailability and binding efficiency of XNA and DNA combinations to target mRNA. The therapeutic effect of ASOs can be increased with alternative modifications such as Locked Nucleic Acid (LNA), phosphorothioate (PS) or 2'-O-methyl RNA. These modifications have the potential to provide intracellular stability, more effective binding to SMN mRNA and increase RNase H activation.

Another important research area is to improve the uptake and intracellular distribution of ASOs. Liposomal carriers, nanoparticles, or advanced versions of polyethylenimine (PEI) can be used to more efficiently enter the cell by XNA-DNA-ASOs. Nanoparticle technology can increase the effect of targeted therapy by directing ASO to specific cells. In addition, endosomal escape mechanisms should be developed to prevent endosomal capture of ASO, and pH-sensitive carrier systems and agents that will allow escape from the endosome should be used.

Combining ASO treatments with gene therapies is also a promising approach for future studies. XNA-DNA-ASOs can provide more potent therapeutic effects by targeting *SMN1* gene re-expression and alternative splicing regulation of *SMN2* gene when combined with gene therapies such as adeno-associated virus (AAV). Such combination strategies may allow the development of personalized treatment protocols for different types of SMA. In addition, combination treatments with ASOs that regulate *SMN2* gene expression may help achieve better results, especially in *SMN1* knockout cells.

Studies should be conducted on animal models to evaluate the effectiveness of ASOs in more complex systems. The pharmacokinetic and pharmacodynamic profiles of ASOs should be tested using SMA mouse models, and their capacity to reach target

tissues such as the brain and spinal cord should be evaluated. Long-term effects of ASOs on neurological functions can be examined in these models. Animal models will provide preclinical validation and constitute an important step in evaluating the effects and possible side effects of ASOs before moving on to human clinical trials.

In terms of clinical trials, appropriate patient groups should be determined to test the effects of XNA-DNA-ASOs. Treatment protocols should be created by considering the genetic profiles of SMA patients, and treatment strategies specific to patient subgroups can be developed according to the degree of *SMN1* gene loss. Biomarker-based evaluations should be conducted to determine patient subgroups in which ASO treatments may be more effective, and patients who respond to treatment should be carefully monitored.

The safety profile of XNA-DNA-ASOs should also be carefully evaluated. During long-term applications, possible side effects such as immune responses, hepatotoxicity or renal toxicity should be investigated and findings obtained at the cellular level should be confirmed in animal models. Conducting such toxicity and safety studies before clinical trials will increase the long-term reliability of the treatment.

Finally, correct dose optimization is required to increase the therapeutic efficacy of XNA-DNA-ASOs. Mild SMN expression increases obtained at low doses can be optimized by monitoring clinical responses. With biomarker-based assessments, the effects of ASO on target tissues can be monitored using data obtained from biological fluids such as spinal fluid or blood. In this way, clinical responses to treatment can be evaluated more clearly.

As a result, chemical modifications, intracellular uptake strategies, combined treatment methods and clinical trial strategies need to be improved in order for XNA-DNA-ASOs to become more effective and reliable therapeutic agents in the treatment of SMA. These approaches will allow more successful results to be obtained in the treatment of SMA and will increase the therapeutic potential of XNA-DNA-ASO.

REFERENCES

Bennett, C. F., & Swayze, E. E. (2010). RNA targeting therapeutics: Molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Annual Review of Pharmacology and Toxicology, 50, 259-293. https://doi.org/10.1146/annurev.pharmtox.010909.105654

Braasch, D. A., & Corey, D. R. (2001). Locked nucleic acid (LNA): Fine-tuning the recognition of DNA and RNA. Chemistry & Biology, 8(1), 1-7. https://doi.org/10.1016/S1074-5521(01)00086-3

Chiriboga, C. A., Swoboda, K. J., Darras, B. T., Iannaccone, S. T., Montes, J., De Vivo, D. C., & Bishop, K. M. (2016). Results from a phase 1 study of nusinersen (ISIS-SMN Rx) in children with spinal muscular atrophy. Neurology, 86(10), 890-897. https://doi.org/10.1212/WNL.0000000000002445

Corey, D. R. (2017). Nusinersen, an antisense oligonucleotide drug for spinal muscular atrophy. Nature Neuroscience, 20(4), 497-499. https://doi.org/10.1038/nn.4502

Crooke, S. T., Witztum, J. L., Bennett, C. F., & Baker, B. F. (2018). RNA-targeted therapeutics. Cell Metabolism, 27(4), 714-739. https://doi.org/10.1016/j.cmet.2018.03.004

De Vivo, D. C., Bertini, E., Swoboda, K. J., et al. (2019). Nusinersen as a therapeutic option for spinal muscular atrophy: A review of clinical efficacy and safety. Developmental Medicine & Child Neurology, 61(5), 656-661. https://doi.org/10.1111/dmcn.14129

Eckstein, F. (2000). Phosphorothioate oligodeoxynucleotides: What is their origin and what is unique about them? Antisense and Nucleic Acid Drug Development, 10(2), 117-121. https://doi.org/10.1089/oli.1.2000.10.117

Finkel, R. S., Mercuri, E., Darras, B. T., et al. (2017). Nusinersen versus sham control in infantile-onset spinal muscular atrophy. The New England Journal of Medicine, 377(18), 1723-1732. https://doi.org/10.1056/NEJMoa1702752

Hagedorn, P. H., Persson, R., et al. (2017). Locked nucleic acid: A potent antisense oligonucleotide chemistry targeting TRIM72 RNA. Scientific Reports, 7, 3672. https://doi.org/10.1038/s41598-017-03850-2

Haque, U. S., & Yokota, T. (2023). Enhancing antisense oligonucleotide-based therapeutic delivery with DG9, a versatile cell-penetrating peptide. Molecular Therapy, 31(1), 89-101. https://doi.org/10.1016/j.ymthe.2022.09.003

Hua, Y., Sahashi, K., Hung, G., Rigo, F., Passini, M. A., Bennett, C. F., & Krainer, A. R. (2010). Antisense correction of *SMN2* splicing in the CNS rescues necrosis in a severe spinal muscular atrophy mouse model. Genes & Development, 24(15), 1634-1644. https://doi.org/10.1101/gad.1941310

Kurreck, J. (2003). Antisense technologies: Improvement through novel chemical modifications. European Journal of Biochemistry, 270(8), 1628-1644. https://doi.org/10.1046/j.1432-1033.2003.03555.x

Lunn, M. R., & Wang, C. H. (2008). Spinal muscular atrophy. The Lancet, 371(9630), 2120-2133. https://doi.org/10.1016/S0140-6736(08)60921-6

Morihiro, K., et al. (2016). Nucleic acid nanocapsules incorporating XNA/DNA hybrid structures for targeting exon splicing in *SMN2*. Journal of Molecular Biology, 428(5), 1179-1192. https://doi.org/10.1016/j.jmb.2016.01.011

Naryshkin, N. A., et al. (2014). *SMN2* splicing modulators enhance SMN protein levels in spinal muscular atrophy patients. Science, 345(6197), 688-693. https://doi.org/10.1126/science.1250127

Nishio, H., Niba, E. T. E., Saito, T., Okamoto, K., Takeshima, Y., & Awano, H. (2022). Spinal muscular atrophy: The past, present, and future of diagnosis and treatment. International Journal of Molecular Sciences, 23(10), 5150. https://doi.org/10.3390/ijms23105150

Petersen, M., & Wengel, J. (2003). LNA: A versatile tool for therapeutics and genomics. Trends in Biotechnology, 21(2), 74-81. https://doi.org/10.1016/S0167-7799(02)00038-0

Prior, T. W. (2010). Spinal muscular atrophy: A time for screening. Current Opinion in Pediatrics, 22(6), 696-702. https://doi.org/10.1097/MOP.0b013e32833f3046

Stein, C. A., & Castanotto, D. (2017). FDA-approved oligonucleotide therapies in 2017. Molecular Therapy, 25(5), 1069-1075. https://doi.org/10.1016/j.ymthe.2017.03.023

Watts, J. K., & Corey, D. R. (2012). Silencing disease genes in the laboratory and the clinic. Journal of Clinical Investigation, 122(8), 3056-3060. https://doi.org/10.1172/JCI63061

Zogg, H., Singh, R., & Ro, S. (2021). Current advances in RNA therapeutics for human diseases. Science Progress, 104(3), 100878. https://doi.org/10.1016/j.spen.2021.100878

Lunn, M. R., & Wang, C. H. (2008). Spinal muscular atrophy. Lancet (London, England), 371(9630), 2120-2133. https://doi.org/10.1016/S0140-6736(08)60921-6