The Application of Clinical Genetics

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REVIEW

Spinal Muscular Atrophy: Mutations, Testing, and Clinical Relevance

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Abstract: Spinal muscular atrophy (SMA) is a heritable neuromuscular disorder that causes degeneration of the alpha motor neurons from anterior horn cells in the spinal cord, which causes severe progressive hypotonia and muscular weakness. With a carrier frequency of 1 in 40–50 and an estimated incidence of 1 in 10,000 live births, SMA is the second most common autosomal recessive disorder. Affected individuals with SMA have a homozygous loss of function of the survival motor neuron gene *SMN1* on 5q13 but keep the modifying *SMN2* gene. The most common mutation causing SMA is a homozygous deletion of the *SMN1* exon 7, which can be readily detected and used as a sensitive diagnostic test. Because *SMN2* produces a reduced number of full-length transcripts, the number of *SMN2* copies can modify the clinical phenotype and as such, becomes an essential predictive factor. Population-based SMA carrier screening identifies carrier couples that may pass on this genetic disorder to their offspring and allows the carriers to make informed reproductive choices or prepare for immediate treatment for an affected child. Three treatments have recently been approved by the Food and Drug Administration (FDA). Nusinersen increases the expression levels of the SMN protein using an antisense oligonucleotide to alter splicing of the *SMN2* transcript. Onasemnogene abeparvovec is a gene therapy that utilizes an adeno-associated virus serotype 9 vector to increase low functional SMN protein levels. Risdiplam is a small molecule that alters *SMN2* splicing in order to increase functional SMN protein. Newborn screening for SMA has been shown to be successful in allowing infants to be treated before the loss of motor neurons and has resulted in improved clinical outcomes. Several of the recommendations and guidelines in the review are based on studies performed in the United States.

Keywords: spinal muscular atrophy, carrier screening, newborn screening, SMA treatment

Clinical Manifestations

Spinal muscular atrophy, an autosomal recessive disorder, is the most common genetic cause of infant mortality, affecting 1 in 10,000 live births.[1] The disorder causes progressive loss of the alpha motor neurons of the ventral spinal cord and motor nuclei of the lower brainstem resulting in hypotonia, muscle weakness and atrophy of variable severity depending on the underlying genotype.[1] Weakness is predominantly proximal with greater involvement of the lower extremities and diffuse areflexia on examination. Bulbar and respiratory muscle weakness can occur particularly in more severe cases. Facial and ocular muscles are generally not involved.[2]

As shown in Table 1, the disorder has traditionally been classified into types 0–4 based on symptom severity and genotype, though with recent disease-modifying

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Table 1 Classification of Spinal Muscular Atrophy

SMA Type	Copies *SMN2*	Percent of Cases	Onset	Motor Milestones	Clinical Features	Natural History Prior to Disease-Modifying Therapy
0	1	Rare, <1%	Prenatal, at birth	Non-sitter, no head control	Generalized weakness, hypotonia, respiratory failure, poor feeding, contractures	Death within weeks of birth
1	1–2	45%	0–6 mo	Non-sitter	Proximal predominant weakness, respiratory insufficiency, poor feeding, tongue fasciculations	Death by age 2
2	3	20%	6–18 mo	Sits independently, never stands or ambulates	Proximal predominant weakness, tongue fasciculations, minipolymyoclonus, scoliosis	Most alive at 25 years
3	3–4	30%	A: 18 mo −3yr B: 3–30 yr	Ambulates independently	Proximal, lower extremity predominant weakness, abnormal gait	Normal lifespan
4	4 or more	<5%	> 30 yr	Ambulates independently	Maintain ability to ambulate	Normal lifespan

therapies, phenotypes have become more diverse and classifications have evolved to focus on functional status (non-sitters, sitters, walkers), or treatment response (decline, no change, improvement).[3]

Under the traditional classification, type 0 SMA represents the most severe phenotype with one copy of *SMN2*. Patients present at birth with generalized weakness and hypotonia, respiratory distress, and poor feeding. Decreased intrauterine movements may be felt prenatally and lead to contractures. Prior to disease-modifying therapy, patients died within weeks of birth without achieving any motor milestones (non-sitters).[2,4]

Patients with type 1 SMA, the most common form and representing approximately 45% of cases, develop symptoms around 0–6 months of age, including flexion, proximal predominant limb weakness, respiratory insufficiency, and poor feeding. Given intercostal and chest wall muscle weakness, relative to preserved diaphragm strength, patients show a bell-shaped chest deformity with breathing and paradoxical breathing. Tongue fasciculations are present, but facial and ocular muscle strength remains intact. Cognitive function is normal to above average. Historically, patients did not achieve the ability to sit independently (non-sitters) with death often prior to age 2.[2,5] Most of the type 1 patients have one to two copies of *SMN2*.

Type 2 SMA, comprising 30% of cases, presents with weakness by 6–18 months and most often have three *SMN2* copies. Patients achieve the ability to sit unsupported (sitters), often by 9 months, though may later lose this ability and are never able to stand or walk independently.[2] Examination shows predominantly proximal weakness, more severe in the lower extremities, tongue atrophy and fasciculations, and sometimes fine distal tremor (minipolymyoclonus). Respiratory insufficiency and dysphagia are common, particularly in more severe phenotypes.[2] Weak axial musculature may contribute to significant scoliosis which may worsen restrictive lung disease and respiratory insufficiency.[3] Aggressive supportive treatments prior to the onset of disease-modifying therapy led to increased lifespan, with 68.5% of this historic cohort surviving to age 25.[3,5]

With onset ranging from 18 months to adulthood, type 3 SMA, representing 15% of cases, is defined by achieving the ability to stand or walk without support (walkers), though this ability may be lost with disease progression.[2] Patients may present with symptoms of proximal weakness such as falls, abnormal gait, and difficulty climbing stairs. Unlike other phenotypes, type 3 patients have a normal life expectancy.[2,6] Most patients do not develop significant respiratory muscle weakness.[6] Walkers may be sub-grouped into type 3a with symptoms onset of 18 months-3 years, type 3b with onset 3–30 years, and type 4 with onset at 30 years or more.[3] The milder type 3 and 4 patients usually present with 3 and 4 copies of *SMN2*.

Although SMA is defined as a disorder of the motor neurons, SMN protein is expressed in most tissues with different organs and tissues varying in their requirements for development and functioning. Research in animal models and patients has shown that SMA is a multisystem disease also affecting the skeletal muscle, heart, kidney, liver, pancreas, spleen, and immune system. Multiorgan features including congenital heart defects, cardiac rhythm abnormalities, sleep disturbances, impaired kidney function, and pancreatic defects, have previously been reported in SMA patients, particularly in the more severely affected type 0 or 1 subtypes. As patients live longer with new therapies, these multisystem comorbidities may become more common.[7–11]

Gene Structure

The majority of SMA cases are caused by mutations in the survival motor neuron 1 (*SMN1*) gene positioned at 5q13.[12] *SMN1* (also called *SMNT*, where T stands for telomere) spans 20-kb and lies in the telomeric portion of an inverted duplication of 500kb, a DNA architecture prone to rearrangements and deletions (Figure 1). The duplicated centromeric element, known as *SMN2* (also called C-BCD541 and *SMNC*, where C stands for centromere) is highly homologous to *SMN1* with more than 99% nucleotide identity. First thought to have 8 exons, both *SMN1* and *SMN2* contain 9 exons that encode the 294-amino acid protein, survival of motor neuron (SMN).[12,13] The exons are numbered 1, 2a, 2b, 3, 4, 5, 6, 7 and 8. The stop codon for SMN occurs in exon 7, and exon 8 is left untranslated. *SMN1* and *SMN2* vary by 8 nucleotides, 5 of which are intronic and 3 of which occur in the last 3 exons. Of the differences, only a C-to-T transition in *SMN2* exon 7 (specifically, c.840C>T) falls in a coding region and disrupts an exonic splice enhancer in exon 7. As a consequence of this change, most of the *SMN2* transcripts lack exon 7, creating an incomplete and degraded SMN protein. An estimated 10% of the protein produced by each *SMN2* copy is functional, making it a modifying gene (Figure 1). SMN is an RNA-binding protein that contributes to many cellular processes and pathways, most notably, playing a critical role in snRNP complex assembly in the cytoplasm.[14]

Pathogenic Variants

The majority of SMA cases are caused by an absence of the *SMN1* gene. Ninety-five percent of SMA affected individuals have a homozygous deletion of *SMN1* exon 7 or gene conversion from *SMN1* to *SMN2*, and most of the remaining 5% are compound heterozygotes for an *SMN1* exon 7 deletion and an *SMN1* point mutation (normal in: Figure 2A, variants in: Figure 2B and C).[5] Other intragenic mutations found in the compound heterozygous state with an *SMN1* deletion include: missense, nonsense, splice site mutations, insertions, deletions and duplications (Figure 2C). Recurrent variants have been found in exons 3 and 6, making these two exons hot spots for small mutations and missense mutations, respectively (Figure 2D).[15,16] Exon 6 codes for a domain in the protein which plays a role in protein oligomerization, and those patients with exon 6 missense mutations have decreased SMN protein self-oligomerization capacity.[15] The exon 6 p.Tyr272Cys

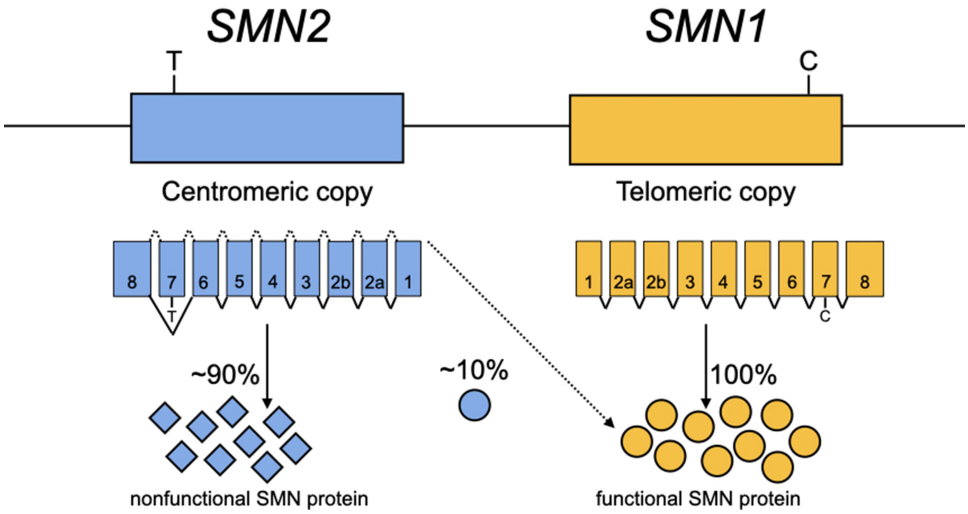

Figure 1 Two genes are responsible for producing the survival motor neuron (SMN) protein, *SMN1* and *SMN2*. SMN1 provides humans with the proper quantity of SMN protein necessary for a normal phenotype. *SMN2* is an inverted duplicate of *SMN1* lying closer to the centromere. A C>T transition in exon 7 of *SMN2* causes the *SMN2* gene to produce mostly (~90%) nonfunctional protein and a small amount (~10%) of the functional SMN protein.

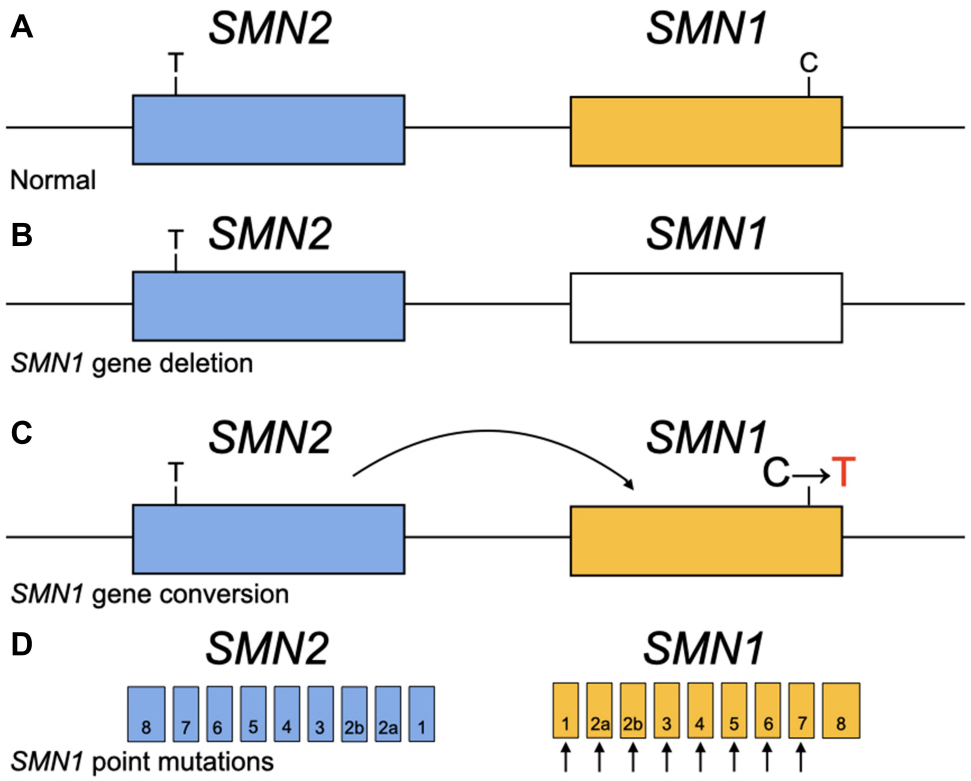

Figure 2 (**A**) A chromosome carrying a normal copy of *SMN1* and *SMN2*. (**B**) The blank box indicates a deleted gene. A deletion can remove part or all of the *SMN1* gene. (**C**) The curved arrow represents a conversion. With the C>T transition in *SMN1*, the *SMN1* copy now closely resembles *SMN2* and is considered *SMN2*-like. (**D**) Point mutations occurring in any of the *SMN1* exons prior to the last exon can affect the SMN protein.

missense mutation is the most frequently reported mutation in the *SMN1* gene. Public archives such as Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/) and Varsome (https://varsome.com/) report relationships between human genome variations and associated phenotypes along with reported clinical significance. Currently, Clinvar shows 49 pathogenic mutations reported in the *SMN1* gene, 20 likely pathogenic, 62 benign, 7 likely benign, in addition to 40 variants of uncertain significance. The majority of pathogenic mutations reported to these databases are substitutions, deletions and duplications with several reports of small insertions.

Testing

Diagnostic Testing

With 95% of affected individuals having a homozygous absence of *SMN1* exon 7, screening for the loss of exon 7 is the first tier in diagnostic testing. There are numerous DNA assays which allow for the detection of absence of *SMN1* exon 7 and are based on the c.840C>T difference between *SMN1* and *SMN2*. Multiplex ligation-dependent probe amplification (MLPA) is one of the most popular methods used as an initial deletion test in laboratories as it is convenient, highly sensitive, and capable of determining both *SMN1* and *SMN2* copy number.[17]

Prenatal testing for SMA for the *SMN1* exon 7 deletion can be performed on DNA extracted from either chorionic villus sampling (CVS) specimens or amniotic fluids. Prenatal diagnosis of SMA often occurs when there is a previously identified homozygous deletion in the index case or a 25% risk of an affected fetus (when both parents are identified as carriers by family history or following carrier testing) or the presence of abnormal findings on fetal ultrasound, such as decreased fetal movements, contractures in-utero, or increased nuchal translucency. The presence of maternal cell contamination of the fetal specimen may result in a false-negative test result and therefore must be tested for and shown to be absent prior to reporting the prenatal test result.

The *SMN1* exon 7 deletion test is currently being utilized as a reliable and accurate confirmatory test for the majority of patients suspected to have SMA. The test is highly sensitive, approximately 95%, and nearly 100% specific. Furthermore, results can be easily reported within 24 hours. The more invasive muscle biopsy test on patients

presenting with an SMA-phenotype is no longer the first tier test and is unnecessary for the majority of cases. Alternative diagnoses should be considered when 2 normal copies of the *SMN1* gene are detected in these individuals. In such cases, other motor neuron disorders that should be considered include congenital myopathies, muscular dystrophies, and metabolic disorders should be considered.

Point Mutation Testing

Although the majority of molecular diagnoses for SMA cases will be through homozygous deletion screening, another 5% of cases will be caused by other subtle mutations in the *SMN1* gene. As a consequence of the high deletion frequency, most of these cases will reveal a single *SMN1* deletion on one allele and an intragenic type of mutation on the other allele. These patients are referred to as compound heterozygotes and have been shown to have a variety of different types of *SMN1* mutations including missense mutations, nonsense mutations, splice site mutations, insertions and small deletions. Due to *SMN1's* small size, it is a relatively straightforward procedure to Sanger sequence the gene and identify mutations in patients who are test negative for the homozygous deletion test. Furthermore, massive parallel DNA sequencing allows one to simultaneously sequence a number of genes involved in neuromuscular disorders along with the *SMN1* gene.[18] There are rare SMA affected patients with a single copy of *SMN1* and an unidentified second mutation. These unidentified mutations may include a mutation in an intron, which could affect splicing or one within a regulatory region of the gene. In these cases, mRNA analysis may be helpful.

Verification of the occurrence of the intragenic mutations is located in the *SMN1* gene, and not the *SMN2* gene, should be performed. *SMN1*-specific long-range PCR amplification followed by either direct DNA sequencing of that long-range product or nested PCR sequencing is necessary when variants or mutations are identified. Direct sequencing of the *SMN* genes includes the following important limitations: 1) allele-specific sequencing requirements of all identified variants, 2) identification of variants of uncertain significance, 3) non-detection of mutations in patients with chromosomal rearrangements or mosaicism for the mutation and 4) non-detection of large deletions or insertions.[19] All novel gene changes should be compared with variants with the human gene mutation database (HGMD) and Clinvar variant database. Additionally, the frequency of gene changes in the gnomAD database (https://gnomad.broadinstitute.org) should be considered during classification and the change should be analyzed by applicable predictive software for the effects of the change on the protein structure and function. If the patient is a compound heterozygote, the *SMN1* deletion and the point mutation should be proven to be in the *trans* configuration, which may require parental testing (Figure 3). Sequence analysis of the *SMN1* gene is of particular importance for patients with an SMA phenotype found to have 2 copies of the *SMN1* gene, who originate from genetically isolated populations or are born to consanguineous parents. In families from genetically isolated populations, the point mutation may have a high frequency whereas in affected children born to consanguineous parents, the mutation would have only arisen once, and the affected received the same familial mutation from each parent. In either case, the affected individuals are homozygous for the mutation. Homozygous subtle mutations have been reported in some of these cases.[20,21] Mutations should be classified following the American College of Medical Genetics and Genomics (ACMG) standards and guidelines.[22]

Newborn Screening

The identification affected infants prior to the presentation of clinical symptoms has been accomplished by newborn screening (NBS) for a number of disorders and has allowed for newborns to be treated prior to irreversible changes that may take place. NBS has become one of the most successful public health initiatives in history and has improved the quality of life of many people with a variety of disorders. In recent years, the number of conditions included in many NBS panels has expanded. For a disorder to be included in an NBS program, they generally have to meet the following criteria: the condition is an important health problem, the disease can be detected in the early newborn period but is clinically silent, the test has appropriate sensitivity and specificity, and most importantly, there are clinical favorable outcomes associated with early therapeutic intervention.

In type 1 SMA infants, rapid loss of motor units has been shown to occur within the first three months of age resulting in severe denervation with loss of more than 95% of motor units within six months.[23] For SMA type I patients, a very small window for beneficial therapeutic intervention exists. Therapies need to be administered within the newborn period for maximum benefit, before the loss of motor neurons, which can only be

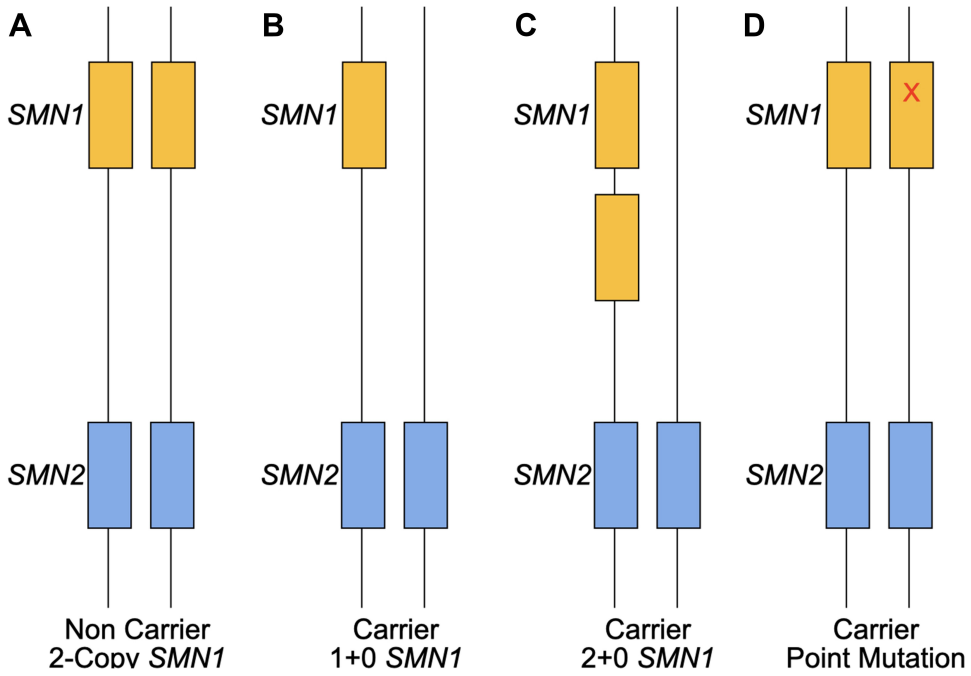

Figure 3 (**A**) A wild type with 2 copies of *SMN1* and *SMN2* on each chromosome. (**B**) SMA carrier with only one copy of *SMN1* on one chromosome and loss of *SMN1* on the other. (**C**) A silent SMA carrier with a duplication of *SMN1* on one chromosome and no *SMN1* on the other chromosome. (**D**) SMA carrier with one normal copy of *SMN1* on one chromosome and one copy that contains a point mutation on the other chromosome.

accomplished by identifying the infants through SMA newborn screening. In addition, identifying SMA in a newborn using NBS saves the family from the pain and cost of unnecessary testing in the future. Furthermore, the early diagnosis and subsequent genetic counseling can help identify other at-risk family members and prevent additional cases.

Treatment of SMA was previously limited to supportive care until December 2016 when the first disease-modifying therapy, nusinersen, was approved by the US Food and Drug Administration following a successful clinical trial. A single affected child was identified via the New York state SMA NBS pilot study and was enrolled in a presymptomatic nusinersen clinical trial.[24] At three years of age, the child was meeting motor milestones appropriate for her age. Nusinersen was also the first drug that received approval for treatment of SMA by the European Medicine Agency (EMA). Gene therapy was subsequently approved by the FDA in May 2019. The most robust responses for both nusinersen treatment and gene therapy have been shown to occur when treatment is initiated presymptomatically.[25] The EMA approved gene therapy for the treatment of patients with SMA having up to three copies of the SMN2 gene or the clinical presentation of SMA type 1.[26]

Since SMA does not have a biochemical marker, newborn patients undergo DNA testing for deletion of exon 7 in *SMN1*. The deletion was previously detected from DNA extracted from newborn blood spots using a liquid microbead array.[27] A feasibility study involving 40,103 newborn blood spots and using the array approach identified four *SMN1* homozygous deletions.[28] Utilizing a high throughput newborn screen and qPCR, DNA from 165,525 blood spots were screened for the *SMN1* deletion in a German Pilot Project and 22 cases of SMA were identified.[29] All of the pre-symptomatic, nusinersen-treated children remained without motor symptoms, whereas the 2 untreated children with 2 *SMN2* copies presented onset of disease before 3 months of age. NBS for SMA was implemented in New York State in 2018. Blood spots from 225,093 infants were tested in the first year, and 8 screened positive for the homozygous deletion. The asymptomatic infants with 2 or 3 *SMN2* copies were treated with either nusinersen or gene therapy and were all asymptomatic at their last follow-up.[30]

An NBS program will also identify exon 7 deletion positive milder later-onset cases of SMA. Since these cases may not require early intervention as the more severe cases, the early diagnosis of the milder types of cases may not be as acceptable from both the medical

community and families who may not desire to have this information. Positive SMA NBS results necessitate genetic counseling for the families; however, if the physicians and parents are aware that a child may develop SMA symptoms in the future, they can monitor for motor problems and prepare for treatment. The genotype/phenotype association between the *SMN2* copy number and clinical severity is well supported in the literature (see *SMN2* Testing Section) and will allow for selection of those SMA-affected individuals who require early therapeutic intervention.[31] Lastly, it is worth reiterating that the clinical sensitivity of identifying a newborn with SMA is about 95%, as those compound heterozygotes with a single *SMN1* exon 7 deletion and a point mutation will not be identified.

Carrier Testing

Quantitative *SMN1* gene dosage analysis is used to identify carriers with one copy of *SMN1* from non-carrier individuals with 2 or more copies of *SMN1*. Carrier testing has been valuable to families with a history of SMA who are at risk of having affected offspring. Learning the carrier status of a couple can allow informed family planning, lead them to test prenatally through non-invasive prenatal or in the setting of in vitro fertilization, preimplantation genetic testing. Genetic counseling is an important aspect of carrier testing, and individuals should receive counsel before the testing takes place. Unfortunately for most parents, their carrier status is discovered upon the birth of an affected child of an autosomal recessive disease. The goal of carrier screening is for all couples, even without a past history of the disease, to identify the risk of conceiving an affected child in advance of a pregnancy. The following criteria are used and considering a population-based carrier screening program: (1) the disorder is clinically severe, (2) there is a high frequency of carriers in the screened population, (3) availability of a reliable test with a high specificity and sensitivity, (4) availability of prenatal diagnosis and (5) and access to genetic counseling. SMA meets these criteria and is recommended by the American College of Medical Genetics and American College of Obstetricians and Gynecologists for inclusion in population-based genetic screening.[32,33]

The carrier test comes with some known limitations, including that approximately 2% of SMA cases are due to de novo rearrangement events rather than inheriting the mutation from a parent.[34,35] Despite the genetic lethality of the disease, the carrier frequency is high owing at least in part to the high rate of de novo mutations in *SMN1*.[34] It has also been shown that about 4% of the population possesses 3 *SMN1* copies.[35] As a result, carriers with two *SMN1* copies on one chromosome and no *SMN1* copies on the other chromosome is relatively common.[36–39] This is referred to as the "2 + 0" genotype (Figure 3). A carrier with two *SMN1* genes on one chromosome and an *SMN1* deletion on the other chromosome cannot be distinguished from a noncarrier with one *SMN1* copy on each chromosome 5. In most populations, approximately 3–4% of carriers have been shown to have the "2+0" genotype.[40] However, in African Americans, the frequency of alleles with two or more copies of *SMN1* has been shown to be 3–8 times more common when compared to other ethnic groups, which results in higher frequency of African American carriers with the "2+0" genotype.[40] In certain populations, most notably in the Ashkenazi Jewish community, the presence of the g.27134T>G variant is associated with chromosomes carrying 2 *SMN1* in cis.[41] This variant is also informative in other specific populations, such as the Spanish population, where it is documented that 19.35% of cis carriers had the g.27134T>G variant.[39] Consequently, the variant cannot be used to identify all cis carriers, as it is not always present. Family studies can also provide evidence toward identifying cis chromosomes. Finally, the dosage testing is a deletion-based test and does not identify carriers of other types of *SMN1* intragenic mutations, which require sequencing. Ultimately, the detection of two *SMN1* copies in an individual significantly reduces the risk of being a carrier, but a residual risk remains for those individuals found to have 2 *SMN1* copies. Calculations using the Bayesian approach should be utilized for the accurate determination of residual risk.[38]

Genetic counseling is a fundamental component of any carrier screening program, and the concept of residual risk is not a new concept for genetic counselors, who regularly counsel couples regarding the cystic fibrosis carrier screening. It is important that individuals being tested acknowledge the limitations of the carrier test: 2 *SMN1* genes in cis on the one chromosome 5, nondeletion types of mutations and the rare occurrence of de-novo mutations. The possibility of these false-negative results must be included in all carrier reports. As is true for all carrier screening programs, the testing must be confidential and voluntary. Preferably, carrier testing should be offered prior to conception when couples have the most reproductive options available.

SMN2 Testing

SMN2 copy number results provide probable information in predicting the likely clinical severity for an affected patient but should not be viewed as definitive (See Genotype Phenotype Association Section). As discussed in the newborn screening section, *SMN2* copy number analysis is valuable for identifying those who require early treatment. The Biogen's NURTURE clinical trial demonstrates the significant impact from early nusinersen treatment, with affected patients under six weeks of age with two or three copies of *SMN2* had significantly better outcomes than when treatment was delayed beyond six weeks of age.[25] Patients with two copies of *SMN2* have a dramatically altered disease, with all patients surviving, sitting, 88% walking with assistance, 77% walking independently, and none requiring permanent ventilation assistance.[25] Patients with three copies of *SMN2* met motor milestones on schedule and did not show clinical SMA symptoms. In the SMA gene replacement therapy, 15 patients with two *SMN2* copies were living event-free at 20 months of age, far better than the 8% rate of survival in a historical cohort.[42]

In 2018 Cure SMA enlisted a group of clinicians and expert scientists who were tasked with developing an algorithm for treatment of infants with positive SMA newborn screening results. A reiterative surveying modified Delphi technique was used.[31] For those individuals with qualifying genotypes (two or three copies of *SMN2*), the decision to treat immediately was unanimously recommended based on the results of pre-symptomatic infants in the NURTURE trial.[25] The working group recently updated their recommendation for infants diagnosed with SMA via NBS with four copies of SMN2. These infants should also receive immediate treatment, as even the loss of a small number of motor neurons is unacceptable when effective treatment is available.[43]

Genotype Phenotype Association

Since the loss of both copies of *SMN1* exon 7 is found in the majority of patients, no phenotype-genotype correlation was initially observed in SMA. Several studies have now shown that the *SMN2* copy number is the most important modifier of the SMA disease severity.[3,35,44–47] At least one copy of *SMN2* is retained in all patients and produces low levels of SMN protein but does not fully compensate for the loss of *SMN1*. Due to the presence of the splice mutation in exon 7, the SMN2 gene is only able to produce about 10% of full-length transcript. The copy number varies from zero to three copies in the normal population with approximately 10–15% of normals having no *SMN2*. The majority of patients with the severe type I form have one or two copies of *SMN2*, most patients with type II have three *SMN2* copies, and milder patients with type III have three or four *SMN2* copies. Three unrelated asymptomatic family members positive for the homozygous *SMN1* deletion were reported to have five copies of *SMN2*.[48] These cases support the modifying role of *SMN2* copy number and reveal that the associated expression levels of five *SMN2* copies may compensate for the loss of the *SMN1* gene expression. This same inverse dosage relationship was shown in an SMA mouse model.[49,50] Mice with two copies of human *SMN2* but lacking the endogenous mouse *Smn* gene develop severe SMA and die within the first week of life; however, mice with multiple copies of *SMN2* do not manifest the disease.

Exceptions to the inverse correlation between the *SMN2* copy number and disease severity have occurred. Three *SMN2* copies are the most common genotype in both type 2 and type 3 SMA, thus indicating that the copy number does not always accurately predict the phenotype in milder patients. The discordance between the *SMN2* copy number and disease severity in some cases is consistent with the existence of disease modifiers that may contribute to the phenotypic outcome. Identification of SMA modifiers not only allows for a better understanding of the pathogenesis of the disorder but also may identify potential targets for therapeutic interventions.

The loss of the *SMN1* gene can occur via deletion or by gene conversion to SMN2. The conversions in effect increase the *SMN2* copy number and often result in milder phenotypes. However, exceptions exist and may be the result of incomplete deletions of the *SMN2*. As a result of the high sequence homology of the SMN loci, exact deletion breakpoints have been hard to identify. In the event *SMN2* genes are truncated, they may not produce any full-length transcripts and thus, only nonfunctional SMN protein. Some of the conversion events are partial and result in different hybrid genes, which often consist of *SMN2* exon 7 and *SMN1* exon 8 but differ in the extent of the conversion.[51] Partial conversions have been observed in approximately 5–7% of SMA patients. The smallest conversion, which was confined to *SMN2* exon 7, with all other sequences remaining as the *SMN1* version, was shown to increase exon 7 inclusion and produced milder phenotypes in five patients with 3 *SMN2* copies. It was

shown that the A-44G in intron 6 was the major positive modifier resulting in improved exon 7 splicing by reducing the RNA binding affinity of the splicing repressor HuR.[52]

It has generally been assumed that all *SMN2* genes are equivalent; however, variants within the gene may also modify the phenotype. Modifiers within the *SMN2* gene may affect splicing by either disrupting a slice silencer or creating a splice enhancer. The *SMN2* exon 7 c.859G>C variant promotes exon 7 inclusion and thus, accounts for a milder phenotype than expected by the SMN2 copy number.[53,54] Although the C to T at +6 in *SMN2* exon 7 normally results in the production of *SMN2* transcripts predominantly lacking exon 7 (by disrupting an exon splicing enhancer or creating an exon splicing silencer), the c.859G>C was shown to partly restore normal exon splicing and produce more full-length *SMN2* transcript. The variant has never been reported in a patient with type 1 SMA but has been seen in patients with type 2 and 3 when the 2 *SMN2* copies are heterozygous or homozygous for the c.859G>C variant, respectively.[53,55] Importantly, these cases again support the therapeutic benefit of increasing the *SMN2* gene expression in order to decrease the severity of the disorder (see in Treatment section).

The clinical severity observed in approximately 5% of patients with *SMN1* intragenic mutations is not only determined by the *SMN2* copy number but also by the type and location of the mutation. The observed phenotype in patients carrying specific point mutations has not always correlated with the *SMN2* copy number. Mendonca et al recently identified 16 patients with the exon 3 nonsense mutation c.460C>T (p.Gln154*).[16] All of the patients presented with a milder phenotype (SMA types 3 and 4), including 2 patients with 1 *SMN2* copy and 10 patients with 2 *SMN2* copies. The missense mutation p.Ala2Val has been found in several type 3 patients in the presence of 1 or 2 copies of *SMN2* while type 1 patients with p.Trp92Ser were shown to have 3 *SMN2* copies.[56]

Other genes outside the *SMN1/SMN2* loci may modify the disease severity. There are very rare families reported in the literature in which the disease severity is not consistent between affected siblings with identical *SMN2* copy number.[35,57–62] These patients are referred to as "discordant siblings," and they have been shown to occur with all types of SMA. Thus, there are type 1 SMA cases where the sibling has type 2 SMA, type 2 cases where the sibling has SMA type 3b, and type 3a cases where the sibling is phenotypically normal. Some of the variability among discordant sibs may be explained by differences in splicing factors, allowing some *SMN2* copies to express more full-length transcripts.[63] It has also been reported that in some families with clinically unaffected females with *SMN1* deletions, plastin 3 (*PLS3*, T-plastin or T-fimbrin; MIM 300,131) expression was higher than in their affected siblings.[64] *PLS3* plays a key role in axonogenesis and may act as another gene modifier. Overexpression of PLS3 rescues the axonal growth defects observed in the zebrafish with reduced SMN levels. However, in a severe SMA mouse model, the transgenic expression of *PLS3* did not restore motor function or lifespan.[65] Another disease modifier identified in at least five asymptomatic individuals with an *SMN1* deletion and 4 copies of *SMN2* is Neurocalcin delta (*NCALD*), a negative regulator of endocytosis.[66] These gene modifiers are significant for understanding the pathogenesis of SMA and may be important targets for future therapeutic efforts.

Treatment

Understanding the underlying genetic mechanism of SMA has led to the development of two targeted therapies that increase functional SMN protein production, the first by altering *SMN2* RNA to produce a full length SMN protein and the second through direct delivery of the *SMN1* gene via a viral vector.[67] Table 2 contains treatment types, mechanism of action, route of administration, related clinical trials and status for each drug used in the treatment of SMA for both SMN dependent and independent pathways.

SMN1 Gene Therapy

Onasemnogene abeparvovec uses an adeno-associated virus capsid to deliver a copy of the *SMN1* gene with a cytomegalovirus enhancer and chicken beta-actin promoter to motor neurons, muscle, and other peripheral tissues where *SMN1* is expressed through a single intravenous injection. It was approved for treatment of SMA by the FDA in May 2019.[42] START, a Phase I trial treated 15 infants with SMA type 1 with varying doses of gene therapy and compared outcomes to a historical control group to evaluate safety, adverse events, time until death or permanent ventilation, motor milestones and motor function. All treated patients by the age of 20 months were alive without requiring permanent mechanical ventilation with significant improvement in motor milestones in the high dose cohort.[42] Longer term follow-up has shown sustained survival and improved motor function of treated

Table 2 SMA Treatments

Type of Treatment	Mechanism of Action	Drug	Route of Administration	Clinical Trials	Current Status
SMN dependent pathway					
SMN1 gene delivery	*SMN1* gene transfer via adenovirus vector	Onasemnogene abeparvovec	Single intravenous injection	START (Phase I) STRONG (Phase I) SPR1NT (Phase III)	FDA approved in May 2019
Act on *SMN2* to increase SMN protein production	Antisense oligonucleotide that binds *SMN2* mRNA to modify splicing	Nusinersen	Intrathecal injection every 4 months	NCT01494701 and NCT01780246 (Phase I) NCT01839656 (Phase II) ENDEAR (Phase III) CHERISH (Phase III) NURTURE (Phase II) SHINE (Phase III)	FDA approved in December 2016
	Small molecule that alters splicing of *SMN2*	Risdiplam	Oral daily medication	FIREFISH (Phase II, III) SUNFISH (Phase II, III) JEWELFISH (Phase II) RAINBOWFISH (Phase II)	FDA approved in August 2020
SMN independent pathways					
Restores mitochondrial homeostasis	Cholesterol like molecule that enhances mitochondrial functioning and inhibits release of apoptotic factors	Olesoxime	Oral daily medication	2 Phase II trials	Development stopped
Enhance muscle function	Skeletal muscle troponin activator that acts to increase the skeletal muscle force response to nerve stimulation.	Reldesemtiv	Oral twice daily medication	Phase II trial	Phase III trial is planned
Promote muscle cell growth and division	Monoclonal antibody inhibits latent myostatin	SK-015	IV injection every 4 weeks	TOPAZ (Phase II)	Ongoing clinical trials
Improve muscle strength and fatigue	Acetylcholine esterase inhibitor	Pyridostigmine	Oral medication, can be taken multiple times per day	Currently trialed in types 2–4 SMA	Currently in clinical trials

patients, with better outcomes and baseline function in patients treated earlier.[68–70] Liver function tests were found to be elevated after drug administration, likely due to an immune response to the viral vector. Daily steroid (1mg/kg) administration for one month and monitoring of liver function tests prevented further liver toxicity. During clinical trials, transient decreases in platelet counts, some meeting the criteria for thrombocytopenia, were also reported.[71] No other adverse effects of the gene therapy have been reported.[42,68–70] STRONG, a Phase I clinical trial, investigated intrathecal gene therapy administration to 28 SMA type 2 patients aged 6 months to 60 years. Review of data 6–12 months after therapy showed no treated patients had lost motor milestones and some had gained milestones.[70,72] The trial was recently placed on a partial hold by the FDA recently given safety concerns from an animal model.[73] SPR1NT is an ongoing Phase III trial evaluating safety and efficacy of gene therapy

administration to pre-symptomatic SMA patients with 2–3 copies of SMN2. Preliminary data have shown that all treated patients are alive without requiring ventilation and are achieving motor milestones.[74]

SMN2 Modulators

Nusinersen is an antisense oligonucleotide that modifies that splicing of *SMN2* pre-mRNA by binding to a specific region to block splicing and promote inclusion of exon 7, thus resulting in production of a full length SMN protein. Nusinersen became the first drug approved for treatment of SMA by the US FDA in December 2016 and the European Medicines Agency in June 2017 after multiple trials supporting its safety and efficacy.[67] As this antisense oligonucleotide does not cross the blood brain barrier, administration is by intrathecal injection.[67]

A Phase I study (NCT01494701 and NCT01780246), which included 28 patients with SMA types 2 and 3, ages 2–14 years, showed safety and tolerability with no serious adverse events found following single intrathecal injections of nusinersen (1–9 mg doses). SMN protein was found to be increased in CSF and patients receiving the higher doses showed improved motor function.[75] A Phase II trial (NCT01839656) with escalating doses of nusinersen administered to 20 SMA type 1 patients, ages 3 weeks to 7 months similarly showed safety, tolerability and efficacy, with improved motor function in patients receiving higher doses and significantly increased overall survival with decreased need for mechanical ventilation.[76] Following these trials, two large, multicenter, randomized controlled, Phase III studies, ENDEAR in SMA type 1 patients (including 122 patients 7 months or younger) and CHERISH in SMA type 2 patients (126 patients, 2–9 years old) showed significant improvement in motor milestones and survival in the treated group, with more benefit seen in infants with shorter disease duration in the ENDEAR trial.[76,77] NURTURE, an ongoing Phase II trial is investigating the optimal timing of nusinersen treatment in 25 pre-symptomatic SMA patients with 2 or 3 copies of *SMN2*. In an interim report, all patients were alive without need for ventilation and able to sit without support. Most had achieved independent ambulation.[25] SHINE, an ongoing Phase III trial, is currently assessing the long-term effects of nusinersen treatment including patients previously enrolled in the abovementioned ENDEAR trial, and has found the treatment to be safe, well tolerated, and most effective when administered early.[78]

Nusinersen is delivered as a loading dose of four 12 mg intrathecal injections over 2 months, followed by maintenance doses every 4 months.[3] Complications can result from the lumbar puncture including rare reports of hydrocephalus.[67] The lumbar puncture may also be challenging in patient with scoliosis or spinal fusion, requiring use of radiographic-image-based guidance and intrathecal catheters.[67] The lack of systemic delivery is also a concern as the SMN protein may have important functions in the peripheral tissues.[11]

Small molecules therapies that modify *SMN2* mRNA splicing to produce increased full length SMN protein have also been developed and in contrast to nusinersen, have benefits of systemic distribution and oral bioavailability. Risdiplam, one of these small molecules taken as a liquid formulation daily, became the third approved disease-modifying treatment for SMA by the US FDA on August 7, 2020 given results of ongoing clinical trials showing safety and efficacy.[79] FIREFISH, an ongoing trial for SMA type 1 patients (set to finish 2023), showed improved motor function, attainment of motor milestones, and lack of dysphagia and ventilatory requirement in treated patients.[80,81] SUNFISH studying SMA types 2 and 3 (ongoing trial finishing in 2023) also showed improvement of motor function in treated patients, particularly in younger patients.[82–84] JEWELFISH, a Phase II open label trial (finishing in December 2024), is currently investigating the safety and tolerability of risdiplam in SMA patients ages 6 months–60 years who have previously been treated with nusinersen or onasemnogene abeparvovec.[85] RAINBOWFISH (finishing in October 2025) is assessing risdiplam safety and efficacy in presymptomatic newborns and infants up to 6 weeks old.[86]

RG7800 small molecule therapy was previously investigated in Phase I trials (MOONFISH), but studies were ended after eye toxicity was found.[3] Phase I–II clinical trials of branaplam were also terminated after evidence of blood vessel, kidney, spinal cord, and peripheral nerve toxicity was found in animal studies.[87,88]

Celecoxib, a cyclooxygenase 2 inhibitor, was shown to increase SMN protein in SMA cells and animal models. A Phase II trial is currently recruiting SMA 2 and 3 patients, in hopes that it may serve as an adjunctive SMA treatment.[89]

SMN Independent and Combination Pathways

Other therapies under investigation have targeted pathways independent of the *SMN1* and *2* genes and focused

on enhancing survival and functioning of motor neurons and muscle through alternative mechanisms.[3]

Olesoxime is a cholesterol-like molecule that preserves mitochondrial functioning and inhibits release of apoptotic factors that lead to motor neuron death. By restoring mitochondrial homeostasis, olesoxime is thought to maintain motor neuron integrity, reduce denervation of muscle tissue, and reduce reactive astroglia and microglia activation.[90] An initial Phase II trial in patients with SMA II and III did not reach the primary outcome of improved motor function and a follow up Phase II trial showed a decline in motor function after 18 months resulting in cessation of drug development.[91–93] Reldesemtiv is a skeletal muscle troponin activator. It slows calcium release from the troponin complex and sensitizes the sarcomere response to calcium in order to increase the skeletal muscle force response to nerve stimulation. A Phase II trial in patients with SMA II–IV showed no safety or tolerability issues. A Phase III trial is planned.[94] SK-015 is a monoclonal antibody that inhibits latent myostatin to promote muscle cell growth and division. It is currently being investigated in SMA2 and 3 patients in the TOPAZ trial.[95,96] Pyridostigmine, an acetylcholinesterase drug used for treating myasthenia gravis, is theorized to improve muscle strength and fatigue in SMA. It is currently being trialed in patients with types 2–4 SMA.[89]

With the development and approval of multiple SMA disease-modifying therapies, the use of combination therapy is being investigated. As adequate functional SMN protein is required for optimal function of many dividing cells, it is unclear if a single dose of onasemnogene abeparvovec or maintenance nusinersen will be sufficient.[3] Given the drugs' different mechanisms of actions, combination use of onasemnogene abeparvovec and nusinersen could maximize therapeutic effect with little risk of drug interaction and toxicity.[89] Small case series and reports showed patients treated with both onasemnogene abeparvovec and nusinersen tolerated the combination, though efficacy and long-term effect remain unclear.[97,98] As mentioned previously, JEWELFISH, a Phase II open label trial, is currently investigating the safety and tolerability of risdiplam in SMA patients' ages 6 months–60 years who have previously been treated with nusinersen or onasemnogene abeparvovec.[85] Combined use of nusinersen and SMN-independent myostatin inhibitor therapy has shown a positive effect in animal models that may translate to another clinical approach.[89]

Conclusion

The most robust response to SMA treatments has clearly been shown to occur in treating presymptomatic patients. Thus, early detection through newborn screening is paramount to ensuring efficient treatment access prior to manifesting symptoms of the disease. Prenatal cases identified as a result of carrier screening will also allow for early treatment. Biomarkers and outcome measures to assess disease response to therapy are needed, particularly in determining use of additional or combination treatments. Future research is needed to understand the long-term effects of the therapies alone or in combination.[67]

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Disclosure

The authors report no conflicts of interest in this work.

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