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Twenty-Five Years of Spinal Muscular Atrophy Research: From Phenotype to Genotype to Therapy, and What Comes Next

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Abstract

Twenty-five years ago, the underlying genetic cause for one of the most common and devastating inherited diseases in humans, spinal muscular atrophy (SMA), was identified. Homozygous deletions or, rarely, subtle mutations of *SMN1* cause SMA, and the copy number of the nearly identical copy gene *SMN2* inversely correlates with disease severity. SMA has become a paradigm and a prime example of a monogenic neurological disorder that can be efficiently ameliorated or nearly cured by novel therapeutic strategies, such as antisense oligonucleotide or gene replacement therapy. These therapies enable infants to survive who might otherwise have died before the age of two and allow individuals who have never been able to sit or walk to do both. The major milestones on the road to these therapies were to understand the genetic cause and splice regulation of *SMN* genes, the disease's phenotype–genotype variability, the function of the protein and the main affected cellular pathways and tissues, the disease's pathophysiology through research on animal models, the windows of opportunity for efficient treatment, and how and when to treat patients most effectively.

This review aims to bridge our knowledge from phenotype to genotype to therapy, not only highlighting the significant advances so far but also speculating about the future of SMA screening and treatment.

THE PHENOTYPE OF SPINAL MUSCULAR ATROPHY

Description and Classification

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by degeneration of alpha motor neurons in the anterior horn of the spinal cord. The characteristic symptoms are hypotonia, muscular atrophy, and weakness of proximal muscles, predominantly affecting the lower extremities.

Before therapies were developed, SMA was classified into three main types (types I–III) based on the age of onset and achieved motor milestones (127). The emergence of additional phenotypes broadened this classification to include congenital (type 0) (43) and adult onset (type IV) (190). However, this classification delivers cross-sectional information and does not adequately address the dynamic changes in the clinical picture after treatment. Emerging therapeutic options and adjacent clinical trials accelerated improvements in natural history studies and led to an update of the consensus statement (54, 118, 178). Today, overlaps among SMA types are commonly observed.

Furthermore, because the predictive value of a classification based solely on motor assessment at the initial presentation is limited (147), the classical classification of SMA is becoming less practical for current clinical studies. To ensure the consistency and uniformity of outcome measures and therapy follow-up, it has been recommended to reclassify the patients as nonsitters, sitters, and walkers (54, 118, 178). This approach acknowledges the SMA phenotype as a continuum and focuses on the current functional status and the therapy response (14, 118, 178). Nevertheless, we recognize that no classification perfectly covers each presentation and disease course of SMA.

Nonsitters. The majority of nonsitters have SMA type I. However, independently sitting individuals with disease onset after six months (type II) may lose that skill and revert to the nonsitter group (**Figure 1**). Bulbar and intercostal muscles are usually affected, resulting in feeding difficulties and respiratory insufficiency with a bell-shaped thorax deformity. The diaphragm is usually spared and is dominantly involved in paradoxical breathing. The involvement of tongue muscles often presents with fasciculations. Facial muscles are not usually affected, except in some individuals with congenital onset. On examination, the vertical suspension test reveals an absent hip flexion, a slip-down through the examiner's hands, and a head lag due to neck flexion weakness. In the supine position, infants show a frog-leg posture and absent head control on traction (130).

Nutritional and respiratory support has significantly reduced mortality in the past two decades. Despite prolonged survival after these supportive measures, affected infants do not achieve any further motor milestones and remain nonsitters (37).

Sitters. This group includes type II individuals and nonambulatory type III individuals (**Figure 1**). They achieve sitting without help at any point in their development but either are not able to walk freely or lose the ability to walk. They exhibit generalized muscular hypotonia and weakness in the first months of life. The proximal muscular weakness is more profound in the legs than in the arms. Over time, they develop joint contractures and mandible ankyloses. Sitters frequently develop scoliosis from sitting in an upright position with weak axial muscles, and without any supportive measures, scoliosis and intercostal muscle weakness may lead to restrictive lung disease and respiratory insufficiency (130). The disease progresses between 5 and 15 years of age, especially

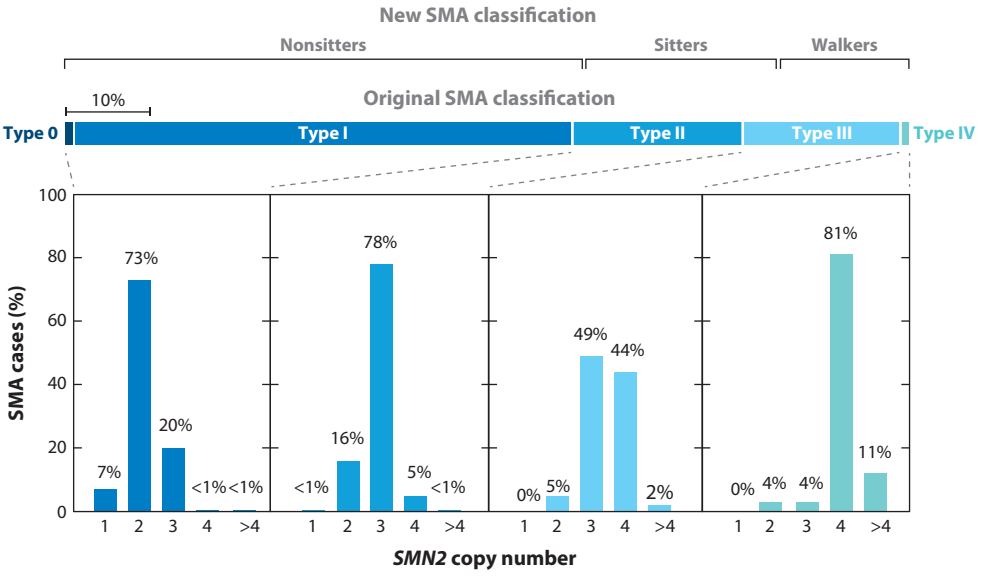

Figure 1

Clinical forms of SMA according to the new and original classifications for therapy follow-up and their correlations with *SMN2* copy numbers. All type 0 and type I patients and some type II patients who are unable to sit independently fall into the new category of nonsitters. The remaining type II individuals and some type III individuals who are unable to walk independently fall into the category of sitters. The remaining type III individuals and all type IV individuals fall into the category of walkers. The correlations between clinical severity and *SMN2* copy numbers are depicted according to the original classification, as no correlation studies for the new classification are available. *SMN2* copy number values and clinical correlations were taken from the most recent and extensive compiled study of SMA (24). Type 0 SMA cases reported so far always carry one *SMN2* copy (63, 148), and in most studies these were included under type I. Abbreviation: SMA, spinal muscular atrophy.

during puberty owing to the associated increases in weight (117). The extent of respiratory problems determines the survival outcome. Sitters have much longer survival than nonsitters (189): The survival probability of type II sitters is 98.5% at 5 years of age and 68.5% at 25 years of age (191).

Walkers. This group includes individuals with SMA type III or IV who gain and keep their ability to walk (**Figure 1**). Achievement of the walking milestone is correlated with the age at onset and the age at independent sitting. Almost 95% of early sitters can walk by the age of 18 months, which is possible only for 50% of late sitters (147). The disease course is usually steady. Ambulant patients typically show no significant disease progression according to the 12-month change in their Hammersmith Functional Motor Scale–Expanded (HFMSE) values (117), and they can even show a slight increase in motor function over a 12-month period according to their HFMS, HFMSE, and Gross Motor Function Measure (GMFM) values (90). Examinations over a 48-month interval showed a slight decrease in motor function measures (HFMSE and GMFM) and spirometry values (91). In contrast to nonambulatory individuals, life expectancy does not change significantly in ambulatory individuals (130).

Biochemistry

The majority of SMA patients show normal or mildly increased levels (<10×) of serum creatine kinase. In contrast to primary muscular disorders, increased creatine kinase levels do not correlate

with disease severity or duration. However, creatine kinase levels in type III patients usually show higher titers than those in SMA type I patients (149). Other biochemical serum markers usually do not show any abnormalities.

Electrophysiology

The assessment of neurophysiological function in SMA is based on electromyography and nerve conduction studies. Due to the loss of lower motor neurons in the anterior horn, electromyography shows neurogenic features such as changes in motor unit action potentials, increased spontaneous discharges, and denervation potentials. In individuals without SMA, the regular appearance of action potentials in healthy motor units [comprising the lower motor neuron, axon, neuromuscular junction (NMJ), and innervated muscle fibers] shows a bi- or triphasic morphology with a normal duration and amplitude for an individual's age and amount of muscle, whereas in individuals with SMA, the increase in spontaneous discharges produces a polyphasic pattern with higher amplitudes and prolonged duration. The electromyography interference pattern is another critical parameter for testing the condition of motor units during voluntary muscle contraction: The loss of functional motor units in SMA means that the dense pattern of interfering waveforms seen in healthy individuals is also partly lost. Motor unit estimation methods reflect actual motor unit loss and are relevant for the estimation of disease severity and progression.

Among increased spontaneous activities, fibrillations and positive sharp waves indicate ongoing denervation and appear mostly in SMA type I. Fasciculations are visible mostly in type III and suggest chronic denervation (8). However, these abnormalities are not specific to SMA and can be present in any spinal motor neuron pathology (123).

Because SMA primarily affects the motor neuron axons, nerve conduction studies should show normal conduction velocities. However, a reduction in the compound muscle action potentials might be observed when the number of functional motor units drops substantially (8).

Pathology

The so-called group atrophy is the typical myopathological feature of SMA. It is characterized by muscle fiber loss, atrophy, and compensatory hypertrophy of the surviving fibers. Both type I and type II muscle fibers are prone to atrophy, but hypertrophy occurs only in type I fibers (12). Despite myopathological features that do not correlate with the clinical picture and disease prognoses (188), fiber type grouping and increased fatty infiltration are more common in type III. Because the efficiency and availability of genetic testing have increased, muscle biopsy is no longer recommended in the diagnosis of SMA.

Postmortem studies are the primary source of information on spinal cord pathology (9, 27, 97). All SMA types show a paucity and atrophy of anterior horn motor neurons and migration of motor neurons along axonal pathways (heterotopy). Degenerative changes (gliosis, chromatolysis, and ballooning), increased empty-cell beds, and the involvement of dorsal root, cortical, or thalamic neurons are variable and regarded as secondary phenomena (33). A recent study with the largest postmortem data set to date demonstrated that the survival of motor neuron (SMN) protein levels in the human spinal cord are highest during the fetal period, which is followed by a 6.5-fold decline in the postnatal period and a further decline after three months of life (139). This study underlines the importance of SMN levels in the early stages of motor neuron development.

Ultrastructural alterations in the NMJ underlie changes in acetylcholine clustering, synaptic vesicle transport defects, and aberrant nerve terminals (110). It is, however, a matter of debate whether NMJ alterations are secondary to motor neuron pathology or damaged motor neurons are the primary pathology (17).

Biomarkers

The significance of biomarker studies has become more apparent as the number of therapeutic options has expanded over the last few years. In addition to the use of *SMN2* copy number as a prognostic biomarker, predictive and pharmacodynamic biomarkers have become paramount in ongoing clinical studies.

SMN-related biomarkers. *SMN2* copy number inversely correlates with disease severity and is considered a sensitive and accurate prognostic biomarker for SMA (48, 181). Despite some exceptions with discordant individuals, *SMN2* copy number determination is a standard step in SMA diagnostics (118). Furthermore, most countries have made *SMN2* copy number testing a requirement for therapy and reimbursement (118, 154). Among other SMN-related biomarkers, low SMN mRNA and protein levels measured from peripheral blood demonstrate some prognostic and potential pharmacodynamic prediction value (95).

Non-SMN-related biomarkers. Electrophysiological methods, such as measurement of compound muscle action potentials, motor unit estimation, and electrical impedance myography, are regarded as putative biomarkers for prognostic, predictive, surrogate endpoint, and pharmacodynamic estimations (11). Although genetic testing has replaced electrophysiological testing for diagnostic purposes, the latter provides reliable and sensitive outcomes for clinical studies. *SMN2* copy number and functional clinical scores do not provide insights into the actual health of remaining motor neurons, their axonal sprouting capacities, or distally innervated muscle groups, which can be estimated by electrophysiological methods (167). The maximum ulnar amplitude and area of compound muscle action potentials correlate with age, disease severity, and motor function and thus provide a strong prediction of functional outcome (95, 102, 167). Furthermore, the latest clinical trials for nusinersen and gene replacement therapy utilize the maximum ulnar amplitude and peak measurements of compound muscle action potentials as a standard outcome measure of therapy response (4, 35, 50).

Electrical impedance myography is a new approach that assesses muscle impedance properties and thus correlates with SMA progression. Although this method is painless and rapid and requires minimal patient cooperation (151), the outcomes from the NeuroNEXT biomarker study did not provide satisfactory evidence for the use of electrical impedance myography in infantile SMA (94).

Non-SMN-related molecular biomarkers are currently under investigation. By using unbiased proteomic, metabolomic, and transcriptomic approaches, the Biomarkers for Spinal Muscular Atrophy trial group identified 200 candidate biomarkers correlating with functional motor scores as assessed by the Modified Hammersmith Functional Motor Scale (MHFMS) (51). Preliminary data from this study and further endeavors have resulted in the development of a multiplex immunoassay panel, Spinal Muscular Atrophy Multi-Analyte Profiling (SMA-MAP), consisting of 27 plasma proteins associated with motor function and other SMA outcomes (93). The NeuroNEXT study tested the utility of SMA-MAP in 18 SMA and 20 control infants and revealed significantly lower concentrations of specific proteins (including cadherin-13, cartilage oligomeric matrix protein, and peptidase D) in SMA infants compared with controls (94). A recent study found that SMA infants had significantly higher levels of phosphorylated neurofilament heavy chain (pNF-H) levels compared with age-matched controls and showed that plasma pNF-H concentrations were reduced in SMA individuals after nusinersen therapy (36). Plasma pNF-H concentration represents the first robust molecular biomarker that reflects both the disease and the response to nusinersen therapy.

SMN GENES, RNA, PROTEIN, AND FUNCTION

Despite the large phenotypic variability of the disease, all types of 5q SMA are caused by homozygous deletions or, rarely, other mutations in the *SMN1* gene (OMIM 600354) (100, 180). The disease severity is determined mainly by a copy gene, *SMN2* (OMIM 601627): The more *SMN2* copies an SMA individual has, the milder the phenotype is (48, 108, 181). Rare *SMN2* variants, as well as independent modifiers such as plastin 3 (PLS3; OMIM 300131) or neurocalcin delta (NCALD; OMIM 606722), can further influence the disease severity (129, 138, 142). In the following sections, we discuss the evolution of the *SMN* genes, *SMN* gene structure, *cis*- and *trans*-regulatory domains affecting *SMN* splicing, and SMN protein function.

Evolution of the *SMN* Gene Region and Differences Among Populations

Humans are the only species that carry two different *SMN* paralogs, *SMN1* and *SMN2*; all other species have only one *Smn* gene. *SMN* duplication has been described in primates (144); however, recent work based on long-read next-generation sequencing has demonstrated that they have only one *SMN* copy (39), which suggests that a duplication of *SMN* and the surrounding genes localized on chromosome 5q13.2 occurred during evolution from primates to humans. The *SMN* duplication allowed one copy to differentiate into the current *SMN2* copy (92).

Significant structural differences among ethnicities have been described. In the black African population, the frequency of individuals with a 2 *SMN1*/0 *SMN2* haplotype is eight times that of the Caucasian population (173). According to the out-of-Africa theory, the duplication likely occurred in the African population. Subsequently, in a subset of the African population, one copy diverged into *SMN2*, which might have been foundational for the rest of the world (92). Consequently, in Caucasian and Asian populations, the most frequent haplotype is 1 *SMN1*/1 *SMN2*. This distortion in the number of *SMN1* copies per haplotype may also be responsible for the higher SMA carrier frequency observed in these populations (**Table 1**).

The *SMN1* and *SMN2* Genes

SMN1 and *SMN2* were identified by Judith Melki's group in 1995 (100). Each *SMN* copy encompasses 34 kb on a genomic level and contains 10 exons (exons 1, 2a, 2b, 3, 4, 5, 6a, 6b, 7, and 8).

Table 1 Spinal muscular atrophy carrier frequency in different ethnicities

Ethnicity	Carriers (one *SMN1* copy)	Total number of tested individuals	Ratio of carriers in the population
European	90	3,704	1:41
US Caucasian	558	26,839	1:48
US Asian	110	6,908	1:63
US black	64	6,183	1:97
US Jewish	115	7,536	1:66
US Hispanic	110	8,968	1:82
US mixed (newborn screening)	38	1,530	1:40
Australian/New Zealand Caucasian	3	147	1:49
Asian	2,407	116,162	1:48
Sub-Saharan African	6	868	1:145
Israeli Jewish	294	14,741	1:50
Total	3,795	193,586	1:51

Table based on Reference 173 and references therein.

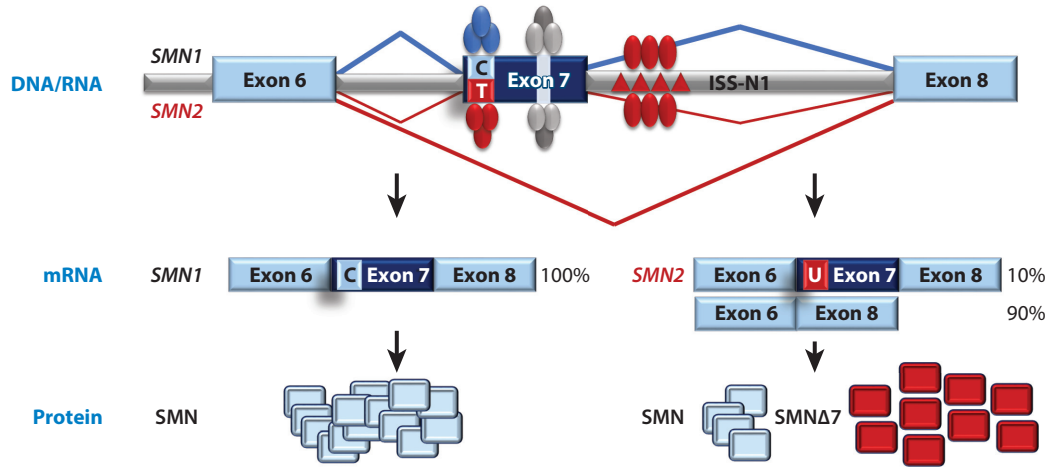

Figure 2

Regulatory elements modulating exon 7 splicing in *SMN* genes. The top section shows the genomic structure of the *SMN1* and *SMN2* genes and their RNA transcripts in the region from intron 6 to exon 8. The critical difference between *SMN1* and *SMN2* is a translationally silent mutation at position 6 of exon 7, which is part of an exonic splicing enhancer in *SMN1* (*blue box with a C*) and an exonic splicing silencer in *SMN2* (*red box with a T*). Exon 7 is correctly spliced in *SMN1* (*upper blue lines*), whereas *SMN2* produces mainly transcripts that lack exon 7 (*lower thick red line*) and only a small proportion of correctly spliced transcripts (*lower thin red lines*). Exon 7 inclusion is determined by the balance between exonic splicing enhancers (*blue* and *gray boxes*), recognized by splicing factors (*blue* and *gray ovals*), that promote exon 7 inclusion. Intronic splicing silencers (*red box* and *red triangles*), recognized by splicing factors (*red ovals*), inhibit exon 7 inclusion. ISS-N1 (*red triangles*) is the most important intronic splicing silencer in intron 7, which, upon blocking by SMN antisense oligonucleotides (nusinersen), facilitates exon 7 inclusion. The left side of the middle section shows full-length *SMN1* mRNA, which produces only full-length functional SMN protein (*blue rectangles* in the *bottom section*). By contrast, the right side of the middle section shows small amounts of full-length *SMN2* mRNA, which produces functional SMN protein, and large amounts of aberrantly spliced *SMN2* transcripts, which produce SMNΔ7 protein, which is unstable, unable to oligomerize, and thereby prone to degradation (*red rectangles* in the *bottom section*). Abbreviation: ISS-N1, intronic splicing silencer N1.

The two *SMN* genes differ by only five nucleotides (22, 157). Of these, only the C>T transition at position +6 in exon 7 (c.840C>T) is within the coding region (**Figure 2**). However, it is a silent variant that causes no amino acid exchange. The remaining variants reported so far cannot be uniquely assigned to one of the two *SMN* copies. Two long noncoding RNAs are transcribed from the *SMN* locus. The antisense transcript SMN-AS1 is 1.6 kb in length and localized in intron 1, and SMN-AS2 starts in exon 8 and ends in intron 5. Both transcripts downregulate the expression of *SMN* (34, 187).

Splicing of *SMN* Genes

SMN1 is nearly always correctly spliced and produces full-length transcripts and protein. *SMN2*, by contrast, produces mainly alternatively spliced transcripts lacking exon 7 (*SMN2*Δ); only approximately 10% are correctly spliced *SMN2* transcripts that contain all exons and produce a full-length protein (68, 100). Moreover, both *SMN1* and *SMN2* produce a small amount of transcripts lacking exon 3, exon 5, or both (58).

SMN exon 7 spans 54 nucleotides and harbors a stop codon at nucleotide positions 49–51. The region encoded by exon 7 is crucial for protein oligomerization and function. Consequently, exon 7 and the surrounding introns are fully packed with *cis*-regulatory domains [exonic splicing enhancers (ESEs) and intronic splicing enhancers] that facilitate exon 7 inclusion in *SMN1* and, to a lesser extent, *SMN2*. However, these domains compete against exonic splicing silencers and intronic splicing silencers (ISSs), which facilitate exon 7 exclusion in *SMN2* (reviewed in 160).

Exon 7 of both *SMN* genes contains a centrally placed ESE with a GA-rich region, and mutations in this ESE abolish exon 7 inclusion in both genes. The main splicing factor that recognizes this ESE is SFRS10 (hTRA2-β1) (69, 70). In addition, the splicing factors SRSF9 (SRp30c), heterogeneous nuclear ribonucleoprotein (hnRNP) G, hnRNP M, TDP43, and PSF promote exon 7 inclusion via direct or indirect binding to this ESE (160 and references therein). This network of splicing factors binding to this particular ESE is likely responsible for the ~10% full-length mRNA generated by *SMN2*. Overexpression of these splicing factors, either separately or in combination, restores the splicing capacity of *SMN2* minigenes up to 80% and increases endogenous SMN protein levels (69, 70). Complete depletion of murine *Sfrs10* had almost no effect on exon 7 inclusion, highlighting the complexity of the in vivo splicing (112). Histone deacetylase inhibitors, such as valproic acid, increase SFRS10 (hTRA2-β1) (21) and enhance *SMN2* exon 7 inclusion and thus the amount of SMN protein. These findings sparked the groundbreaking idea to modulate exon 7 splicing in vivo as a strategy to treat SMA (69). Similarly, tailed 5′GGA antisense oligonucleotides (ASOs) facilitate recruitment of SFRS10 to the tail, markedly increasing exon 7 inclusion and SMN levels (161).

The most important difference between the two *SMN* genes is the C6U substitution (c.840C>T) in exon 7. Twenty years ago, in a time when silent mutations were mostly underestimated, Wirth's and Androphy's groups showed that SMA is caused by a silent mutation that disrupts an ESE, causing exon 7 skipping in *SMN2* (106). This seminal finding opened the door to therapy based on restoring *SMN2* splicing. Next, Krainer's group found that this C6U substitution disrupts a heptamer motif of ESE, CAGACAA, recognized by the SR-rich splicing factor SRSF1 (ASF/SF2) (25). By contrast, Manley's group showed that the C6U substitution creates a new exonic splicing silencer, which is bound by the splicing repressor hnRNA A1 (89). The latter, together with an ISS localized in intron 6 from −112 to −68 base pairs (bp) (element 1) and intron 7 from +10 to +24 bp (ISS-N1), facilitates exon 7 skipping (124, 158). Among all *cis*-regulatory domains, the discovery of ISS-N1 (158) and its targeting by specific ASOs (79) opened a completely new therapeutic era for the treatment of SMA individuals.

SMN Protein and Cellular Function

SMN protein is essential for every cell and species. The depletion of SMN is early embryonic lethal (155). Since only humans have two *SMN* copies, SMA occurs naturally only in humans; all other species developing SMA are genetically engineered by introducing human *SMN2* copies, *SMN2* cDNA, or mutations that promote exon 7 skipping or decrease SMN functionality (19, 75, 125, 126). SMN participates in different protein complexes involved in small nuclear ribonucleoprotein (snRNP) biogenesis, translation, transcription, microRNA metabolism, stress granule formation, cell survival, ubiquitin homeostasis, DNA damage response, actin cytoskeleton dynamics, endocytosis, vesicular transport, vesicle trafficking along neurons, and energy homeostasis (extensively reviewed in 72, 159, 168, and references therein).

Ribonucleoprotein Function and Splicing

The canonical function of SMN is snRNP biogenesis and splicing (103, 104, 135). SMN depletion mainly affects the U12 minor spliceosome (57). Consequently, an accumulation of U12-dependent intron retention transcripts has been observed, but only a few were reproducibly confirmed, such as TMEM41B (Stasimon) (42, 107). Nonetheless, the restoration of TMEM41B expression level failed to rescue the SMA phenotype in mice (171). Other significantly misspliced transcripts impair calcium homeostasis and voltage-gated calcium channel clustering, which are hallmarks of

SMA (83, 150). This finding fits well with the three SMA protective modifiers, PLS3, NCALD, and CHP1, which bind Ca^{2+} and are either Ca^{2+} sensors or regulators (71, 84, 142). Calcium is essential for many processes at the NMJ level, including exocytosis and endocytosis. Interestingly, all three SMA protective modifiers are able to rescue impaired endocytosis in SMA cells and animal models (40, 71, 84, 142).

Cytoskeletal Dynamics, Endocytosis, Mitochondrial Function, and Local Translation

Although SMN is a housekeeping protein, reduced levels of 20–40%, as found in SMA individuals (68, 101), primarily affect the NMJs innervated by alpha motor neurons. Therefore, cellular pathways other than snRNP biogenesis and splicing might be tissue-specifically impaired in alpha motor neurons (62 and references therein). Whether a motor neuron–specific splicing defect is responsible for reduced SMN levels is still an enigma. Moreover, it remains unknown why some spinal motor neurons and certain regions of the spinal cord are more vulnerable than others. In general, it seems that larger neurons are more vulnerable, as they might rely on higher SMN amounts. Indeed, the gene name "survival of motor neuron" has been perfectly chosen.

One of the earliest findings in SMA pathology was defective NMJ development and maturation accompanied by neurofilament accumulation (29). Studies have also reported synaptic vesicle formation and transmission defects; these defects depend heavily on actin dynamics, which is disturbed in SMA (1, 20, 40, 71, 145). F-actin, which is reduced in SMA, is essential for all types of endocytosis, a crucial process in neurotransmission. Indeed, reduced SMN levels impair endocytosis at the NMJ level and can be restored or ameliorated by the SMA modifiers PLS3, NCALD, and CHP1 (71, 84, 142).

Transport and local translation of mRNAs such as ACTB, GAP43, and CARM1, which are crucial for axonal development and function, are reduced in SMA (3, 98, 145, 152). Since late endosomes are associated with local translation and mitochondrial function in axons (30), and these processes are also affected in SMA (2, 13, 122), one can envisage that SMN reduction disturbs the supply of specific mRNAs in the axon, impairing a plethora of processes connected to proper NMJ function.

GENETIC TESTING AND PHENOTYPE–GENOTYPE CORRELATION

The mutation spectrum in SMA patients is remarkably unique, with 96% showing a homozygous absence of *SMN1* and 4% carrying point mutations (180). Due to the complex genomic structure, gene conversion and de novo rearrangements occur quite frequently. Besides the inverse correlation between disease severity and *SMN2* copy number, additional variants within the *SMN2* gene or independent modifiers, such as PLS3 or NCALD, can further influence disease severity (129, 138, 142).

Genetic Testing

The gold standard genetic testing for SMA is multiplex ligation-dependent probe amplification of *SMN1* and *SMN2* (10, 118). This method allows the identification of SMA patients with a homozygous *SMN1* deletion; SMA patients with one *SMN1* copy, who might be compound heterozygous for a second, subtle *SMN1* variant; the exact number of *SMN2* copies; and healthy heterozygous carriers. It fails to differentiate between individuals with two *SMN1* copies on each chromosome 5 (*cis* version) and individuals with one *SMN1* gene on each chromosome 5 (*trans*

version). It also does not identify subtle mutations in *SMN1* (6% of SMA patients). Therefore, an individual with two *SMN1* copies can still be an SMA carrier (false-negative rate of ~5%) (180).

The existence of two *SMN* genes in the human genome hinders the search for variants in *SMN1*. There are two ways to identify subtle variants in *SMN1*: (*a*) long-range PCR of the entire *SMN1* genomic fragment of approximately 28 kb (exon 1 to exon 8) using primers that specifically amplify *SMN1*, followed by reamplification and sequencing of each exon (96), or (*b*) amplification and cloning of *SMN* cDNA products and PCR-based identification of those carrying the *SMN1* gene. The laborious second version allows the identification of exonic and intronic variants that may cause splicing defects (106, 165). The first method, although fast, fails to detect deep intronic variants that affect splicing.

The Mutation Spectrum of *SMN1*

Regardless of the disease severity, all 5q SMA patients have biallelic *SMN1* mutations (100). In approximately 96% of SMA patients, the genetic cause is an *SMN1* deletion or gene conversion of *SMN1* into *SMN2*, which results in a homozygous loss of *SMN1* exon 7 or exons 7 and 8 (48). While the majority of SMA type I patients have a true *SMN1* deletion, in SMA types II and III, gene conversion of *SMN1* leads to increased *SMN2* copy number. Incomplete gene conversion results in hybrid *SMN1*/*SMN2* genes, with exon 7 of *SMN2* origin and exon 8 of *SMN1* origin (23, 65, 172, 180). A compiled study on *SMN1* deletion screening in SMA patients revealed 96% homozygous deletions in SMA type I, 94% in type II, and 86% in type III (180). Consequently, patients with the milder SMA forms carry a subtle mutation more often than patients with the severe form (6, 85, 106).

In approximately 4% of SMA patients, subtle mutations can be found in combination with *SMN1* loss on the second chromosome 5 (6, 106). Very rarely, and only in consanguineous families, two subtle *SMN1* variants have been reported (23). Currently, 108 different pathogenic *SMN1* variants have been described across *SMN1* (32). The most frequently found subtle mutations are p.Tyr272Cys in the German population and p.Thr274Ile in the Polish population (85, 180); the frameshift mutation p.Arg133fs*148, caused by a 4-bp deletion (c.399_402delAGAG), in the Spanish population; and the frameshift mutation p.Gly261fs*269, caused by an 11-bp duplication (c.770_780dup11), in the Spanish, French, and US populations (6, 23, 31, 133).

De Novo Mutations

The SMA region on 5q13 is highly unstable due to a repeat unit of approximately 500 kb, which is duplicated and inverted and includes several genes other than *SMN1* and *SMN2* (100, 146). Physical and genetic maps of the region and, recently, long-read next-generation sequencing have shown that this region is extremely polymorphic. The various units vary not only in number (from zero to four per chromosome) but also in their orientation (39, 120, 153).

The region is prone to unequal recombination and gene conversion, leading to frequent de novo mutations. Thus, 2% of SMA cases result from de novo mutations, which are more often due to unequal recombination than to gene conversion events (48, 185).

Phenotype–Genotype Correlation

The severity of the SMA phenotype is influenced mainly by the *SMN2* copy number, with more copies resulting in a milder phenotype. Although this correlation is strong, it is not absolute. Thus, 73% of SMA type I patients carry two *SMN2* copies, 78% of SMA type II patients carry

three copies, 50% of SMA type IIIa patients carry three copies, 61% of SMA type IIIb patients carry four copies, and 75% of SMA type IV patients have four copies. The predictive value of three *SMN2* copies is less than that of two or four copies; three *SMN2* copies can be found in 20% of type I, 78% of type II, and 51% of type III patients (24, 48, 181) (**Figure 1**).

Some of the missense mutations, such as p.Tyr272Cys, are associated with a severe phenotype, while p.Thr274Ile is associated with a milder phenotype (85, 180). However, the severity of the phenotype is additionally dependent on the *SMN2* copy number accompanying the subtle mutation (180). Variants within *SMN2* can also influence severity; for example, the missense variant c.859G>C (p.Gly287Arg) increases full-length *SMN2* transcripts and thus positively influences the SMA phenotype (48, 138).

Carrier Detection and Frequency

Based on molecular genetic data, the worldwide SMA carrier frequency is 1:51, or 3,795/193,586 (173 and references therein) (see **Table 1**). All studies included only SMA carriers with one *SMN1* copy (1:0 genotype); SMA carriers with two *SMN1* copies on one chromosome (2:0) or with a point mutation or small deletion within *SMN1* ($1:1^{D}$) were omitted. In the German population, 4.8% of control individuals had two *SMN1* genes per chromosome, and 1.7% of SMA parents carried a subtle *SMN1* mutation (48, 183). Consequently, these individuals will not be recognized by a quantitative *SMN1* screening analysis and are false negatives. The highest frequency seems to occur in the European population (1:41) and the lowest in the sub-Saharan African population (1:145).

Interestingly, a pilot newborn screening study in Germany identified 30 out of 213,276 babies as having homozygous *SMN1* deletions, giving an SMA incidence of 1:7,109 and a carrier frequency of 1:42 (175). This carrier frequency is very close to the previously calculated frequency for a smaller number of European individuals (1:41). The first newborn screening in the United States identified 38 out of 1,530 newborns to be SMA carriers (1:40), which is also very close to the European frequency but higher than the reported frequencies for various specific US ethnicities, which range from 1:48 to 1:97 (174). However, the total number of individuals in this US newborn screening is smaller than those in the other studies (173).

Modifiers of Spinal Muscular Atrophy in Humans

Various modifiers of the SMA phenotype have been found in humans, animal models, and genetic screenings (reviewed in 182, 184). Here, we focus only on positive SMN-independent genetic modifiers found in asymptomatic *SMN1*-deleted individuals in SMA families. Two modifiers have been reported: overexpression of PLS3 and downregulation of NCALD (67, 129, 142). Both were identified by differential expression analysis comparing the transcriptomes of lymphoblastoid cell lines from homozygously *SMN1*-deleted asymptomatic and symptomatic family members. The underlying molecular mechanism responsible for the upregulation of PLS3 or downregulation of NCALD is still unsolved. In both cases, long-distance upstream or downstream regulatory elements seem to be the cause (B. Wirth, unpublished data). No variants within the two genes seem to be responsible, making direct genetic testing on the DNA level impossible.

SMA animal models including mice, zebrafish, flies, and worms in which PLS3 overexpression was induced either genetically or via AAV9-PLS3, or NCALD was downregulated either genetically or via small interfering RNAs or ASOs, have been shown to rescue or ameliorate the SMA pathology. Importantly, these protective modifiers also helped to reveal impaired endocytosis as the primary mechanism that is disturbed by reduced SMN levels and restored by both modifiers (71, 84, 142).

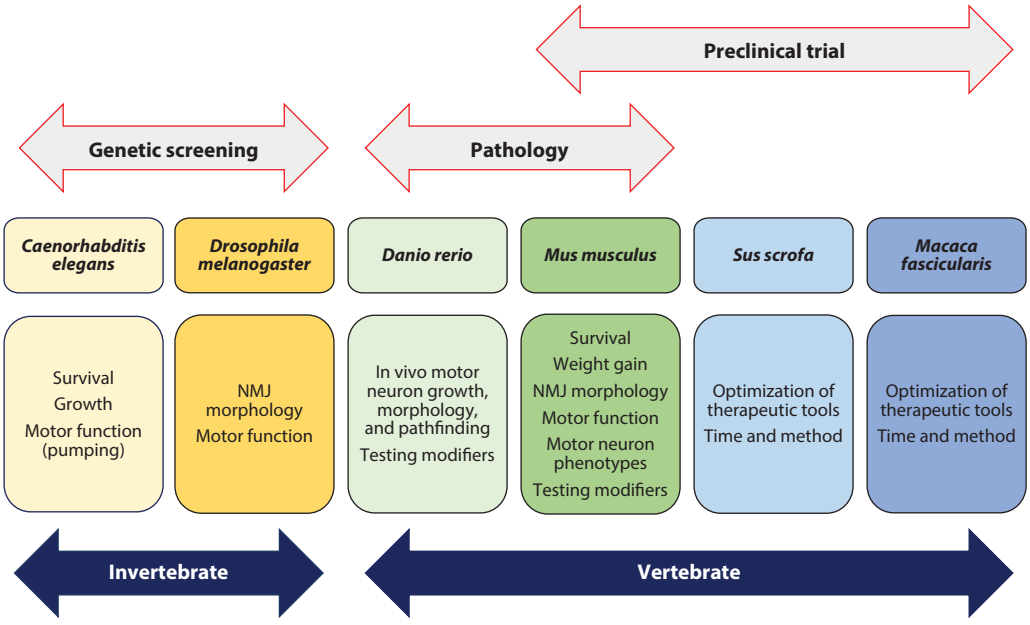

Figure 3

Animal models of SMA. The various models have been the pillar of SMA translational research, and each one has unraveled unique aspects of SMA pathophysiology and/or defined the direction of SMA drug development. The boxes summarize the most important informative aspects that each model contributed to in the SMA field. Abbreviations: NMJ, neuromuscular junction; SMA, spinal muscular atrophy.

ANIMAL MODELS OF SPINAL MUSCULAR ATROPHY

Various SMA animal models have been produced and characterized, including *Caenorhabditis elegans*, *Drosophila*, zebrafish, mice, and pigs, in order to understand basic SMA pathology, determine the target tissues and the time window for effective treatment, identify disease-modifying genes and pathways, and validate therapeutic approaches at a preclinical stage. As each of these animals has only one *Smn* gene in its genome, none of them naturally develop SMA. Different approaches have been used to overcome this problem, including conditional *Smn* knockout, introduction of the human *SMN2* gene, or introduction of splice or missense mutations (46 and references therein). In the following sections, we discuss the animal models and their contributions to our knowledge of SMA (**Figure 3**).

Unraveling the Pathophysiology of Spinal Muscular Atrophy

As SMN is a crucial protein for cell survival, mice with complete *Smn* deletion fail to develop during the early stages of embryogenesis (155). Numerous mouse models have been produced to recapitulate SMA phenotypes by introducing human *SMN2* and/or SMNΔ7 (75, 99, 126). These mouse models showed short survival (6–14 days), impaired motor function, loss of spinal motor neurons, and defects in NMJs and muscle tissues (reviewed in 62 and references therein). They have been used to identify defects in RNA metabolism, protein synthesis, endocytosis, protein homeostasis, the actin cytoskeleton, and Ca^{2+} homeostasis (71, 83, 98, 186, 192). Interestingly, the leading cause of early death in SMA mice might be severe impairment of internal organs, including the lung, heart, pancreas, intestine, vessels, and bones, suggesting that other organs or tissues contribute to SMA pathology (reviewed in 66 and references therein).

Zebrafish SMA models have been used to visualize motor neuron morphology in vivo. SMN protein in zebrafish has a 66% similarity with human SMN (16). Because SMN deficiency in zebrafish causes defects in axonal outgrowth and pathfinding of motor neurons, they have been actively used to identify pathomechanisms and to validate the effect of genetic modifiers (84, 111, 129, 142, 179).

Determining the Target Tissues and the Time Window for Effective Treatment

Transgenic mice with tissue-specific modifications of SMN levels have been generated to identify additional contributors to SMA pathology. First, motor neuron–specific SMN-deficient mice survived 25 days with severe motor impairment (56). Second, muscle-specific deletion of exon 7 caused muscle atrophy, suggesting that SMN is indispensable for muscle (28). However, a tissue-specific deletion and replacement study showed that SMN proteins produced from two copies of *SMN2* might be sufficient for healthy muscle function, suggesting that muscle requires less SMN than motor neurons do (82). Third, liver-specific SMN loss (only with SmnΔ7) caused neonatal lethality with severe defects in liver development, while patients with two copies of *SMN2* did not show any sign of liver dysfunction, suggesting that the liver requires relatively less SMN (177). Thus, complete *Smn* knockout in any organ is detrimental, given the housekeeping role of SMN in snRNP biogenesis and splicing. While SMN levels below 15–20% in mice seem to cause internal organ impairment, levels above 20–25% impair only motor neuron function.

Knowledge from mouse models strongly suggests that different cell types have different levels of susceptibility to SMN deficiency and that restoring SMN levels in peripheral tissues and internal organs will be necessary for SMA patients (162). Even in the nervous system, it seems that motor neurons are not the only ones affected by SMA. Defects in proprioceptive sensory neurons have been reported together with defects in spinal circuitry (115). Whether the impairment of sensory neurons is due to intrinsic defects or is a consequence of motor neuron dysfunction is still uncertain (60).

In mice, SMN levels are higher in neonatal days (around postnatal day 5) and then are gradually reduced (around postnatal day 15), which underlines their importance during the neonatal period (61). Indeed, tamoxifen-induced SMN reduction confirmed the pivotal role of SMN protein in NMJ formation during the neonatal period (until postnatal day 17). Once the NMJs are matured, the amount of SMN protein required for maintenance is significantly lower (88). Together, these data narrow the time window for effective treatment, mainly corresponding to the NMJ developmental period.

Identifying Disease-Modifying Genes and Pathways of Therapeutic Relevance Using Invertebrate Models

The *C. elegans* SMA model shows delayed development and defects in motor functions such as motility and pharyngeal pumping. Genome-wide genetic screenings of *C. elegans* have been performed and revealed the genetic modifiers and affected pathways of SMA: grk-2 as a modifier of SMA pathology and endocytosis as an affected cellular function of SMA (40, 41). Genetic screens in *C. elegans* or *Drosophila* SMA models unraveled more than 300 genetic modifiers (156).

Validating Therapeutic Tools for or During Preclinical Studies

Splice-modifying ASOs were first tested in an SMA mouse model with *SMN2* to confirm their effectiveness in vivo (78, 80). Recently, combinatorial therapy with two different ASOs—an *SMN2*

splice corrector and an inhibitor of the NCALD genetic modifier—was tested in an SMA mouse model and showed promising outcomes (169). Gene therapy with self-complementary adeno-associated virus (scAAV) delivery of *SMN1* alone or with other genetic modifiers, including IGF, PLS3, and STMN1, has also been tested in SMA mouse models (45, 55, 87, 121, 134, 170, 176). The efficiency and safety of gene therapy were later confirmed in large animals, such as pigs and monkeys (121, 134). Pigs have been used as a model organism for preclinical trials because the size and morphology of their spinal cords are similar to those of humans (47). For example, the efficiency of scAAV vector serotypes for spinal motor neuron transduction has been tested in pigs (163). A porcine SMA model has been produced and used to optimize the time window of treatment and to test functional outcomes of scAAV9-mediated SMN gene therapy (44). This study showed that even after the disease manifests, SMN restoration could still ameliorate SMA phenotypes. However, restoring SMN levels in presymptomatic stages showed impressive effects on motor function, implying that neonatal screening is indeed crucial to protect newborns from the disease. Needless to say, monkeys are used as nonhuman primate models to validate the efficiency of gene therapy before clinical trials. The monkey models confirmed that intrathecal injection is the most efficient method of delivering scAAV9 to spinal cords (134). Reference 86 provides a nice overview of preclinical studies.

THERAPY OF SPINAL MUSCULAR ATROPHY

SMN-Dependent Therapies

The unique genetic scenario of SMA patients (lack of functional *SMN1* and variable *SMN2* copy numbers, short cDNA length, and a uniform mutation spectrum) provided an avenue for therapy development and became the epicenter of SMA translational research. Over the past few years, efforts aiming to modulate both *SMN* genes led to the first effective therapies for SMA: the up-regulation of full-length *SMN2* transcripts (and consequently of functional SMN protein) and the replacement of the defective *SMN1* gene.

***SMN2* modulators.** New agents designed to correct the missplicing of the *SMN2* gene product are at the forefront of SMA clinical trials (**Table 2**). Specifically, *SMN2* splicing modulators target the *SMN2* pre-mRNA and promote the retention of the exon 7 in *SMN2* transcripts, thereby enhancing the translation of fully functional *SMN2*-encoded SMN protein.

Nusinersen, an antisense oligonucleotide therapy. ASOs are short, synthetic, single-stranded, chemically modified nucleic acids designed to complementarily bind to a specific mRNA target. ASOs exert their final effect by promoting RNA degradation, interfering with pre-mRNA processing, blocking RNA binding motifs, or disrupting target mRNA structure (143) (**Figure 4**).

Nusinersen is an 18-mer modified 2′-*O*-2-methoxyethyl phosphorothioate ASO that blocks the binding of hnRNP A1 to the ISS-N1 motif, thereby promoting exon 7 inclusion in *SMN2* (80). The first preclinical studies in severe SMA mice treated with nusinersen showed its impressive effects on SMN protein levels (exon 7 inclusion higher than 90% in *SMN2* transcripts), increased survival, and improved muscle physiology (77, 143), which led to expedited approval of the *SMN2* ASO (ISIS SMNRx) and the initiation of the first clinical trial (NCT01494701).

Since its introduction into clinical practice on December 23, 2016, nusinersen has been commercialized under the name Spinraza (Ionis Pharmaceuticals and Biogen) and is the only treatment licensed by the US Food and Drug Administration and European Medicines Agency for all types of SMA in pediatric and adult patients. Encouraging results from the ENDEAR

Table 2 Spinal muscular atrophy therapies undergoing clinical development studies

Type of therapy	Strategy	Drug name	Organization	Identifier(s)	Current clinical stage
SMN dependent					
SMN2 modulators	*SMN2* ISS-N1–targeting ASO	Nusinersen (Spinraza)	Biogen/Ionis	NCT02386553 (NURTURE) NCT02052791 NCT02865109 NCT01780246 NCT02462759 NCT01703988 NCT02193074 (ENDEAR) NCT02292537 (CHERISH) NCT01839656 NCT02594124 (SHINE) NCT01494701	Administered to patients
	SMN2-targeting small molecules	Risdiplam (RG7916)	Hoffmann–La Roche/Genentech	NCT03032172 (JEWELFISH) NCT02913482 (FIREFISH) NCT02908685 (SUNFISH) NCT02240355 (MOONFISH) NCT02633709	Ongoing phase 3
		Branaplam (LMI070)	Novartis	NCT02268552	Ongoing phase 2
SMN1 gene therapy	*SMN1* gene transfer	Onasemnogene abeparvovec [AVXS-101, Zolgensma (IV)]	AveXis/Novartis	NCT02122952 (START) NCT03381729 (STRONG)	Approved by US Food and Drug Administration
SMN independent					
Neuroprotection	Enhancement of mitochondrial function	Olesoxime (TRO19622)	Hoffmann–La Roche	NCT01302600 NCT02628743 (OLEOS)	Drug development terminated
Muscle enhancement	Troponin-dependent increase of muscle contraction	Reldesemtiv (CK-2127107)	Cytokinetics/Astellas	NCT02644668	Phase 2 completed
	Inhibitor of latent myostatin	SRK-015	Scholar Rock	NCT03921528 (TOPAZ)	Ongoing phase 2

Abbreviations: ASO, antisense oligonucleotide; ISS-N1, intronic splicing silencer N1.

study (NCT02193074) in infant-onset SMA (53) and the CHERISH study (NCT02292537) in childhood-onset SMA (116) accelerated the approval of Spinraza. Preceding these trials and further supporting the clinical efficacy, tolerability, and safety of Spinraza were two phase 1 and 2 studies (26, 50) and two open-label studies: NURTURE (NCT02386553), a phase 2 trial

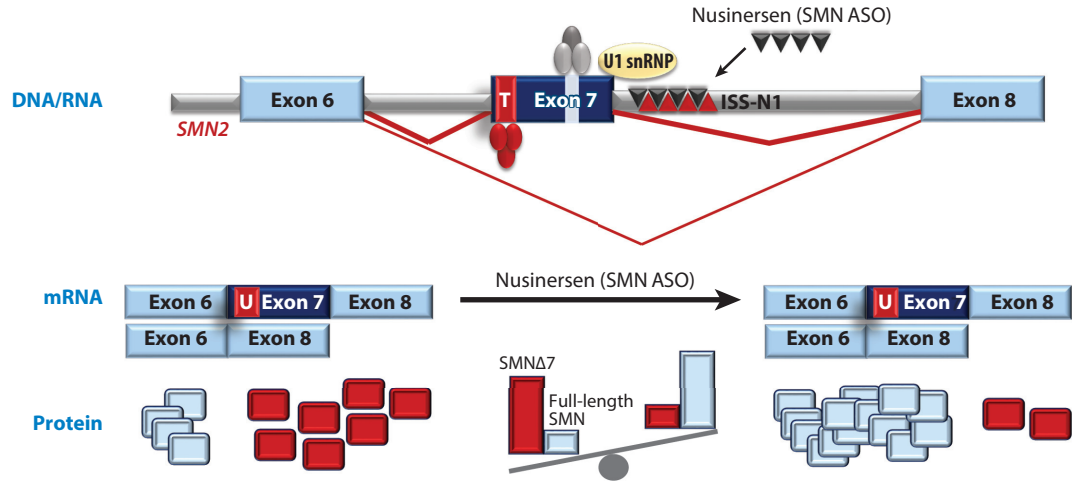

Figure 4

Nusinersen-mediated restoration of *SMN2* splicing. The ASO nusinersen (*black triangles*) blocks the ISS-N1 site in intron 7 (*red triangles*), impairing the access of negative splicing factors (hnRNP A1/2) and stabilizing the U1 snRNP machinery that recognizes exon 7 in *SMN2* pre-mRNA. It thereby promotes exon 7 inclusion in *SMN2* mature transcripts and shifts the balance toward increased full-length SMN protein levels. Abbreviations: ASO, antisense oligonucleotide; hnRNP, heterogeneous nuclear ribonucleoprotein; ISS-N1, intronic splicing silencer N1; snRNP, small nuclear ribonucleoprotein.

in presymptomatic infants (38), and SHINE (NCT02594124), an ongoing phase 3 study that aims to assess the long-term clinical effects of Spinraza treatment (for a summary of studies, see **Table 3**). The treatment protocol includes an initial loading dose period consisting of four 12-mg intrathecal injections over two months, followed by a maintenance period with drug injections every four months (73). In general, nusinersen has been found to be safe and well tolerated (64, 73). The reported side effects are consistent with either the expected symptomatology of SMA or the related effects of a lumbar puncture. To date, more than 10,000 patients have undergone therapy with nusinersen worldwide.

Small molecules. Small-molecule therapies that modulate *SMN2* splicing are being developed and will be a key part of the future clinical landscape of SMA. Perhaps the most significant benefit of these molecules is their ease of administration, as they are orally bioavailable and, unlike nusinersen, have systemic distribution, targeting not only the central nervous system but also other organs. The latter is of crucial importance, considering the contribution of peripheral organs and tissues to the pathology of SMA (76, 78). Besides the limited ability of these molecules to effectively cross the blood–brain barrier, their major drawback is the potential risk of off-target effects (18).

To circumvent the unspecificity of small molecules, PTC Therapeutics and Hoffmann–La Roche performed a high-throughput chemical screening of *SMN2* splicing modifiers and identified two molecules, RG7800 and RG7916, that selectively shifted *SMN2* splicing by stabilizing the U1 snRNP complex and, remarkably, were able to cross the blood–brain barrier (128, 137). Oral administration to severe SMA mice extended their life spans, led to increased SMN levels, protected the NMJ, and improved motor function (128, 140, 141). The first phase 1 clinical trial (MOONFISH, NCT02240355), which aimed to test the safety, tolerability, and pharmacological properties of RG7800 in pediatric and adult SMA, was terminated soon after the enrollment phase due to the eye toxicity detected in long-term tolerability studies.

Table 3 Overview of important nusinersen clinical trials

Study	Design and objective	Patients	Time period	Primary outcome	Observations
SMA type I					
NURTURE (NCT02386553)	Phase 2, open label, single group assignment Assess the efficacy, safety, tolerability, and pharmacokinetics of multiple doses of nusinersen	Infants (six weeks or younger) genetically diagnosed with presymptomatic SMA with two or three *SMN2* copies	2015–2022[a]	Time to death or respiratory intervention (i.e., ventilation for six or more hours per day continuously for seven or more days or tracheostomy)	All infants were alive and none required respiratory intervention after one year of nusinersen delivery. All showed improved HINE motor milestones and appropriate age-related developmental gains (38).
SHINE (NCT02594124)	Phase 3, open label, nonrandomized, parallel assignment Evaluate the long-term safety and tolerability of nusinersen	SMA patients who previously participated in investigational studies of nusinersen (ENDEAR)	2015–2023[a]	Adverse events and/or serious adverse events based on neurological examination, laboratory assessment, coagulation parameters, and weight and 12-lead ECG abnormalities	Interim evaluation: Treatment was safe and well tolerated. Motor skills improved in all patients, with greater motor and developmental improvements in patients who started nusinersen therapy in ENDEAR.
ENDEAR (NCT02193074)	Phase 3, randomized, sham-procedure controlled Assess the clinical efficacy and safety of intrathecal-administered nusinersen	Infants (210 days or younger) with SMA and two *SMN2* copies	2014–2016	Percentage of motor milestone responders and time to death or permanent ventilation	Overall survival and probability of event-free survival were significantly higher in the treated group. The treated group had a lower risk of death (63%) than the sham-procedure control group. Nusinersen-treated infants (53%) achieved more HINE motor milestones (i.e., 22% achieved head control, 10% were able to roll, 8% were able to sit without assistance, and 1% were able to stand). Control group infants did not gain any milestones (53).
SMA types II and III					
CHERISH (NCT02292537)	Phase 3, randomized, sham-procedure controlled Assess the clinical efficacy and safety of intrathecal-administered nusinersen	SMA patients aged 2–12 years with onset of clinical symptoms after six months of age	2014–2017	Change from baseline in HFMSE score at month 15 of treatment	Interim evaluation: Nusinersen treatment increased HFMSE score by a mean of 4 points, whereas the sham-procedure control group had a decline of 1.9 points. Final trial analyses: Children treated with nusinersen showed definite motor improvement and higher survival likelihood than the control group (116).
CS12 (NCT02052791), including CS2 (NCT01703988) and CS10 (NCT01780246)	Phase 1, open label, single group assignment Assess the safety and tolerability of nusinersen in patients from the CS2 and CS10 studies	CS12: children, adults, and older adults with SMA CS2 and CS10: SMA patients aged 2–15 years with an estimated life expectancy of more than two years from screening	2014–2017	Adverse events and/or serious adverse events based on neurological and physical examination, laboratory and cerebrospinal fluid laboratory assessment, and weight and ECG abnormalities	Nusinersen treatment over approximately three years led to motor function improvements and disease stabilization not observed in historical SMA cohorts. Nusinersen showed long-term benefit in later-onset (type III) SMA.

Abbreviations: ECG, electrocardiogram; HFMSE, Hammersmith Functional Motor Scale–Expanded (≥3 points indicates an improvement in at least two motor skills); HINE, Hammersmith Infant Neurological Examination; SMA, spinal muscular atrophy.

[a]Estimated end of ongoing trial.

By contrast, the second Hoffmann–La Roche molecule, RG7916 (risdiplam), managed to reach phase 2 clinical studies and Priority Medicines (PRIME) designation by the European Medicines Agency after the successful completion of the phase 1 study (NCT02633709). Currently, risdiplam is under evaluation in three ongoing clinical trials in Europe: one in 1–7-month-old infants with severe SMA (FIREFISH, NCT02913482), one in 2–25-year-old patients with SMA type II or III (SUNFISH, NCT02908685), and one in children and adults with SMA type II or III who had already received daily doses of risdiplam for two years (JEWELFISH, NCT03032172). Interim analyses of the FIREFISH study reported improved motor function and event-free survival in infants with SMA type I compared with the infants from historical cohorts. In addition, interim analyses of the SUNFISH study reported that risdiplam has led to a sustained increase of SMN with no drug-related safety issues leading to study withdrawal (119; E. Mercuri, unpublished results presented at the 23rd International SMA Researcher Meeting, Anaheim, California, USA, June 27–30, 2019).

Another small molecule promoting exon 7 inclusion in *SMN2* is LMI070 (branaplam), developed by Novartis Pharmaceuticals. Preclinical analyses of branaplam in severe SMA mice showed not only an increase in SMN levels but also an extended life span in orally treated animals (131). Phase 1–2 clinical trials of branaplam administration in SMA type I patients (NCT02268552) were discontinued after more than a year due to evidence of toxicity to blood vessels, kidney, spinal cord, and peripheral nerves from animal studies conducted in parallel (132).

***SMN1* gene replacement.** Despite the breakthrough effects of nusinersen in the SMA therapy field, some major limitations remain, including the need for periodic intrathecal administration, the fact that nusinersen does not address the upregulation of SMN in other relevant tissues (e.g., muscle and the NMJ), and the questionable sustainability of low peripheral levels of SMN in the long run. This last item is of crucial importance, considering that approximately 80% of patients with SMA type I carry one or two copies of the *SMN2* gene (48). The availability of nusinersen is also contingent on its elevated price and particular hospital specifications.

Being a monogenic disorder, SMA offers an excellent constellation for gene replacement therapy. The relatively small *SMN1* cDNA can be successfully packed into a nonreplicating scAAV9 vector that can be systemically delivered. Thus, it can efficiently transduce spinal motor neurons, as it can cross the blood–brain barrier, and reach the muscle or other peripheral tissues where the SMN protein is abundantly expressed.

Preclinical studies in severe SMA animal models (i.e., mouse and pig) showed that intravenous injection of scAAV9-*SMN1* restored SMN levels, extended survival, and restored motor function and neurophysiology (44, 55, 121). This preliminary evidence was foundational for a phase 1 open-label clinical trial (START, NCT02122952). In this study, a single intravenous dose of AVXS-101 (scAAV9-*SMN1*, driven by the cytomegalovirus enhancer and the chicken β-actin promoter) was administered to 15 infants with SMA type I carrying a biallelic mutation of *SMN1* and two *SMN2* copies (113). Patients were stratified in two cohorts that received either a high dose [12 patients, 2.0×10^{14} vector genomes (vg)/kg body weight] or a low dose (3 patients, 6.7×10^{13} vg/kg body weight) of the medication. The scAAV-treated group was matched against historical cohorts from the NeuroNEXT SMA infant biomarker study (94).

Patients who received the gene therapy showed, in a dose-dependent effect, superior and sustained achievement of motor milestones and better motor function, in contrast to the motor decline observed in historical cohorts. Specifically, 11 out of 12 patients in the high-dose group were able to sit unaided, 9 could roll over, 11 could be orally fed, and 2 walked independently. Most importantly, all 15 patients were alive and independent of mechanical ventilation at 20 months of age, in contrast to 8% survival in historical cohorts resembling the natural course of the disease

(113). AVXS-101 (onasemnogene abeparvovec) administration increased liver transaminase levels 35 times above reference levels, probably as a consequence of the massive immune response against viral peptides, which could be successfully reversed by daily glucocorticoid administration (1 mg/kg) for one month.

In the long term, the beneficial effect of onasemnogene abeparvovec on motor function appears to be sustained (4, 5, 114). At 3.7 years after the initial gene replacement, all patients from the higher-dose group were alive, and none required permanent ventilation support. Moreover, achieved motor milestones and motor improvements were also sustained over time. At the time of analysis (March 8, 2019), seven patients were not hospitalized or receiving additional care, and three patients had started adjuvant nusinersen therapy (114). Interim results of an ongoing open-label phase 1–2 clinical trial of intrathecal administration of onasemnogene abeparvovec in patients with three *SMN2* copies (STRONG, NCT03381729) showed a sustained gain of motor milestones and, as the primary outcome, treatment safety (52).

On May 24, 2019, onasemnogene abeparvovec (marketed under the name Zolgensma) became the first gene therapy to be approved in the United States for the treatment of pediatric SMA patients (up to two years of age). The recommended dose (1.1×10^{14} vg/kg body weight, estimated retrospectively from the first dose of 2.0×10^{14}) is delivered in a single intravenous injection (74). The relevant safety information for Zolgensma includes severe acute liver injury as the main risk; however, the recommended dose appeared to be well tolerated both short and long term in patients with SMA type I or II and presymptomatic SMA infants (reviewed in 74).

Primary outcomes from the clinical trials for onasemnogene abeparvovec (AVXS-101-CL-101, NCT02122952) and nusinersen (ENDEAR, NCT02193074) were compared using frequentist and Bayesian approaches. This indirect analysis suggested that onasemnogene abeparvovec could have a superior benefit relative to nusinersen in terms of overall survival, independence from assisted ventilation, motor function, and achieved motor milestones. Onasemnogene abeparvovec also appears to offer a more favorable cost–utility procurement (74, 109).

SMN-Independent Therapies

A significant body of evidence has substantiated that SMA is a systemic disorder that goes beyond motor neurons. Indeed, other organs and cell types are subclinically affected in SMA patients and animal models (66 and references therein). Moreover, mouse models corroborate that the disease is a non-cell-autonomous defect of the motor neurons (76). In this context, the implementation and further development of SMN-independent therapies are extremely relevant, considering that SMN-dependent therapies reduce disease severity but do not cure the disease. Moreover, targeting only the central nervous system will not address the multiorgan impairment of SMA patients, especially those with the most severe presentation of the disease (66). SMN-independent therapies hold the key to enhancing the beneficial effects of SMN-dependent strategies if used in a combinatorial therapy approach.

Neuroprotection. Enhancing motor neuron survival and function presents an attractive therapeutic target for SMA patients. Olesoxime (TRO19622) is an orally active cholesterol-like molecule that preserves mitochondrial function by targeting components of the mitochondrial permeability complex and preventing the release of proapoptotic factors that lead to motor neuron death. At the preclinical level, olesoxime-mediated restoration of mitochondrial homeostasis preserves motor neuron integrity and reduces muscle denervation, astrogliosis, and microglial activation (166).

Olesoxime safety and tolerability were assessed in a phase 2, randomized, placebo-controlled clinical trial (NCT01302600) in which 165 patients with SMA type II or III and ranging in age from 3 to 25 years received a twice-daily oral administration of olesoxime (10 mg/kg) for 24 months. Although the trial did not meet the primary outcome of improved motor function, it did show that olesoxime delays the typical functional decline observed in untreated SMA patients (15, 117). However, Hoffmann–La Roche announced that it would not pursue further studies of olesoxime for the treatment of SMA, as a later phase 2 trial (OLEOS, NCT02628743) yielded a decline in motor function after 18 months of treatment.

Restoration of muscle function. Skeletal muscle enhancement therapies aim to counteract muscle atrophy by improving muscle performance and increasing muscle mass. One of these enhancers is the fast skeletal muscle troponin activator reldesemtiv (CK-2127107), which not only slows down calcium release from the troponin complex but also sensitizes the sarcomere response to calcium (81). Most importantly, it amplifies the skeletal muscle force–frequency response upon nerve stimulation (7). Reldesemtiv administration (single doses up to 4,000 mg) proved to be safe and tolerable in phase 1 studies conducted in healthy individuals. A consecutive phase 2, randomized, placebo-controlled trial (NCT02644668), which enrolled 70 patients with SMA type II, III, or IV, showed a significant drug concentration–dependent increase in aerobic capacity and endurance and a significant improvement in respiratory muscle strength (S. Rudniki, unpublished update of clinical trial CY5021 presented at the 22nd International SMA Researcher Meeting, Dallas, Texas, USA, June 14–16, 2018).

SRK-015 (Scholar Rock) is a highly selective monoclonal antibody that acts as an inhibitor of latent myostatin (136). Preclinical studies demonstrated that it efficiently promotes muscle cell growth and differentiation, increases muscle function, and improves the bone phenotype of SMA mouse models (49, 105). The safety, pharmacokinetics, and pharmacodynamics of increasing SRK-015 doses are being assessed in a phase 1, randomized, placebo-controlled trial. Recent interim results validate its safety and efficacy in a once-every-four-weeks dosing regimen. Specifically, after a single dose of SRK-015 (10, 20, or 30 mg/kg), serum levels of latent myostatin were significantly increased and sustained over almost three months (Y. Chyung, unpublished results reported at the 23rd International SMA Researcher Meeting, Anaheim, California, USA, June 27–30, 2019).

THE FUTURE OF SPINAL MUSCULAR ATROPHY SCREENING AND TREATMENT

Newborn Screening

All preclinical and clinical studies have shown that early therapeutic intervention, preferably presymptomatically, achieves the best results. The most recent studies on autopsy material from SMA and control individuals have shown that SMN levels are highest prenatally, drastically decline postnatally, and further drop after three months of age. These findings emphasize the need for SMN immediately after birth, or even prenatally (139). Strikingly, neurofilament levels at birth are already greatly increased in SMA patients with two *SMN2* copies, strongly hinting at increased axonal degradation (36). Therefore, newborn screening is essential to identify individuals carrying homozygous *SMN1* deletions and to start treatment in the presymptomatic phase. Based on the neurofilament data, for individuals with two *SMN2* copies, the notion of "presymptomatic" is already questionable.

In the United States, Belgium, Australia, and Germany, newborn screening either has been introduced nationwide or will be introduced soon. In the United States, newborns with four *SMN2*

copies are not directly included in therapy but are followed in a watch-and-wait strategy (59). However, based on several considerations, immediate treatment is crucial to allow proper development and maturation of NMJs, which occurs before two years of age. In mice, conditional depletion of *Smn* before postnatal day 17 caused SMA, while after that time, no phenotype was observed (88). Therefore, even if the therapy is discontinued or reduced, enough SMN should be available during the critical period of NMJ development and maturation. The amount of SMN might be insufficient in individuals with four *SMN2* copies, as some patients with four copies developed SMA as early as eight months of age (175). Onasemnogene abeparvovec is approved only for children under two years of age in order to allow proper blood–brain barrier penetration of scAAV9-*SMN1*, and individuals with four *SMN2* copies will also significantly benefit, since they most likely will never develop SMA under therapy.

New Emerging Phenotypes

With two approved drugs (nusinersen and onasemnogene abeparvovec) and others in the pipeline (risdiplam), one can envisage that almost every infant will be treated presymptomatically as soon as a homozygous *SMN1* deletion is detected during newborn screening. However, even in countries where an SMA newborn screening has been implemented, there are still gaps in the health-care system (home birth, clinical capacity, infrastructure, and drug availability) that might prevent immediate treatment in the neonatal period, before the irreversible loss of motor neurons and the development of the first symptoms. Moreover, while it is very likely that individuals with three or four *SMN2* copies will never develop any phenotype if treated presymptomatically, it is still questionable whether treatment of patients with two *SMN2* copies will be sufficient to maintain the function of motor neurons and other cell types throughout their lives. Presymptomatically, nusinersen-treated newborns with three *SMN2* copies show motor development similar to that of healthy children, in contrast to newborns with only two *SMN2* copies. The latter underperformed in comparison with the natural development of healthy controls, clearly demonstrating that intrathecal injection of nusinersen is not sufficient to fully counteract SMA (38).

Moreover, there is still little knowledge of the long-term effects of nusinersen or onasemnogene abeparvovec therapy. Therefore, patients need to be clinically followed over a reasonable period (6–10 years), with special emphasis on cognitive abilities, social integration, and heart development. Doing so would allow the identification of potential problems and the adoption of precautionary measures if needed.

Approximately 10,000 SMA patients are currently being treated with nusinersen, and several hundred have been treated with onasemnogene abeparvovec. All patients appear to have benefited from the therapy, by either stabilizing or improving the phenotype. However, patients with only two *SMN2* copies in particular might develop new phenotypes not previously seen due to the early lethality of SMA type I and may need additional SMN-independent therapy.

Combinatorial Therapies

SMN protein is essential for every cell. Therefore, it is likely that in individuals with only two *SMN2* copies, even if they are treated presymptomatically with nusinersen or systemically with onasemnogene abeparvovec (which is diluted with every cell division), the SMN may be insufficient for dividing cells. Risdiplam may be an option to support all other cell types systemically. However, clinical trials are not yet complete, and therefore it is premature to conclude that a combination of nusinersen, onasemnogene abeparvovec, and/or risdiplam is the ultimate solution. It still might be that SMN-independent therapies are necessary, such as a decrease of the protective

SMA modifiers NCALD or CHP1 or an increase of PLS3 (71, 84, 87, 142, 169) or the restoration of the calcium level by the troponin activator reldesemtiv (7).

Carrier Screening

More and more carriers will be identified, either by screening programs such as those in Israel and Taiwan or in diagnostic settings based on exome and genome screens in the future. How a couple with a 25% a priori risk of having a baby with SMA will make a decision in the future is difficult to predict. However, the decision might strongly depend on whether SMA becomes curable (as opposed to treatable) along with, most likely, the future costs of therapy.

Ethical and Financial Issues

Currently, the cost of Spinraza is approximately $400,000–500,000 (or €400,000–500,000) in the first year and $250,000–300,000 (or €250,000–300,000) per year for the duration of the patient's lifetime. The cost of Zolgensma is $2 million for a single injection. These prices are not affordable for any health-care providers in the long run. With the increasing number of therapies for rare diseases, in the near future, an ethical and thoughtful consideration of all players (industry, the health-care system, patients, and caregivers) will be crucial.

DISCLOSURE STATEMENT

B.W. and N.M.-F. are inventors on patent applications related to SMA gene modifiers, NCALD, and/or CHP1.

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