SPINAL MUSCULAR ATROPHY: CLINICAL CHARACTERISTICS, GENETICS AND MANAGEMENT

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Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is a neurodegenerative disease, following the autosomal receive mode of inheritance and it is characterized by degeneration of anterior horn Motor Neurons (alpha MNs) in the lower spinal cord, leading to progressive symmetrical muscular weakness and atrophy. The worldwide incidence of SMA is approximately 1-2 in 11.000 [1] and the prevalence is 1-2 per 100.000. The carrier frequency ranging from 1:91 in African Americans to 1:35 in Caucasians and 1:54 in United States [2, 3] could be slightly different based on the ethnic group [3, 4]. Werdnig and Hoffman in the early 1890s were the first to describe a mild phenotype of SMA; but only in the early 1900s did Beevor describe for the first time a severe form of SMA with the involvement of the respiratory system due to intercostal muscle weakness [5, 6]. SMA is caused by loss of the Survival Motor Neuron 1 (*SMN1*) gene on chromosome 5q (in 95% of SMA cases) which encodes for the *SMN* protein [7]. Interestingly, in humans on the same chromosome there is a second centromeric form of *SMN1*, called *SMN2*, which translates approximately 10% of the functional *SMN* protein and can be present in different copy numbers [7], which is relevant for the clinical manifestation as *SMN2* copy number significantly correlates with disease severity (see below).

Clinical Picture

SMA pathology is heterogeneous, with a wide range of phenotypic spectrum. It is classified into five different types according to the age of onset and maximum function (Table 1). Briefly, the most severe types are SMA 0 (Congenital SMA) and SMA type I (Werdnig Hoffmann disease). Regarding SMA type 0, the onset is before birth (in utero) and death occurs immediately after or within two weeks from birth. In SMA type I, children never achieve the ability to sit independently and they present also fasciculations of the tongue. Oculomotor and mimic muscles seem not to be involved; but in cases with cardiogenesis, defects have been reported [8]. Of further note, cognitive and sensory functions are not compromised. If not treated or unsupported, death usually occurs before reaching the second year of life [9]. In contrast to SMA1 patients, SMA type II patients are able to sit but unable to walk independently and may survive into adulthood - though with shorter life expectancy. SMA type III and type IV are the mildest forms. In particular, SMA type III patients are ambulant, even though some of them might lose autonomous de-ambulation over time. Meanwhile SMA type IV is considered an "adult-onset" form of SMA, with age-of-onset typically occurring after 30 years and ranging from normal motor function to mild motor impairment and preserved life expectancy. All SMA patients lack the *SMN1* gene, therefore the amount of *SMN* protein produced depends entirely on the number of *SMN2* copies that each individual carries. The broad phenotypic spectrum associated with *SMN1* deficiency is driven by the number of the *SMN2* gene copies (see Table 1).

SMA can also be classified into 4 groups based on the severity of the disease and on the number of *SMN2* copies carried by patients [10].

SMN Gene And Protein

SMN1 is a telomeric gene comprised of eight exons: denoted 1, 2a, 2b, 3, 4, 5, 6 and 7. This gene is located on the chromosome 5 in a complex region (5q13.2) characterized by the duplication of an approximate 500 kb element containing several genes [7].

The *SMN1* gene encodes a 294 amino acid RNA-binding protein (38 kDa), which is ubiquitously expressed in all the human body, with higher levels detected in the spinal cord, brain, kidney and liver [7, 11]. Immunohistological staining of *SMN* protein revealed its localization in the nucleus, in the shape of foci, and diffuse staining in the cytoplasm [12]. The dot-like structures formed by *SMN*, similar and adjacent to coiled bodies (CBs or Cajal bodies), are called "Gemini of coiled bodies", simplified as Gems [11]. Gems are a dynamic nuclear structure and their number, like the expression level of *SMN* protein, is directly correlated to the SMA phenotype [11, 13].

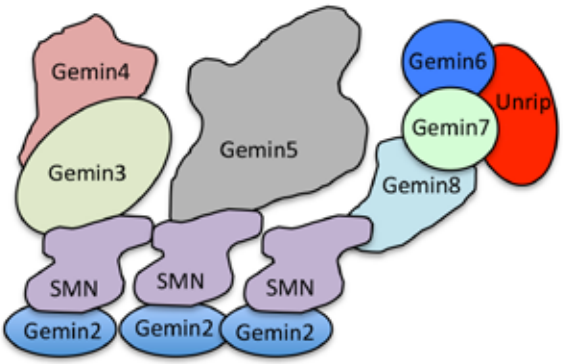
Table 1. SMA phenotype classification

Phenotype	Age at Onset	Life span	Motor Milestones	SMN2 Copies
Healthy Carrier	/	Normal	Normal	0
SMA 0 (Congenital SMA)	Prenatal	<6 months	None Achieved	1
SMA I (Werdnig-Hoffmann disease)	< 6 months	Most often ≤ 2 years, but may live longer	Sit with support only	2
SMA II (Dubowitz disease)	6-18 months	70% alive at age 25	Independent sitting when placed	3-4
SMA III (Kugelberg-Welander disease)	> 18months	Normal	Independent ambulation	3-4
SMA IV	Adulthood	Normal	Normal	4-8

SMN is composed by five domains: (i) N-Terminal-Gemin 2 domain, (ii) Tudor domain, (iii) basic-lysine-rich domain, (iv) the YG box domain, and (v) C-terminal proline-rich domain [14] (Fig. 1). In particular, the basic/lysine-rich region, encoded by exon 2, has been demonstrated to interact with *SMN*-Interacting Protein 1 (SIP1), also known as Gemin2, RNAs and other proteins, such as p53, both *in vitro* and *in vivo* [15-17]. The Tudor domain is another important region present on *SMN*. It is a highly conserved motif codified by exon 3 that binds to the terminal glycine and symmetrical dimethylated arginine-rich tails of Sm ribonucleoproteins, facilitating the assembly of the spliceosomes [18-20]. Moreover, it is responsible for the interaction with other proteins, such as Fused-in-Sarcoma (FUS), Histone 3 and Fragile X Mental Retardation Protein (FMRP) RNA polymerase II 21. Mutations in this domain are often found in SMA patients [18-21].

The other domains of *SMN* are YG box (tyrosine-glycine-rich region), which interacts with zinc-finger protein (ZPR1), amphipathic helix protein SIN3A (transcription co-repressor) and Gemin3 [14], and the proline rich domain that interacts with Profilins, a family of proteins involved in actin dynamics [22]. It has been reported that *SMN* protein, through the YG domain, is able to oligomerize with itself and that the loss of exon 7 decreases the efficiency and stability of self-oligomerization [16, 23] (Fig. 2).

Fig. 1. Schematic representation of *SMN* mRNA and corresponding *SMN* protein binding domains



SMN1 possess four domains which allowed the binding to other proteins or with itself. Lysine-rich domain, Proline-rich domain and YG box domain bind to other *SMN1*, Tudor domain allows the binding to Sm and Lysin-erich domain binds to Gemin2 protein

Fig. 2. Schematic representation of SMN complex. SMN complex is composed by nine proteins, SMN, Gemin family from 2-7 and Unirip

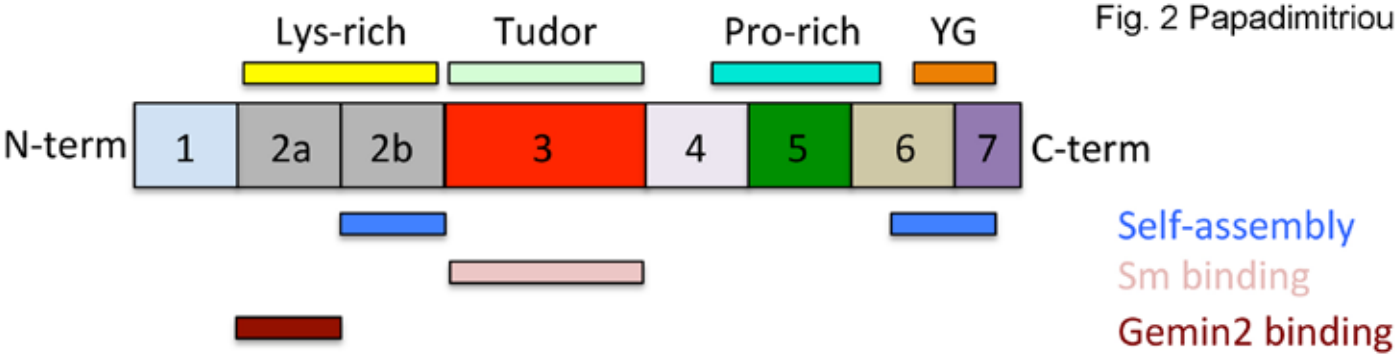
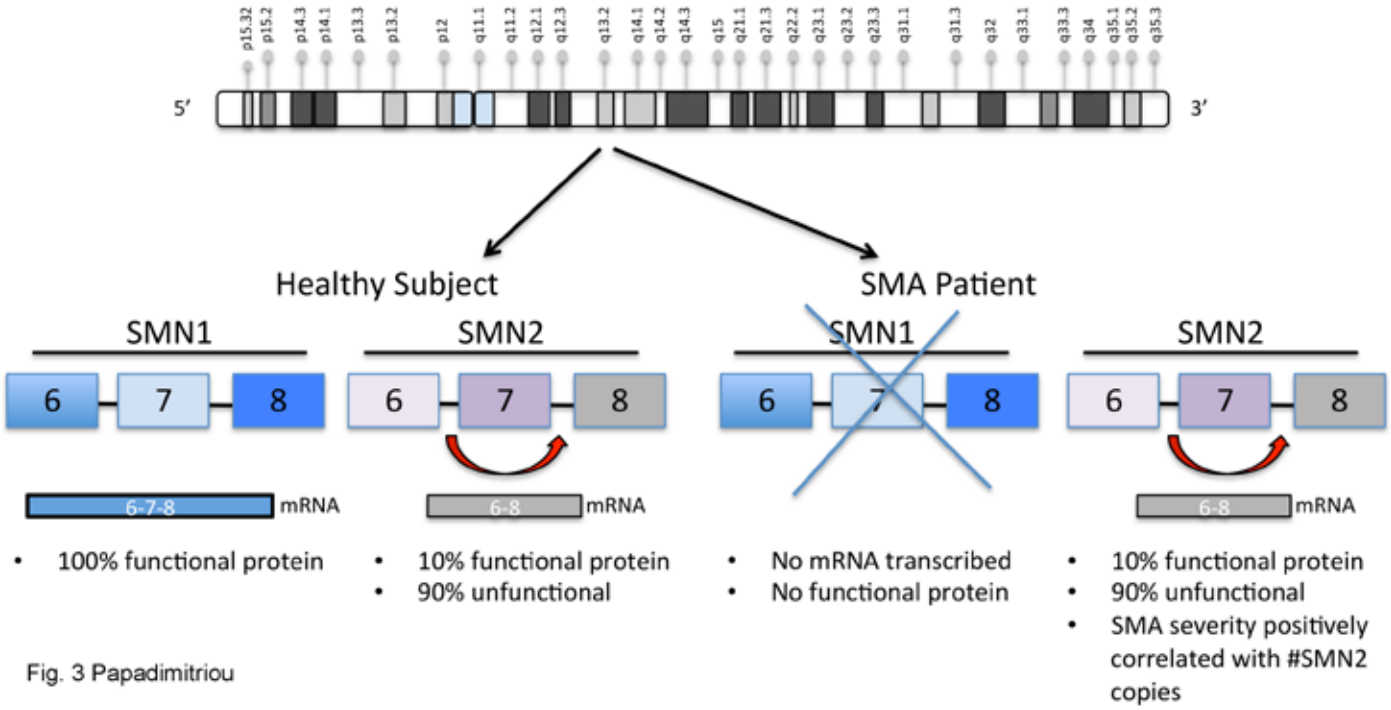


Fig. 3. Schematic representation of *SMN1* and *SMN2* in healthy and affected subjects



SMN (also referred to as Gemin1) can also be recruited in a group of protein called *SMN* complex [24-28]. This complex is composed by members of the Gemin family of proteins, in particular from Gemin2 (formerly SIP1), Gemin3/DP103 (a DEAD-box RNA helicase), Gemin4, Gemin5/p175 (a WD repeat protein), Gemin6, Gemin7 and UNRIP (UNR-interacting protein) [29]. In the middle of the complex there are *SMN*, Gemin7 and Gemin8, while the other components are bound to the complex through different interactions [30] (Fig. 2).

On the same chromosome, the *SMN2* gene shares more than 99% nucleotide identity with *SMN1*; indeed, the sequence differs only in five nucleotides (two exonic and three intronic) [31]. Nevertheless, a single point mutation (840C ≥ T substitution) in the last codon (exon 7) of *SMN2* modifies its splicing, resulting in a truncated and unstable form of *SMN* protein lacking 16 amino acids and the carboxyl terminus. The *SMN2* gene encodes for an *SMN* protein also called *SMN*Δ7 – as the 7th exon is missing in this case. In most of the cases (90-95%) *SMN*Δ7 protein is functionally defective, and is rapidly degraded (two-fold shorter half-life compared to the full-length), while only 5-10% is a functional full-length (FL) protein [16] (Fig. 3). Healthy subjects express 100% FL-*SMN* protein as a result of *SMN1* translational process and 10% functional FL-*SMN* protein as a result of *SMN2* translation. SMA subjects lack both *SMN1* alleles and consequently *SMN2* plays a pivotal role in the definition of the phenotype. Indeed, several genotype/phenotype analyses confirmed an inversed correlation between the phenotypic appearance of SMA and the *SMN2* copy numbers (e.g. higher *SMN2* copy number correlates with a milder SMA phenotype [32]).

The *SMN* protein is implicated in a few different functions, such as RNA metabolism (pre-mRNA splicing, transcription through its interaction with CTD of pol II, translation and stress granule (SG) formation), signal transduction, intracellular trafficking (endocytosis, cytoskeleton), and DNA recombination/repair [14]. In particular, with respect to RNA metabolism, the *SMN* complex is important not only for pre-mRNA splicing, but it is also involved as a chaperon in maturation, assembly and function of spliceosomal small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, and U5, which are the core component of the spliceosomal complex [33-35]. Spliceosomal snRNPs are U-rich snRNPs composed by seven Sm proteins (B/B', D1, D2, D3, E, F, and G) that remove introns from pre-RNA. Their biogenesis is a multi-step process that begins in the nucleus, translocates to the cytoplasm, and then back to the nucleus. It starts with transcription, regulated by RNA polymerase II (pol II), of pre-snRNAs that are co-transcriptionally processed at their 5'-end, with the inclusion of 7- methyl guanosine (m7G) cap and cleaved at their 3'-end. Through an export complex, pre-snRNAs are transported in the cytoplasm where they are further processed by *SMN* complex and heptameric Sm ring. These protein interactions facilitate the hypermethylation, 3'-end trimming,

and subsequently the snRNPs translocate together into the nucleus; here the complex dissociates and *SMN* transiently localizes in Cajal Bodies (CBs), where snRNAs undergo to full maturation [14, 36].

Another important role played by *SMN* is the trafficking of mRNAs along the axon and in the growth cone of primary MNs [37]. Indeed, it has been shown that *SMN* modulates the localization of β-actin within the growth cones [38]. In particular, *SMN* has been found to colocalize with profilin 2a, which is an actin-binding protein [39]. Moreover, *SMN* complex seems to interact also with candidate plasticity gene 15 (Cpg15). Indeed, it has been showed that the depletion of *SMN* causes Cpg15 and β-actin reduction in distal axons [40]. In a recent study, Rage and colleagues performed a genome-wide study in MN-like cells (NSC-34) of *SMN*-associated RNAs. The authors identified over 200 mRNAs associated with *SMN*, of which 30% localized to the axon in an *SMN*-dependent manner [41]. Further evidence concerning *SMN* and mRNA interaction in the axons, is provided by Fallini and colleagues; in fact, they present a potential non-canonical function of *SMN* in axons through the binding of the *SMN* Tudor domain to a neuronal-specific mRNA-binding protein, HuD, which has a role in neuronal development and plasticity [42]. Results showed that impairment in *SMN* expression caused the reduction of HuD protein levels in the axons, and this decrease could impair the axonal localization and the interaction of mRNAs with mRNA-binding proteins like HuD, KSRP, and hnRNP-R/Q resulting in a defective subcellular localization of transcripts likely necessary for MNs maintenance. They also demonstrated that *SMN* is required for axonal localization of poly(A) mRNA-containing granules. Although these data suggest a possible involvement of *SMN* in mRNA transport, it has not yet been demonstrated if mRNA trafficking impairment in SMA is due to *SMN* deficiency, or whether the MN degeneration is caused by the damaged transport of specific transcripts.

There are several mechanisms that regulate *SMN* protein levels, such as post-translational modifications (sumoylation, ubiquitination and phosphorylation), *SMN* mRNA stabilization, genomic and sequence integrity, transcription regulation, transregulatory splicing factors and subcellular localization. Moreover, other external factors could also be involved in *SMN* expression regulation, such as oxidative stress, hypoxia and starvation [43]. While *SMN* and *SMN*Δ7 proteins are expressed ubiquitously, and there is evidence of dysfunction in other systems [e.g. 8], the reason(s) why MN's exhibit greater vulnerability in SMA patients remains to be determined.

Therapeutics

In the past decade the majority of therapeutic approaches to rescue the pathological and clinical phenotype in SMA have been focusing on *SMN* protein level restoration. This can be achieved either through administration of a functional *SMN1* gene, or by acting on *SMN2* protein levels. Currently there are three commercially available treatments for SMA in Europe: Nusinersen, Risdiplan and Zolgensma, described in detail below.

SMN2 Targeting Approaches

As mentioned above, *SMN2* gene differs from *SMN1* by only one base change and *SMN2* is present in all SMA patients in different copy numbers. Compounds that target *SMN2* could potentially benefit the SMA phenotype through stabilization of *SMN2* mRNA or protein, by increasing *SMN2* transcription or enhancing exon 7 inclusion [44].

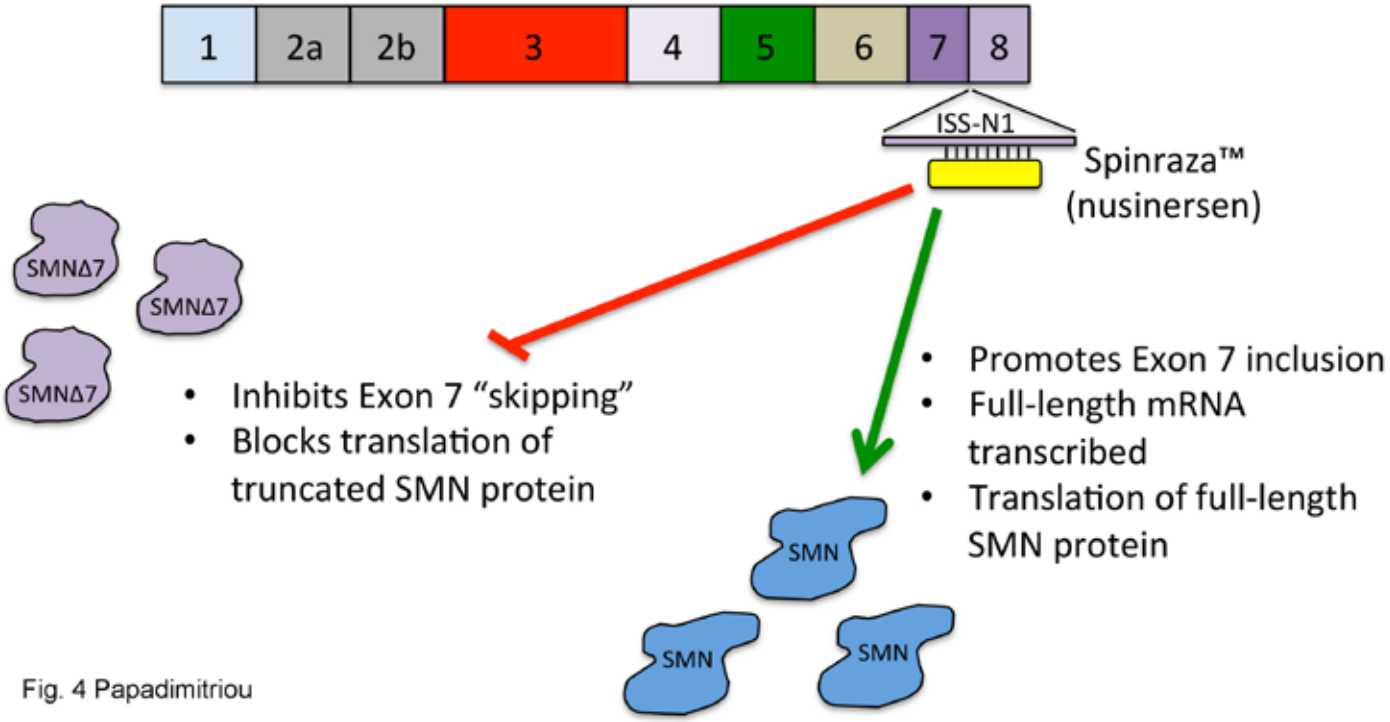
NUSINERSEN

In SMA patients, as previously described, around 90% of the produced *SMN2* protein lacks exon 7, resulting in a non-functional and unstable protein. The use of antisense oligonucleotides (ASOs) has been shown to restore, reduce or modify protein expression. This specific approach is engineered to specifically bind to the cis-acting splicing regulatory motif, promoting the exon 7 inclusion in *SMN2* [45] (Fig 4). In particular, the inclusion of exon 7 is modulated by two intronic splicing enhancers, both located on intron 7, and two intronic splicing silencer sequences (ISSs), in intron 6 and 7 (ISS-N1) [46]. Deletion of ISS-N1 within intron 7 leads to a significantly enhanced incorporation of exon 7 in *SMN2* minigenes [47].

Nusinersen, marketeded as Spinraza®, is the first FDA (FDA, 2016) and EMA-approved (EMA 2017) drug for SMA treatment based on ASO technology. Indeed, hybridization of Nusinersen to ISS-N1, causes rearrangements of DNA structure masking site where the splicing machinery binds (hnRNP A1/A2) promoting the inclusion of the exon 7 in *SMN2*, and therefore resulting in the production of FL-*SMN* protein [14] (Fig. 4).The schedule of the treatment consists of four loading doses; the first to the third should be delivered at 14-day intervals, while the fourth dose should be administrated 30 days following the third. It is further recommended that a maintenance dose be administrated once every 4 months lifelong (FDA, 2016).

The phase I and II clinical trials of Nusinersen for children with SMA type 2 and 3 [48, 49] were promising, followed by three phase III studies (ENDEAR, CHERISH, & NURTURE). The ENDEAR study, included 121 infants (younger than 7 months of age) with SMA type 1 who underwent: a) repeated intrathecal administration of Nusinersen; or b) a sham-infusion. The group that received Nusinersen demonstrated

Fig. 4. Schematic representation of Nusinersen's mechanism of action



Nusinersen binds the ISS-N1 sequence on *SMN2* gene and modulate its splicing, leading to the production of a functional FL-*SMN2* protein

significantly prolonged survival or delayed need for permanent ventilation compared to the sham-control group [50]. Fifty-one percent of the Nusinersen group reached the criteria of "motor-milestone-responders" (achievement of motor milestones in HINE-2 scale; *Hammersmith Infant Neurological Examination*) compared to 0% in the sham-control group. Even though the motor development of the ASO treated group significantly modified the disease's natural course, the critical milestone of independent sitting was achieved only by a minority of patients (6/73) at one year of treatment [50]. The CHERISH trial, included 126 older children with a median age of 4 years with SMA type 2. As with the ENDEAR study, the Nusinersen group exhibited a significant gain in motor function (mean increase of 4.0 points in the HFMSE scale); while the sham control group deteriorated slightly (mean *decrease* of 1.9 points in the HFMSE scale) [51]. The results were so encouraging after an interim analysis, that both studies were terminated prematurely and all participants were switched to the treatment group. Subsequently the NURTURE study included 25 pre-symptomatic infants, 15 of them carrying 2 *SMN2* copies and 10 of them with 3 copies, all under 6 weeks of age. Interestingly, the ability to sit independently was acquired by all 25 patients while 22 out of 25 patients achieved independent walking [52, 53]. After completion of these studies, Nusinersen was approved in December 2016 by the FDA and in May 2017 by the EMA. The schedule of the treatment consists of four loading doses. The first, second, and third doses should be delivered at 14-day intervals, while the fourth dose should be administrated 30 days following the third. It was further recommended that a maintenance dose be administrated once every 4 months for the remainder of the patients life.

RISDIPLAM

Risdiplam (Evrysdi) is a small molecule (RG7916) developed by Roche. This compound is a pyridazine with high binding affinity to exons 5 and 7. The mechanism of action of this compound is rooted in its ability to modify the splicing pattern at these exons and ultimately increase the amount of functional *SMN*-protein. It is an orally administered compound that can cross the blood-brain barrier; and leads to an elevation of FL-*SMN* levels both in the CNS and in peripheral tissues [54].

Originally in the FIREFISH-study, 21 infants with a diagnosis of SMA type 1 between 1 to 7 months of age received either Risdiplam in a low-dose (Part 1, n = 4) primary aiming to assess safety, or in a high dose (Part 2, n = 17) to assess efficacy [like independent sitting after 1 year (12 months) of treatment]. 33% of infants (n =7/21), and 41% of those infants in the higher dose group in Part 2 (n = 7/17)

obtained independent sitting after a median time of 14.8 months of treatment. No safety issues were reported. The SUNFISH-study SMA type 2 and 3 older patients receiving RG7916. Like the FIREFISH trial, this study addressed dose-finding in Part 1 and efficacy in part 2 in a double-blind, placebo-controlled design. In this trial 58% of the patients demonstrated an improvement of at least 3 points according to the Motor Function Measure-32 (MFM32) scale [55].

The JEWELFISH trial is an ongoing open-label study involving all types of SMA non-naïve patients and previously treated with therapies targeting to *SMN*, olesoxime, or gene therapy, and subsequently given Risdiplam with a wide age range from 6 months to 60 years. The study aimed to assess mostly pharmacokinetics and pharmacodynamics data to determine if prior treatment affected the response to Risdiplam. To date, there are 174 patients enrolled. So far the adverse effect profile is very consistent with what was reported in the SUNFISH trial. There was a 2-fold elevation in *SMN* protein levels reported. The most recent data announced at the MDA [Muscular Dystrophy Association] [56] conference showed that the increase in the *SMN* protein was higher in those who had the lowest levels at baseline: patients with SMA type 1. Particularly encouraging have been the preliminary results of the RAINBOWFISH-study. In this trial, pre-symptomatic infants with SMA were treated with Risdiplam for at least one year. The preliminary results of this ongoing trials showed that treated infants gained the ability to sit, stand and walk. This trial is also currently ongoing.

SMN1 Gene Replacement

The primary goal in gene therapy approaches for the treatment of SMA is to directly target the dysfunctional *SMN1* gene. The adaptation of Adeno-associated viruses (AAV) into gene therapy vectors have opened up a new field for the development of therapeutic strategy for diseases of the CNS. These viruses can cross the BBB and serotype 9 in particular, has a particular tropism for CNS and MNs [57, 58].

It has been demonstrated that a single intravenous injection in neonatal mice of self-complementary AAV9 (scAAV9) can transduce around 60% of lumbar MNs and it is also able to transduce brain neurons, dorsal root ganglia, astrocytes both in the spinal cord and in the brain, as well as cardiac and skeletal muscle tissue. The persistence of the virus in these tissues was up to 5 months [59].

One approach, developed by AveXis, takes advantage of scAAV9 encoding wild type *hSMN1*. This new drug, called Zolgensma, is currently produced and marketed in a partnership with Novartis, was approved in May 2019, by the FDA for children with SMA under 2 years of age. Murine studies had previously showed significant changes in the disease's course [60, 61].

ZOLGENSMA

Fifteen infants with SMA type 1 carrying 2 copies of *SMN2* and less than 8 months of age were included in the first Zolgensma (AVXS-101) clinical trial [62]. All the participants received a single intravenous administration of either high- (n = 12) or low-(n = 3) dose of AVXS-101. The high dose group showed a significant improvement in Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) scores, with 11 participants attaining scores >40 points – a milestone rarely achieved in the natural history of SMA-1. During the follow-up period, 9 of the 12 participants receiving Zolgensma in high-dose could sit for >30 seconds, without support. When compared to a control cohort with a natural disease progression improvement of motor function and survival motor and achievement of milestones by AVXS-101 therapy were largely confirmed [63]. Moreover, the phase-3 STR1VE study involved 22 participants with the type 1 of SMA and age under the 6 months at the time of administration, who achieved independent sitting for 30 s or longer at the 18 month of age study visit while 3 of them presented serious side effects (one with hydrocephalus, and two with increased hepatic aminotransferases) [64].

While the route of administration for infants undergoing gene therapy involves *systemic* intravenous injections regardless of the target, for older patients intrathecal application might result a more efficient technique in terms of motor neuron transduction [65]. Initial trials comparing intrathecal to iv gene therapy in pigs and mice have shown improved gene expression [66, 67]. Moreover, the STRONG trial examines the effects of intrathecal administration of Zolgensma in patients with SMA type 2 (under the age of 6 years).

Children in which a titre of antibodies against the viral vector was detected, were excluded from the study. Two of the main concerns are: a) efficacy decline, indeed, a repeated injection would not be feasible due to the formation of the antibodies against scAAV9; and b) the deficiency of *SMN* in peripheral tissue, which could reveal a previously hidden non-cell autonomous mechanism and phenotype. In fact, AAV9 has a high affinity for post-mitotic cells such as MNs, but not for highly proliferative cells like muscle cells; which are also affected in SMA [68]. Although gene therapy seems to be the most promising, results are consistent with oligonucleotide therapy, confirming that the therapeutic window is very narrow, and that it is very important the timely intervention for best therapeutic improvement.

Upregulation of muscle function

As an alternative approach to the therapeutic approaches that target cell autonomous mechanisms in MNs in SMA, there is a parallel treatment strategy that specifically targets skeletal muscle function. The most studied and clinically advanced compounds are Myostatin-inhibitors and Fast Skeletal Muscle Troponin Activators (FSTA). During development, myostatin, which is primarily expressed in skeletal muscle, inhibits muscle over-growth. Administration of the myostatin-inhibitor SRK-015 in SMA-mice led to improved muscle function [69], while the safety profile of SRK-015 in humans is being evaluated in a phase II clinical study. Another example of a compound targeting muscle is Reldesemtiv (CK-2127107), which is a FSTA-class compound which leads to improved contraction of skeletal muscle fibers by slowing the release of calcium from the troponin complex [69]. Its use as a therapeutic for SMA was assessed in 70 participants that had been diagnosed with SMA type 2-4. While the final results of this trial are pending, a mild improvement (that reached the statistically significant threshold), in a motor performance test (six-minute walk test; 6MWT) after both 4 or 8 weeks of treatment, revealed after the interim analyses.

Conclusion

With a deep pipeline for SMA therapeutics, and currently three FDA and EMA approved treatments for the disease, the era of SMA therapeutics has completely changed over the past years achieving a fundamentally altered natural course of a fatal disease. New therapeutic options, such as those targeting splicing events or other gene therapy approaches, shed light on the clinical management of a so far untreatable disease; which could also open up a number of possibilities for other genetic disorders. As the treatment window, particularly in diseases like SMA, is critical to achieve maximum therapeutic effects, it is imperative for early diagnosis and treatment initiation; dictating the need for a broader prenatal/newborn *SMN* screening.

Additional therapeutic approaches at advanced stages of clinical development are currently being evaluated and are likely to provide additional treatment options for people suffering from SMA. The widening of the therapeutic interventions should increase, on one hand, the level of awareness; and on the other hand, the focus on interdisciplinary clinical management. Despite widening new drug treatment options, there is still a lifelong disease burden to consider. Upon approval, the data concerning safety and long-term effects of the novel compounds are limited, therefore additional collection and analyses of real-world data are indispensable for more meaningful and long-lasting effects.

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