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Research

The AAV Vector as a SMA Disease Modifier

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GENE THERAPY AND THE VIRAL VECTORS: AN OVERVIEW

The discovery of DNA as the biomolecule of genetic inheritance and disease opened the prospect of therapies in which mutant and damaged genes could be altered for the improvement of the human condition [1,2]. Genome-editing is a technology that allows specific changes in the genes of interest. This approach is capable of manipulating the genome of living cells or organisms in various ways: insertions or deletions of chosen genes, introduction of point mutations, knockout or correction of specific genes [3]. Gene therapy provides a unique approach to treat a variety of both inherited and acquired diseases by delivering a therapeutic gene material to correct the loss-of-function caused by mutation or to express the deficient gene product [4].

Despite years of preclinical studies, it was not until the early 1990s that the first gene therapies were studied in humans [5]. In fact, the first clinical trial to gain approval for transfer of a foreign gene into humans was conducted at the National Cancer Institute in Bethesda in 1990 [6]. In spite of numerous setbacks, efficacious gene-based therapies still hold the great promise to revolutionize the clinical management of human diseases [7].

Numerous preclinical and clinical studies of gene therapy strategies for preventing or treating a wide range of neurodegenerative diseases have been carried out in recent decades however, safety concerns remain one of the biggest barriers to successful clinical application. Potential gene-based therapeutic strategies to treat neurodegenerative disorders should therefore be carefully scrutinized for clinical development, including evaluation of available safety profiles and pharmacological effects, and identification of individuals who can benefit [8]. Safe and efficacious gene delivery requires a suitable vector and viruses are designed by nature for in vivo gene delivery [9]. The role of viral vectors in gene delivery is primary due to their function in the delivery of genetic material into host cells [10,11]. The suitability of a viral vector for a given application depends on multiple factors, including target cells or tissues, tropism, use for *ex vivo* versus *in vivo* gene transfer, packaging capacity, potential for genome integration (insertional mutagenesis) and also the propensity for immunotoxicities.

Gene therapy may cause severe toxicity due to overexpression of the transgene in targeted tissues or expression in off target cells [8]. Nonetheless, insertional mutagenesis and genotoxicity are probably also concerning when certain transgenes are injected with high-dose vectors. Toxic effects have included impaired ambulation, ataxia, damaged dorsal root ganglia, elevated transaminases, and proprioceptive deficits. Host responses can also affect the duration and safety of every gene therapy strategy. Moreover, patients with adaptive immune responses can produce corresponding neutralizing antibodies, which may prevent the vectors from reaching their intended tissues or cells.

A key challenge to be overcome when designing an efficient gene therapy approach for treating neurodegenerative disorders is access to the central nervous system (*CNS*), which must be mediated by either crossing the blood–brain barrier (*BBB*) or by direct administration into the *CNS*. The success of the CNS gene therapy approaches greatly depends on the selected delivery system and it is wildly recognized that poor gene delivery is the limiting factor for most *in vivo* gene therapies. In addition, gene therapy relies on the use and optimization of safe nonreplicating viral vectors and the choice of the viral vector depends on the tropism of the virus and its ability to allow sustained therapeutic gene expression in the target cells.

Among the various types of vectors for gene therapy products, such as plasmid *DNA*, viral, bacterial vectors and genetically modified cells, the viral vectors have been widely developed, and several kinds of viruses have been used to deliver genes to target cells. Past and ongoing clinical trials have utilized several viral vector systems, including *Adenovirus* (Ad), *Adeno-Associated Virus* (AAV), *Lentivirus* (LV), murine g-retrovirus and *Herpes Simplex Virus* (HSV) [9,10,11]. These vectors differ in cell tropism, payload capacity and their ability to integrate into the host genome [4].

AAV was discovered over 50 years ago and has since become one of the leading gene delivery vectors in clinical development [1].

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Nevertheless, the supply of *AAV* vectors for clinical studies for the proof-of-concept remains challenging, thus limiting the number and duration of clinical trials.

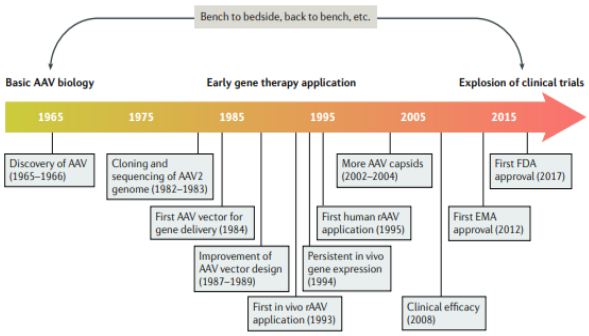

Figure A: Key milestones in adeno-associated virus (AAV) gene therapy development.

Adeno-associated virus (*AAV*) are composed of a non-enveloped icosahedral capsid (protein shell) that contains a linear single-stranded *DNA* genome [10,11]. The *AAV* genome contains three open reading frames (*ORFs*) encoding for replication proteins (*Rep*), capsid proteins (*Caps*), the assembly activating protein (*AAP*) and is flanked by two inverted terminal repeats (*ITRs*) [10,11]. The life cycle of *AAV* is dependent on the presence or absence of a helper virus, hence its name. In the absence of helper functions, *AAV* genomes integrate into the host genome with a preference for a specific site on chromosome, and *AAV* can be rescued from this latent state by infection with a helper virus.

Ideally, vectors for *CNS* gene delivery should present: i) an effective transduction and no off-target effects; ii) suitable transgene expression levels and duration, in order to induce a therapeutic effect in the absence of cellular toxicity; iii) lack of pathogenicity and immunogenicity, leading to no adverse responses to the treatment; and iv) large-scale efficient vector production, with high purity levels.

Extensive pre-clinical studies have successfully shown the therapeutic potential of *AAV* and *Lentiviral vectors* in neurological disorders, since they have emerged as the vectors of choice for *CNS* gene transfer and although both exhibit a limited packaging capacity when compared to *Adenoviruses* and *Herpes Simplex Viruses*, they present significant advantages, including stable transgene expression in post-mitotic cells, neuronal tropism, and diminished immune responses [9,10]. Moreover, when comparing these two options, AAVs exhibit important advantages, including a better safety profile due to the non - pathogenic nature of their wild - type form [11]. In addition, AAV cell infection results mainly in episomal transgene expression, consequently reducing the risk of insertional mutagenesis, an important safety concern for integrating viral vectors such as *Lentiviruses*.

Successful transduction by *AAV* vectors starts with cell surface receptor binding and depends on several subsequent steps, such as endocytic uptake, escape from the endosomal pathway, entry into the cell nucleus; virus uncoating and single-stranded genome release, second-strand synthesis and finally transcription (Figure B).

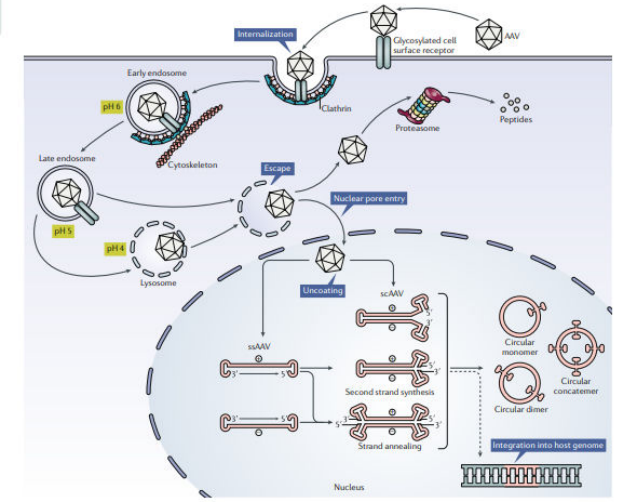

Figure B: Diagram of AAV transduction pathway.

However, AAVs also present some limitations, stressing the importance of further optimization methods. One of these disadvantages concerns the delay in transgene expression (around 2 weeks for maximum expression) when compared to other vectors, since it needs a secondstrand synthesis. Nonetheless, self-complementary AAV vectors (scAAV) overcome this limitation as they carry double-stranded DNA genomes that become transcriptionally active immediately upon decapsidation in the nucleus. These sc AAV vectors carry a mutated ITR, missing the terminal resolution (TR) site where Rep proteins cut the genomes during replication to generate ssDNA. In the absence of the TR site in one ITR, the replication continues in the opposite direction without resolution of the previous strand resulting in the synthesis of a double-stranded DNA vector genome.

AAV serotypes are the major determinant of several crucial characteristics of successful AAV-based gene therapy, including biodistribution, tissue tropism, and susceptibility to neutralizing antibody generated in vivo [8]. Discovering how the specific serotypes distribute gene cargos to their intended tissues for vector delivery was vital for developing a reliable and predictable gene therapy strategy. More than one hundred AAV variants consisting of 13 serotypes have been identified from humans and nonhuman primates (NHP). AAV2 was the first serotype to be modified into a recombinant vector for gene delivery [4]. Because of its relative safety profile and its sustained expression in neurons, AAV2 has been used in numerous clinical trials and is currently considered a satisfactory vector for gene therapy of neurodegenerative disorders.

Vector particles containing viral proteins that are identical or similar to antigens that humans are exposed to as a result of natural infection may be neutralized by antibodies upon injection into in some humans because of pre-existing immunity [11]. Recognition of viral structures (capsids or nucleic acids) by innate immune sensors may cause tissue infiltration by innate immune cells, may trigger the production of interferon (IFN)-a/b, thereby inducing an antiviral state in the tissue, reducing transduction and providing an activation signal for adaptive immune responses. Activation of, and subsequent antigen

presentation by, dendritic cells (DCs) is a critical step in linking innate to adaptive immunity, leading to activation/differentiation and expansion of T cells (the immunological barriers to successful AAV gene delivery are portrayed in Figure C).

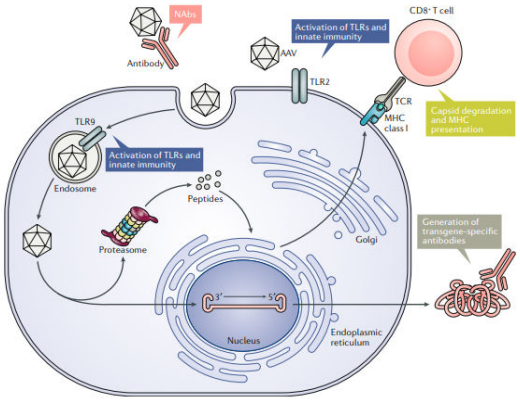

Figure C: Immunological barriers to successful rAAV gene delivery.

Regardless, non-viral vectors (liposomes, exosomes and polymeric nanoparticles) are considered a promising option, due to their simple and cost-effective production methods, as well as their safety profile. However, they present a relatively low efficiency and mediate a transient effect, requiring repeated administrations with the potential risk of triggering an immune response [5,8,11]. Gene therapies based on non - viral delivery vectors can be sorted into lipid-based vectors and polymeric vectors [2,9] and the most extensively applied non - viral gene carriers are lipid-based vectors.

Neutral lipids, like cholesterol, DOPE and DSPE, have served as the 'helper lipid' among liposomal components to improve liposome stability and transfection capacity [3,5]. The prominent features of cationic lipids, such as DOTAP, DODAP, DOTMA, and DC-cholesterol, which have been used for gene therapy, include three major domains: hydrophobic tails, linking groups, and cationic cap groups. The main shortcomings of cationic lipids are their unsatisfactory pharmacokinetic biodistribution due to nonspecific binding and rapid clearance, and their cytotoxicity [8].

Lipidoids (lipid-like materials), magnetic nanoparticles, and exosomes have also shown promise as gene delivery carriers for neurodegenerative disorders. Furthermore, cationic polymers provide another kind of non-viral vector that is extremely attractive for gene therapy due to their capacity for endosomal/lysosome escape, which is the result of their sponge-proton effect, fine spherical architecture, and tremendous chemical diversity. Overall, non-viral gene therapy has improved substantially in recent decades, thus, additional insights into the relationship between structure and function of gene delivery material and fuller understanding of the critical factors that restrict effective gene delivery are likely to advance the clinical treatment of neurodegenerative disorders [8].

RAAV VECTOR MANUFACTURING

Recombinant adeno-associated viruses (rAAVs) are replication-defective belonging to the Parvoviridae family, particularly the Dependovirus genus since it needs co-infection with a helper virus to replicate and complete its life cycle24 and are ideal for functional studies in vivo [1,3]. Recombinant AAV (rAAV) is increasingly becoming the vector of choice for many gene therapy protocols [2,11] as it possesses several attractive features including its ability to be concentrated to high titre, its robust nature and perhaps most importantly its lack of innate pathogenicity [5,6]. In addition, since rAAV vectors do not express viral genes and do not transduce antigen-presenting cells, they are less immunogenic than other viral vectors for gene therapy.

Biotech manufacturing has responded to the demand of AAV vectors for en mass delivery of complementary genes for large-scale and cost-efficient rAAV production. However, contamination of the helper virus in samples could become a health and safety concern. In mammalian cell-based production systems, the assembly of rAAV vectors requires: (1) the recombinant vector genome composed of the gene of interest (GOI) and the regulation elements for the GOI expression in target cells (promoter, poly A, introns) flanked by AAV ITRs [3,5] (2) the AAV rep and cap genes and (3) helper functions from Ad or HSV for efficient replication and rescue of the recombinant genome.

Despite the many advantages for using rAAV vectors for gene therapy, a major limitation to date has been the inability to generate vector at a scale suitable to supply a human clinical trial [9,5]. Traditional laboratory scale systems using adherent cells that have routinely been used to produce LVs and rAAVs, are struggling to produce viral vectors in sufficient quantities for clinical applications and are not sufficiently scalable or cost-effective to meet expected future demands [3,6]. This is partly due to the use of adherent cell lines, for which traditional systems are not scalable due to the large surface area and high levels of manual handling required. Additionally, transient transfection methods cannot be easily adapted to large scale because efficient transfection of large amounts of cells is difficult to achieve and is susceptible to variation.

The safety and efficiency profiles of the AAV-based drugs depend on the upstream and downstream steps that ensure complete removal of process-derived impurities and on the development of robust and precise assays for the detection and quantification of these impurities [2,10,11]. In addition, one approach to developing a scalable process to produce rAAV vectors has been to generate stable packaging cell lines in which the AAV rep and cap genes are integrated into the host chromosome [3,5]. Moreover, transient delivery of rep/cap genes in the presence of helper genes can also contribute to product heterogeneity, including AAV vectors lacking a transgene [1]. These "empty capsids" represent a significant proportion of virus produced in transient transfection assays [4]. Thus, it is critically important to develop robust analytical quality control (QC) methods that can distinguish between these viral variants in order to ensure similarities between production lots.

Nevertheless, the key obstacle to the development of stable packaging cell lines is toxicity of the AAV Rep protein [3,7] but this toxicity can be diminished by regulated expression of the rep gene at the translational level limiting the production of the toxic Rep proteins [5,10,11]. This down - regulation of the Rep proteins leads to an increased production of rAAV by the transient transfection process.

A major advantage of transient transfection is that serotype-specific vectors of AAV can be easily generated in a short period of time by supplying a capsid/replication plasmid of choice, or in a combination of plasmids and a helper virus [3,10,11]. Therefore, transient transfection technology, offers a rapid generation of material for the proof-of concept [4]. Transient transfection has been done using adherent cells in cell plates, cell factories or roller bottles. When the scaling up of the process is desired in a short period of time to generate pre-clinical/clinical material or material required for the proof-of-concept, methods using adherent cells may need more incubator space and are time consuming and labor-intensive processes.

Following transfection, rAAVs are collected from the media or cell lysate and subjected to numerous purification steps. The complexity of purification and length of time spent on steps often prevents small laboratories from preparing their own rAAV samples. Moreover, budget limitations restrict research plans to what is achievable based on commercially available rAAV stocks, since customized rAAV preparations are commercially packaged at a significant cost.

The success of generating a scalable production technology relies heavily on understanding the basic biology of AAV in regard to generating reagents such as cell lines, plasmids, or recombinant viral vectors that when used together, will closely mimic wild-type (wt) AAV production [2,7,10]. Potential problems in the production of rAAV stocks are both the limited amount of recombinant virus that is produced by traditional methods and the possibility of wild-type replication competent adeno-associated virus (wtAAV) contamination [11]. Whilst wtAAV is not a pathogen, the presence of contaminants is undesirable as they may affect experiments concerning the biology of rAAV. Additionally, as protocols using rAAV with altered tropism are becoming more prevalent [4,5,8,9] it is important that no recombination be permitted that may cause the creation of a replication competent AAV with modified capsids.

To scale-up the production of any clinical grade biological material, it is particularly important to have a cell line that maintains a high yield of the product, and in a sufficient quantity as a Master Cell Bank (MCB) with full characterization [10,11]. For larger-scale manufacturing efforts, transient delivery of plasmid requires excess quantities of DNA, adding to the overall cost of production and purification [1,4]. One major challenge is establishing large-scale manufacturing technologies in accordance with current good manufacturing practices (cGMP) to yield the purified vector quantities needed for the expanding clinical need [7] and several technological platforms are competing for this niche. Also, another approach using the insect cell-baculovirus system for AAV manufacturing [2,3,5] has recently attracted attention and has shown great potential for the large-scale manufacturing of clinical material.

There are some pharmacopeias and guidelines that address the quality of rAAV products, including the United States Pharmacopeia (USP) and two FDA Guidance for Industry in the US, European Pharmacopoeia (EP), EMA reflection paper, EMA Guideline in EU and notification in Japan. Although these Pharmacopeias and Guidelines describe the required properties of rAAV, there is no established standard to design appropriate quality for human use. Therefore, there is a need for a scientific approach for Quality-Risk-Management to produce rAAV of appropriate quality.

For efficient and rapid development of the manufacturing process and quality control strategy, the quality by design (QbD) approach [4,5] can be as effective for gene therapy products as it is for gene recombinant proteins, which have been developed for decades. However, prior available knowledge required for the QbD approach is limited in the field of gene therapy [3,6]. The quality by design (QbD) approach is commonly endorsed in the development of gene recombinant proteins to obtain desired levels of quality, safety and efficacy. The QbD approach is defined as, "A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and Quality-Risk-Management" (QRM). The QbD approach is comprehensive and involves setting a quality target product profile (QTPP), identifying critical quality attributes (CQAs), developing processes including establishing design space (DS) and determining control strategies.

However, there are some difficulties involved in applying the general CQA identification methods used for biopharmaceuticals to gene therapy owing to the following: (1) Prior knowledge from the literature is limited; (2) Manufacturing experience based on production batches tends to be limited owing to a small number of patients, as gene therapy often targets rare diseases; (3) Developers do not have sufficiently accumulated information regarding the previous development of similar products; (4) Accumulation of knowledge through product development is insufficient because many products remain in their early development phase.

Zolgensma® Manufacturing: The Transient Triple Transfection

The most well-established methods for producing lentiviral and adenoviral vectors rely on transient transfection of plasmid DNA into host cells, typically HEK293, HEK293T adherent cells, PER . C6 or sf9 insect cells. The transformation of human embryonic kidney (HEK) cells following exposure to sheared fragments of human adenovirus DNA, generated the widely used expression tool known today as the HEK293 cell line.

Human embryonic kidney 293 (HEK293) host cells are often used for transient transfections because they are highly transfectable and their transfection processes are scalable. Despite the availability of scalable methods and protocols for production, **transient transfection** of adherent HEK293 cells remains the most commonly used method to produce AAV vectors for pre-clinical research and large-scale manufacturing [2,3]. Zolgensma® is manufactured by the transient triple

transfection of HEK293 cells [5,10,11] and a typical manufacturing process is presented in Figure D.

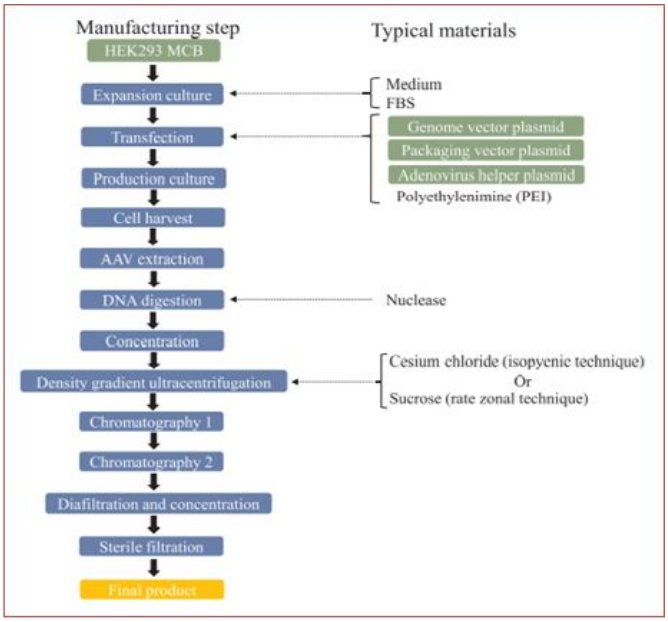

Figure D: Manufacturing flow chart for recombinant adeno-associated virus (rAAV) [11].

First, the HEK293 cells are simultaneously transfected by the genome vector plasmids (containing GOI), packaging vector plasmids and adenoviral helper plasmids using polyethylenimine. The packaging vector plasmids provide three viral capsid protein genes (VP1, VP2 and VP3) and the AAP gene. The helper plasmids provide the adenoviral helper genes E2A, E4 and VA that are necessary for the replication and propagation of rAAV. Other essential adenoviral helper factors (E1A/E1B) are expressed in the HEK293 production cells that are used for the rAAV manufacturing.

After the HEK293 cells transfected with the three plasmids are expanded, the viral particles inside the HEK293 cells are extracted using hypotonic shock, freeze-thaw cycles of cell pellets or Microfluidizer®. Then, the cells and cell debris are removed from harvested supernatants or cell lysates by centrifugation or microfiltration.

In addition, as viral particles are enriched both inside and outside the producer cells, both the cellular fraction and supernatant are recovered. The DNA derived from the HEK293 cells is digested by a nuclease to reduce the viscosity of the cell lysate. Subsequently, rAAV is concentrated by ultrafiltration before proceeding to the purification steps. Density-gradient ultracentrifugation with cesium chloride (CsCl) or sucrose is widely used for viral particle purification to separate the rAAV from other contaminants based on size, shape and density

Chromatography is another commonly used technique for purifying rAAV based on surface properties or size. The rAAV is further purified by a combination of affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and/or size exclusion chromatography. The buffer exchange and concentration by ultrafiltration are performed following the purification steps; thereafter, the rAAV are sterilized by filtration and decanted into vials [15,27].

SMA ETIOLOGY AND PATHOPHYSIOLOGY

Spinal Muscular Atrophy (SMA) is an autosomal recessive motor neuron disorder characterized by rapidly progressive hypotonia and weakness with respiratory complications [1]. SMAs is caused by degeneration of alpha motor neurons of the spinal cord anterior horns (and sometimes also brainstem motor nuclei) [2,5,7,10] and is the most frequent lethal neurodegenerative disorder in infants [3,6,11]. Patients with SMA have a homozygous disruption of the Survival Motor Neuron 1 (SMN1) gene on chromosome 5q13 by deletion, rearrangement or mutation. Mutations include nonsense, frame-shift, missense, deletions, inversions and splicing site changes.

SMN has been implicated in several functional processes, including pre-mRNA splicing, mRNA transport and axon growth. The wild-type SMN protein has an established function in small nuclear ribonucleoprotein (snRNP) assembly suggesting a role for pre-mRNA splicing in SMA disease progression. Alternatively, because motor neurons have highly specialized, far-extending axons, it has been postulated that the localization of mRNAs to these distal processes is affected in SMA, which may be a driver of the selectivity for motor neuron degeneration.

Genetic linkage has mapped the SMA locus to chromosome 5q13 and due to the large inverted repeats in the 5q13 region, the SMA locus is divided into telomeric and centromeric parts. Two highly homologous copies of the genes are present in the telomeric and centromeric parts of the SMA locus: SMN1 (telomeric SMN), SMN2 (centromeric SMN), telomeric NAIP and centromeric NAIP (NAIPΨ). However, it is also known that telomeric NAIP deletion is often accompanied by a decrease of SMN2 copy number in SMA type I and the presence of the NAIP gene is accompanied by an increase of SMN2 copy in patients with type II-III.

In addition, the copy number of SMN2 modifies disease severity, as gradually increased amounts of SMN protein become available in patients with more copies, resulting in milder SMA types II-IV. Although the expression of an SMN1 duplicated gene, SMN2, is preserved in SMA patients, SMN2 expression cannot fully compensate for the loss of SMN protein and ultimately fails to prevent the disease [1]. SMN2 harbors a silent mutation in exon 7 that alters the splicing of the mRNA leading to the predominant production of a truncated, unstable protein along with a minority of correctly spliced transcripts, generating low levels of full-length protein [10,11]. Disease severity is usually inversely proportional to SMN2 gene copy number, therefore, milder phenotypes are associated with the presence of three or more copies of SMN2 [5]. Figure E shows the location of exons 7 and 8 of the SMN genes (SMN1 and SMN2) and exons 5 and 13 of the NAIP genes (telomeric and centromeric NAIP).

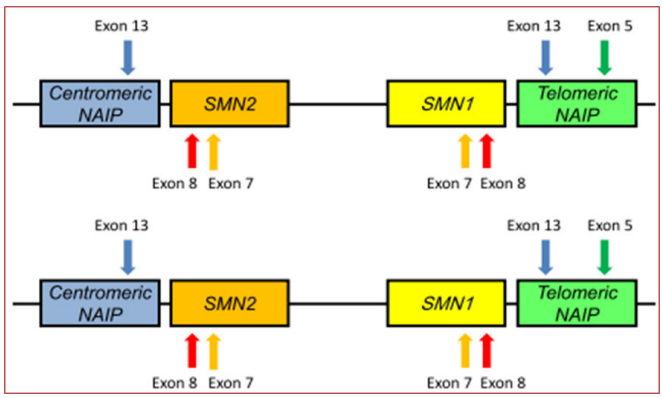

Figure E: Location of exons 7 and 8 of the SMN genes (SMN1 and SMN2) and exons 5 and 13 of the NAIP genes (telomeric and centromeric NAIP). This schema shows the most common genotype of SMN1 alleles, [1 + 1]. As for the NAIP genes, this schema is based on the model of"centromeric NAIP and telomeric NAIP" [6].

Several clinical investigations demonstrate that early diagnosis and intervention are essential for improved response to treatment and better prognosis [5,10,11]. Therapeutic interventions that are effective at pre-symptomatic or early stages of the disease creates the need for awareness, expedite diagnosis and consideration of newborn screening programs. Fetal SMA studies have revealed that neuropathology begins prenatally during neuromuscular development. Then, it is assumed that the fetal period appears to play an essential role in SMA pathogenesis. Moreover, data in animal models as in humans indicate that higher levels of SMN are required prenatally in comparison with the postnatal period [6]. A neonate with SMA may be asymptomatic, but neonatologists should look for early signs and manifestations of the disease. In general, areflexia precedes hypotonia and muscle weakness albeit in some cases manifestations are observed altogether, particularly when a delay of initial suspicion postpones the indication of SMN1 test that will confirm the disease in the vast majority of the cases.

Nevertheless, traditional SMA types alone are not sufficient to define patient populations who might benefit most from gene therapy. In symptomatic patient's age at onset, disease duration and motor function status at the start of treatment are the most important factors that predict response to treatment. For example, the clinical condition of a patient with SMA type II in advanced stages of disease can be significantly more severe compared with a patient in early stages of SMA type I [3]. Additionally, since the introduction of disease-modifying treatments, several patients originally belonging to type I or type II have acquired sitting position or ambulation, respectively and thus cross the boundaries of the traditional classification. In fact, disease stage and duration might be more important predictors of outcomes than the subtype of SMA.

In presymptomatic patients, SMN2 copy number is the most important predictor of clinical severity and age of onset. As long as no better biomarkers or predictors are available, treatment decisions for presymptomatic patients should primarily be based on SMN2 copy number [5,6,10]. Figure F exhibit the key characteristics of SMA diagnostic algorithm.

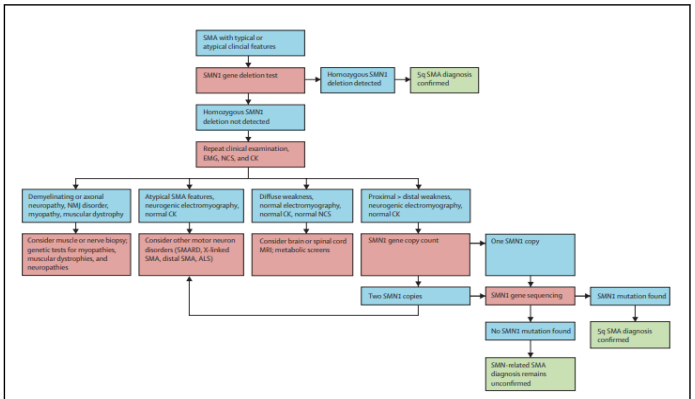

Figure F: Diagnostic Algorithm for SMA.

Several factors can contribute to a potential delay of treatment initiation and include clinical diagnosis, genetic confirmation, drug availability and reimbursement decisions. In newly diagnosed patients, any delay of treatment should be avoided. Ideally, the time frame between diagnosis and initiation of a disease modifying treatment should be no longer than 14 days

While SMA type 0 represents the most severe form displaying symptoms at birth, SMA Type I (also known as Werdnig–Hoffman disease) has an onset before the age of six months and floppiness is evident in affected infants. It is impossible for these children to sit without support, and difficulty with nursing and inability to swallow, aspiration, respiratory failure and tongue fasciculation are seen [5,6]. These children require feeding support measures such as nasogastric tube feeding. The average life expectancy is 8 months, and mortality is 75–95% within 24 months in the absence of respiratory support.

Those with SMA Type II have a later onset between 6 and 18 months [6] are able to maintain a sitting position, though they never gain the ability to stand or to walk without support and tongue fasciculation is seen. Arthrogryposis and scoliosis become increasingly prominent with growth and respiratory failure is likely to develop after an airway infection. Type III (or Kugelberg–Welander disease), with childhood onset, is characterized by the ability to walk independently at first, with gradual deterioration, as individuals fall easily, lose the abilities to walk and get up and elevating the arms becomes more difficult [10,11]. Individuals with type IV, a slowly progressive lower motor neuron disorder, have their onset in adulthood. Muscle weakness and atrophy are seen in all SMA types, along with diminution and, ultimately, disappearance of deep tendon reflexes.

Furthermore, patients with severe SMA also develop congenital heart defect and arrhythmias, vascular abnormalities such as digital necrosis and mild hyperglycemia, suggesting pancreatic dysfunction. Besides that, no clinical guidelines have been established for the management of swallowing function deterioration in patients with SMA type I[10,11].The progression of swallowing dysfunction should be considered not only from the perspective of prevention of aspiration pneumonia and/or malnutrition, but also from the developmental perspective and quality of life, in light of the life expectancy increasing beyond 2 years. The progressive deterioration of swallowing function in

patients with SMA type I should be the focus of greater attention considering these patients' increased life expectancy. In addition, patients with SMA suffer from esophageal reflux, constipation and delayed gastric emptying, which might be a direct consequence of SMN deficiency in neurons of the enteric nervous system (ENS), rather than a secondary event [5]. In particular, SMN deficiency causes interruption of ENS signaling to colon smooth muscle.

SMA Disease Modifiers

Great progress has been made in the clinical translation of several therapeutic strategies for SMA, including measures to selectively address SMN protein deficiency with SMN1 gene replacement or modulation of SMN2 encoded protein levels, as well as neuroprotective approaches and supporting muscle strength and function [6,7]. Examples of new therapeutic approaches for SMA include acceleration of the SMN2 gene transcript by histone deacetylase (HDAC) inhibitor and splicing modification of the SMN2 gene by Antisense Oligonucleotides Nusinersen (Spinraza®) the intra - thecally administered Antisense Oligonucleotide (ASO) - was approved as first-ever therapy of SMA [8,9]. Nusinersen works by binding to an intronic sequence in exon 7 in SMN2 mRNA55, thereby blocking aberrant SMN2 RNA transcription, thus increases the expression of full-length SMN2 mRNA transcript and functional SMN protein levels in the central nervous system (CNS) [5]. Over the past three years, many patients of all ages with SMA worldwide have been treated with Nusinersen (Spinraza®) and data are just emerging about its tolerability and efficacy in different clinical settings and age groups [10,11]. Nusinersen was shown to significantly improve motor function and survival in infants and motor function in children with later-onset SMA and was successfully approved by the US Food and Drug Administration in 2016 and European Medicines Agency in 2017. Risdiplam (Evrysdi ®) is another RNA splicing modifier that enables the SMN2 gene to produce a full length and functional SMN protein [1] and received its first approval in the US for the treatment of SMA in patients 2 months of age and older. One of the advantage of this drug is the oral route of administration. While the intrathecal administration route of Nusinersen mainly limits its effect to motoneurons of the central nervous system (ASOs do not cross the BBB), the systemic distribution demonstrated in preclinical studies with Risdiplam by oral administration allows to hypothesize a possible effect in other tissues.

For pediatric patients with monogenic disorders, viral-mediated gene replacement therapy (GRT), which can address the root cause of neurogenetic disorders by encoding specific genes in viral vectors, is a promising treatment option.

Intravenously administered AAV9-mediated single-dose SMN1 gene replacement therapy (onasemnogene abeparvovec-xioi, Zolgensma®), which increases SMN protein expression both in the CNS and peripherally [7] was recently approved by the US Food and Drug Administration and the European Medicines Agency for children with SMA who are younger than two years of age [10]. In addition, Zolgensma ® uses self-complementary DNA technology, which enables the vector, delivered as double-stranded DNA, to rapidly form a functional episome, resulting in rapid onset of effect [8]. While this broad indication provides new opportunities, it also triggers discussions on the appropriate selection of patients in the context of limited available evidence [3]. Along with self-complementary AAV technology, onasemnogene abeparvovec is designed with a hybrid CMV enhanced chicken β-actin (CBA) promoter [10] to drive high, sustained human SMN expression by increasing the onset of transgene translation and avoiding the rate-limiting step of cell-mediated second-strand synthesis typically required by recombinant AAV, promoting rapid and efficient transduction [11]. A key biological property of the onasemnogene abeparvovec vector is the AAV9 serotype, which has been shown to cross the blood-brain barrier, permitting the targeting of critical cells in the pathogenesis of SMA motor neuron. As motor neurons are long lived, one-time administration of AAV9 GRT is thought to be sufficient for lifetime episomal transgene expression in the cell. Furthermore, SMA I is rapidly progressive, thus, early delivery and onset of high transgene expression with minimal delay is critical to arrest further motor neuron loss.

Although the proportion of the population that is positive for the anti-AAV9 antibody is smaller than for most other AAV serotypes and children have low anti-AAV9 antibody titer frequencies, anti-AAV9 antibody levels are an important safety and efficacy consideration for AAV-mediated GRT studies.

Interestingly, infants with SMA I treated previously with Nusinersen have shown shorter disease duration and experienced greater benefit [7]. Nonetheless, before more evidence is available, combination of both approved therapies should not be part of routine care. In severe symptomatic patients, irreversible degeneration of motor neurons and muscle tissue are probably the most important factors for any lack of efficacy or rescue of the phenotype regardless of the (higher) amount of SMN protein available from any treatment.

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