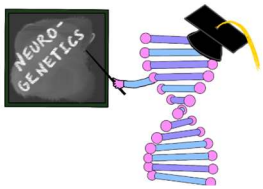



**Teaching Guide**

**Module 14: Therapies for Neurogenetic Diseases, 3 (ASOs and Virally Delivered Therapies)**

This module is more theoretical compared to some of the phenotype-based modules. Facilitator should have reviewed the teaching modules and suggested reading prior to the session. Facilitator should also be cognizant of time management so that all the slides can be covered.

Slide 1: Title

Slide 2: Learning objectives. After completion of this module, the learning will be able to…

Slide 3: Chief complaint

Slide 4: Interactive, get responses from audience for a couple minutes. Generally, want to get the **patient seen in neurology within a few days, if possible, to counsel family, obtain blood work in anticipation for treatment.**

At that first visit, patient would typically see: Neurology, OT/PT, genetic counselor.
Get Blood work (troponin I, AAV9 antibody, Genetic testing confirmation CBC CMP, coags)
Obtain Insurance (can be difficult with newborn that does not have a social security # yet)

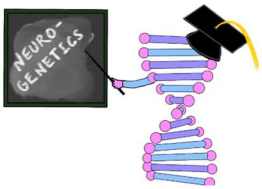
Slide 5: High-yield answers include:

- Family history (negative)
- Physical examination (mild neck weakness when prone)
- Confirmatory genetic testing
    - Confirmation of biallelic SMN1 pathogenic variants
    - SMN2 gene copy number
- Discussion about treatment options
- Baseline blood tests (troponin-I, AAV9 antibody titers, CBC, liver function tests)

General goal to get treatment started within 2 weeks from first neurology visit **(AAV9 antibody titers generally valid for 2 weeks).**

Treatment options can include (as of 5/2023):

- Nusinersen: At baseline and prior to each dose, obtain a platelet count, coagulation laboratory testing, and quantitative spot urine protein testing.
    - The recommended dosage is 12 mg (5 mL) per administration.



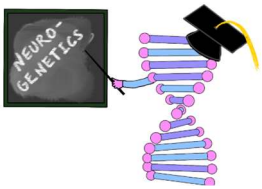



    - Initiate SPINRAZA treatment with 4 loading doses; the first three loading doses should be administered at 14-day intervals; the 4th loading dose should be administered 30 days after the 3rd dose; a maintenance dose should be administered once every 4 months thereafter.
- Onasemnogene abeparvovec
    - Testing for the presence of anti-AAV9 antibodies
    - The safety and efficacy of ZOLGENSMA in patients with anti-AAV9 antibody titers above 1:50 have not been evaluated.
    - Retesting may be performed if anti-AAV9 antibody titers are reported as positive or elevated[1.]
    - Baseline evaluations of liver function (clinical exam, AST, ALT, total bilirubin, albumin, prothrombin time, PTT, and INR), creatinine, complete blood count (including hemoglobin and platelet count), and troponin-I.
    - Liver function, platelet count, and troponin-I will need to be monitored following infusion.
    - Due to the increased risk of serious systemic immune response, administer ZOLGENSMA to patients who are clinically stable in their overall baseline health status (e.g., hydration and nutritional status, absence of infection) prior to infusion. Postpone ZOLGENSMA in patients with infections until the infection has resolved and the patient is clinically stable. Clinical signs or symptoms of infection should not be evident at the time of ZOLGENSMA infusion.
- Risdiplan
    - Daily oral small-molecule medication that can alter splicing of SMN2, like nusinersen.

Slide 6: This was the confirmatory genetic testing report, which used **multiplex ligation-dependent probe amplification (MLPA**), a variation of polymerase chain reaction (PCR) to get amplification of multiple targets and the ability to distinguish genotypes that differ by 1 base. So, this can detect copy number of SMN1 (to diagnose SMA) as well as SMN2 (as SMN2 copy number correlates with type/severity of SMA). *Discuss how the newborn screen looks for common SMN1 deletion, so it will miss point mutations/ single nucleotide variants which will involves a different testing strategy.*

Slide 7: Five minutes in breakout rooms/small groups to find the answers to the questions. Then 5 minutes for groups to report back. Keep this exercise brief to be able to cover all slides. Answers below, and on slides 9-12:

- Group A: *What does the SMN1 gene encode? Where is this gene expressed?*
    - SMN1 encodes Survival motor neuron protein.
    - Expressed mostly in lower motor neurons (slide 9)





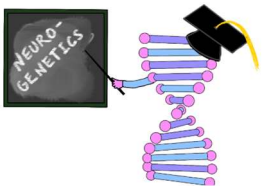

- Group B: *What is most common genetic cause of SMA? What is the mode of inheritance?*
    - Biallelic loss of function (deletion in 95%) of SMN1
    - Autosomal recessive inheritance in 98% (slide 9)
- Group C: *What are the clinical subtypes of SMA? How do these correlate with SMN2 copy number?*
    - Type 0 – prenatal SMA. Non sitters. Rare. Decrease or loss of fetal movement in late pregnancy. Symptoms are apparent at birth and include severe weakness/hypotonia
    - Type I – Non-sitters. Most common. Infantile onset. AKA Werdnig-Hoffman disease.
    - Type II – Sitters. Onset of symptoms between 6-18 months.
    - Type III – Walkers. AKA Kugelberg-Welander disease and resembles muscular dystrophy. Some can walk, but most have difficulty with walking.
    - Type IV – mild motor impairment. Onset in young adulthood. Very rare.
    - SMN2 copy number correlation in slide 13.
- Group D: *What's the difference between SMN1 and SMN2 genes?*
    - **SMN1 and SMN2 differ by 1 base**, such that SMN2 mostly makes a transcript without exon 7, and therefore the protein product from SMN2 that lacks exon 7 is not fully functional. A small amount of transcript (about 10%) from SMN2 includes exon 7 and can make full-length functional SMN protein. (Slides 10-12).

Slide 8: Overview of SMA symptoms mostly pertaining to Type 1. Note that it is important to be able to clinically recognize SMA, as newborn screen that relies on QPCR to determine SMN1 copy number will miss 5% of SMA cases that are caused by single nucleotide variant on 1 allele and loss of SMN1 on other allele.

Slide 9: Overview of SMN1 gene and SMN protein product, inheritance, and the difference between SMN1 and SMN2.

Slide 10: This shows the difference in the single base **(C vs T) in SMN1 vs SMN2**, respectively, at the start of exon 7. Having a T there in SMN2 results in 90% of transcripts having exon 7 spliced out (so 90% of the transcripts omit exon 7). The exons are represented by blue boxes, and the introns are in thinner gray rectangles. The blue (SMN1) and red (SMN2) lines connecting the exons represent how splicing happens when the mature mRNA transcript is made from DNA.

Of note, the red triangles labeled ISS-N1 represent an intronic splicing silencer in intron 7, and this is where nusinersen binds, **resulting in decreased binding of splicing factors there** (in red ovals), so that exon 7 is included more.






Slide 11: SMN1 includes exon 7 in all transcripts while SMN2 only includes exon 7 in 10% of transcripts.

Slide 12: **Lack of having exon 7 results in a shorter protein that is not fully functional.** Individuals with SMA have no SMN1, therefore without treatment, they rely on the small amount of full length SMN protein produced from SMN2, with more copies of SMN2 being more beneficial. **Nusinersen and risdiplam increase the amount of full-length SMN protein made from SMN2**. **Onasemnogene abeparvovec is gene replacement therapy** for SMN1, delivered by an AAV9 vector.

Slide 13: Genotype (SMN2 copy number) and phenotype correlation for SMA.

Slide 14: As of 2/22/23, NBS for SMA is in almost all states (all except NV and HI)

Slide 15: Note: the newborn screen (in Michigan) uses quantitative PCR to detect SMN1 copy number, and two results showing zero copies of SMN1 are needed for a positive screen. It's very rare to have false positives, but there can be false negatives.

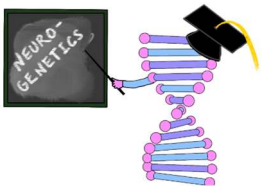
The SMN2 copy number is not tested, and this is done at another laboratory. For SMA, the newborn screen test modality and confirmation varies by state.

Multiplex ligation-dependent probe amplification (MLPA), a variation of polymerase chain reaction (PCR) to get amplification of multiple targets and the ability to distinguish genotypes that differ by 1 base. So this can detect copy number of SMN1 (to diagnose SMA) as well as SMN2 (as SMN2 copy number correlates with type/severity of SMA).

Slide 16: **The newborn screening tests are set by each individual state in the US**. There are national recommendations, Determined by ACHDNC (Advisory Committee on Heritable Disorders in Newborns and Children) to make it onto Recommended Uniform Screening Panel https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp
The criteria on this slide is from an older publication from the WHO that outlines general guidelines for inclusion on the NBS.

Slide 17: Three treatments for SMA.

1. OA - children with SMA <2 years old. Weight based dosing.
2. Nusinersen - any type of SMA at any age. Not weight based. 12 mg or 5 mL dose.
3. Risdiplam - oral, daily, initially for patients 2 months and up.






Slide 18: Additional info: OA was FDA-approved in 2019 for children <2 yrs old, and then approved in other jurisdictions. $2.125 M per treatment initial list price. **Most common side effects are elevated liver enzymes and vomiting.** Get steroids with dose (may alter vaccination schedule). The AAV9 vector can cross blood brain barrier, so IV delivery can result in widespread gene expression in the CNS. AAV9 can also transduce lung, liver, heart, and muscle.

Slide 19: Nusinersen: **First approved treatment for SMA**. ASO = anti-sense oligonucleotide. FDA approved for pediatric and adult patients with SMA. ASOs, as currently formulated, don't cross the blood-brain barrier. Do not spend too much time on this slide as this has been already discussed before.

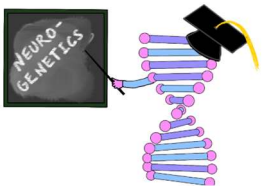
Slide 20: hnRNP (heterogeneous ribonucleoprotein particle, part of mRNA splicing machinery) usually binds to a region 3' of exon 7 of SMN2, to cause splicing that skips exon 7. This leads to a truncated transcript and non-functional SMN protein. **Nusinersen binds to this region of the unspliced transcript to block hnRNP from binding. This results in inclusion of exon 7**, and the production of full length, functional SMN protein.

Slide 21: Risdiplam oral, daily, initially FDA approved in 8/2020 for patients 2 months and up. Summer 2022 FDA indication expanded to include any age.
Crosses blood brain barrier (so gets into CNS), and also distributes into peripheral tissues addressing the concern from some that SMA is a whole-body disease rather than one just of motor neurons

Slide 22: Emphasize that with SMA, the natural history data were key for making SMA ready for clinical trials, so that there's something to compare to. **And SMA has a relatively predictable natural history.** For severe, life-limiting diseases with new potentially helpful disease-modifying therapy, randomized, placebo-controlled trials are very unlikely to get patients/families excited, as they typically do not want to be in the placebo arm.

Different trials have been done for different indications (presymptomatic infants found on newborn screen, early-onset SMA, later-onset SMA, and previously treated SMA).

Slide 23: **Viruses are the main way we deliver genetic material (nucleic acids) to cells.** When a virus delivers genetic material to a cell, this is termed transduction. There are many different types of viruses, and these differ in their carrying capacity (how much DNA they can carry), their tropism (what cells they infect), toxicities/immune response, and how they can introduce DNA that gets expressed long-term (by integrating into the host genome or staying separate as episomes). Most viruses are engineered taking elements from naturally occurring viruses, removing virulent factors, removing the ability to replicate, and combining different viruses






together to direct tropism. Other techniques to introduce genetic material to cells exist, such as gold nanoparticles.

**One of the most common viruses used/developed for therapy in neurology is the adenoassociated virus (AAV),** which is not the same as adenovirus. Adenovirus is the one that was used in 2003 for OTC deficiency, and caused death in an 18-year-old man—this unfortunate event paused gene therapy development for a while.

X-linked severe immunodeficiency disease (**SCID**-**X1**)
OTC = Ornithine transcarbamylase

Slide 24: Lentiviruses are mostly used for ex-vivo gene therapy, for example when T-cells are harvested from a patient, and then the **lentivirus transduces the cells outside the body** (e.g. for CAR T-cell therapy), and then the cells are expanded and then given back to the patient.

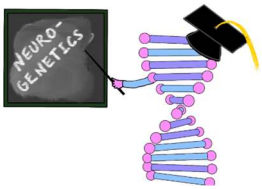
Slide 25: AAV has a lot of pros—broad, long-term transduction. But carrying capacity is small and can cause liver and dorsal root ganglia toxicity.

Slide 26: **ASOs can act to decrease protein production by binding to RNA and causing cleavage/degradation of RNA** (top), or by blocking translation (bottom). This can be useful if the mutation causes a toxic gain of function. ASOs can also modulate splicing, which can lead to more productive mRNA transcripts (middle, nusinersen).

First FDA approved in 1990s for CMV retinitis.

Slide 27:
DSB = double stranded DNA break
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
PAM (explain briefly if time allows): The protospacer adjacent motif (or PAM for short) is a short DNA sequence (usually 2-6 base pairs in length) that follows the DNA region targeted for cleavage by the CRISPR system, such as CRISPR-Cas9. The PAM is required for a Cas nuclease to cut and is generally found 3-4 nucleotides downstream from the cut site.

The CRISPR/Cas9 system is taken from bacteria that use this to recognize and cleave DNA sequences from viruses (as a defense mechanism). **The Cas9 is a protein that cleaves DNA, and the guide RNA is delivered to recognize the piece of DNA to be cleaved and to recruit the Cas9 protein.** With this system, we can precisely cut DNA sequences. When this happens, a double-stranded DNA break occurs, and then one of two DNA repair mechanisms go into action. One results in non-homologous end joining (NHEJ, orange) and before the ends join back together






there can be insertions/deletions created at the DSB site, and this can result in a frameshift mutation. NHEJ is an efficient process. Gene editing to make a precise change in the DNA sequence can be done via a separate repair mechanism, termed homology-directed repair (HDR, teal). In this situation, a piece of donor DNA that incorporates into the DSB site is introduced into the cells as well. The donor DNA contains homology arms that allow recombination into the genomic DNA and can contain sequence differences to fix point mutations. However, HDR is very inefficient.

Base editing and prime editing, which have a single strand DNA break and therefore no NHEJ, may be solutions to edit DNA precisely and more efficiently without introducing undesired insertions/deletions. But delivery of the CRISPR/Cas9 is challenging. Viruses can deliver the guide RNA and the sequences to encode the Cas9 protein, and other methods of gene delivery, like gold nanoparticles can be used as well.

Slide 28: Pros and cons to various methods. **Gene replacement** usually refers to infusion of virally-delivered genetic material that encodes a protein, to replace a dysfunctional gene (e.g. onasemnogene abeparvovec. **Gene editing** may refer to CRISPR/Cas9 or related systems (base or prime editing) to change a single nucleotide. ASOs can be delivered intrathecally (nusinersen), and there are methods to chemically modify ASOs to get blood-brain barrier penetration for IV delivery. **Protein replacement** includes enzyme replacement, like in the case of cerliponase alfa infusion into the cerebral ventricles for Batten disease.

Slide 29: Another comparison chart for ASOs vs gene therapy. Again, note that some studies have **modified ASOs to get better penetration across the blood-brain-barrier (BBB).**

Slide 30: Equity is a big issue with the cost of these treatments.

Slide 31: Treatment with OA has resulted in impressive outcomes for SMA. And hopefully there will be many more gene-directed therapies to come for children with neurological disease!

Slide 32: Suggested Reading.

Slide 33: Acknowledgements.