Uncovering peripheral organ defects in spinal muscular atrophy: insights from AAV9-SMN gene therapy

Aoife Reilly

This thesis is submitted as a partial fulfillment of the Ph.D. program in Cellular and Molecular Medicine of the University of Ottawa

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

Authorization

The content of this document consists of Aoife Reilly's original work. All previously published work as produced by Aoife Reilly et al. obtained the following permissions from the publishers for publication within this document:

1. **Reilly A, Beauvais A, Al-Aarg M, Yaworski R, Sutton E, Thebault S, Kothary R. Peripheral defects precede neuromuscular pathology in the *Smn*$^{2B/-}$ mouse model of spinal muscular atrophy. Journal of Neuromuscular Disease. 2024. (In press).**

According to Sage's Author Archiving and Sharing Policy:
"You may use the Final Published PDF (or Original Submission or Accepted Manuscript, if preferred) in the following ways:

- in relation to your own teaching, provided that any electronic distribution maintains restricted access
- to share on an individual basis with research colleagues, provided that such sharing is not for commercial purposes
- in your dissertation or thesis, including where the dissertation or thesis will be posted in any electronic Institutional Repository or database
- in a book authored or edited by you, at any time after the Contribution's publication in the journal."

2. **Reilly A, Deguise MO, Beauvais A, Yaworski R, Thebault S, Tessier DR, Tabard-Cossa V, Hensel N, Schneider BL, Kothary R. Central and peripheral delivered AAV9-SMN are both efficient but target different pathomechanisms in a mouse model of spinal muscular atrophy. Gene Therapy. 2022 Sep;29(9):544-54.**

According to Springer Nature's Author reuse guidelines:

"Ownership of copyright in original research articles remains with the Author, and provided that, when reproducing the contribution or extracts from it or from the Supplementary Information, the Author acknowledges first and reference publication in the Journal, the Author retains the following non-exclusive rights:

To reproduce the contribution in whole or in part in any printed volume (book or thesis) of which they are the author(s)."

3. **Reilly A, Yaworski R, Beauvais A, Schneider BL, Kothary R. Long term peripheral AAV9-SMN gene therapy promotes survival in a mouse model of spinal muscular atrophy. Human Molecular Genetics. 2024 Mar 15;33(6):510-9.**

According to Oxford University Press' Rights and permission for authors:

"As part of your copyright agreement with Oxford University Press you have retained the right, after publication, to use all or part of the article and abstract, in the preparation of derivative works, extension of the article into a booklength work, in a thesis/dissertation, or in another works collection, provided that a full acknowledgement is made to the original publication in the journal. As a result, you should not require direct permission from Oxford University Press to reuse your article."

Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease characterized by motor neuron loss and skeletal muscle atrophy. SMA is caused by the loss of the *SMN1* gene and low SMN protein levels. Although lower motor neurons are a primary target, there is evidence that peripheral organ defects contribute to SMA. Motor neurons are nevertheless considered the primary target for treatment, and therapies directed to the central nervous system (CNS) are used on SMA patients and in clinical trials. Here, we aimed to explore the contributions of the peripheral organs to SMA by first performing a systematic characterization of the peripheral and neuronal defects in the $Smn^{2B/-}$ mouse model of SMA and then comparing the rescue effect of either intravenous (IV) or intracerebroventricular (ICV) delivery of scAAV9-cba-SMN on the mice. $Smn^{2B/-}$ mice displayed several peripheral defects prior to motor neuron loss. ICV injections increased SMN in peripheral organs and the CNS while IV administration increased SMN in peripheral tissues only, largely omitting the CNS. Further, ICV injections provided better motor neuron and motor function protection. Surprisingly, both delivery routes resulted in an equal long-term rescue effect on survival, weight, and peripheral defects. These results demonstrate the early and independent contributions of the peripheral organs to SMA pathology. Our work also emphasizes a need for a holistic approach to SMA management, including treatments that target motor neuron-independent pathways and the use of a set of biomarkers that monitors multiple systems.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AAV – adeno associated virus

ALS – amyotrophic lateral sclerosis

ASO – antisense oligonucleotide

ChAT – choline acetyltransferase

CHOP INTEND – Children's Hospital Of Philadelphia Infant Test Of Neuromuscular Disorders

CMAP – compound muscle action potential

CNS – central nervous system

COPI – coat protein I

CSF – cerebrospinal fluid

DAPI – 4′,6-diamidino-2-phenylindole

DNA – deoxyribonucleic acid

DPPIV – dipeptidyl peptidase 4

DRG – dorsal root ganglia

FDA – Food and Drug Administration

GFP – green fluorescent protein

H&E – hematoxylin and eosin

HDAC – histone deacetylase

HFMSE – Hammersmith Functional Motor Scale Expanded

ICV – intracerebroventricular

IGF1– insulin-like growth factor 1

IHC – immunohistochemistry

IT – intrathecal

IV – intravenous

MN – motor neuron

MS – multiple sclerosis

MUNE – motor unit number estimation

NAFLD – non-alcoholic fatty liver disease

NAIP - NLR family apoptosis inhibitory protein

NF - neurofilament

NfL – neurofilament light chain

NHP – nonhuman primate

NMJ – neuromuscular junction

NT – no treatment

PBS – phosphate buffered saline

PFA - paraformaldehyde

PLS3 – plastin 3

pNF-H – phosphorylated neurofilament heavy chain

qMRI – quantitative magnetic resonance imagine

RNA – ribonucleic acid

SC – spinal cord

SMA – spinal muscular atrophy

SMN – survival motor neuron

TA – *tibialis anterior*

TBS – tris-buffered saline

TRITC – tetramethylrhodamine

TVA – *transversus abdominis*

X-SCID – x-linked severe combined immunodeficiency

XLMTM – x-linked myotubular myopathy

ZPR1 – zinc finger protein

Acknowledgments

I'd firstly like to thank my supervisor, Dr. Rashmi Kothary, for supporting me in so many ways throughout this long and exciting journey. From my early days in the lab, you have provided me with unconditional support and a trust in my abilities that I had yet to develop for myself. Your belief in me allowed me to develop independence and a strong sense of pride and self-confidence. I appreciate your compassion during challenging times and your recognition of my success when things are going well.

Next, I'd like to acknowledge my lab members, whose support, mentorship, and friendship have been invaluable in helping me to reach this milestone. Thank you first to Ariane, for showing me the ropes of the lab and for supporting and helping with my projects over the years. I appreciate your eagerness to help and the kindness and positivity you bring to the lab. Thank you also to Sabrina and Yves, who also helped form the backbone of the lab throughout my time, providing assistance and expertise along the way. Thank you also to my mentor and good friend Sarah, for making me feel welcome during my early days in the lab, teaching me how to navigate grad school, and offering me unwavering guidance and support with my research. I am also so grateful for your friendship, which made coming into work especially fun. Thank you to my other lab friends over the years: Lucia, Becca, Niko, Emma, Charlie, Chyna, and Monique, who have also provided me with important mentorship, collaboration, and companionship. And to all other lab members that I have worked with during my time here: Majd, Kelsea, Morgan, Anisha, Sam, Ally, and Marco – thank you. I could not have completed this journey without the help of everyone.

I would also like to thank my thesis advisory committee: Dr. Robin Parks, Dr. Michael Schlossmacher, and Dr. Nadine Wiper-Bergeron. Your thoughtful feedback and advice

throughout this process have been essential to the success of my projects. Thank you as well to the funding agencies who supported me throughout my degree, including the Canadian Institutes of Health Research and the University of Ottawa Centre for Neuromuscular Disease.

Finally, thank you to the friends and family who have provided me with love and support over the past 6 years. Thank you to my parents for believing in my potential and encouraging me to pursue graduate studies. Thank you to my friends and roommates Bridget and Alie, who always helped me to decompress and made being locked down together in 2020 a fun adventure. Thank you as well to Anna, Kiara, Kaitie, Emma, and Michelle, for being the most incredibly supportive and caring friends. And thank you to my partner Ellie, for your patience and confidence in me, and for creating a safe space where I am always heard and understood.

Chapter 1: General Introduction

Spinal Muscular Atrophy: a genetic neuromuscular disorder

Prevalence and genetic basis

Spinal Muscular Atrophy (SMA) is a devastating autosomal recessive neurodegenerative disorder. The disease is classically characterized by lower motor neuron degeneration and subsequent atrophy of skeletal muscle. SMA is estimated to affect 1-2 in 100,000 individuals worldwide, with an incidence of 1 in 10,000 births (1,2). Patients suffer from progressive muscle weakness, leading to complications such as scoliosis, respiratory failure, and early death.

SMA is caused by the loss or mutation of the survival of motor neuron 1 (*SMN1*) gene on chromosome 5 at position 5q13 (3). This gene, along with its paralogous copy *SMN2*, was first identified in 1995 and is responsible for the production of the Survival Motor Neuron (SMN) protein (3,4). The SMN protein is ubiquitously expressed and is essential for human life. *SMN2* is mostly identical to the *SMN1* gene, save for five mutations, one of which is a critical C to T transition at position 6 of exon 7 (5,6). This mutation creates an exon splicing suppressor, causing exon 7 to be skipped during splicing and excluded from most *SMN2* mRNA transcripts. The protein product is thus unstable and quickly degraded (7). For this reason, the *SMN2* gene only produces a limited amount of protein and is not able to compensate for the loss of *SMN1* (Figure 1.1).

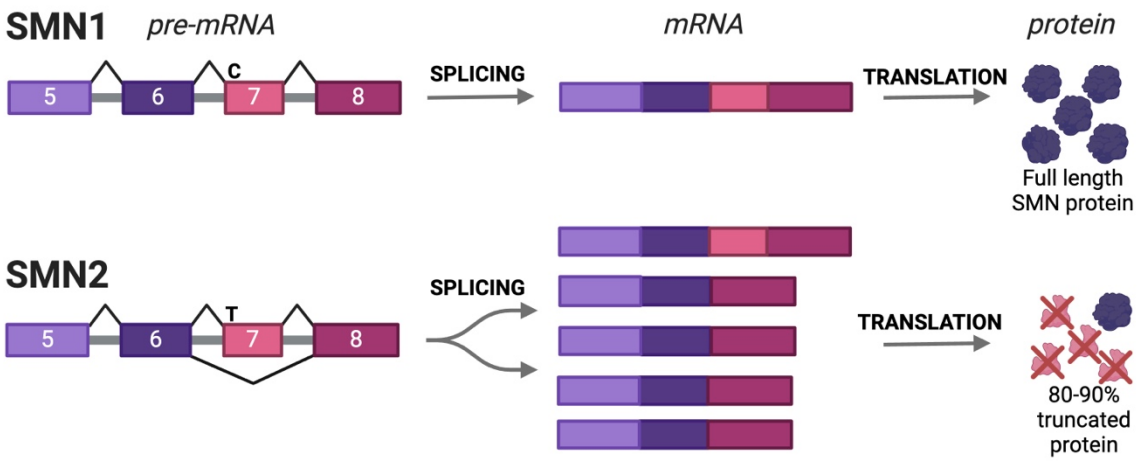


Figure 1.1 Pre-mRNA splicing of SMN1 and SMN2 RNA and the resulting effects on SMN protein production. Created with BioRender.com.

Clinical Features of SMA

SMA severity varies widely between individuals, primarily due to the variation in *SMN2* gene copy number from person to person. Humans usually possess somewhere between 0-8 copies of the gene. As a result, the severity of SMA disease, ranging from types 0 to 4, is largely mediated by *SMN2* copy number. Type 0 SMA patients usually possess one copy of *SMN2*. These patients present with decreased prenatal movement and succumb to the disease before 6 months of age. Type 1 patients are diagnosed in the first 6 months of life and typically possess 2-3 copies of *SMN2*. These patients present with severe muscle weakness in the arms, legs, and trunk. Type 1 patients never achieve the ability to sit. If untreated, they suffer from significant respiratory muscle weakness, requiring ventilatory support, and die from respiratory failure within their first two years. Type 2 SMA is diagnosed between 6-18 months. These patients are able to sit and usually possess 3 copies of *SMN2*. They require mobility support and will typically need respiratory support as the disease progresses over time. Type 3 patients are

diagnosed after 18 months of age, presenting with symptoms of progressive weakness in the proximal muscles. These patients can walk but often require mobility aids as the disease progresses. Type 3 patients typically have 3-4 copies of *SMN2*. Finally, type 4 patients present with mild muscle weakness in adulthood and possess around 4-5 copies of *SMN2*. This is the slowest progressing SMA type, but mobility aids are sometimes necessary (6).

While severity and progression rates vary between individuals, several clinical features are consistent across all SMA types. Before the discovery of the genetic basis of SMA, patients were diagnosed based solely on clinical findings and muscle biopsies (6). Early indicators of the disease include progressive proximal muscle weakness, reduced muscle tone, and delayed reflexes despite normal social engagement and cognitive abilities (8). Motor function and milestones, typically measured using standardized tests such as the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND), are also delayed in SMA patients (9). Patients show reduced motor nerve function and motor unit numbers, as measured using Compound Muscle Action Potential (CMAP) testing (10).

Pathological Features of SMA

Most pathological data have been collected from the autopsy of severely affected SMA patients after they have succumbed to the disease. Early autopsy reports described the disease by a characteristic loss of motor neurons in the anterior horn of the spinal cord and small myofiber size in skeletal muscle (11). Typical features of an SMA patient’s spinal cord include motor neuron loss, empty cell beds, the presence of glial bundles in the ventral spinal roots, and heterotopic motor neurons (6). Though mostly restricted to motor neurons, some reports have identified abnormal neuronal morphology in the dorsal root ganglia, thalamus, and Clarke’s

column (12,13). SMA patient ventral roots show reduced number and size of myelinated axons as well as an abundance of glial bundles (14–16). Skeletal muscle biopsies reveal a characteristic pattern of large areas of small myofibers with occasional hypertrophic fibers (17,18). Empty sections of basal lamina can be observed due to myofiber loss (19). At the level of the neuromuscular junction, endplates show immature morphology, changes in acetylcholine receptor clustering, and axonal swelling, much of which can also be observed in prenatal muscle (6,20). Autopsy reports of peripheral organ defects including hepatic and cardiovascular abnormalities have also been reported and are described in detail in a later section.

Mouse Models of SMA

The *SMN2* gene arose from a gene duplication event exclusive to humans (21). As the SMN protein is essential for life, a loss of the *SMN1* gene in other animals leads to embryonic death (22). For this reason, only humans suffer from SMA. Several animal models, including zebrafish, drosophila, and mice, have been generated to more closely study the disease and evaluate treatment strategies. Among these, mouse models are the most widely used (summarized in Table 1.1).

As mice only possess one *Smn* gene, compared to humans' *SMN1* and *SMN2*, knockout of *Smn* results in embryonic lethality (23). Interestingly, knockout of only one copy of *Smn*, producing $Smn^{+/-}$ mice, results in a normal phenotype (24). The first mouse model of SMA was developed by creating an *Smn* null allele on a genetic background that harboured two copies of the human *SMN2* gene ($Smn^{-/-};SMN2^{+/+}$) (22). These mice, known as the "severe" or "Line89" model, quickly develop motor neuron loss by post-natal day 5 (P5) and die soon after. This model is commonly used to study the most severe forms of SMA. A similar model was also

created by another group, which uses an *Smn* with an exon 7 deletion instead of an *Smn* null allele (25). These mice, known as the Taiwanese model, survive about 10 days. A milder version of this model has also been generated, which possesses 4 copies of the *SMN2* gene. This model is often known as the Taiwanese mild model and it exhibits muscle atrophy, motor function deficits, and motor neuron loss at the age of 9 to 12 months (26).

A less severe version of the $Smn^{-/-};SMN2^{+/+}$ mouse was generated by the addition of a human SMNΔ7 cDNA ($Smn^{-/-};SMN2^{+/+};SMN\Delta7^{+/+}$). These mice, known as the "Delta 7" or "Δ7" model develop decreased weight, motor function, and motor neuron loss by P9 and survive for a mean of 13.5 days (27). Delta 7 mice are commonly used for the study of severe SMA and provide a longer time frame than the severe model that can be useful for the study of disease progression and therapeutic efficacy.

Mouse models of SMA have also been generated by the genetic alteration of the mouse *Smn* gene to produce less SMN protein. The $Smn^{2B/-}$ mouse model possesses a mutated version of the *Smn* allele called Smn^{2B}. This allele was generated by mutating an exonic splice enhancer within exon 7, leading to the production of mostly unstable SmnΔ7 transcripts (28). These mice produce about 15% of the SMN protein produced by wild type mice and represent a less severe form of the disease than Delta 7 mice. Our laboratory's $Smn^{2B/-}$ mice are bred on a C57/BL6 background and demonstrate typical SMA characteristics, including motor neuron loss, neuromuscular junction (NMJ) pathology, muscle weakness and atrophy, weight loss, and a mean survival of 25 days (29). The $Smn^{2B/-}$ mouse also displays a unique peripheral phenotype consisting of delayed muscle development (30,31), fatty liver disease (32,33), glucose

metabolism defects (34), immune dysregulation (35), and gastrointestinal defects (36), that allows for a closer examination of the non-neuronal aspects of SMA.

While the above mouse models represent the more severe types of SMA, our laboratory has recently developed a mild model of SMA with a longer lifespan than the most commonly used pre-clinical models. This mild mouse model was generated by crossing homozygous *Smn*$^{2B/2B}$ mice and mice homozygous for the human *SMN2* gene, to eventually produce *Smn*$^{2B/-}$*;SMN2*$^{+/-}$ animals. These mice demonstrate a normal survival with mild muscle atrophy, motor weakness, and NMJ dysfunction in adulthood. Interestingly, these SMA-like characteristics are seen more prominently in male mice of this model (37).

Table 1.1. Summary of mouse models of SMA

Mouse model	Common name	Lifespan	Main findings	Reference
***Smn*$^{-/-}$*;SMN2*$^{+/+}$**	Severe	5-7 days	MN loss, denervation, muscle atrophy	(22)
***Smn*$^{H7/H7}$*;SMN2Hung*$^{+/-}$**	Taiwanese model	10 days	MN loss, denervation, muscle atrophy	(25)
***Smn*$^{H7/H7}$*;SMN2Hung*$^{+/+}$**	Taiwanese mild model	9-15 months	MN loss, muscle atrophy, tail/ear/foot deformity	(26)
***Smn*$^{-/-}$ *;SMN2*$^{+/+}$*;SMN*Δ*7*$^{+/+}$**	Delta 7, Δ7	14 days	MN loss, denervation, muscle atrophy, NMJ pathology	(27)
***Smn*$^{2B/-}$**	*Smn*$^{2B/-}$, 2B/-	28 days	MN loss, denervation, muscle atrophy, NMJ pathology, peripheral and metabolic features	(38)
***Smn*$^{2B/–}$*;SMN2*$^{+/–}$**	n/a	Normal survival	MN loss, muscle weakness, NMJ pathology later in life	(37)

Genetic Modifiers of SMA

Despite the monogenic nature of SMA, patients with the same number of *SMN2* copies can present with varying degrees of disease progression and severity. This has led to the discovery of over a dozen modifier genes associated with SMA. These genes can affect SMN expression directly through processes like transcription and splicing, as well as through post-translational modifications such as the phosphorylation of SMN. Conversely, modifiers can also indirectly affect the SMA phenotype through impacting axonal transport, cytoskeleton dynamics, and neurotransmission.

A well-known indirect protective modifier of SMA, plastin 3 (*PLS3*), was discovered through transcriptomic profiling of affected and unaffected siblings who possessed identical *SMN2* copy numbers (39). PLS3 is an F-actin binding and bundling protein involved in cell migration, adhesion, vesicle trafficking, and endocytosis. It is thought to be essential for axonogenesis, and thus higher levels of PLS3 can provide a protective effect against the axonal defects and degeneration associated with SMA. PLS3 is also shown to rescue impaired endocytosis in SMN-deficient cells. Through examination of the PLS3 interactome, two other proteins, calcineurin-like EF-hand protein 1 and coronin1C, were found to directly interact with plastin 3 and to produce similar protective effects on endocytosis when overexpressed (40).

Zinc finger protein 1 (ZPR1) is an SMN-interacting protein that is expressed at lower levels in SMA patients (41). Reduction of ZPR1 levels in mouse models show progressive motor neuron loss and mislocalization of spliceosomal proteins, while overexpression of ZPR1 in motor neurons improves SMN localization in the nucleus and enhances axonal growth (42,43).

Another modifier gene, Neuronal Apoptosis Inhibitory Protein (*NAIP*), has been closely associated with SMA since early studies on the genetic basis of the disease (44). *NAIP* is situated close to *SMN1* and *SMN2* and its deletion is associated with an earlier age of onset and more severe clinical phenotype (45,46).

The SMN Protein

Though the genetic basis of the disease has been well described, the function of the SMN protein and its connection to SMA pathogenesis remains elusive. The protein was originally given its name due to high levels of expression in motor neurons, the apparent vulnerability of these cells to the depletion of SMN, and a higher requirement for SMN during neuronal development (47,48). However, early studies of the SMN gene discovered that it was actually expressed in all cell types studied (49). Upon the identification of the protein and the production of the first SMN antibodies, SMN was observed in both the cytoplasm and the nucleus. Within the nuclei, SMN was found to aggregate in tight structures that were called “gems”, or “gemini of coiled bodies”, due to their apparent association with coiled bodies of the nucleus (50). Coiled bodies, or Cajal bodies, are nuclear structures that facilitate the assembly of small ribonucleoproteins (snRNPs), which play an essential role in pre-mRNA splicing (51). This discovery thus began the characterization of the most recognized function of the SMN protein, its role in small ribonucleoprotein (snRNP) assembly.

Immunoprecipitation studies of the SMN protein revealed that it forms a complex with several other proteins. This complex, called the SMN complex, consists of SMN, Gemins2-8, and Unrip (6). All components of the SMN complex associate with Sm proteins, an essential component of snRNPs (52). The complex was thus shown to facilitate the assembly of snRNAs

and Sm proteins into snRNPs in the cytoplasm, then traffic snRNPs to the nucleus for spliceosome assembly (53–55). This role is made evident by splicing defects that have been found in SMA cell lines and animal models (56–58). However, splicing defects have been shown to mostly occur in later stages of the disease (59), suggesting that SMN's role in snRNP assembly may not be the most important role of SMN in SMA pathogenesis.

Although the role of SMN in snRNP assembly is clear, this process is not motor neuron-specific and thus does not provide an explanation for why motor neurons are so vulnerable to the loss of SMN. Interestingly, while SMN protein is localized to the nucleus during development, it is seen in the cytoplasm and axons of mature motor neurons (47). Further, the SMN protein's nucleic acid binding domain and its interactions with several RNA binding proteins in motor neurons have suggested a role for the protein in mRNA trafficking (60–62). Live cell imaging with green fluorescent protein (GFP)-tagged SMN granules has revealed the bidirectional movement of SMN protein throughout axons and localization at the growth cone (63). Growth cones were also found to be reduced three-fold in Taiwanese SMA mice (64). Further, SMN protein has been shown to bind with COPI (coat protein I) vesicles, which are major components of the COPI system responsible for intracellular trafficking and neuronal cell maturation (65). Subsequent studies using advanced imaging methods found that SMN acts as a chaperone for RNA binding proteins (RBPs), facilitating the assembly of messenger ribonucleoproteins (mRNPs) that regulate trafficking at the level of the cytoskeleton (66). This theory is supported by several findings of mislocalization of mRNA transcripts within the axons of SMN-deficient motor neurons (67,68). Overall, this evidence points to a role for SMN protein in axonal transport and growth, and may explain why motor neurons display a greater sensitivity to reduced SMN levels.

Through further investigation of SMN's RNA binding properties, a role for SMN in translational regulation has also been proposed. SMN protein was found to be associated with polyribosomes, the machinery that facilitates mRNA translation (69). When SMN was introduced to an *in vitro* translation system, translation was reduced in a dose-dependent manner, implying that SMN plays an important role in translation control. SMN has also been shown to indirectly affect translation through the miR-183/mTOR pathway. SMN-deficient cultured neurons showed an upregulation of miR-183, an miRNA essential for axonal growth and survival through the translational regulation of *mTor*. Knockdown of miR-183 in the Taiwanese mouse model of SMA prolonged survival and protected motor function (70). More recently, translatome profiling has confirmed these findings, describing widespread translational deficiencies in Taiwanese SMA mice (71).

Overall, SMN appears to play several important roles in axonal outgrowth and survival. The large size of motor neurons as well as their limited regenerative capacity likely also contributes to the selective vulnerability of motor neurons to SMN deficiency. Nevertheless, SMN deficiency appears to affect all cell types and a role for the protein in cytoskeletal dynamics, endocytosis, autophagy, and protein homeostasis have also been described that may be responsible for other tissue-specific vulnerabilities.

Therapeutic Landscape

Historically, the treatment of SMA has centred around symptom management and palliative care. It is not until recently, due to the discovery of the genetic basis of the disease, that SMN-altering treatments have been developed and become available to patients (Treatment landscape summarized in **Figure 1.2**).

Figure 1.2 Summary of the therapeutic landscape for SMA patients. Created with BioRender.com.

Symptom Management Strategies

Typical management of SMA requires support with mobility, respiration, and nutrition. Increasing muscle weakness is addressed with the use of mobility aids, physical therapy, and muscle stretching. Orthopedic management of spinal deformity, joint instability, and muscle contractures is also beneficial. Braces can be used to support muscles and joints, while surgery can be necessary in the case of severe scoliosis. Weakened respiratory muscles are supported through assisted airway clearance and ventilation in severe cases. SMA patients are also at risk

of nutritional deficiencies due to chewing and swallowing difficulties and gastrointestinal issues. Their diets are closely managed and supplementation or assistive feeding is implemented if needed (72–74).

Repurposing of drugs for SMA

Several commonly used drugs have been explored in pre-clinical and clinical trials as potential treatments for SMA. Clinical trials in the 1970s and 80s explored the use of guanidine hydrochloride, due to its acetylcholine releasing effects and success in the treatment of myasthenic syndrome (75). However, results of therapeutic benefit were mixed and side effects were often severe (76,77). In the 1990s and 2000s, gabapentin and thyrotropin releasing hormone were explored for their known neurotrophic and neuroprotective effects. While both showed promise in anecdotal cases and uncontrolled trials (78,79), neither showed a significant therapeutic benefit in randomized controlled trials (80,81). Creatine has also been explored as a potential treatment for SMA due to its known roles in increasing muscular mass and strength, but a randomized controlled trial of 40 SMA patients found no therapeutic effect of creatine treatment (82).

Histone Deacetylase Inhibitors

Once SMA was established as a disease of SMN protein deficiency, research began to explore how SMN protein levels could be increased. In 2001, sodium butyrate, a histone deacetylase (HDAC) inhibitor, was identified as a candidate SMA treatment through a screen of several drugs on their ability to influence SMN levels in human SMA lymphoid cell lines (83). The group then demonstrated sodium butyrate's ability to increase SMN levels by changing the alternative splicing pattern of exon 7 in the *SMN2* gene, and found that treatment with sodium

butyrate improved SMA-like pathology in Taiwanese SMA mice (83). Several other HDAC inhibitors, including valproic acid, phenylbutyrate, and trichostatin A produced a modest increase in SMN levels in preclinical models of SMA (84–86) .

Despite these drugs' success in preclinical trials, randomized controlled clinical trials of valproic acid and phenylbutyrate showed no therapeutic effect and no effect on SMN mRNA levels in the blood of treated patients (87–89). HDAC inhibitors are also non-specific to SMN, raising concerns of off-target effects when used as a treatment for SMA. Other agents that showed promise at increasing SMN expression in pre-clinical studies but failed to produce significant placebo-controlled clinical trial results include hydroxyurea, a drug used to treat sickle-cell disease and cancer (90,91), and albuterol, a beta-adrenergic agonist (92,93).

Nusinersen: The first approved treatment for SMA

With the knowledge that the *SMN2* gene could be targeted to increase functional SMN protein production, researchers set out to create an antisense oligonucleotide (ASO) treatment that altered the splicing of *SMN2* pre-mRNA. In 2007 and 2008, Dr. Krainer and colleagues used cultured cells derived from patients with SMA to develop several potential ASO candidates that bound to exon 7 or to sequences up or downstream of exon 7 (94,95). Of this group, ASO 10-27 emerged as the most promising candidate. Over the following years, tests of the ASO candidate by Dr. Krainer's group in partnership with IONIS Pharmaceuticals showed promising therapeutic efficacy in animal models of the disease, extending survival and improving motor function (96–98).

These studies also provided insights into better understanding the disease. In one study, an embryonic injection of ASO 10-27 provided a greater rescue to peripheral necrosis than

injection at P1, emphasizing the importance of early treatment (96). When different routes of administration were explored, a subcutaneous injection of ASO 10-27 improved survival by 10-fold in a severe mouse model, while a CNS-directed injection only improved survival by 2-fold, indicating that peripheral restoration of SMN is important for long-term treatment (97). However, as ASOs are incapable of penetrating the blood-brain-barrier when delivered systemically, ASO 10-27 was developed for intrathecal (IT) delivery to patients using standard lumbar puncture. In 2015, the first in-human, open-label, single-ascending dose study of ASO 10-27 was performed under the drug's new name, nusinersen. Following the success of this and subsequent trials, nusinersen, now marketed as Spinraza by Biogen, was approved in 2017 as the first U.S. Food and Drug Administration (FDA) approved therapy for all ages and types of SMA in pediatric and adult patients.

In the first year of treatment with nusinersen, patients receive four loading doses followed by maintenance doses every 4 months (99). Nusinersen has demonstrated safety and efficacy in the treatment of SMA in patients of all ages, improving and preserving motor milestones, though rare adverse events such as infections and post-lumbar puncture complications have been reported (100). Most patients with 2 or 3 copies of *SMN2* treated presymptomatically with nusinersen have achieved motor milestones such as sitting and walking that would previously be unheard of in this group (101). Nusinersen can also stabilize or improve motor function in older patients, but proves to be more effective in earlier stages of the disease (102).

Onasemnogene Abeparvovec

As nusinersen was emerging, a gene-replacement therapy strategy was also being developed as a promising alternative to the invasive and frequent dosing schedule required for ASO delivery.

Viral gene-replacement therapy is a therapeutic approach that attempts to harness the infectious power of a virus to insert a therapeutic gene into the cells of sick patients. The concept of genetic engineering was developed in the late 1960s based on the discovery that viruses, such as the shope papilloma virus, were capable of integrating their own genes into a host-cell's genome (103). The use of retrovirus and adenovirus-mediated gene transfer was explored in humans in the late 1990s (104). While initial results were promising, the tragic death of a patient receiving adenovirus-based treatment for ornithine transcarbamylase deficiency alerted researchers to the dangers of a patient's immune response to viral vectors (105).

Despite these initial setbacks, several adenovirus and retrovirus-based gene therapy strategies were developed in the early 2000s. However, another safety concern soon emerged, that of the potential genotoxicity of inserting therapeutic genes directly into the host's genome. Three patients developed leukemia after treatment in a clinical trial for a retrovirus-based gene therapy for severe combined immunodeficiency (X-SCID), as the virus integrated next to and activated an oncogene (106). Meanwhile, an intramuscularly-injected retrograde-transported lentiviral gene transfer system was being developed and showed promise in preclinical amyotrophic lateral sclerosis (ALS) trials (107). Researchers studying SMA harnessed this system to deliver SMN protein to motor neurons, increasing survival by 3-5 days in Delta 7 mice (108). However, the multiple intramuscular injections required to target motor neurons and the

safety concerns around the integrating properties of lentiviruses made this therapy less suitable for clinical translation.

As research progressed in the field of gene therapy, treatment strategies for neurological disorders began to prefer adeno-associated virus (AAV) vectors due to their lower immunogenicity and minimally integrative properties (109–111). Further, newly discovered serotypes AAV6, AAV8, and AAV9 allowed for better widespread transduction of skeletal muscle, heart, and liver (112–115). In 2009, a self-complementary AAV9 (scAAV9) was developed that crossed the blood brain barrier through intravenous injection, transducing the motor neurons in neonatal and adult mice and cats (116,117). With these promising results, scAAV9 delivery of an *SMN1* transgene was explored through intravascular, intramuscular, and intracerebroventricular (ICV) injection. All routes of scAAV9-SMN injection showed transduction of the motor neurons, rescued motor function and extended survival by upwards of 100 days in Delta 7 mice (118–121).

As a result of the promising pre-clinical data, Avexis launched the first clinical trial of scAAV9-SMN under control of a hybrid cytomegalovirus enhancer–chicken beta-actin promoter (scAAV9.CB.hSMN) also known as AVXS-101, in 2014 (122). In this Phase 1 trial, known as the START trial, infants with Type 1 SMA received a one-time intravenous infusion of AVXS-101 and were compared with historical data sets as controls. Twenty months after treatment, all patients showed improvements in motor function and none required ventilatory support, a milestone usually achieved in only 8% of Type 1 SMA cases (122). The treatment showed a favourable safety-profile, extended patient survival, and led to motor function improvements which were maintained in subsequent long-term follow up studies (122,123). Some children

reached motor milestones that were unprecedented for SMA patients, such as standing and walking. All children from the highest dose group were alive and had maintained motor milestones 7.5 years later. The following Phase 3 trials, STR1VE and SPR1NT, demonstrated a similar efficacy and safety of the treatment in more severely affected patients and presymptomatic infants, respectively (124–127). As with nusinersen, presymptomatic treatment was more effective at protecting motor function and survival (124,125). Onasemnogene abeparvovec, named Zolgensma by its new owner Novartis, was approved by the FDA in 2019 and by Health Canada in 2020 for use in pediatric SMA patients (128,129).

Emerging safety concerns of onasemnogene abeparvovec

While the benefit-risk profile of onasemnogene abeparvovec was determined to be favourable in clinical trials, use of the drug carries the risk of serious adverse events. Hepatotoxicity is a common side-effect of treatment, as the liver is known to be a primary target for AAV9 vectors (130,131). In the initial START trial, serum aminotransferase elevations were detected in several patients, leading to the administration of oral prednisolone alongside onasemnogene abeparvovec from that point forward (122). A recent study found that 90% of patients had elevated serum transaminase concentrations after treatment with onasemnogene abeparvovec, despite receiving oral prednisolone for a minimum of 30 days after treatment (130). In 2022, two onasemnogene abeparvovec patients from Russia and Kazakhstan tragically died of acute liver failure after beginning to taper off of corticosteroids (132). Other serious adverse events that have been reported include thrombocytopenia and thrombotic microangiopathy, one case of which was fatal for a 6-month-old patient (133,134).

Though not observed in humans, other toxicity concerns from onasemnogene abeparvovec treatment have arisen from pre-clinical trial data. Studies on mice found evidence of cardiac toxicity, including inflammation and intracardiac thrombi, as well as fatal atrial thrombosis after intravenous onasemnogene abeparvovec treatment (135). Another preclinical study explored the effects of an AAV9 variant (AAVhu68) carrying a human SMN transgene on nonhuman primates (NHPs) and piglets. Degeneration of the dorsal root ganglia (DRG) was observed in the treated NHPs, while the piglets displayed severe proprioceptive deficits and ataxia (136). These results may be attributed to the high dose of AAV used, as a study that administered high-dose intravenous injections of an AAV vector (AAV-PHP.B) to NHPs showed similar severe toxicity (137).

Intrathecal delivery of onasemnogene abeparvovec

Due to the weight-dependent dosing of onasemnogene abeparvovec, there are challenges associated with treating older and heavier patients. Currently the largest weight treated is up to 21 kg, meaning that most older children and adult patients cannot receive the treatment. A high dose of the drug is required for intravenous treatment as only a limited amount of the virus can cross the blood-brain-barrier when administered systemically. However, as the majority of vector genomes distribute to the liver after onasemnogene abeparvovec administration, a higher intravenous treatment dose increases the previously described risk of hepatotoxicity (138). Further challenges arise from the high cost of the drug. Upon its release, Zolgensma was designated as the most expensive drug in the world, with a cost of $2.1 million per dose (139). Adjusting the cost for heavier patients could inflate the cost drastically, making it less affordable and less likely to be covered by patients' insurance providers.

To avoid the high doses required to treat older patients intravenously, IT administration was proposed as an alternative method of delivery for this group of patients. The effectiveness of a CNS-directed therapy had been demonstrated in preclinical trials, where a 10-fold lower dose of scAAV9-cba-SMN was required to achieve a comparable rescue of survival in mice when treating ICV compared to IV (140). IT delivery of a 30-fold lower dose of the vector was then determined to be safe and effective at transducing the spinal cord of NHPs to a comparable degree to IV injection (140). The Phase 1 STRONG trial began in 2017 to evaluate the safety and optimal dose of a one-time IT infusion of onasemnogene abeparvovec in sitting nonambulatory patients who were 6 to <60 months old (141).

In 2019, the trial was halted due to newly emerging toxicity concerns from animal studies, including the previously mentioned DRG toxicity seen in intravenously treated NHPs (142). In a follow up study, mononuclear cell inflammation and neuronal degeneration were observed in the DRGs and trigeminal ganglion of NHPs treated IT with onasemnogene abeparvovec (143). Two years later, after no evidence of DRG toxicity in treated patients, the hold was lifted and the study proceeded, this time with the elimination of the highest dose cohort (141). Patients in the STRONG trial were divided into two groups of older and younger patients. Patients in both groups achieved clinically meaningful improvements in motor function scores, but almost all patients did not meet the primary and secondary endpoints of standing independently or walking (141). As with intravenous treatment, instances of hepatotoxicity, hepatomegaly, thrombocytopenia, cardiac events were reported. Interestingly, the number of hepatotoxic-related adverse events was slightly lower than in intravenous clinical trials (21% vs 34%) (130). According to a 4-year follow up report, all patients had either maintained or achieved new motor milestones (144). Based on the success of the STRONG trial, the medium

dose from this trial was used for the Phase 3 STEER trial. This trial is currently underway to determine the efficacy of a fixed dose of IT onasemnogene abeparvovec for patients aged 2 to <18 years old.

Risdiplam

Risdiplam, developed by Genentech and sold under the brand name Evrysdi, is the latest treatment available for SMA patients. Risdiplam is an orally administered small molecule drug that was developed as an alternative to the more invasively administered nusinersen and onasemnogene abeparvovec.

Based on the therapeutic benefit of altering *SMN2* pre-mRNA splicing by nusinersen treatment, researchers aimed to identify an orally available small molecule that could increase SMN protein levels by promoting the inclusion of exon 7 in *SMN2* mRNA. A promising candidate, RG7800, was identified through a screen of a library of small molecules against a human embryonic kidney cell line harboring an *SMN2* minigene (145). Daily administration of the compound to Delta 7 mice increased SMN protein levels throughout the CNS and peripheral organs. RG7800 also extended survival and protected mice against motor neuron loss, NMJ denervation, and skeletal muscle atrophy (145).

RG7800, as well as several other small molecules that were identified in high-throughput screening experiments, were evaluated in Phase 1 clinical trials. While early results of the RG7800 trial showed that the drug was well tolerated by patients and healthy adults, the study was halted due to reports of irreversible off-target adverse effects on the retina in animal toxicology studies (146). Researchers then altered the chemistry of RG7800 to create a comparably effective compound called RG7916, or risdiplam (147). Risdiplam was successful in

clinical trials, providing improvements in motor function and muscle strength in children and adults with all types of SMA (148,149). However, the treatment demonstrated limited improvement in motor function in older individuals and was more likely to stabilize symptoms in this group (150).

Risdiplam was approved by the FDA in 2020 and Health Canada in 2021 for use in patients two months of age and older (151,152). Preclinical studies have raised concerns about potential adverse effects such as growth impairment, male infertility, retinal toxicity, inflammation of epithelial tissues, and mutagenic effects, but none have been observed in humans to date (Evrysdi prescribing information).

Gaps in the treatment landscape

Despite remarkable advances in the treatment of SMA, the quality of life achieved by current SMA therapies remains unclear (current therapies summarized in Figure 1.2). Treatment responses vary widely between individuals, with some patients experiencing significant improvement while others may show little response. Treatment efficacy is also limited in adult patients. In nusinersen trials, only a mild treatment effect is seen in adult patients, coupled with several lumbar puncture-related adverse events (153). Given the high cost of treatment and repeated lumbar puncture procedures, there is hesitancy among the older SMA population about whether nusinersen's limited efficacy justifies the invasive nature of the treatment. Meanwhile, onasemnogene abeparvovec is presented to SMA patients as a one-dose lifesaving drug. However, while it has extended the lives of many patients, less than half of Type 1 SMA patients in the Phase 3 SPR1NT trial maintained an ability to thrive over the course of the treatment (124,125). Further, long term data are not yet available to determine the longevity of this

treatment. A large portion of patients were also nonresponsive to risdiplam treatment. In a trial of Type 1 patients, 41% of patients were not able to reach the primary endpoint of sitting unassisted after 24 months (148). In a trial of symptomatic Type 2 and 3 patients, older patients did not show improvement but only a stabilization of symptoms (150).

Many patients who showed a limited response to therapy are now receiving multiple therapies in an attempt to maximize the benefits. While the effectiveness of combinatorial therapy is not yet clear, dual therapy of any combination of nusinersen, onasemnogene abeparvovec, and risdiplam appears to be well tolerated (154,155). However, one study of Type 1 SMA infants treated with nusinersen and onasemnogene abeparvovec found that combinatorial therapy was only as effective as nusinersen therapy alone (156). Overall, the shortcomings of the available treatments are clear, and a better understanding of the pathogenesis of SMA is needed to optimize the treatment landscape and improve patient outcomes.

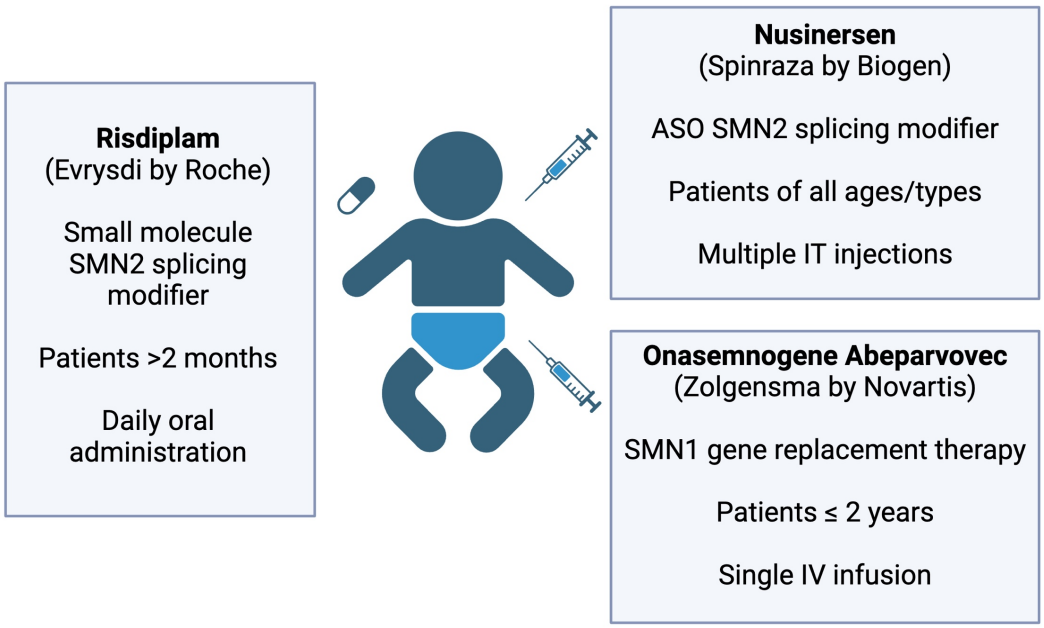


Figure 1.3 Summary of the FDA and EMA approved SMN-targeted therapies for SMA. Created with BioRender.com.

Biomarkers to monitor treatment response

As the therapeutic landscape continues to expand for SMA, there is a greater need for objective and reliable ways to monitor disease progression and treatment efficacy. In clinical trials, treatments are mainly evaluated by symptoms-based standardized scales such as the Children's Hospital of Philadelphia Test of Neuromuscular Disorders (CHOP-INTEND) and the Hammersmith Functional Motor Scale (HFMSE) tests or basic motor milestones such as sitting and standing. Outcomes vary from trial to trial and studies are conducted at various sites by different evaluators, making the results highly subjective and preventing comparisons between treatments or trials.

Electrophysiology and imaging

Electrophysiology and imaging techniques play an important role in the assessment of SMA. The CMAP technique measures the electrical output of a motor unit and can allow for the calculation of a motor unit number estimate (MUNE). CMAP has been shown to correlate with CHOP-INTEND scores in SMA patients and has improved with treatment in Type 1 SMA patients treated with onasemnogene abeparvovec (157–159). In a nusinersen trial, pre-treatment CMAP amplitude was predictive for how well patients would respond to treatment, and a higher change in CMAP correlated with a better response to treatment (160). However, in children with later onset SMA, CMAP and MUNE were generally unchanged over 3 years of nusinersen treatment (161). Muscle volume and physiology can also be monitored by quantitative magnetic resonance imaging (qMRI) and ultrasonography. While these techniques serve as important

diagnostic tools and can detect differences between SMA patients and healthy controls, they have not been effective at monitoring disease progression over time (162,163). Further, all these techniques can be invasive, tedious, and cumbersome to the patient. Researchers are therefore seeking to identify sensitive and accurate molecular biomarkers for the objective assessment of treatment response.

SMN protein and transcripts

SMN protein and *SMN2* transcript levels in blood have been explored as potential molecular biomarkers for SMA. However, results are mixed on whether SMN protein and *SMN2* transcript levels are actually lower in patients with SMA, and expression levels overlap greatly between disease types, making them unsuitable as markers of clinical severity (6,164,165). However, one study has correlated SMN protein levels in blood with denervation based on CMAP values (166). Further, a clinical trial of Nusinersen reported that cerebrospinal fluid SMN protein levels correlated with an increase in motor function (167). SMN protein in serum-derived extracellular vesicles (EVs) has also been explored as a potential biomarker. Elevations of SMN protein levels in isolated exosomes correlated with genotype in mice, and correlated with disease subtype in SMA patients (168). In a nusinersen trial, SMN transcripts in EVs increased after 14 months of treatment, and increased to a higher degree in younger patients, but did not show a statistically significant correlation with motor function scales (169). Overall, SMN protein and transcripts hold potential as a biomarker of treatment response, but the inconsistencies in study findings warrant caution in their use as biomarkers.

Neurofilament Protein

Given that the hallmark of SMA disease is the degeneration of motor neurons, there is a growing interest in utilizing neuronal proteins as biomarkers of treatment response. Neurofilament (NF) is a cytoskeletal protein abundant in the axons of neurons. It is released from axons and elevated in the blood and CSF following injury (170,171). NF elevations have been reported in several neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) (172–174), Multiple Sclerosis (MS) (175,176), and Parkinson's disease (177,178).

Neurofilaments, specifically phosphorylated neurofilament heavy chain (pNF-H) and neurofilament light chain (NfL), are the most frequently utilized experimental biomarkers in clinical trials for SMA. In early nusinersen trials, plasma pNF-H was found to be elevated 10-fold in infants with SMA compared to healthy controls, and a higher pNF-H was associated with lower age at symptom onset and lower baseline CHOP-INTEND score (179). Nusinersen treated infants also demonstrated a greater and more rapid decline in blood pNF-H than placebo-treated infants (180). In a study of presymptomatic infants treated with nusinersen, blood pNF-H concentration was higher in infants predicted to develop SMA than healthy controls, correlated with SMN2 copy number, and declined rapidly with treatment (181). These results are however difficult to interpret because the SMA patients were younger than the healthy controls in the study and blood pNF-H is known to be elevated in healthy infants and decline with age (179).

Interestingly, studies have found no differences in NF concentrations in blood or CSF of adults with SMA compared to healthy controls, nor any changes in NF levels with treatment with nusinersen (182–184). Our laboratory found similar results in a mild model of SMA, where no

elevations in NfL, or the neuronal proteins Tau and UCHL1, were observed at any time point over the course of disease progression (Appendix A).

While NF shows potential as a useful biomarker of treatment response for nusinersen, few studies have explored its usefulness in the context of other treatments. Surprisingly, two onasemnogene abeparvovec trials identified a spike in serum NfL levels of Type 1 and 2 SMA patients one month after treatment, despite evidence of reinnervation (185,186). However, this trial also demonstrated the utility of NF as a predicitive biomarker as the least responsive patient to the treatment had higher presymptomatic NF levels. Overall, there is a need for further research into molecular biomarkers for SMA and into the use of NF as a biomarker of treatment response in treatments other than nusinersen.

Non-neuronal aspects of SMA

SMA has been classically described as a motor neuron disorder, characterized by lower motor neuron degeneration and neuromuscular junction dysfunction leading to progressive muscle weakness. However, accounts from patients and observations of mouse models have suggested the independent vulnerability of several non-neuronal organs to the loss of SMN protein (summarized in Figure 1.3). The development of novel SMN-enhancing treatments has also allowed for further insights into the tissue specific impacts of SMN loss on animal models of the disease.

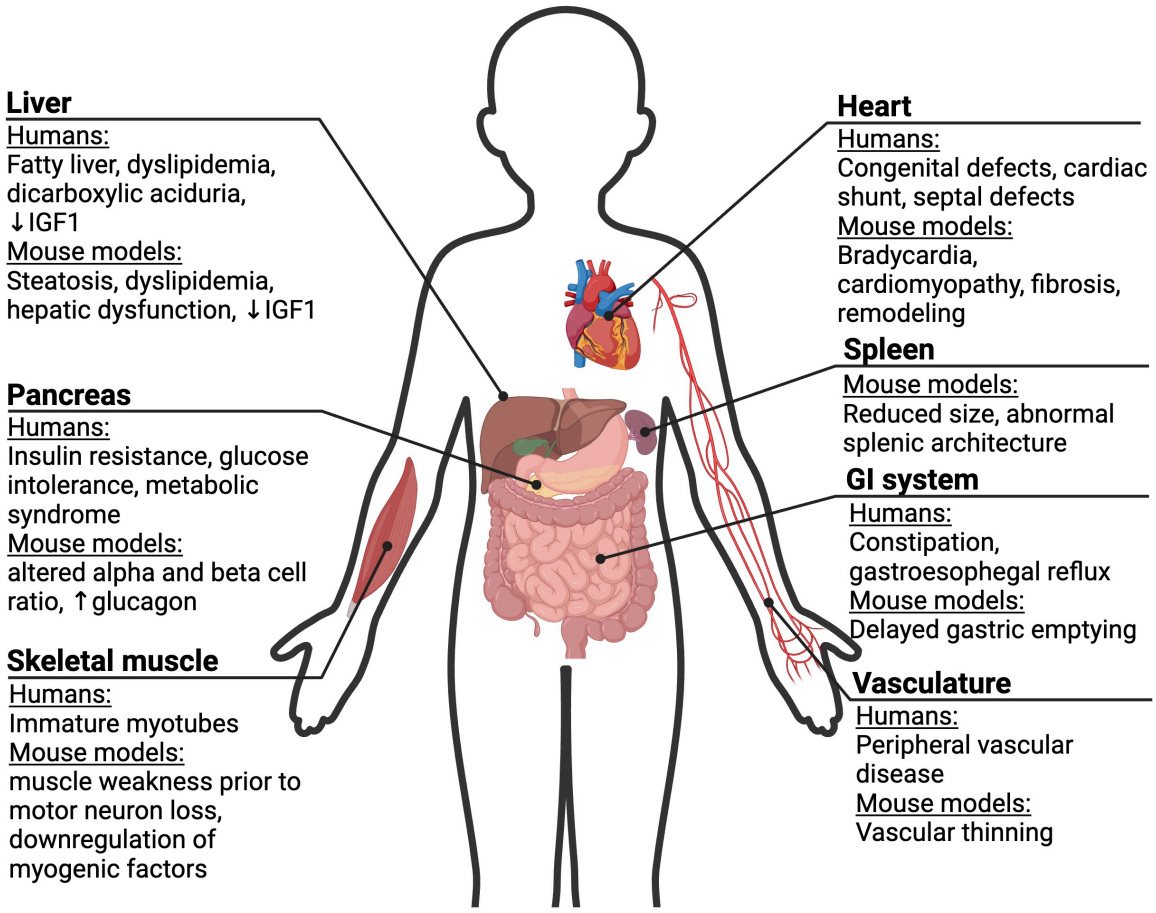


Figure 1.4 Observations of non-neuronal tissue defects in SMA patients and mouse models of SMA. Created with BioRender.com.

Skeletal Muscle

The earliest indications of non-neuronal intrinsic defects were described in skeletal muscle, which shows delayed development in SMA patients independent of denervation. Myotubes from SMA Type 1 fetuses are smaller and less mature, and show early defects in synaptic development (20,187). Further, histochemical and molecular analysis of muscle from

SMA Type 1-3 patients showed downregulations of myogenic factors and mitochondrial biogenesis (188). Cell and animal models have been used to further study the effect of SMN loss on muscle cells independently from degenerating motor neurons. An SMN-depleted myoblast cell line revealed decreased proliferation and dysfunction in myotube fusion, confirming cell-specific defects (189). In mouse models of SMA, muscle weakness is apparent prior to motor neuron loss (30). Molecular data also shows proteomic alterations and downregulation of myogenic factors in skeletal muscle prior to the onset of neuronal degeneration (31,190). Further, muscle-specific SMN knockdown models reveal a severe muscular dystrophy phenotype (191). Overall, skeletal muscle has an independent role in the pathogenesis of SMA. Of note, a muscle-specific SMN restoration is not sufficient to rescue a mouse model of SMA (192), but systemic treatment has been shown to provide a more holistic rescue than treatment directed to the CNS, (97,193) likely due to the contributions of the muscle and other peripheral tissues to SMA pathogenesis.

The Heart and Vasculature

Severe SMA patients present with congenital heart defects, including atrial and ventricular septal defects and hypoplastic left heart syndrome (194,195). Insurance claims from Type 1-4 SMA patients reveal higher instances of cardiac shunt, septal defects, and peripheral vascular disease (196). Though originally considered coincidental, abnormalities appear to increase with the severity of disease. One in 4 SMA patients with 1 copy of *SMN2* present with congenital heart defects, as compared to 1 in 50 million in the general population, suggesting that the SMN protein may play an important role in cardiac development (194). Not surprisingly, the Delta 7 mouse model of SMA displays similar developmental and functional defects, including bradycardia, cardiomyopathy, fibrosis, remodeling, and vascular thinning (197–200). One study

associated the bradycardia to a reduction in sympathetic innervation and nervous system dysfunction (200). Interestingly, the heart was only partially rescued upon treatment with gene-replacement therapy, suggesting that a lack of SMN during embryonic development results in potentially irreversible congenital defects (199).

SMA patients present with defects of the vasculature including capillary bed depletion, microvascular injury, peripheral vascular disease, and chronic venous insufficiency (201,202). Capillary dysfunction is also thought to be the cause of distal necrosis observed in the *Smn*$^{2B/-}$ mouse model and in rare cases of severe SMA (203).

Gastrointestinal system

Gastrointestinal symptoms are common among SMA patients and are also seen in several mouse models of the disease. This can include constipation, delayed gastric emptying and gastroesophageal reflux (72). Similarly to the cardiovascular system, results from mouse models have associated impaired enteric nervous system signalling with these symptoms (36). Interestingly, a CNS-specific restoration of SMN protein in the Delta 7 mouse model did not rescue gastrointestinal dysfunction, suggesting that these defects are the result of tissue specific or enteric nervous system specific mechanisms (36).

The Liver

Early patient studies showed indications of fatty acid metabolism defects including abnormal esterified carnitine and dicarboxylic aciduria (204,205). Macro and microvesicular steatosis have also been described in the liver of SMA patients (32,206). More recently, lipid profiling of 72 pediatric SMA patients revealed higher instances of dyslipidemia compared to the general population (32). Liver and fatty acid metabolism defects have also been observed in

mouse models of the disease. The *Smn*$^{2B/-}$ mouse model displays steatosis, dyslipidemia, elevated triglycerides and a phenotype resembling metabolic dysfunction–associated steatotic liver disease (MASLD, formerly non-alcoholic fatty liver disease or NAFLD). These mice show molecular markers of damage and fibrogenesis. Hepatic dysfunction is also apparent as measured by reductions in protein production, iron homeostasis and insulin-like growth factor (IGF1) metabolism (33). Similar dysfunction has been observed in SMA patients, seen as reduced serum IGF1 and overall reduced serum proteins (207,208). Mouse models revealed that liver pathology was likely not a result of denervation, as livers in denervated SOD1^{G93A} mice, a model of ALS, do not demonstrate an abnormal phenotype (32). Further, a liver-directed AAV9-mediated restoration of SMN rescues the fatty liver phenotype in *Smn*$^{2B/-}$ mice, suggesting that these defects are the result of liver-intrinsic mechanisms (209). The underlying molecular mechanisms are yet to be defined, but several indicators of hepatic mitochondrial dysfunction were identified by proteomic analysis and enzymatic assays, indicating that defects in the catabolism of fatty acids may contribute to accumulation of fat within the liver (33).

The Pancreas

Glucose metabolism defects are present in SMA patients and have been characterized in detail in mouse models. SMA patients report insulin resistance, glucose intolerance, and metabolic syndrome (34,210,211). Patients also show reduced lean mass and increased fat mass compared to the general population (212). The *Smn*$^{2B/-}$ model of SMA displays an imbalance in pancreatic islet cell composition, with a higher than usual proportion of glucagon-producing alpha cells to insulin-producing beta cells (34). These defects are reflected by elevated serum glucagon levels in the mice. Interestingly, after the discovery of these defects, similar findings were observed in pathological samples from SMA type 1 patients, including a disorganized

appearance of pancreatic islets and more alpha cells compared to control samples (34). These defects imply that the SMN protein may play a role in pancreatic development.

Immune Organs

Immune organ defects have been characterized in SMA patients and several mouse models of SMA. In three models, spleen size is markedly reduced and the splenic architecture is disturbed (35,213,214). Splenic abnormalities, such as increased white pulp, congested red pulp, and accessory spleens, were also present in a subset of Type 1 SMA patients (214). The thymus is also atrophied in SMA patients (215), while both $Smn^{2B/-}$ mice and severe SMA mice show decreased thymus size, cortex thinning and increased apoptotic bodies (35).

Rationale and Hypothesis

SMA has traditionally been characterized as a disease of muscle wasting as a result of motor neuron degeneration. However, SMN is a ubiquitously expressed protein that plays a role in several cell types. There has been much debate in the field on whether restoration of SMN to the motor neuron is sufficient to treat SMA. While several studies have demonstrated that a motor neuron specific SMN increase can improve the phenotype of a mouse model of SMA (216–218), the rescue of survival achieved by these models is limited compared to that achieved by systemic restoration of SMN. Further, selective depletion of SMN protein to the motor neuron results in a milder phenotype than systemic depletion (219). Moreover, increasing reports of peripheral organ-specific defects suggest that SMA may not be a disease restricted to the motor neuron.

Despite the evidence of peripheral tissue defects in SMA, CNS-specific treatments like nusinersen continue to be used in SMA patients and IT delivery of onasemnogene abeparvovec is

being explored in clinical trials. Current therapies show limited effects in a large portion of patients and peripheral organ defects may become more apparent as patients age. It is thus important to understand in detail the contributions of the peripheral organs to SMA pathogenesis to optimize the therapeutic landscape. We used the *Smn*$^{2B/-}$ mouse model of SMA to further investigate the involvement of the peripheral organs in SMA.

Hypothesis

SMA is a multi-system disorder, with peripheral tissue defects playing a critical role in disease pathogenesis alongside motor neuron degeneration

Aims

1. Characterize the onset of neuronal and peripheral phenotypes in *Smn*$^{2B/-}$ mice throughout development
2. Investigate the impact of CNS-directed and peripheral-directed AAV9-SMN treatment routes on developing *Smn*$^{2B/-}$ mice
3. Investigate the long-term impact of CNS-directed and peripheral-directed AAV9-SMN treatment routes on *Smn*$^{2B/-}$ mice

Chapter 2: Peripheral defects precede neuromuscular pathology in the *Smn*$^{2B/-}$ mouse model of spinal muscular atrophy

Peripheral defects precede neuromuscular pathology in the *Smn*$^{2B/-}$ mouse model of spinal muscular atrophy

Aoife Reilly[a,b,c], Ariane Beauvais[a], Majd Al-Aarg[a,e], Rebecca Yaworski[a,b,c], Emma Sutton[a,c], Simon Thebault[f], Rashmi Kothary[a,b,c,d,*]

[a] Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Canada

[b] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Canada

[c] Centre for Neuromuscular Disease, University of Ottawa, Ottawa, Canada

[d] Department of Medicine, University of Ottawa, Ottawa, Canada

[e] Faculty of Science, University of Ottawa, Ottawa, Canada

[f] Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA

Published in: Journal of Neuromuscular Disease, 11, 6, 510-519. |

DOI:10.1177/22143602241288036

Author contributions

AR and RK designed the research. AR performed experiments for all figures. AB contributed to Figures 2.2 and 2.6, MAA contributed to Figures 2.2 and 2.3, ST contributed to Figure 2.2C, ES contributed to Figure 2.4, and RY contributed to Figure 2.3. BLS provided material support. AR analyzed the data. AR wrote the manuscript with input from all authors.

Abstract

Background: Spinal Muscular Atrophy (SMA) is an inherited neurodegenerative disease caused by the loss or mutation of the survival motor neuron 1 *(SMN1*) gene. Though classically regarded as a motor neuron disorder, reports are increasingly describing the involvement of non-neuronal organs in SMA. The $Smn^{2B/-}$ mouse is a model of SMA that displays a peripheral phenotype including metabolic defects.

Objective: Here, we characterized several neuronal and non-neuronal defects in the $Smn^{2B/-}$ mouse throughout development to better understand the progression of the disease and the relationship between tissue defects.

Methods: We collected tissues from mutant $Smn^{2B/-}$ mice and $Smn^{2B/+}$ littermate controls at several timepoints and evaluated spinal cord motor neuron loss, neuromuscular junction pathology, muscle fiber size, liver steatosis, and pancreatic islet cell composition. Blood glucose and plasma neurofilament light chain (NfL) were also measured.

Results: $Smn^{2B/-}$ mice displayed several peripheral defects prior to motor neuron loss and showed early elevations in neurofilament light chain (NfL) protein.

Conclusions: This work provides an important framework for guiding future research with this mouse model and demonstrates that the liver may be an early target in the development of SMA.

KEYWORDS (4-10): metabolism, muscles, motor neurons, neuromuscular junction, liver, pancreas, animal models, neurofilament protein, biomarkers, blood glucose

Introduction

Spinal Muscular Atrophy (SMA) is a deadly childhood neurodegenerative disorder characterized by the wasting of skeletal muscle due to lower motor neuron death. The worldwide prevalence of SMA is estimated to be 1-2 in 100,000 individuals, with an incidence of about 1 in 12,000 live births (220,221). This inherited disease is caused by a homozygous deletion or mutation of the survival of motor neuron 1 (*SMN1)* gene. *SMN1* produces the survival motor neuron (SMN) protein, which is required for motor neuron development and maintenance (222,223). The closely similar *SMN2* gene produces only a small amount of full-length SMN protein and is unable to fully compensate for the loss of *SMN1* (224). *SMN2* gene copy number varies between individuals, and can thus influence the severity of disease in patients with SMA (225).

While patients with severe SMA previously had a life expectancy of less than 2 years, they now have access to several life-prolonging treatments. Three drugs: nusinersen, risdiplam and onasemnogene abeparvovec, are currently authorized for use by the United States Food and Drug Administration (FDA) and European Medicine Agency (EMA), while several other therapies are in pre-clinical and clinical development. Interestingly, therapeutic strategies are more effective when delivered systemically instead of directly to the central nervous system, likely due to the contributions of peripheral organs to SMA disease (226–228).

SMA has been historically studied as a motor neuron disorder. However, SMN is a ubiquitously expressed protein and a growing body of evidence describes the effects of SMN depletion in non-neuronal tissues. Patients with SMA report higher instances of cardiovascular, gastrointestinal, metabolic, reproductive, and skeletal defects than healthy peers, which occur prior to the first signs of neuromuscular disease (196). Further, mouse models of SMA have been

used to characterize the involvement of non-neuronal organs in SMA. The *Smn*$^{2B/-}$ mouse has a genetically modified mutant *Smn* allele that produces only a small amount of SMN protein, similar to the human *SMN2* gene (38,229). This model represents a severe SMA phenotype, displaying motor neuron degeneration, muscle weakness, and neuromuscular junction pathology. These mice display motor function deficits starting at postnatal day 9 (P9), and generally live for a median of 25 days (229,230). Interestingly, several non-neuronal phenotypes have also been described in *Smn*$^{2B/-}$ mice including immune organ defects, gastrointestinal dysfunction, liver steatosis, and pancreatic pathology (32,34). While it is becoming clear that the depletion of SMN affects several systems within the body, the relationship between the affected systems and their impact on the overall development of disease in SMA is not well understood.

To better outline the relationship between affected tissues and their contributions to disease development, we characterized several neuronal and non-neuronal phenotypes in *Smn*$^{2B/-}$ mice throughout development. We show that several non-neuronal symptoms arise before motor neuron loss, and that liver pathology occurs early in disease progression.

Materials and Methods

Animals

Smn$^{2B/-}$ and *Smn*$^{2B/+}$ mice were generated by crossing *Smn*$^{+/-}$ and *Smn*$^{2B/2B}$ mice (both on the C57BL/6J genetic background). All mice were housed at the University of Ottawa Animal Facility and experimental protocols were approved by the Animal Care Committee of the University of Ottawa. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care, and the Animals for Research Act. One litter of mice was monitored daily for 60 days for survival, while additional litters were weighed and sacrificed for blood and tissue collection at P5, P7, P9, P11, P13, P15, P17, and P19. Four to five mice were used for each genotype at each time point. For plasma NfL concentration assays, blood was taken at P4, P7, P11, P15, and P19. Five to ten mice were used at each time point.

Tissue processing and staining

Spinal cords were collected and choline acetyltransferase (ChAT) immunostaining was performed as previously described to visualize motor neurons (37,230). For each animal, 10 sections were stained and 20 ventral horns were quantified by the counting of ChAT positive motor neurons. *Transversus abdominis* muscle was collected, stained with TRITC-conjugated bungarotoxin, and immunostained for neurofilament and synaptic vesicle protein to visualize neuromuscular junctions as before (230). Endplates were marked as either positive or negative for displaying neurofilament accumulation and/or denervation. Liver and *tibialis anterior* muscle were processed and stained with hematoxylin and eosin (H&E) as previously described (230). The average cross-sectional area of 100 muscle fibers was measured using ImageJ to quantify muscle fiber size. Pancreas was collected and immunostained for insulin and glucagon to

visualize alpha and beta cells of the pancreatic islets as before (230). Cells from at least 5 islets per animal were counted as either insulin or glucagon positive in order to establish an average ratio of alpha to beta cells. Antibodies are listed in Table 2.1.

Table 2.1. List of antibodies used for immunohistochemistry

Method	Antibody/ reagent	Species	Dilution	Company (Catalog #)
IHC – motor neurons	ChAT	Goat	1:100	Millipore (AB144P)
IHC – motor neurons	anti-goat Alexa Fluor 555	Donkey	1:200	Invitrogen (A21432)
IHC - NMJ	TRITC conjugated bungarotoxin	Mouse	1:1 000	Invitrogen (T1175)
IHC - NMJ	Neurofilament (NF-M)	Mouse	1:100	Developmental Studies Hybridoma Bank, (P12839)
IHC - NMJ	Synaptic vesicle glycoprotein 2A	Mouse	1:250	Developmental Studies Hybridoma Bank, (Q7L0J3)
IHC - NMJ	Anti-mouse Alexa Fluor 488	Goat	1:250	Invitrogen (A11001)
IHC - Pancreas	Insulin	Rabbit	1:50	Abcam (ab181547)
IHC - Pancreas	Glucagon	Mouse	1:200	Abcam (ab10988)
IHC - Pancreas	Anti-rabbit Alexa Fluor 488	Goat	1:500	Invitrogen (A11034)
IHC - Pancreas	Anti-mouse Alexa Fluor 555	Goat	1:500	Invitrogen (A28180)

Blood collection and plasma analysis

Blood samples were collected upon euthanasia. Plasma was extracted and NfL protein concentration was determined using the Simoa NF-Light® assay (Quanterix, Billerica, MA) on a Simoa HD-1 analyzer as previously described (231). Blood glucose was measured as before using a Freestyle Precision Neo meter (Abbott, Lake County, IL) (230).

Statistical analysis

Data are presented as the mean ± standard error of the mean. Unpaired t-tests were used to compare the means of each genotype at each time point. Samples sizes ranged between 4-5 mice. Significance was indicated by * for $P \leq 0.05$, ** for $P \leq 0.01$, and *** for $P \leq 0.001$.

Results

$Smn^{2B/-}$ mice survive 22 days and show decreased weight gain starting at P11

To characterize the progression of disease in the $Smn^{2B/-}$ mouse, we first performed a survival analysis and measured weight over time in comparison to $Smn^{2B/+}$ littermates. $Smn^{2B/-}$ mice showed a median survival of 22 days while all $Smn^{2B/+}$ mice survived the 60-day monitoring period (Figure 2.1B). The first evidence of the diseased phenotype arises at P11, when $Smn^{2B/-}$ mice are ~~s~~maller in weight than $Smn^{2B/+}$ controls. Following this, $Smn^{2B/-}$ mice begin to lose weight and $Smn^{2B/+}$ continue to gain weight (Figure 2.1C). As this timepoint is the first evidence of the SMA-like phenotype in the $Smn^{2B/-}$ model, subsequent characteristics were evaluated beginning at P11.

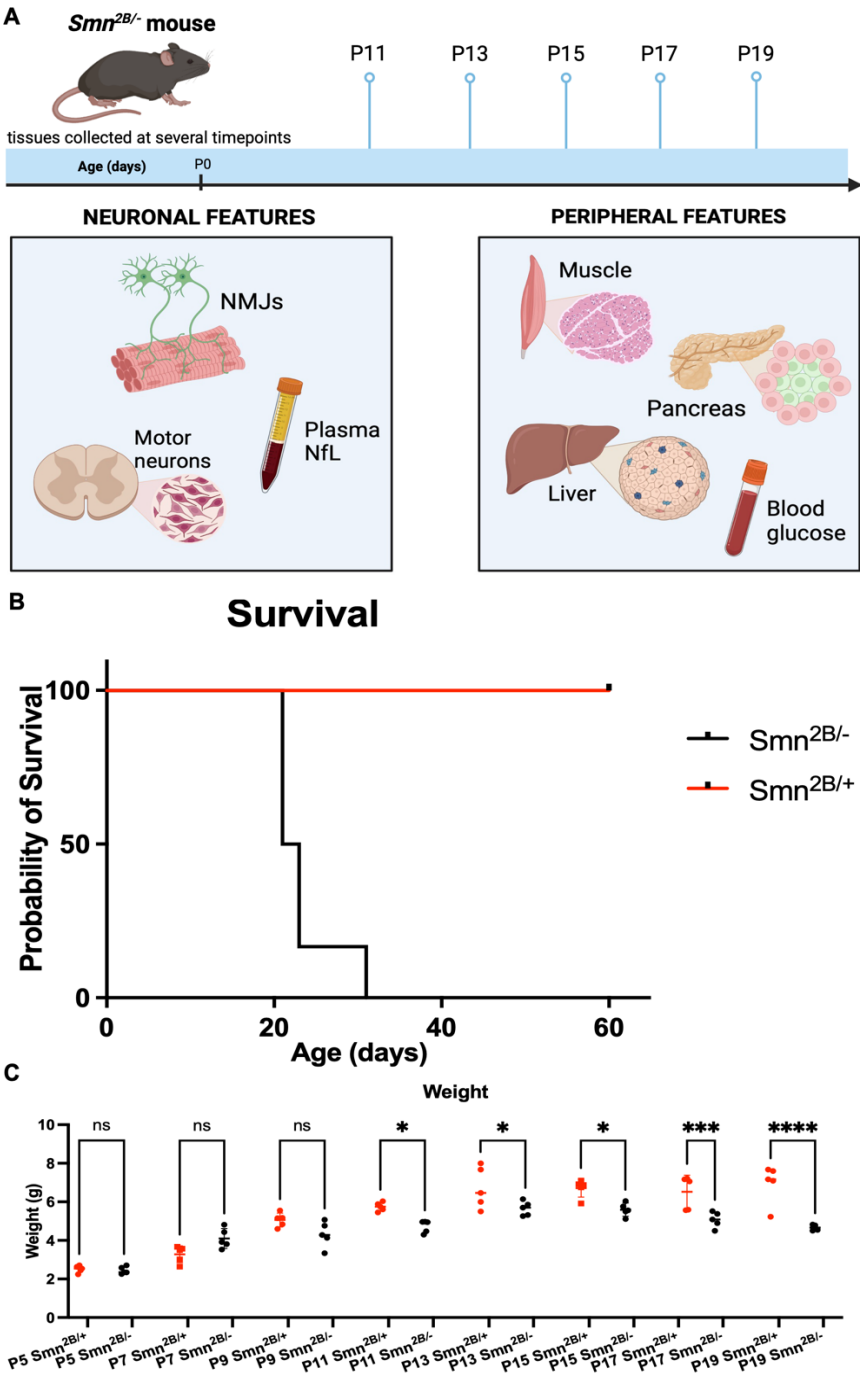


Figure 2.1. Weight and survival of *Smn*$^{2B/-}$ mice throughout development. (A) Schematic representation of experimental design for temporal evaluation of SMA-like characteristics in *Smn*$^{2B/-}$ mice. Created with BioRender.com. (B) Kaplan-Meier survival curve comparing *Smn*$^{2B/-}$

and *Smn*$^{2B/+}$ mice up to 60 days. (C) Comparison of weights between *Smn*$^{2B/-}$ and *Smn*$^{2B/+}$ mice from P5 to P19.

Motor neuron degeneration begins at P19 in Smn$^{2B/-}$ *mice and NfL is elevated at P11*

We next measured the degree of motor neuron degeneration, a hallmark feature of SMA, in *Smn*$^{2B/-}$ mice throughout development. Motor neuron number was determined by ChAT immunostaining of motor neuron cell bodies within the anterior horn of the lumbar spinal cord. Motor neuron cell body number was lower in *Smn*$^{2B/-}$ than *Smn*$^{2B/+}$ mice beginning at P19 (Figure 2.2A,B). We also measured plasma NfL, a neuronal protein that shows potential as a biomarker of axonal damage and disease progression in SMA (185,232). Plasma NfL was first elevated in *Smn*$^{2B/-}$ mice at P11, several days earlier than the first appearance of motor neuron degeneration (Figure 2.2C).

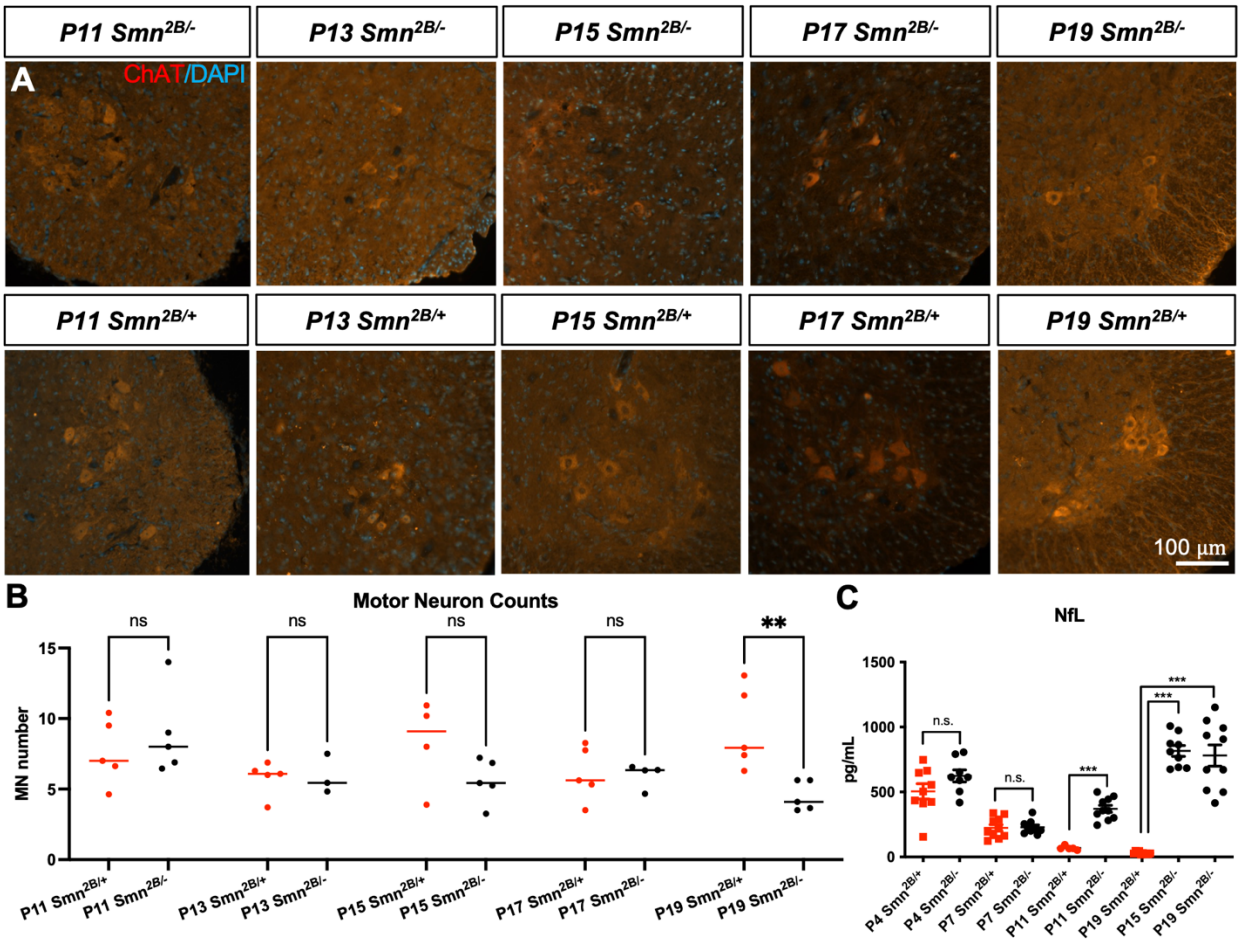


Figure 2.2. Temporal analysis of motor neuron degeneration in *Smn$^{2B/-}$* mice. (A) Representative immunofluorescent images of sections of lumbar spinal cord anterior horns from *Smn$^{2B/-}$* mice stained for ChAT (red) and DAPI (blue). (B) Quantification of motor neuron cell body numbers. (C) Plasma NfL levels. (n=4-10, mean ± SEM, individual t-tests at each time point, $p \leq 0.01$ for **, $p \leq 0.001$ for ***). Scale bar = 100 µm.

Presynaptic neurofilament protein accumulation is first apparent at P13 in $Smn^{2B/-}$ mice, and denervation is apparent at P17

We next assessed neuromuscular junction (NMJ) pathology to further investigate how motor neurons are affected throughout development in the $Smn^{2B/-}$ model. $Smn^{2B/-}$ mice show defects at the site of the NMJ, including altered endplate morphology, accumulation of neurofilament protein at the nerve terminal, and denervation of endplates (233). Upon investigation of the NMJs of the *transversus abdominis* muscle through neurofilament and synaptic vesicle protein immunostaining, presynaptic neurofilament protein accumulation was first apparent at P13, while denervation of endplates was first apparent at P17 (Figure 2.3). NMJs are therefore affected prior to the loss of motor neuron cell bodies in the spinal cord.

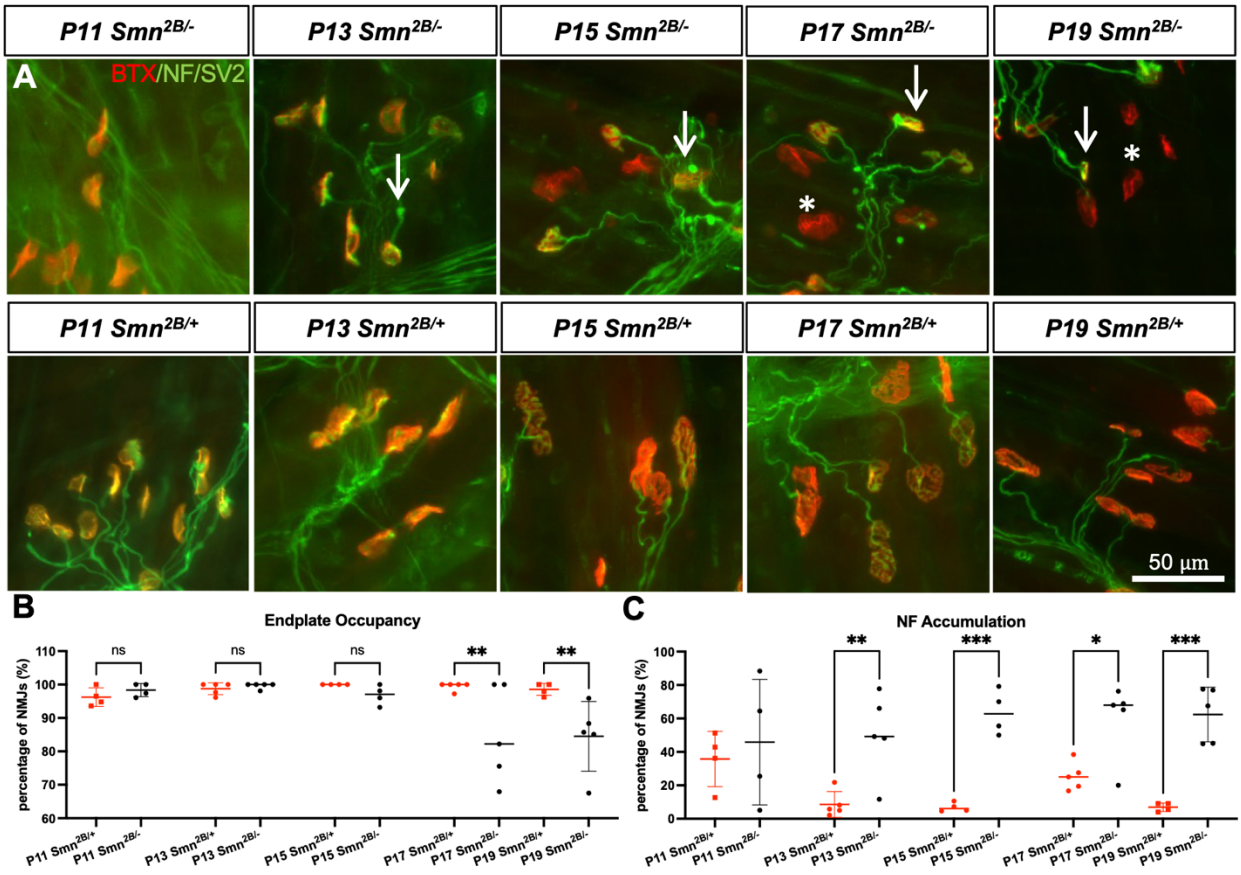


Figure 2.3. Temporal analysis of neuromuscular junction pathology in *Smn$^{2B/-}$* mice. (A) Representative immunofluorescent images of *transversus abdominis* (TVA) muscle from *Smn$^{2B/-}$* mice stained with bungarotoxin (red), and for neurofilament (NF) (green) and synaptic vesicle protein 2 (green). Quantification of endplate occupancy (B) and neurofilament accumulation (C) in stained NMJs (asterisks depict unoccupied endplates; arrows indicate NF accumulations; n=4-5, mean ± SEM, individual t-tests at each time point, $p \leq 0.05$ for *, $p \leq 0.01$ for **, $p \leq 0.001$ for ***). Scale bar = 50 µm.

Cross-sectional muscle fiber area is reduced in tibialis anterior muscle from Smn2B/- mice starting at P17

To determine when muscle atrophy is first occurring in *Smn*$^{2B/-}$ mice, we measured the cross-sectional area of H&E-stained muscle fibers from the *tibialis anterior* muscle. Muscle atrophy is another hallmark characteristic of SMA, likely caused by the degeneration of motor neurons as well as intrinsic mechanisms affecting muscle development (31,189,234). Muscle fiber size was first reduced in *Smn*$^{2B/-}$ mice compared to *Smn*$^{2B/+}$ mice starting at P17 (Figure 2.4). Muscle atrophy thus coincides with denervation at P17, following neurofilament accumulation at the NMJ at P13 and preceding motor neuron degeneration in the spinal cord at P19.

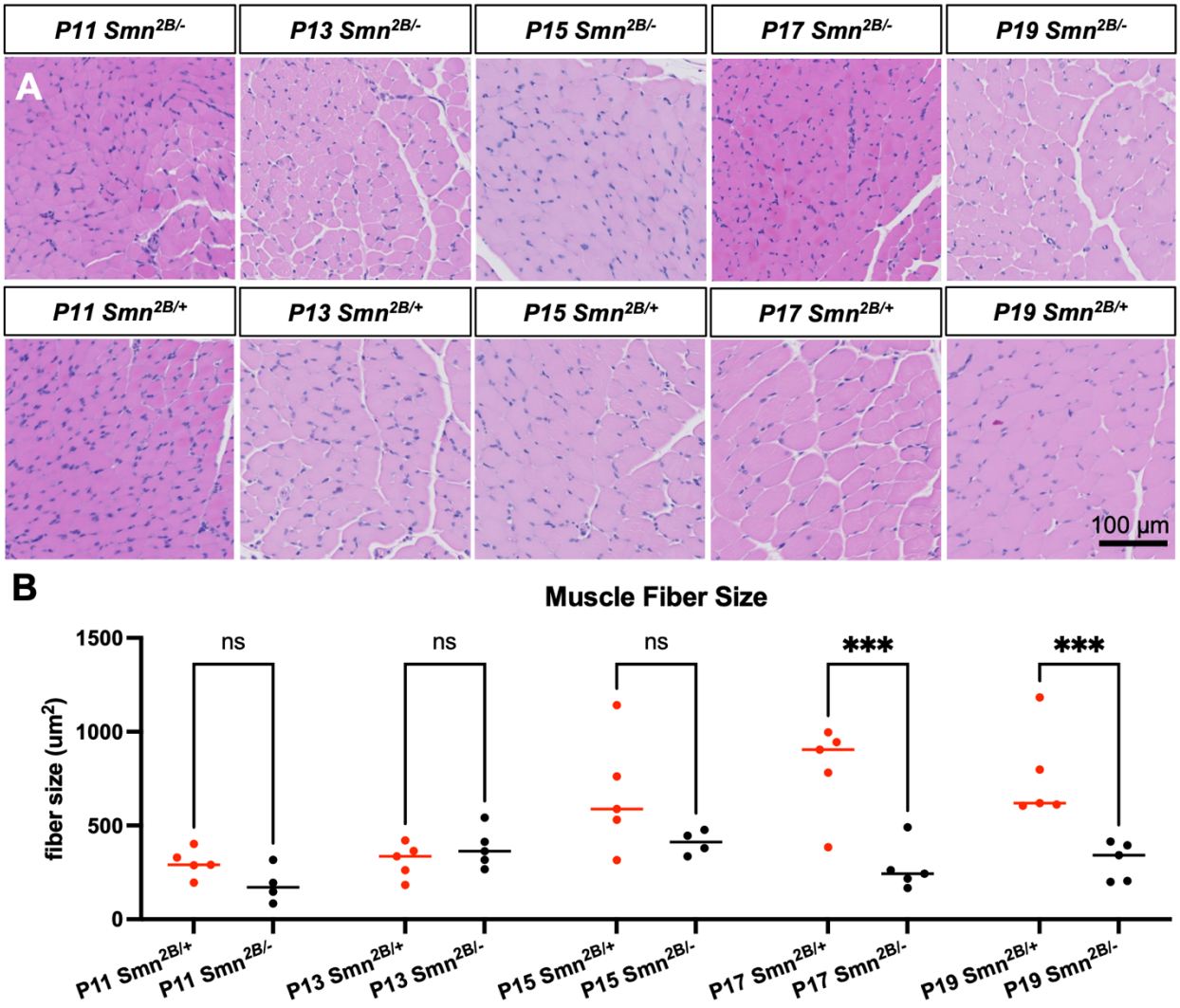


Figure 2.4. Temporal analysis of muscle fiber size in *Smn*$^{2B/-}$ mice. (A) Representative images of H&E stained *tibialis anterior* sections from *Smn*$^{2B/-}$ mice. (B) Quantification of muscle fiber size (n=4-5, mean ± SEM, individual t-tests at each time point, $p \leq 0.001$ for ***). Scale bar = 100 µm.

Liver steatosis is first apparent at P13 in $Smn^{2B/-}$ mice

In addition to neuromuscular pathology, $Smn^{2B/-}$ mice display an extensive number of metabolic abnormalities including liver steatosis and dysfunction (32,33). Steatosis was qualitatively observed using H&E-stained liver cross sections to determine the onset of the development of liver pathology in $Smn^{2B/-}$ mice. Fat was first observed within hepatocytes of $Smn^{2B/-}$ mice starting at P13 (Figure 2.5). The liver is therefore affected early in the $Smn^{2B/-}$ model, prior to neuromuscular pathology.

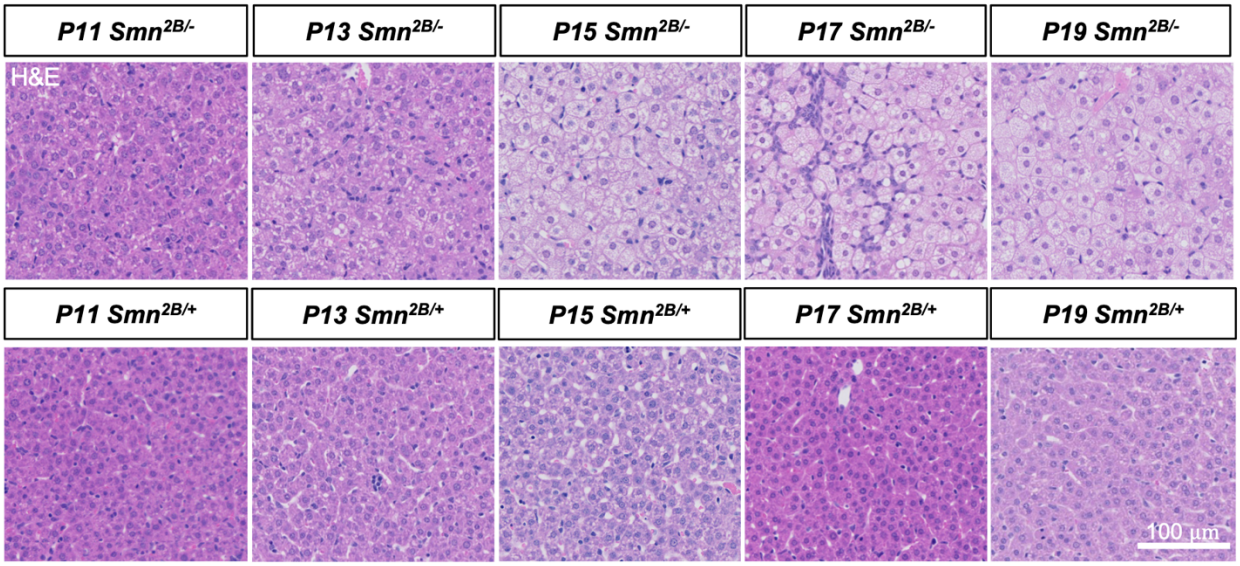


Figure 2.5. Temporal analysis of liver steatosis in $Smn^{2B/-}$ mice. Representative images of H&E stained liver sections from $Smn^{2B/-}$ mice. Scale bar = 100 µm.

Ratio of alpha to beta cells in pancreatic islets is disrupted beginning at P19

Smn$^{2B/-}$ mice display glucose metabolism defects including hyperglucagonemia, glucose resistance, and an increased ratio of glucagon-producing alpha cells to insulin-producing beta cells in the pancreas (34). To determine the onset of pancreatic pathology during development, pancreata were immunostained for insulin and glucagon and the ratio of alpha to beta cells within the pancreatic islets was measured. The ratio of alpha to beta cells was first disrupted in *Smn*$^{2B/-}$ mice at P19 (Figure 2.6). Blood glucose was also measured as another representation of glucose metabolism defects in the *Smn*$^{2B/-}$ model. Blood glucose was decreased in *Smn*$^{2B/-}$ mice compared to *Smn*$^{2B/+}$ mice starting at P15 (Figure 2.7). Glucose metabolism defects thus likely precede pancreatic pathology, as does liver steatosis.

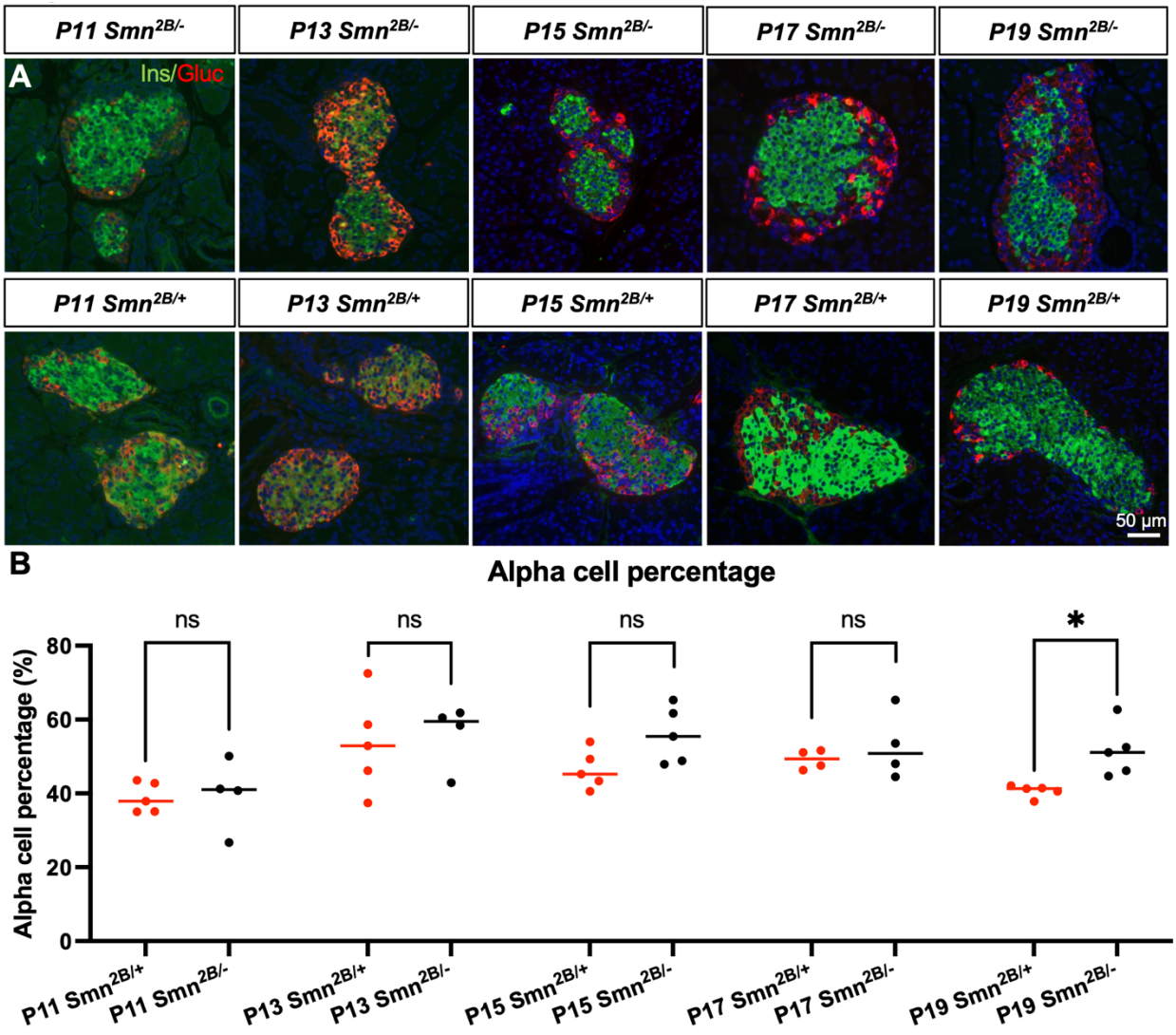


Figure 2.6. Temporal analysis of pancreatic pathology in *Smn*$^{2B/-}$ mice. (A) Representative immunofluorescent images of sections of pancreatic islets from *Smn*$^{2B/-}$ mice stained for glucagon (red) and insulin (green). (B) Fraction of glucagon-positive alpha cells related to total number of pancreatic islet cells (n=4-5, mean ± SEM, individual t-tests at each time point, $p \leq 0.05$ for *). Scale bar = 50 µm.

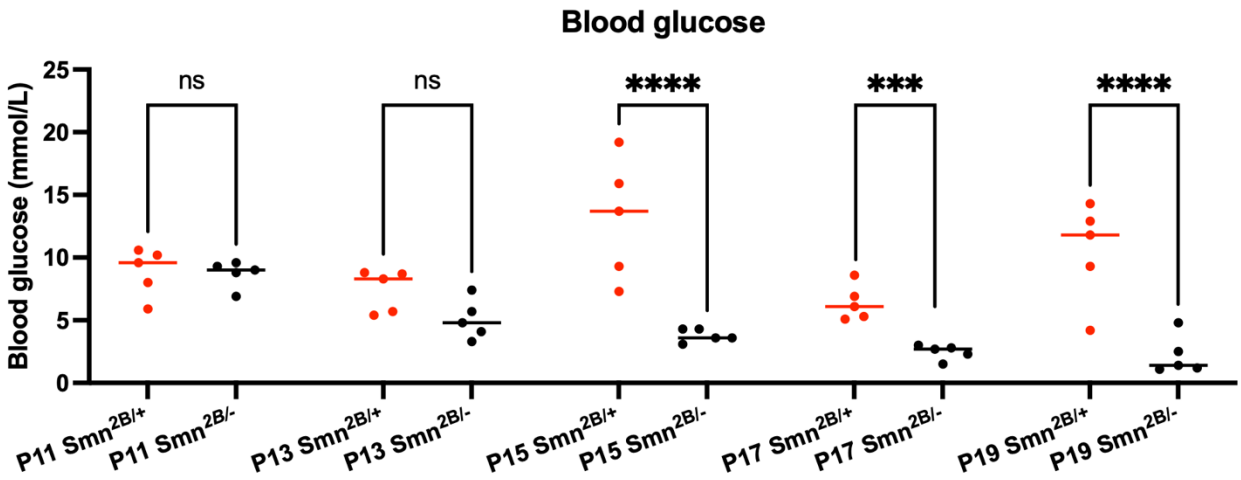


Figure 2.7. Temporal analysis of blood glucose in *Smn*$^{2B/-}$ mice. Quantification of blood glucose levels in *Smn*$^{2B/-}$ mice over time. (n=5, mean ± SEM, individual t-tests at each time point, $p \leq 0.001$ for ***, $p \leq 0.0001$ for ****).

Discussion

We have presented an in-depth temporal characterization of the *Smn*$^{2B/-}$ mouse model of SMA. While many of these characteristics have been previously described in *Smn*$^{2B/-}$ mice (29,32–34), this is the first study to identify the initial occurrence of each phenotype during development. Mice show slowed growth beginning at P11, coinciding with elevated NfL protein in plasma. They display liver steatosis and neurofilament accumulation at the endplates of neuromuscular junctions starting at P13. Muscle fiber size and blood glucose are decreased from P15 onwards. NMJ endplates are denervated starting at P17. Loss of motor neuron cell bodies in the spinal cord and a disruption of the ratio of alpha to beta cells in the pancreas are seen at P19. These mice survive to a median of 22 days (summarized in Figure 2.8).

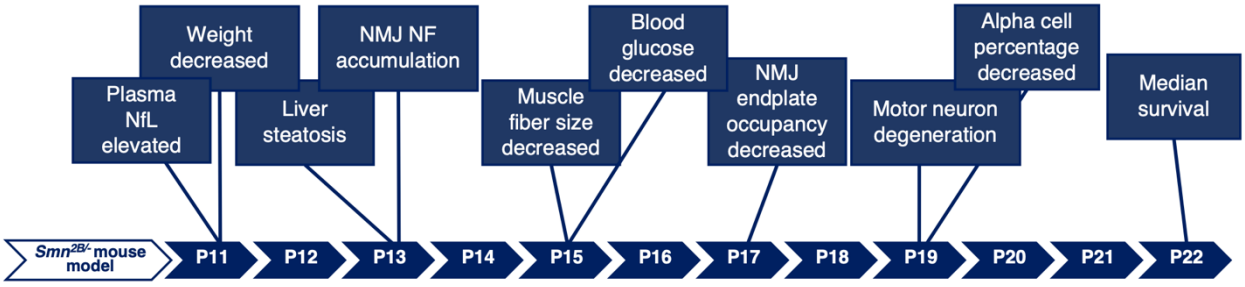


Figure 2.8. Graphical summary of the age of onset of SMA-like characteristics in *Smn*$^{2B/-}$ mice.

Smn$^{2B/-}$ mice display a non-alcoholic fatty liver disease (NAFLD) phenotype, consisting of steatosis, liver damage, liver dysfunction, and dyslipidemia at P19 (32,33). Here, we show the emergence of liver steatosis early in the development of the disease, at P13, several days prior to motor neuron degeneration. These results support findings that liver pathology occurs prior to neuromuscular symptoms, though a previous finding identified fat accumulation even earlier, at P9 (32). The mechanism of the liver phenotype is unclear, though its early emergence would suggest that it is the result of SMN-dependent molecular pathways within the liver. The pancreas was previously theorized to be implicated, due to the unusually high number of glucagon-producing alpha cells and high levels of blood glucagon (33,34). However, we showed that the alpha/beta cell ratio was altered late in *Smn*$^{2B/-}$ mice, several days after the emergence of the liver phenotype. In fact, the pancreatic cell ratio could be a result of metabolic stress, as beta cells are more prone to death than alpha cells when exposed to hyperlipidemia (235). This is supported by the impacts of a liver-specific depletion of SMN, which revealed pancreatic defects similar to those in *Smn*$^{2B/-}$ mice, emphasizing the liver's impact on the pancreas in this mouse model (de Almeida *et al,* unpublished data). Further, glucagon resistance and hyperglucagonemia are known to be associated with NAFLD (236). High glucagon could thus be a result of liver steatosis rather than a contributing factor. More insight into the liver-intrinsic nature of these defects was provided by a study describing a liver-directed restoration of SMN in *Smn*$^{2B/-}$ mice. An adeno-associated virus-mediated restoration of SMN restricted to the liver provided a rescue of the fatty liver phenotype and pancreatic defects (Sutton *et al.,* unpublished data). The liver-directed treatment also partially protected survival, motor function and NfL protein levels, emphasizing the influence of the liver on neuronal development through the regulation of neurotrophic factors like IGF1 (237).

We also showed that liver steatosis occurs prior to NMJ denervation and muscle atrophy in *Smn*$^{2B/-}$ mice. This agrees with previous studies, which found no steatosis in livers from mutant SOD1^{G93A} mice, a model of ALS with similar denervation and wasting of skeletal muscle (32). Steatosis is therefore likely not the result of denervation or reduced muscle use. In fact, hyperlipidemia associated with the fatty liver phenotype could affect muscle function and exacerbate muscle atrophy through lipotoxicity. This is supported by a previous study that identified lipid droplets on muscles from *Smn*$^{2B/-}$ mice (238).

Muscle atrophy begins before motor neuron loss in *Smn*$^{2B/-}$ mice, likely due to the involvement of muscle intrinsic pathways and NMJ defects. Intrinsic defects in muscle development have been observed previously in SMN depleted myoblast cultures, myoblasts from patients with SMA, and in muscle-specific SMN knockout mouse models (31,189,234). Muscle atrophy was also preceded by neurofilament accumulation at the NMJ, indicating that neurofilament accumulation could be an early contributor to muscle atrophy, preceding denervation and the loss of motor neuron cell bodies. However, the consequences of neurofilament accumulation at the nerve terminal are unclear. Accumulations are present in denervation resistant muscles from SMA mice, suggesting that it may not be the cause of denervation (239). Neurofilament accumulations could nevertheless affect the function of the NMJ or be representative of other issues with axonal transport within the neuron.

Neurofilament protein is also being explored as a potential biomarker for SMA. Neurofilament light chain (NfL) protein is elevated in plasma and CSF of pediatric SMA patients and is believed to act as a marker of neuronal degeneration (185,232). Here, we provide the first systematic characterization of plasma NfL throughout development in *Smn*$^{2B/-}$ mice. We observed progressive elevations of NfL that increased with the development of the neuromuscular

phenotype. However, the first NfL elevations were observed prior to any of the pathological findings, suggesting that NfL could also be useful as a prognostic biomarker. Interestingly, NfL was elevated several days prior to denervation and motor neuron degeneration. These elevations could be representative of axonal transport dysregulation and NMJ neurofilament accumulation rather than motor neuron degeneration. NfL levels could also be elevated as a reflection of other neuronal populations. Astrocytes, microglia, interneurons, and sensory neurons have all been shown to be affected in animal and cells models of SMA (240–243). Alternatively, the NfL assay may be a more sensitive method for measuring motor neuronal degeneration than immunohistochemistry.

The *Smn*$^{2B/-}$ model is a unique model of SMA that shows several peripheral phenotypes present in human SMA patients. While a distinctive metabolic phenotype is seen in all *Smn*$^{2B/-}$ mice, the human population is more heterogeneous in its presentation of non-neuronal SMA symptoms. A portion of SMA patients present with higher instances of dyslipidemia, elevated markers of fatty acid oxidation defects, and microvesicular steatosis of the liver (32,206). Some patients also present with insulin resistance, impaired glucose tolerance, and metabolic syndrome (34,212,244). We have displayed that the liver may be an early target of SMA, with the potential to impact several other systems affected in the disease. The liver performs an important role in neuronal development, metabolism, and detoxification that should not be overlooked in the care and treatment of SMA patients. A holistic treatment strategy will provide the greatest outcome for patients.

With the increasing use of therapeutics, SMA patients' lives are being extended and peripheral symptoms are becoming more apparent. It is crucial to understand the impact of individual tissues on the overall picture of the disease to treat patients as effectively as possible.

Our work has provided an in-depth systematic characterization of the *Smn*$^{2B/-}$ mouse phenotype to allow for a better understanding of the connections between the systems affected in SMA and to guide future research with this model.

Acknowledgements

The authors thank the University of Ottawa Histopathology Core and the Eastern Ontario Regional Laboratory Association (EORLA). We are grateful to the Kothary laboratory for helpful discussions.

Funding

This work was supported by Muscular Dystrophy Association (USA) [grant number 963652 to R.K.]; the Canadian Institutes of Health Research [grant number PJT-186300 to R.K.]; the Fredrick Banting and Charles Best Canadian Institutes of Health Research Doctoral Research Award to A.R.; and the University of Ottawa Brain and Mind Institute CNMD STAR Awards to A.R., R.Y., and E.S.

Conflict of interest statement

The authors have no conflict of interest to report.

Data availability

The data supporting the findings of this study are available on request from the corresponding author.

Chapter 3: Central and peripheral delivered AAV9-SMN are both efficient but target different pathomechanisms in a mouse model of spinal muscular atrophy

Central and peripheral delivered AAV9-SMN are both efficient but target different pathomechanisms in a mouse model of spinal muscular atrophy

Aoife Reilly[1,2,3], Marc-Olivier Deguise[1,2,3], Ariane Beauvais[1], Rebecca Yaworski[1], Simon Thebault[4,5], Daniel R. Tessier[6], Vincent Tabard-Cossa[6], Niko Hensel[1,3], Bernard L. Schneider[7,8], Rashmi Kothary[1,2,3,5,*]

[1]Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Canada

[2]Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Canada

[3]Centre for Neuromuscular Disease, University of Ottawa, Ottawa, Canada

[4]Division of Neurology, Department of Medicine, The Ottawa Hospital, Ottawa, Canada

[5]Department of Medicine, University of Ottawa, Ottawa, Canada

[6]Department of Physics, University of Ottawa, Ottawa, Canada

[7]Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

[8]Bertarelli Platform for Gene Therapy, Ecole Polytechnique Fédérale de Lausanne, Geneva, Switzerland

Published in: Gene Therapy, 29, 544-554 (2022) | DOI: 10.1038/s41434-022-00338-1

Author contributions

AR and RK designed the research. AB and MOD performed experiments for Figure 3.1F. RY assisted with ICV injections. ST, VTC, and DRT performed experiments for Figure 3.2F. AR performed experiments for all other figures. BLS provided material support. AR analyzed the data. AR, NH, and RK wrote the manuscript with input from all authors.

Abstract

Spinal muscular atrophy (SMA) is a neuromuscular disease caused by loss of the *SMN1* gene and low SMN protein levels. Although lower motor neurons are a primary target, there is evidence that peripheral organ defects contribute to SMA. Current SMA gene therapy and clinical trials use a single intravenous bolus of the blood-brain-barrier penetrant scAAV9-cba-SMN by either systemic or central nervous system (CNS) delivery, resulting in impressive amelioration of the clinical phenotype but not a complete cure. The impact of scAAV9-cba-SMN treatment regimens on the CNS as well as on specific peripheral organs is yet to be described in a comparative manner. Therefore, we injected SMA mice with scAAV9-cba-SMN either intravenously (IV) for peripheral SMN restoration or intracerebroventricularly (ICV) for CNS-focused SMN restoration. In our system, ICV injections increased SMN in peripheral organs and the CNS while IV administration increased SMN in peripheral tissues only, largely omitting the CNS. Both treatments rescued several peripheral phenotypes while only ICV injections were neuroprotective. Surprisingly, both delivery routes resulted in a robust rescue effect on survival, weight, and motor function, which in IV treated mice relied on peripheral SMN restoration but not on targeting the motor neurons. This demonstrates the independent contribution of peripheral organs to SMA pathology and suggests that treatments should not be restricted to motor neurons.

Introduction

Spinal muscular atrophy (SMA) is a devastating childhood neurodegenerative disorder characterized by the loss of lower motor neurons and skeletal muscle atrophy. Untreated and severely affected patients suffer from proximal and progressive muscle weakness, leading to complications such as scoliosis, respiratory failure, and early death (245). Recent advances in treatment development are bringing hope to SMA patients, but the benefits of therapy on the peripheral organs are not yet understood.

SMA is caused by a deletion or mutation in the *Survival of Motor Neuron 1* (*SMN1*) gene (222). The SMN protein product is essential and a complete SMN loss results in early embryonic lethality. Thus, SMA severity is mediated by a second SMN-producing gene, *SMN2,* which acts as a modifier of disease phenotype (224,246). Due to insufficient *SMN2* pre-mRNA splicing, this gene produces about 5-10% of the amount of full-length protein compared to *SMN1* (223). Humans possess a varying number of *SMN2* copies, thus copy number inversely correlates with disease severity and age of onset (247).

SMA has been classically regarded as a motor neuron disorder due to the preferential vulnerability of motor neurons to the loss of SMN protein. However, the SMN protein is ubiquitously expressed, and a wide range of non-neuronal tissues are affected in SMA patients and animal models. Muscle intrinsic defects (248–250), cardiac defects (251,252), fatty acid metabolism defects (253,254), glucose metabolism defects (255,256), immune organ defects (257–259) and gastrointestinal disfunction (260,261) have been observed in SMA patients and characterized in mouse models of the disease. In fact, the most effective experimental treatments in SMA pre-clinical models were delivered systemically, indicating that targeting the peripheral organs is necessary for a robust treatment effect (262–264).

There are currently three FDA-approved therapies available for SMA patients: nusinersen, risdiplam, and onasemnogene abeparvovec. While nusinersen and risdiplam act on the *SMN2* gene to correct pre-mRNA splicing and increase SMN protein production, onasemnogene abeparvovec uses a blood-brain-barrier penetrant adeno-associated virus serotype 9 (AAV9) vector carrying SMN cDNA to encode for the missing SMN protein. This gene therapy treatment is administered as a one-time intravenous (IV) injection, aiming to induce long-term expression of SMN protein in the periphery as well as the CNS (264,265). Moreover, there are current ongoing clinical trials using IT injections as a more CNS directed approach (266). However, the biodistribution of a systemic versus a CNS-directed scAAV9-cba-SMN treatment in CNS and peripheral organs as well as the subsequent impact on overall pathology is yet to be described in detail.

Therefore, we used the $Smn^{2B/-}$ mouse, a model that demonstrates peripheral abnormalities, to explore the impact of peripheral and CNS-directed treatment routes on the overall pathology. At P1, the $Smn^{2B/-}$ mice have no detectable pathology. At this stage, the mice are considered pre-symptomatic. We injected mice with scAAV9-cba-SMN employing either intravenous (IV) or intracerebroventricular (ICV) delivery to generate two different patterns of viral transduction. ICV application resulted in a modest SMN increase in the periphery and the spinal cord. IV scAAV9-cba-SMN delivery induced a strong SMN-expression in the periphery while it surprisingly omitted the spinal cord. Consistently, both treatments rescued several peripheral phenotypes while only ICV injections were neuroprotective. The lack of neuroprotection in IV treated mice became clearly apparent since (i) there was no amelioration of motor neuron numbers, (ii) no rescue of neurofilament light (NfL) chain plasma levels, and (iii) no increase in neuromuscular junction (NMJ) endplate occupancy compared to ICV-injected

animals. Surprisingly, both delivery routes resulted in a robust rescue effect on survival, weight, and motor function, which therefore in IV treated mice relies on peripheral SMN restoration and not on motor neurons. These results show that restoration of SMN to the peripheral organs can provide rescue to various features independently of motor neuron rescue and suggest that SMA therapies should not be restricted to the CNS.

Materials and Methods

Animals

Smn$^{2B/-}$ (C57BL/6J background) mice were developed by our laboratory (229,267) and housed at the University of Ottawa Animal Care Facility. *Smn*$^{2B/-}$ mice were produced by breeding *Smn*$^{+/-}$ mice (C57BL/6J) to *Smn*$^{2B/2B}$ mice (C57BL/6J). The *Smn*$^{2B/-}$ mouse is a model of SMA and asymptomatic heterozygous *Smn*$^{2B/+}$ mice are used as controls in these experiments. Animals were cared for according to the Canadian Council on Animal Care. All experiments were performed in accordance with ARRIVE guidelines and were approved by the University of Ottawa Animal Care Committee.

scAAV9-cba-SMN treatment

The self-complementary AAV9-cba-SMN vector was produced as previously described and was titred by real time qPCR (268). The scAAV9-cba-SMN expresses human SMN under the control of a chicken beta actin (cba) promoter and was produced at a titre of 9.62 x 10^{13} viral genomes (vg)/mL. Mice at post-natal day 1 (P1) were administered scAAV9-cba-SMN through facial vein injection (5x10^{10} vg per pup administered in a volume of 20 µL; four litters of 8-12 mice) or ICV injection (5x10^{10} vg per pup in a volume of 3 µL; three litters of 6-12 mice). Two litters from each treatment were monitored for motor function, weight, and survival and sacrificed at P60. Mice were tattooed by Animal Care Facility staff around P4-6 to allow for a specific animal's growth and motor function to be tracked over time. Mice were weighed every 2 days. Other litters were sacrificed at P19 for collection of blood and various tissues.

Motor function tests

Three motor function tests were performed. Righting reflex test (Treat-NMD SOP D_M.2.2.002, treat-nmd.org) evaluated overall body strength, pen test (Treat-NMD SOP SMA_M.2.1.001) assessed motor balance and coordination, and mesh grip test (Treat-NMD SOP SMA_M.2.1.002) evaluated limb strength. Motor function tests were performed as reported previously (269). Briefly, righting reflex test was performed from P7 to P19. In this test, the mouse is placed on its back on a flat surface and the time to right itself is measured (up to a maximum of 30 sec). One feature of the righting reflex test is that all mice, regardless of genotype, will improve their performance as they get older. In general, the righting reflex test is most useful in the first few days of life. The pen test involves placing the mouse on a pen and recording the length of time they can balance. This test was performed from P19 to P25. Mesh grip test was performed from P13 to P25. This test measures the strength of a mouse's limbs by timing their latency to fall when suspended from a mesh. Tests were performed every 2 days. Of note, tests were performed by two different evaluators using the lab's controlled and established protocol.

Blood collection and plasma analysis

Mice were euthanized at P19 by decapitation after anaesthesia in a CO_2 chamber. Upon decapitation, one blood sample was collected from each mouse using Microcuvette CB 300 K2E tubes (Sarstedt, Newton, NC) coated with K2 EDTA (*Smn*$^{2B/+}$ n=10; *Smn*$^{2B/-}$ n=7; *Smn*$^{2B/-}$ ICV n=5; *Smn*$^{2B/-}$ IV n=13). Samples were spun at 2,000 g using 5424R centrifuge (Eppendorf, Hamburg, Germany) for 5 min at room temperature. Forty-five µL of the plasma supernatant was then collected in a 1.5 mL Eppendorf tube and stored at -80°C. Samples were thawed on ice and stored at 4°C the day before the assay was to be performed. Samples were analyzed using the

Simoa NF-Light ® assay (Quanterix, Billerica, MA) on a Simoa HD-1 analyzer to determine the concentration of NfL protein. Blood glucose was measured upon blood collection using a Freestyle Precision Neo meter with Freestyle Precision Blood Glucose Test Strips (Abbott, Chicago, IL). About 1 µL of blood was extracted from the collection tube and applied to the test strip to measure blood glucose concentration (*Smn*$^{2B/+}$ n=6; *Smn*$^{2B/-}$ n=6; *Smn*$^{2B/-}$ ICV n=6; *Smn*$^{2B/-}$ IV n=8).

Tissue processing and staining

After euthanasia at P19, liver and *tibialis anterior* (TA) muscles (one per mouse) were fixed in 1:10 dilution buffered formalin (Thermo Fisher Scientific, Waltham, MA) for 48 h at 4ºC and then transferred to 70% ethanol at 4ºC until processing. Pancreata were fixed in 4% paraformaldehyde (PFA) for 48 h at 4ºC and then transferred to 70% ethanol at 4ºC until processing. Lumbar spinal cord was fixed in 4% PFA overnight at 4ºC then prepared for cryosectioning as previously described (269). The abdominal musculature was dissected and fixed in 4% PFA and the *transversus abdominis* (TVA) muscle was separated from the abdominal musculature as per (270).

TA, liver, and pancreas samples were processed at the University of Ottawa Department of Pathology and Laboratory Medicine and embedded in wax using a LOGOS microwave hybrid tissue processor (Milestone Medical, Kalamazoo, MI). Paraffin block tissues were cut with a microtome at 3-4 µm thickness. Sections of TA and liver were stained with hematoxylin and eosin (H&E) using an XL CV5030 autostainer (Leica, Wertzler, Germany). Samples stained with H&E were scanned with a MIRAX MIDI digital slide scanner (Zeiss, Oberkochen, Germany). Images were acquired using Panoramic Viewer 1.15.4 (3DHISTECH, Budapest, Hungary) at

different magnifications. Muscle fibre size was quantified using ImageJ (version 1.53). Approximately 100-200 fibres were counted in different areas of the muscle section to ensure appropriate coverage. The area of each fibre was measured to calculate the distribution of fibre sizes for each animal (*Smn*$^{2B/+}$ n=8; *Smn*$^{2B/-}$ n=8; *Smn*$^{2B/-}$ ICV n=6; *Smn*$^{2B/-}$ IV n=4). Sections of pancreas were deparaffinized in 3 washes of xylene substitute Histo-Clear (National Diagnostics, Atlanta, GA) for 5 min each followed by 2 washes of a 50/50 mixture of absolute ethanol and Histo-Clear for 5 min each. Slides were gradually rehydrated in 100% (v/v), 95% (v/v), 70% (v/v), 50% (v/v), and 0% (v/v) ethanol. Slides were incubated in 0.5% Triton-X-100 (Millipore Sigma, Burlington, MA) in PBS for 5 min, washed 3× with PBS, then blocked in 20% goat serum, 0.3% Triton-X-100 in TBS for 2 h. Slides were incubated with primary antibodies for insulin and glucagon **(Table 3.1)** in 2% goat serum, 0.3% Triton-X-100 in TBS overnight at 4ºC and then washed 3× with PBS. Slides were incubated with secondary antibodies **(Table 3.1)** in 2% goat serum, 0.3% Triton-X-100 for 1 h and then washed 3× with PBS. DAPI (1:1,000) was added to the last PBS wash, followed by the slides being mounted in Dako Fluorescent Mounting Medium (Agilent, Santa Clara, CA). Images were taken with an Axio Imager M2 microscope (Zeiss), with a 20x objective, equipped with filters suitable for DAPI/ fluorescence. The number of glucagon and insulin positive cells per islet were counted for each mouse (*Smn*$^{2B/+}$ n=6; *Smn*$^{2B/-}$ n=6; *Smn*$^{2B/-}$ ICV n=6; *Smn*$^{2B/-}$ IV n=7).

Lumbar spinal cord (SC) was prepared for choline acetyltransferase (ChAT) staining of motor neurons as previously described (269) (*Smn*$^{2B/+}$ n=4; *Smn*$^{2B/-}$ n=5; *Smn*$^{2B/-}$ ICV n=4; *Smn*$^{2B/-}$ IV n=3). The number of ChAT positive motor neurons per ventral horn was recorded for ten different sections per animal, each separated by at least 100 μm to avoid counting the same motor neuron twice. TVA muscles were dissected and stained for neurofilament and synaptic

vesicle protein to visualize NMJs as before with slight alterations (270) (*Smn*$^{2B/+}$ n=10; *Smn*$^{2B/-}$ n=10; *Smn*$^{2B/-}$ ICV n=4; *Smn*$^{2B/-}$ IV n=5). Briefly, the abdominal musculature was dissected and fixed in 4% PFA and the TVA was separated from the abdominal musculature under a dissection microscope. The TVA was incubated with a tetramethylrhodamine (TRITC) conjugated bungarotoxin for 30 min at room temperature. The tissue was then incubated overnight at 4°C with primary antibodies for neurofilament and synaptic vesicle protein 2 **(Table 3.1)**. Tissues were then incubated in secondary antibodies **(Table 3.1).** Tissues were mounted with Dako Fluorescent Mounting Medium (Agilent) and imaged using Axio Imager M2 microscope (Zeiss) with Z-stack feature at 40x magnification. At least 40 NMJs were counted for each animal. Each NMJ was quantified as either normal or displaying neurofilament accumulation, and endplates were noted as either occupied or unoccupied.

Table 3.1. List of antibodies used for immunofluorescence studies

Method	Antibody	Species	Dilution	Company (Catalog #)
IHC	ChAT	goat	1:100	Millipore (AB144P)
IHC	anti-goat Alexa Fluor 555	Donkey	1:200	Invitrogen (A21432)
IHC	TRITC conjugated bungarotoxin	Mouse	1:1 000	Invitrogen (T1175)
IHC	Neurofilament (NF-M)	Mouse	1:100	(Developmental Studies Hybridoma Bank, P12839)
IHC	synaptic vesicle glycoprotein 2A	Mouse	1:250	(Developmental Studies Hybridoma Bank, Q7L0J3)
IHC	Anti-mouse Alexa Fluor 488	Goat	1:250	Invitrogen (A11001)
Western Blot	SMN	Mouse	1:2 000	BD Transduction (610647)
Western Blot	Alpha-tubulin	Rabbit	1:10 000	Abcam (ab4074)
Western Blot	IRDye 680 anti-mouse IgG	Goat	1:10 000	Li-Cor (926-68070)
Western Blot	IRDye 800 anti-rabbit IgG	Goat	1:10 000	Li-Cor (926-32211)
Western Blot	Anti-mouse IgG HRP conjugate	Goat	1:3 000	Bio-Rad (1706516)
Western Blot	Anti-rabbit IgG HRP conjugate	Goat	1:3 000	Bio-Rad (1706515)

Western blot

Tissue processing and immunoblotting was performed as previously described with slight alterations (257). After euthanasia at P19, thoracic spinal cord, liver, and TA muscle were dissected, and flash frozen in Microvette CB 300 Z tubes (Sarstedt) in liquid nitrogen (IV blot: $Smn^{2B/+}$ n=3; $Smn^{2B/+}$ IV n=3; $Smn^{2B/-}$ n=3; $Smn^{2B/-}$ IV n=3. ICV blot: $Smn^{2B/+}$ n=3; $Smn^{2B/-}$ n=3;

Smn$^{2B/-}$ ICV n=6). Protein was extracted from frozen tissue by homogenization of tissue with RIPA lysis buffer and PMSF (Cell Signalling, Danvers, MA). Protein concentrations of samples were determined using Bradford Assay (Bio-Rad, Hercules, CA). Twenty mg of protein was loaded onto a 12% acrylamide gel and subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to a PVDF membrane (Immobilon-P or Immobilon-FL, Millipore, Burlington, MA) and blocked for 1 h at room temperature in 5% (w/v) milk powder in TBS-T or Odyssey blocking buffer (Li-Cor, Lincoln, NE). See **Table 3.1** for primary and secondary antibodies. Signals were detected with Odyssey CLx (Li-Cor) or using Pierce ECL Western Blotting Substrate (Thermo Scientific) and SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific). Densitometry of western blot bands was performed using ImageJ (v. 1.53). Raw values were normalized by geometric mean and used for subsequent housekeeping normalization (α-tubulin values) from the same blot.

Statistical analysis

Data are presented as the mean ± standard error of the mean. One-way ANOVA with Tukey's post-test or Two-way ANOVA with Bonferroni post-test were used to compare multiple means. Statistical tests were performed using GraphPad Prism 5. Significance was indicated by * for $P \leq 0.05$, ** for $P \leq 0.01$, and *** for $P \leq 0.001$. Images were blinded prior to quantification.

Results

Intracerebroventricular scAAV9-cba-SMN administration to $Smn^{2B/-}$ mice results in a mild increase in SMN in the CNS and the periphery while intravenous injection results in restoration of SMN in peripheral tissues only

Our objective was to directly compare the scAAV9-cba-SMN expression and the subsequent phenotypic rescue of SMN replacement focused on the periphery with a replacement strategy targeting the whole body. Therefore, we used a lower dose of a blood-brain-barrier penetrant scAAV9 viral vector either administered IV or ICV to $Smn^{2B/-}$ mice at P1. $Smn^{2B/-}$ have no detectable pathology at this time point and are considered pre-symptomatic. This virus expresses SMN under the control of a chicken beta actin promoter like the commercially available SMA treatment onasemnogene abeparvovec **(Figure 3.1A)**. To evaluate the tissue tropism of each application route, SMN protein levels in treated and untreated $Smn^{2B/+}$ and $Smn^{2B/-}$ mice were obtained by western blot to compare between the neuromuscular system compartment (spinal cord and muscle) and the liver (the major peripheral target of AAV9 in patients). Both application routes lead to increased SMN protein, demonstrating the functionality of the virus (**Figure 3.1B,F)**. However, there was a difference in the tissue specific increases between routes of injection. ICV treatment produced a substantial yet mild increase of SMN protein levels in liver and spinal cord, and a trend towards an increase in muscle **(Figure 3.1B-E)**, while IV treatment completely restored SMN protein to the muscle and liver but did not show a detectable change in the spinal cord **(Figure 3.1F-I)**. Not surprisingly, these results indicate that systemic scAAV9-cba-SMN administration more efficiently restores peripheral SMN levels compared to a CNS-directed approach. Importantly, systemic IV delivered scAAV9-cba-SMN application omitted spinal cord transduction at the titre used **(Figure 3.1I)**.

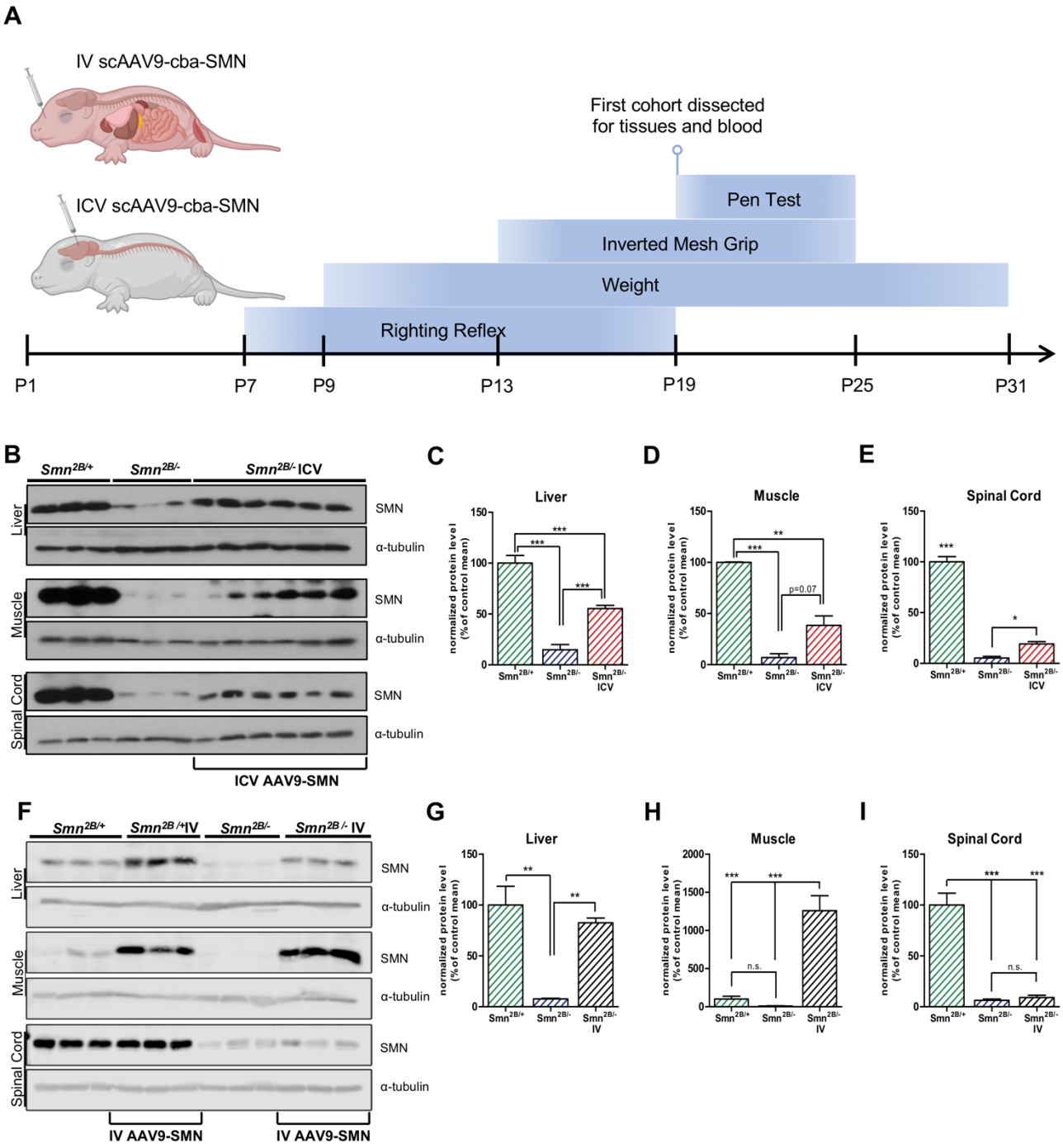


Figure 3.1. Peripheral and spinal cord SMN levels in response to ICV and IV scAAV9-cba-SMN injection. (A) Schematic representation of experimental design for AAV9-SMN treatment and evaluation of SMA-like pathophysiology. Created with BioRender.com. (B-E) Western blots of SMN protein levels in liver, skeletal muscle, and spinal cord following ICV injections in

Smn$^{2B/-}$ mice. (F-I) Western blots of SMN protein levels in liver, skeletal muscle, and spinal cord of IV treated *Smn*$^{2B/-}$ mice. Mice were injected at P1 with $5x10^{10}$ vg of scAAV9-cba-SMN and tissues were dissected at P19. (n=3-6, mean ±SEM, bar graphs represent one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$ for *, $p \leq 0.01$ for **, $p \leq 0.001$ for ***, and n.s.= non-significant).

CNS but not peripheral scAAV9-cba-SMN delivery partially rescues spinal cord motor neuron degeneration in Smn2B/- mice

Motor neuron degeneration and plasma NfL were investigated to further confirm the patterns of SMN restoration **(Figure 3.2)**. Consistent with the CNS transduction pattern, ICV treatment partially protected against motor neuron degeneration, while there was no difference in motor neuron number between IV treated *Smn*$^{2B/-}$ mice and untreated *Smn*$^{2B/-}$ mice **(Figure 3.2A-E)**. Additionally, we measured NfL concentrations in plasma. NfL is a common outcome measure of neurodegeneration in the CNS and a candidate biomarker of neurodegeneration, disease state and therapeutic efficacy in SMA (271). We have established plasma NfL as a marker of disease progression in *Smn*$^{2B/-}$ mice as NfL levels spike and stay elevated with the onset of motor neuron pathology in this model (unpublished). As expected, blood plasma NfL levels were elevated in *Smn*$^{2B/-}$ mice, reflecting motor neuron degeneration **(Figure 3.2F)**. Importantly, ICV treatment resulted in a reduced elevation in plasma NfL **(Figure 3.2F)**. In contrast, there was no protection afforded by IV treatment as evidenced by the relatively high plasma NfL levels. Again, these results are in line with a lack of SMN restoration in the spinal cord in IV treated mice.

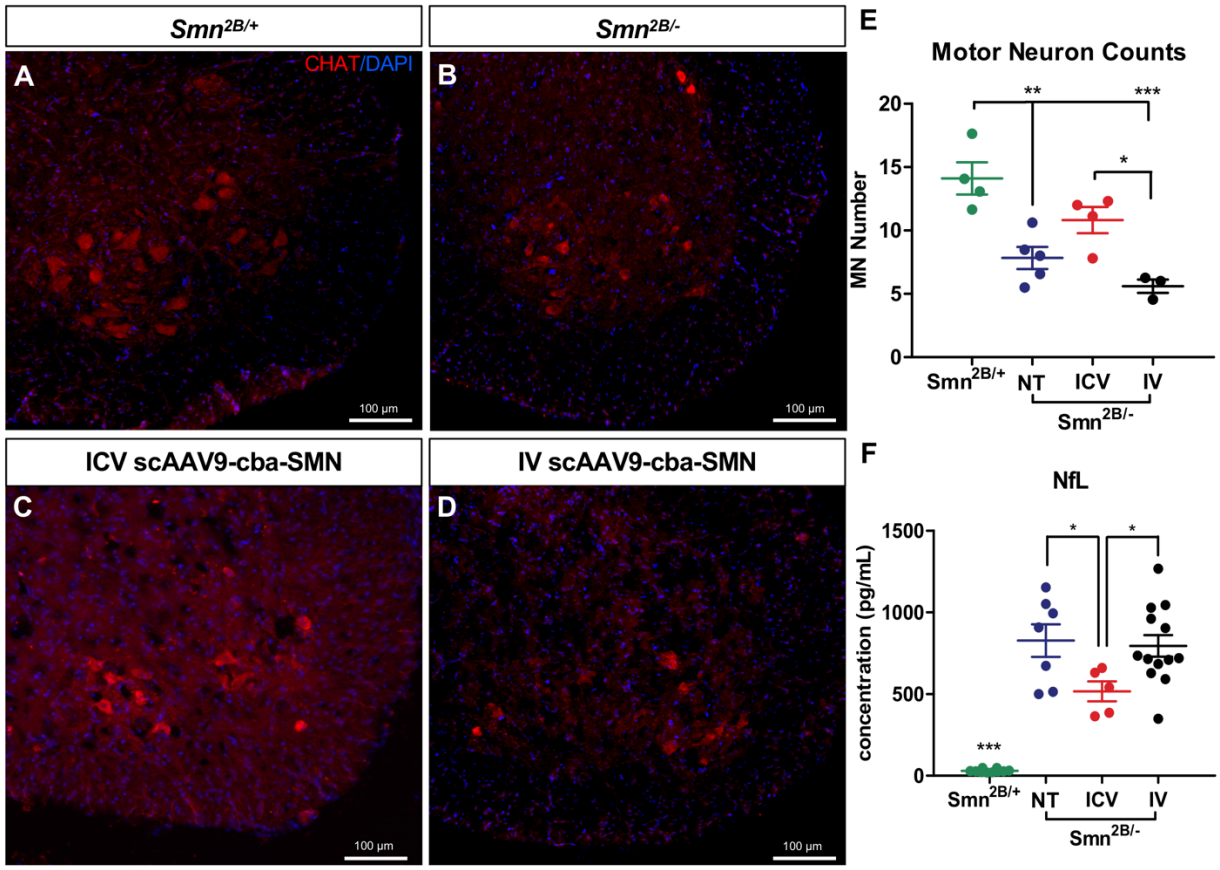


Figure 3.2. Impact of ICV and IV scAAV9-cba-SMN injection on motor neuron degeneration in *Smn*$^{2B/-}$ mice. (A-D) Representative immunofluorescent images of sections of lumbar spinal cord anterior horns stained for ChAT (red) and DAPI (blue) from P19 mice. (E) Quantification of motor neuron cell body numbers. (F) Plasma NfL levels were assessed using single molecule array (Simoa) technology (NT=no treatment, n=4-13, mean ±SEM, one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$ for *, $p \leq 0.01$ for **, $p \leq 0.001$ for ***).

CNS-directed scAAV9-cba-SMN injection in Smn2B/- mice rescues neuromuscular junction pathology better than IV treatment

Next, we analysed the impact of each treatment on muscle function. While there was no change in fibre size in the TA muscle by any of the scAAV9-cba-SMN injections **(Supplementary Figure 3.1)**, our in-depth analyses of the NMJ pathology revealed significant differences **(Figure 3.3)**. A variety of NMJ defects are present in *Smn*$^{2B/-}$ mice including abnormal endplate morphology, neurofilament accumulation, and denervation (272). Neurofilament accumulation and NMJ denervation were quantified to determine the degree of NMJ pathology after both routes of scAAV9-cba-SMN injection. When evaluating endplate occupancy, only ICV injection resulted in a partial rescue while there was no effect in IV injected animals **(Figure 3.3E-H, J)**, which is in line with an ICV mediated rescue of motor neuron numbers and no rescue in IV injected animals. However, both IV and ICV treatments partially rescued neurofilament accumulation in the TVA muscle of P19 *Smn*$^{2B/-}$ mice, yet with a higher efficacy in ICV-injected animals **(Figure 3.3A-D,I)**. This points towards a mechanism which is at least partially muscle-intrinsic since there is no rescue in motor neuron numbers but an increased muscle SMN expression in this group (**Figure 3.2 and Figure 3.1H)**.

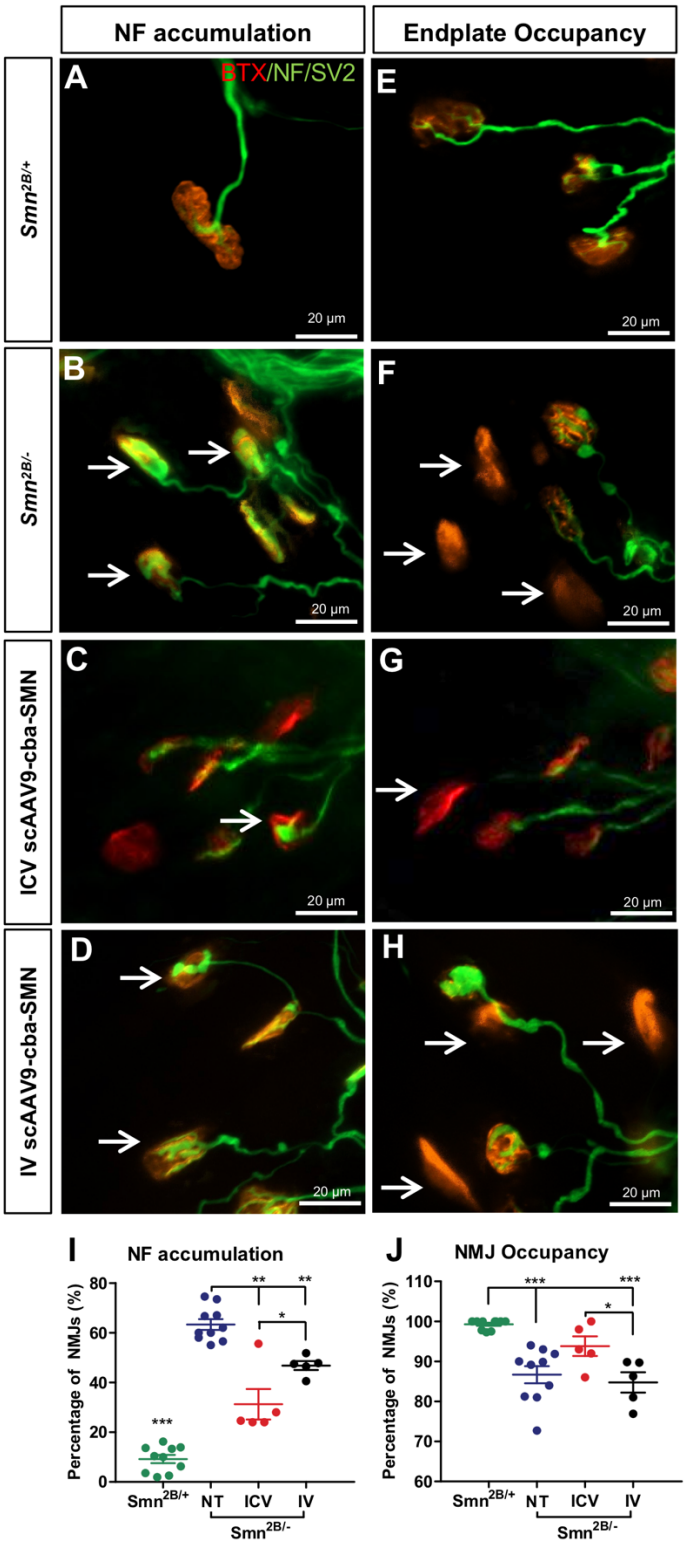
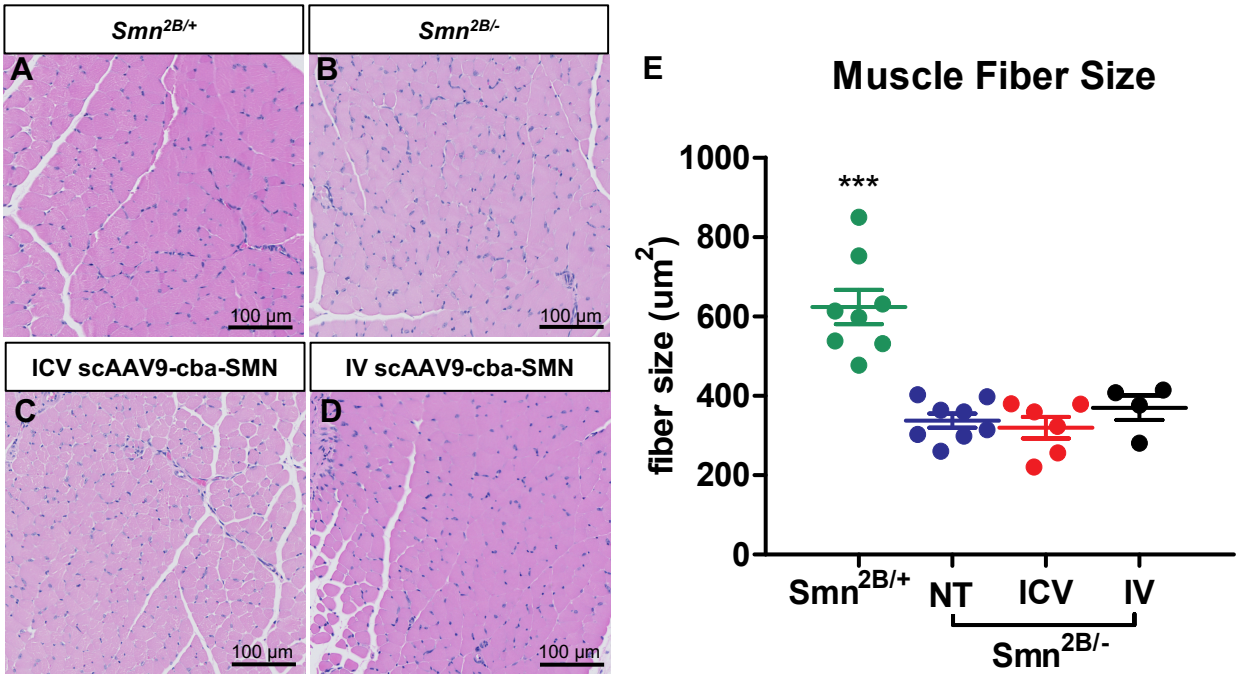


Figure 3.3. Impact of ICV and IV scAAV9-cba-SMN injection on neuromuscular junction pathology in *Smn$^{2B/-}$* mice.

(A-H) Representative immunofluorescent images of *transversus abdominis* (TVA) muscle stained with bungarotoxin (red), and for neurofilament (NF) (green) and synaptic vesicle protein 2 (green) from P19 mice. Quantification of NF accumulation (I) and endplate occupancy (J) in stained NMJs to compare *Smn*$^{2B/-}$ mice, *Smn*$^{2B/+}$ mice, ICV treated *Smn*$^{2B/-}$ mice, and IV treated *Smn*$^{2B/-}$ mice. (NT=no treatment, n=5-10, B-D: arrow shows NF accumulation; F-H: arrow shows unoccupied endplate; I,J: mean ±SEM, one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$ for *, $p \leq 0.01$ for **, $p \leq 0.001$ for ***).



Supplementary Figure 3.1. Impact of ICV and IV scAAV9-cba-SMN injection on muscle fibre size in *Smn*$^{2B/-}$ mice. (A-D) Representative images of H&E stained TA muscle sections from P19 mice. (E) Muscle fibre size distribution of TA muscles comparing *Smn*$^{2B/+}$ mice, *Smn*$^{2B/-}$ mice, ICV treated *Smn*$^{2B/-}$ mice, and IV treated *Smn*$^{2B/-}$ mice (n=4-8, two-way ANOVA with Tukey's post-hoc test).

Both routes of scAAV9-cba-SMN delivery significantly ameliorate SMA-like pathophysiology in $Smn^{2B/-}$ mice with more pronounced effects after systemic administration

To compare the pathophysiological impact of peripheral SMN restoration (IV treatment) vs. partial CNS and peripheral SMN restoration (ICV treatment), we evaluated survival, body weight, and motor function. Mice were monitored every 2 days for survival and weight gain and were subjected to three motor function tests to assess muscle strength **(Figure 3.4)**. Both IV and ICV treated $Smn^{2B/-}$ mice showed a full rescue of survival within the observational period, while untreated $Smn^{2B/-}$ mice had a median survival of 23 days **(Figure 3.4A)**. Both treatments produced a partial rescue in weight gain with no differences between treatment groups **(Figure 3.4B)**. IV and ICV treatment also significantly improved motor function scores. Though not significant, both treatments trended towards improved righting times. This became apparent at P19, where ICV and IV treated mice were able to immediately right themselves while $Smn^{2B/-}$ mice generally were not **(Figure 3.4C)**. In the pen test, both IV and ICV treated $Smn^{2B/-}$ mice had significantly longer balancing times than untreated $Smn^{2B/-}$ mice from P19 to P25 **(Figure 3.4D).** Interestingly, in the mesh grip test, times were improved in IV treated $Smn^{2B/-}$ mice but not ICV treated mice compared to untreated $Smn^{2B/-}$ mice **(Figure 3.4E)**, suggesting greater strength recovery in distal muscles of IV treated mice. This indicates that important aspects of the SMA-like pathophysiology are influenced regardless of the locus of SMN restoration. Moreover, amelioration of pathophysiology in IV treated animals - which at the same time are not neuroprotected - indicates a contribution of peripheral pathologies.

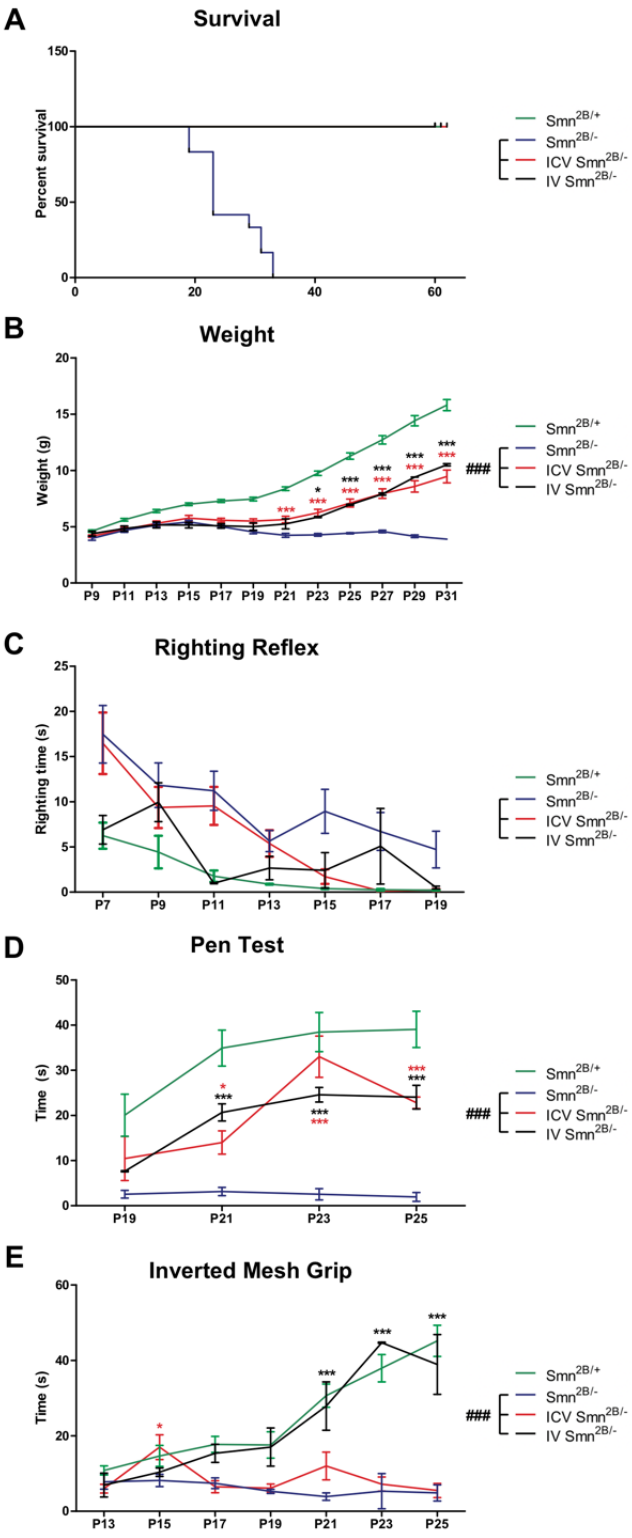


Figure 3.4. Impact of ICV and IV scAAV9-cba-SMN on SMA-like pathophysiology. (A) Kaplan Meier survival curve comparing survival of ICV and IV scAAV9-cba-SMN treated

Smn$^{2B/-}$ to saline treated *Smn*$^{2B/-}$ and *Smn*$^{2B/+}$ mice up to 60 days. Analysis of: (B) weight beginning from P9 to P31, (C) righting reflex from P7 to P19, (D) pen test from P19 to P25, and (E) mesh grip test from P13 to P25. Motor function and weight were evaluated every second day to compare IV treated, ICV treated, and untreated *Smn*$^{2B/-}$ mice. (n=1-12, mean ±SEM, A: Kaplan-Meier survival analysis; B-E: two-way ANOVA, $p \leq 0.001$ for ###, with Bonferroni post-hoc test, $p \leq 0.05$ for *, $p \leq 0.01$ for **, $p \leq 0.001$ for ***).

IV and ICV scAAV9-cba-SMN treatment partially rescue peripheral organ defects in Smn$^{2B/-}$ *mice*

We examined peripheral tissues to evaluate if scAAV9-cba-SMN injections affect pathological aspects that may be independent of the neuromuscular disease mechanisms. Several metabolic defects have been observed in SMA patients and mouse models of SMA. Fatty acid metabolism defects can be observed in *Smn*$^{2B/-}$ mice through lipid accumulation in the liver (253), while glucose metabolism defects are observed through abnormal glucose homeostasis and pancreatic defects (255). Both IV and ICV scAAV9-cba-SMN administration prevented hepatic microvesicular steatosis in *Smn*$^{2B/-}$ mice **(Figure 3.5A-D)**. Of note, the gastrointestinal tract of IV and ICV treated mice also appeared to be healthy, unlike in untreated *Smn*$^{2B/-}$ mice where it shows signs of low motility and malfunction (data not shown). Moreover, *Smn*$^{2B/-}$ mice pancreata show a higher percentage of glucagon-producing alpha cells compared to insulin-producing beta cells and both treatments resulted in a partial rescue in the percentage of alpha cells within the islets compared to *Smn*$^{2B/-}$ mice **(Figure 3.5F-J)**. Accordingly, IV as well as ICV scAAV9-cba-SMN injections restored blood glucose levels to normal in *Smn*$^{2B/-}$ mice **(Figure 3.5E)**. In summary, ICV and IV injections both resulted in the same degree of peripheral rescue,

which is in line with the overall increase of SMN in the periphery in response to both application routes.

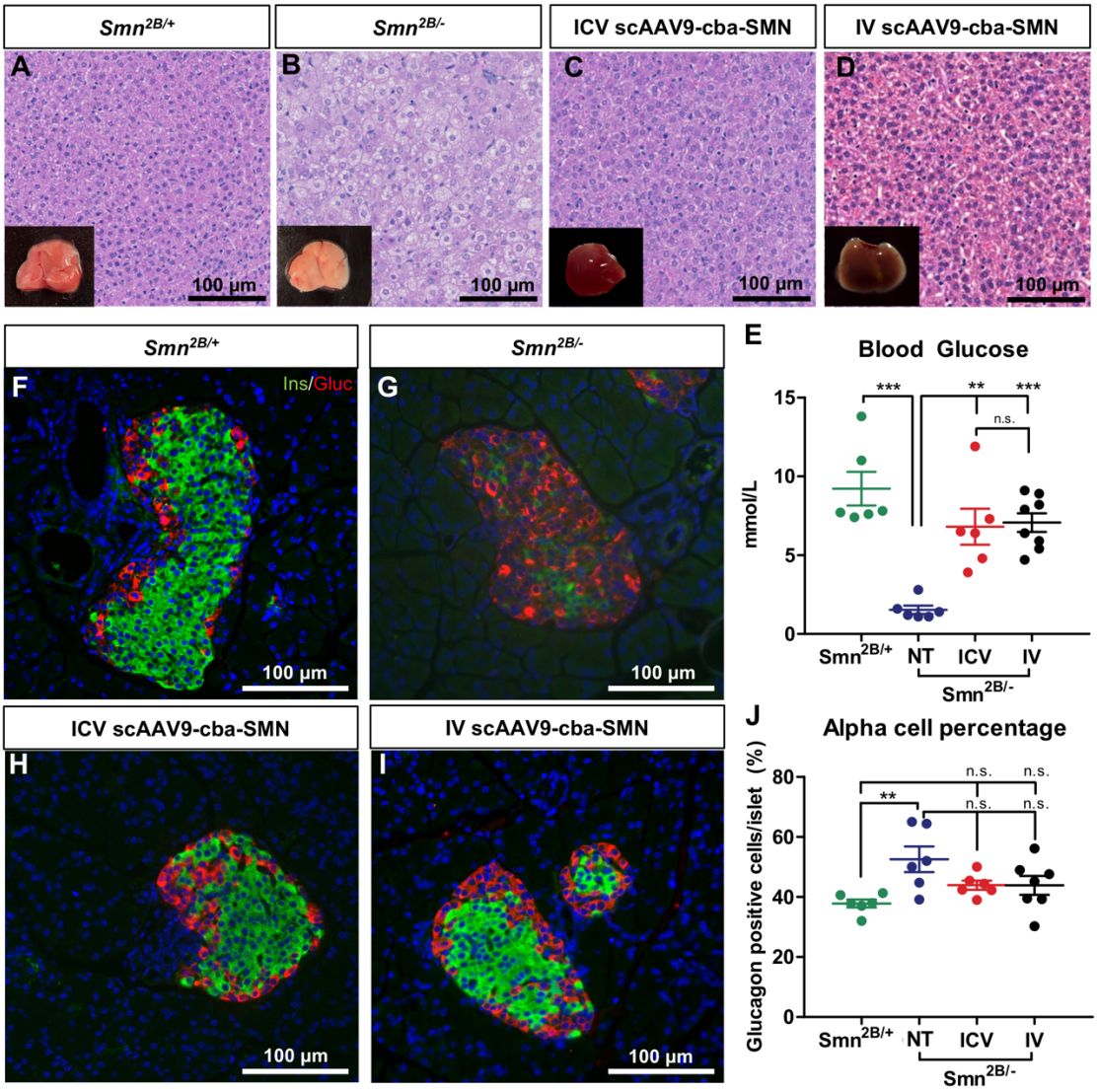


Figure 3.5. Impact of ICV and IV scAAV9-cba-SMN injection on peripheral organ defects in *Smn*$^{2B/-}$ mice. (A-D) Representative images of H&E stained liver sections from P19 mice. Insets show overall appearance of the liver upon dissection. (F-I) Representative immunofluorescent images of sections of pancreatic islets stained for glucagon (red) and insulin (green) from P19 mice. (J) Fraction of glucagon-positive alpha cells related to total number of pancreatic islet cells from *Smn*$^{2B/-}$ mice, *Smn*$^{2B/+}$ mice, ICV treated *Smn*$^{2B/-}$ mice, and IV treated *Smn*$^{2B/-}$ mice. (E) Blood glucose levels from P19 mice (NT=no treatment, n=6-8, mean ±SEM,

one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$ for *, $p \leq 0.01$ for **, $p \leq 0.001$ for ***)

Discussion

Here, we evaluated the beneficial effects of IV and ICV scAAV9-cba-SMN injection in *Smn*$^{2B/-}$ mice to compare a peripheral-centred SMN restoration to a systemic SMN restoration. ICV injection produced a mild increase in SMN levels within the CNS as well as in peripheral tissues of these mice, although SMN was not fully restored to wild type levels. However, the mild increase in SMN was sufficient to partially protect against neuronal degeneration and NMJ pathology, resulting in a significant amelioration of survival, weight and motor function. IV injection of the scAAV9-cba-SMN omitted transduction of the CNS, but fully restored SMN levels in peripheral tissues. In accordance with a lack of central SMN restoration, there was no rescue of motor neuron degeneration. The significant rescue observed in weight, survival, motor function, and metabolic defects in IV-treated animals was therefore likely a result of increased SMN in peripheral tissues. The modest rescue of NMJ neurofilament accumulation in IV-injected mice was possibly due to muscle-intrinsic SMN restoration. Both application routes showed the same degree of rescue of liver and pancreatic phenotypes. Overall, these results, summarized in **Figure 3.6**, emphasize the importance of SMN restoration to the peripheral organs and suggests further investigation into the motor neuron independent mechanisms contributing to weight, survival, motor function, and metabolic defects in SMA.

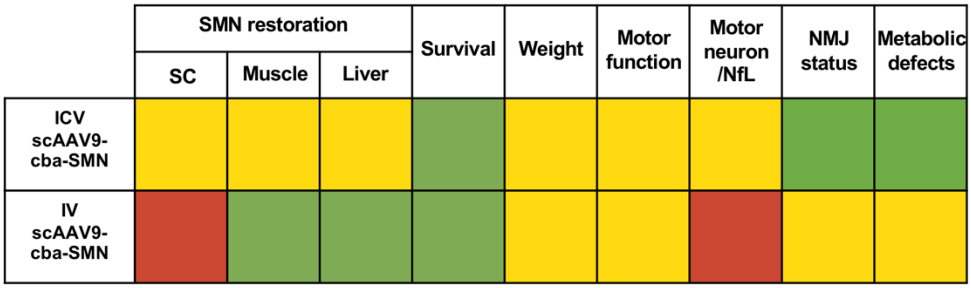


Figure 3.6. Graphical summary of the rescue of SMA-like pathology after ICV and IV scAAV9-cba-SMN injection of *Smn*$^{2B/-}$ mice. Results are classified according to a colour code, where red indicates no rescue, yellow indicates some rescue, and green indicates a full rescue.

With the increasing use of SMN replacement therapy, it is important to understand the independent contributions of peripheral tissues to disease as well as the SMN protein requirements for these tissues. Our results agree with other published data that demonstrate the importance of treating the peripheral organs to achieve a full rescue. Knockdown of SMN protein in motor neurons was shown to cause an SMA-like phenotype in mice that was milder than that produced by ubiquitous knockdown (273), while pre-clinical nusinersen trials demonstrated 25-fold greater survival in mice receiving systemic treatment compared to CNS-restricted treatment (226). Further, an SMN-restoring nusinersen-like treatment restricted to the peripheral tissues provided a robust rescue of survival in a preclinical trial (227). Here, we show that this also applies to blood-brain-barrier penetrant AAV9 based gene therapy. Moreover, for the first time, we report that those pro-survival effects are completely decoupled from the neurodegeneration but instead rely on peripheral phenotypes such as liver and pancreas.

A recent pre-clinical study demonstrated that AAV9-SMN gene therapy in SMNΔ7 mice restricted to neurons does not rescue the SMA phenotype in SMA mice, while ubiquitous expression has a stronger rescue effect (228). Interestingly, this latter report demonstrated a mild

increase in SMN within the spinal cord after IV injection of 4.5 x 10^{10} vg/mouse, a dose similar to our study. However, it is important to note that a different promoter, the phosphoglycerate kinase gene promoter, was used and that experiments on SMA mice using the cba promoter typically use a dose of at least 1x10^{11} vg/mouse to achieve transduction of the CNS (263,274). The efficacy of systemic treatment is also emphasized by clinical data, where patients treated with the CNS-specific treatment nusinersen can often remain severely disabled despite improvements in motor function, requiring ventilatory or nutritional support (275,276). Our results describe how a rescue of the peripheral organs is responsible for the higher efficacy achieved in systemic SMA treatments. However, it is important to note that mice were treated at P1, which would correspond to a prenatal timepoint in humans, limiting the translatability of our study. Treatment would likely be less efficacious in older mice as it has been shown that early delivery of SMN-inducing treatments leads to a greater rescue (263,277). The present study nonetheless emphasizes the importance of targeting the peripheral organs when treating SMA and demonstrates the independent contributions of these tissues to SMA disease.

We were surprised to observe a slight rescue of NMJ neurofilament accumulation in our IV-injected mice, despite no restoration of SMN protein to the spinal cord in this group. This finding hinted towards possible muscle-intrinsic mechanisms of NMJ pathology, as IV injection fully restored SMN protein to muscle. However, studies have demonstrated a rescue of survival in SMA mice after muscle-specific expression of SMN, but no effect on NMJ pathology or synaptic function (278)·(279). NMJ pathology is instead likely related to SMN protein levels in motor neurons, and a consequence of motor neuron dysfunction (280). Interestingly however, motor neuron specific restoration of SMN in SMNΔ7 mice partially rescued endplate size and denervation, but not neurofilament accumulation (281). This is in line with our results that

neurofilament accumulation may not be fully determined by the motor neuron, but also by other cells. Though neurofilament accumulation in neurodegenerative diseases is thought to be caused by hyper-phosphorylation of the protein within neurons (282), it is possible that terminal Schwann cells may have an effect on neurofilament phosphorylation and axonal transport (283). One explanation for the rescue of neurofilament accumulation in IV-injected mice may be that the scAAV9-cba-SMN was capable of transducing terminal Schwann cells to impact the degree of neurofilament accumulation in NMJs.

Both ICV and IV administration seemed to partially rescue motor function, though ICV treatment only rescued performance on the pen test and righting reflex test, not the mesh grip test. These results were surprising considering that IV treated mice showed no rescue in MN degeneration or muscle fiber size, and only a slight rescue in NMJ NF accumulation. Mesh grip test measures the strength of fore and hind limbs, though it can also be impacted by the amount of lean or fat mass, circulation, and neuromuscular factors (284). It is possible that the robust peripheral rescue achieved by IV treatment may have affected other systems that impacted the performance of mice during this test. Further, the western blots showed that SMN expression in skeletal muscle was restored far above baseline in IV treated mice, while it was increased to levels below baseline in ICV treated mice. This restoration may have produced a muscle-intrinsic rescue that led to better hindlimb or forelimb strength in IV treated mice.

We observed a rescue in liver steatosis, with no apparent difference between the degree of rescue across the two methods of delivery. In $Smn^{2B/-}$ mice, liver steatosis and fatty acid metabolism defects lead to several functional defects including reduced protein production, impaired hemostasis, and reduced insulin like growth factor 1 (IGF1) levels (268). Rescue of liver function therefore likely allowed for overall better health and survival, and a possible

restoration of IGF1 levels may also have contributed to improved growth and weight gain, although this needs to be experimentally verified. We also observed a rescue in blood glucose levels and a partial rescue in pancreatic defects. *Smn*$^{2B/-}$ mice demonstrate an imbalance between alpha and beta cells in the pancreatic islets, as well as glucose intolerance, hyperglucagonemia, and elevated glucose at P19 (253,255). Rescue of these defects likely improved survival, as these metabolic changes are associated with higher mortality and sudden cardiac death (285). Further, the metabolic rescue observed in IV-treated *Smn*$^{2B/-}$ mice provides important evidence that the metabolic defects in SMA are motor neuron independent.

Unfortunately, systemic (IV) gene therapy presents significant risks for SMA patients due to liver toxicity and is limited in real world application due to the required high viral doses in older patients. IT application has been explored to allow for a lower dose to be used, reducing the risk of liver toxicity as well as the overall cost of the drug, but significant toxicity concerns have also been flagged for this method of delivery (286). This treatment method also may prevent transduction of the periphery, limiting the effectiveness of the therapy. Based on the results of this study and others, it is becoming more and more apparent that targeting the peripheral organs is essential for complete treatment of SMA and that intravenous treatment is therefore likely worth the risk of liver toxicity. An alternative approach could suggest pairing a low dose systemic administration with a low dose IT administration, to target the periphery while allowing for a less potent dose to be delivered to motor neurons and avoiding the potential toxicity associated with overexpression of SMN.

This study also provided interesting insights into NfL as a biomarker of treatment response in SMA. Despite the rescue of the SMA-like phenotype achieved through IV treatment, no change in NfL levels was observed. Lower NfL was measured in ICV-treated mice, which

showed a similar rescue of growth, survival, motor function, and peripheral features to IV mice. NfL is therefore not a suitable pharmacodynamic biomarker for SMA, as it is likely only representative of motor neuron pathology. These results enforce the importance of understanding the multi-organ and muscle specific pathology present in SMA. As neuronal proteins only represent one aspect of SMA pathology, a diverse set of biomarkers may be necessary for monitoring therapeutic response in SMA patients.

As treatments extend the lives of SMA patients and continue to change the landscape and natural history of the disease, it will be important to focus on the quality of life of SMA patients. Current treatments are by no means a cure for SMA. Treated patients may begin to develop new symptoms and face obstacles that have not been faced before by untreated patients. The data herein highlight the importance of adopting a whole-body approach to SMA treatment, focusing on each specific tissue and its independent SMN requirements. As treated patients continue to age, it is likely that peripheral symptoms will become more apparent and have a larger impact on a patient's health. Our results display that focusing on treating the peripheral organs will not only improve patients' quality of life but also likely impact survival and overall health.

Data Availability

Additional data are available from corresponding author upon request.

Acknowledgements

We thank Dr. Ronald Booth and Adrienne Rowan from the Eastern Ontario Regional Laboratory Association at The Ottawa Hospital for assistance with some of the NfL assays.

Funding

RK was supported by Muscular Dystrophy Association (USA) (grant number 575466); Muscular Dystrophy Canada; and Canadian Institutes of Health Research (CIHR) (grant number PJT-156379). AR was supported by a CNMD STAR Award from the University of Ottawa Brain and Mind Institute. MOD was supported by Frederick Banting and Charles Best CIHR Doctoral Research Award.

Competing Interests

RK received honoraria and travel accommodations from Roche as an invited speaker at their global and national board meetings in 2019. RK and the Ottawa Hospital Research Institute have a licensing agreement with Biogen for the *Smn*$^{2B/-}$ mouse model. MOD received honoraria and travel accommodations from Biogen for speaking engagements at the SMA Summit 2018 held in Montreal, Canada and SMA Academy 2019 held in Toronto, Canada. These COI are outside the scope of this study. All other authors have no competing interests to declare.

Chapter 4: Long term peripheral AAV9-SMN gene therapy promotes survival in a mouse model of spinal muscular atrophy

Long term peripheral AAV9-SMN gene therapy promotes survival in a mouse model of spinal muscular atrophy

Aoife Reilly[1,2,3], Rebecca Yaworski[1], Ariane Beauvais[1], Bernard L. Schneider[4,5], Rashmi Kothary[1,2,3,6,*]

[1]Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Canada

[2]Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Canada

[3]Centre for Neuromuscular Disease, University of Ottawa, Ottawa, Canada

[4]Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

[5]Bertarelli Platform for Gene Therapy, Ecole Polytechnique Fédérale de Lausanne, Geneva, Switzerland

[6]Department of Medicine, University of Ottawa, Ottawa, Canada

Published in: Human Molecular Genetics, 33, 6, 510-519. | DOI: 10.1093/hmg/ddad202

Author contributions

AR and RK designed the research. AR performed experiments for all figures. RY contributed to Figure 4.4. AB contributed to Figure 4.2. BLS provided material support. AR analyzed the data. AR and RK wrote the manuscript with input from all authors.

Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease characterized by motor neuron loss and skeletal muscle atrophy. SMA is caused by the loss of the *SMN1* gene and low SMN protein levels. Current SMA therapies work by increasing SMN protein in the body. Although SMA is regarded as a motor neuron disorder, growing evidence shows that several peripheral organs contribute to SMA pathology. A gene therapy treatment, onasemnogene abeparvovec, is being explored in clinical trials via both systemic and central nervous system (CNS) specific delivery, but the ideal route of delivery as well as the long-term effectiveness is unclear.

To investigate the impact of gene therapy long term, we assessed SMA mice at 6 months after treatment of either intravenous (IV) or intracerebroventricular (ICV) delivery of scAAV9-cba-SMN. Interestingly, we observed that SMN protein levels were restored in the peripheral tissues but not in the spinal cord at 6 months of age. However, ICV injections provided better motor neuron and motor function protection than IV injection, while IV-injected mice demonstrated better protection of neuromuscular junctions and muscle fiber size. Surprisingly, both delivery routes resulted in an equal rescue on survival, weight, and liver and pancreatic defects. These results demonstrate that continued peripherally restricted AAV9-SMN gene therapy is beneficial for disease improvement even in the absence of SMN restoration in the spinal cord.

Introduction

Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disorder characterized by motor neuron loss and muscle atrophy (287). SMA patients suffer from proximal and progressive muscle weakness, which leads to scoliosis, respiratory failure, and early death if not treated (288). SMA is caused by a loss or mutation of the Survival Motor Neuron 1 gene (*SMN1*), a gene responsible for producing Survival Motor Neuron (SMN) protein (3). SMN is ubiquitously expressed and is important for the maintenance and development of motor neurons. A similar gene to *SMN1*, *SMN2*, produces a small but insufficient amount of SMN protein (5). *SMN2* copy number varies between individuals and is inversely correlated to symptom severity, thus contributing to a wide range of disease severity in SMA (225).

Before the development of SMA therapies, severe SMA patients would usually succumb to the disease within months of birth. Now, families of SMA patients can have access to several therapies after diagnosis. The available therapies work by increasing SMN protein to reduce symptoms. Nusinersen, the first FDA-approved therapy for SMA, is an antisense oligonucleotide that binds to *SMN2* pre-mRNA to restore normal splicing and thereby increase the production of SMN protein (289). Subsequently, alternative treatments were developed such as onasemnogene abeparvovec (290), a gene replacement therapy, and risdiplam, a small molecule that is taken orally and that impacts splicing of *SMN2* pre-mRNA (291).

Onasemnogene abeparvovec treatment consists of a single-dose intravenous infusion of an adeno-associated virus 9 vector containing *SMN1* cDNA (AAV9-SMN) that is designed to cross the blood brain barrier to target motor neurons (122). While onasemnogene abeparvovec has improved survival in many patients, concerns exist around the safety of the treatment as well as the long-term efficacy. A high dose of AAV is required to penetrate the blood brain barrier

intravenously, but the high affinity of AAV for the liver has caused liver toxicity and death in some patients (292),(293). Clinical trials are also exploring administering the virus intrathecally to avoid liver toxicity, but concerns of dorsal root ganglia toxicity in non-human primates and mice have raised concern about this method of delivery (136,294). Long-term follow up data from onasemnogene abeparvovec clinical trials has shown that intravenous treatment improved motor function in patients. However, there is limited data on the long-term safety of each route of delivery as clinical trials have only followed patients for up to 4.3 years (144). This presents challenges for parents and doctors making decisions about the ideal treatment for patients. It is thus important to further explore questions of the long-term effectiveness and safety of onasemnogene abeparvovec in animal models of SMA.

While the ideal route of delivery of onasemnogene abeparvovec is unclear, it is apparent that targeting the peripheral organs is essential to providing the optimal treatment in mouse models. While SMA is considered a motor neuron disorder, several non-neuronal tissues appear to be affected independently of motor neuron loss. This has been reported in muscle (30,31,295), heart (197,296), liver (32,206), pancreas (34,210), and immune organs (35,213,214) from mouse models and humans with SMA. Further, early nusinersen trials demonstrated 25-fold greater survival in mice receiving a systemic treatment versus a CNS-restricted treatment (97) and AAV9-SMN restricted to neurons has been shown to be less effective than systemic delivery (193). We previously showed that a peripheral-specific AAV9-SMN therapy in *Smn*$^{2B/-}$ mice provides an equal rescue of many features, including survival, to a systemic treatment (230). Interestingly, this rescue was achieved without protecting motor neurons, indicating that peripheral tissues are likely important contributors to overall SMA pathology. However, the

long-term effectiveness of this treatment, and thus the level of contribution of the peripheral organs during aging in SMA, remains unknown.

The present study explores the long-term efficacy of a peripherally delivered AAV9-SMN gene therapy in a mouse model of SMA. Neonatal *Smn*$^{2B/-}$ mice were treated with scAAV9-cba-SMN through IV or ICV delivery and the protective effect on neuronal and non-neuronal SMA-like pathophysiology was compared up to 6 months of age. We show that a peripherally-directed IV injection provided equal rescue of survival, weight, liver and pancreatic pathology to a systemic ICV injection, despite limited rescue of motor neurons.

Results

IV and ICV administration of scAAV9-cba-SMN to $Smn^{2B/-}$ mice leads to increased SMN protein in peripheral tissues but not in the spinal cord at 6 months post-injection

We aimed to evaluate the long-term effectiveness of a scAAV9-cba-SMN gene-replacement therapy in $Smn^{2B/-}$ mice and to compare the longevity of rescue produced by two different routes of administration. Mice were injected with scAAV9-cba-SMN ($5x10^{10}$ vg per pup) at P1, a time point at which $Smn^{2B/-}$ mice are considered presymptomatic, with either IV or ICV routes of virus delivery. Previous work using IV and ICV injection of scAAV9-cba-SMN has demonstrated a robust rescue of this mouse model up to 60 days post injection(230). The latter study showed an interesting pattern of SMN restoration: a mild increase of SMN protein in the spinal cord (SC) and peripheral tissues in ICV injected mice, and an increase of SMN in the peripheral tissues in IV injected mice. In the present study, we aimed to evaluate the longevity of transgene expression in peripheral and CNS tissues after scAAV9-cba-SMN injection. Western blot analysis was performed to compare SMN protein levels in liver, muscle, and SC between the two routes of delivery at 6 months post-injection. Both routes of delivery produced an increase of SMN levels, indicating that the virus was functional. Interestingly, both IV and ICV injection maintained a restoration of SMN protein in the liver and an overexpression of SMN in skeletal muscle at 6 months, while neither showed a maintenance of SMN restoration in the SC at this stage (Figure 4.1). These results indicate that the AAV mediated expression of SMN persisted in peripheral tissues but not in the SC. It is unclear whether expression ever occurred in the SC with either treatment, but it is likely that SMN expression was increased over the short-term in ICV-treated mice as was shown with previous work using this model (230).

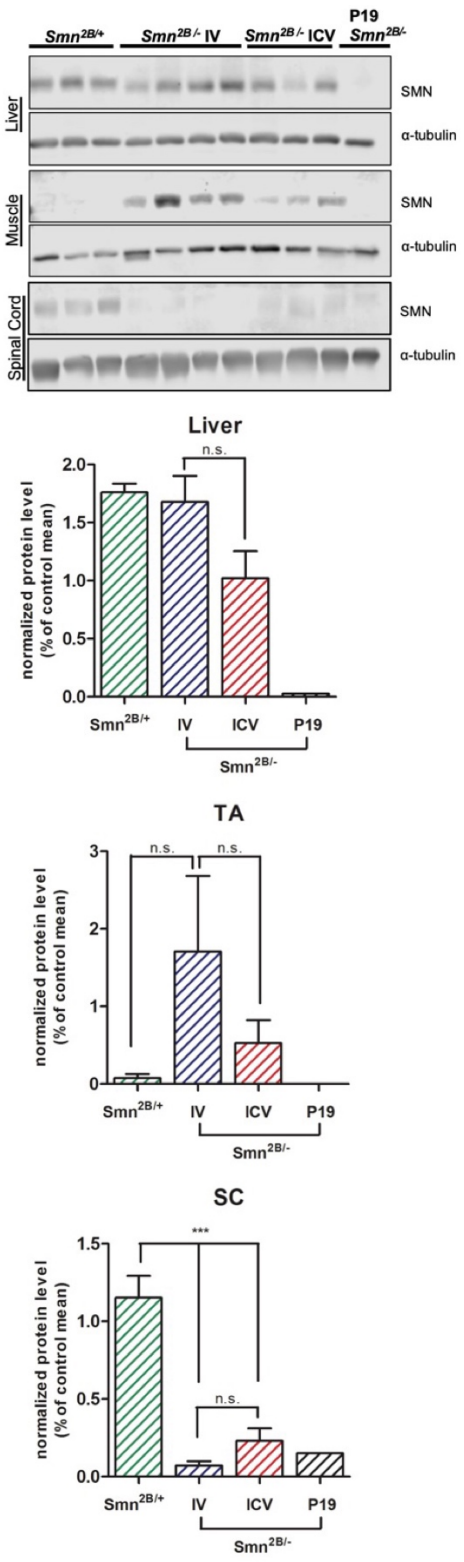


Figure 4.1. Peripheral and spinal cord SMN levels after IV and ICV scAAV9-cba-SMN injection. (A) Western blot analysis was performed to identify SMN protein at 6 months in liver,

tibialis anterior muscle, and spinal cord of IV and ICV treated *Smn*$^{2B/-}$ mice. Mice were injected at P1 with 5x10^{10} vg of scAAV9-cba-SMN. Protein extracts from the tissues of 6-month-old *Smn*$^{2B/+}$ heterozygous and P19 *Smn*$^{2B/-}$ mice were also loaded for comparison. Alpha-tubulin levels were assessed as loading control. (B-D) Quantification of the SMN signal from the western blot normalized to tubulin levels. (B-D: mean ± SEM, one-way ANOVA with Tukey's post-hoc test; *Smn*$^{2B/+}$: n=*3, Smn*$^{2B/-}$ IV: n=4, *Smn*$^{2B/-}$ ICV n=3 (P19 *Smn*$^{2B/-}$ excluded from analysis); $p \leq 0.001$ for ***, n.s. = not significant).

Both routes of scAAV9-cba-SMN delivery significantly ameliorate SMA-like pathophysiology with more pronounced long-term effects after ICV administration

To compare the long-term impact after either IV or ICV injection of scAAV9-cba-SMN, mice were initially characterized for survival, weight, and motor function over the course of 6 months. Weight and motor function were assessed every 2 weeks starting at 3 weeks of age, then every month starting at 3 months. While untreated *Smn*$^{2B/-}$ mice displayed a mean survival of 25 days, all mice in our treatment group survived up to 6 months (Figure 4.2A), at which point they were sacrificed for tissue collection. Note that one mouse in the IV-treated group was euthanized at 2.5 months for reasons unrelated to the SMA-like phenotype (Figure 4.2A). Mice from both treatment groups gained weight equally over time, but never attained the weights of age-matched *Smn*$^{2B/+}$ mice (Figure 4.2B). IV-treated mice were less active than *Smn*$^{2B/+}$ mice in the open field test, while there was no difference in activity level between ICV-treated mice and *Smn*$^{2B/+}$ mice (Figure 4.2C). IV-treated mice displayed motor impairment in the rotarod test, while ICV-treated mice did not (Figure 4.2D). Both treatment groups had significantly weaker grip strength

compared to *Smn*$^{2B/+}$ mice, but ICV-treated mice scored significantly higher than IV-treated mice (Figure 4.2E). Thus, while both treatments equally extended survival and improved weight gain, ICV treatment better improved motor function scores. This contrasts with previously published short-term motor function data from these treatments, which suggested better recovery of muscle strength in IV-treated animals from P21 to P25 (230). This suggests that treatments targeting the motor neuron may be more important for long-term motor function rescue.

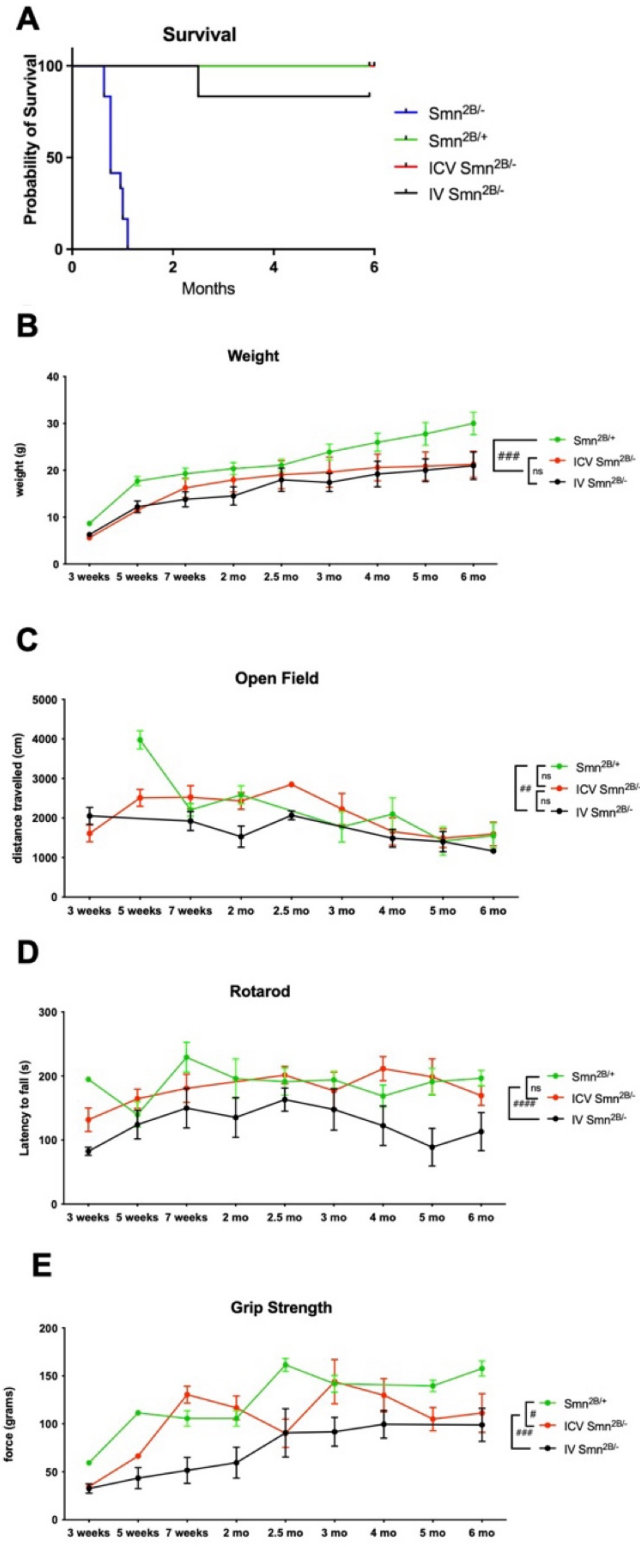


Figure 4.2. Impact of IV and ICV injected scAAV9-cba-SMN on SMA-like pathophysiology in *Smn$^{2B/-}$* mice. (A) Kaplan Meier survival curves comparing IV and ICV scAAV9-cba-SMN

treated *Smn*$^{2B/-}$ mice to untreated *Smn*$^{2B/-}$ and *Smn*$^{2B/+}$ mice up to 6 months. Analysis of: (B) weight, (C) open field test, (D) rotarod test, and (E) grip strength test from 3 weeks to 6 months (*Smn*$^{2B/-}$: n=12, *Smn*$^{2B/+}$: n=*4, Smn*$^{2B/-}$ IV: n=5, *Smn*$^{2B/-}$ ICV n=4; mean ± SEM, A: Kaplan-Meier survival analysis; B-E: two-way ANOVA, p≤ 0.001 for ###, with Bonferroni post-hoc test, p ≤ 0.05 for *, p ≤ 0.01 for **, p ≤ 0.001 for ***).

ICV scAAV9-cba-SMN injection better protects against spinal cord motor neuron degeneration in Smn$^{2B/-}$ *mice*

Motor neuron number and plasma neurofilament light chain (NfL) levels were measured at 6 months to investigate the long-term rescue of motor neuron pathology after either IV or ICV route of delivery of scAAV9-cba-SMN (Figure 4.3). ChAT staining of motor neuron cell bodies in the anterior horn of the SC revealed that IV-treated mice had fewer alpha motor neurons than in the control *Smn*$^{2B/+}$ mice (Figure 4.3A,C), while there was no difference in motor neuron number between ICV-treated mice and *Smn*$^{2B/+}$ mice (Figure 4.3A,B). The quantification of the motor neuron cell bodies is shown in Figure 4.3D. This difference in motor neuron protection was further confirmed by plasma NfL levels, which were significantly elevated in IV-treated mice compared to both ICV-treated mice and *Smn*$^{2B/+}$ mice (Figure 4.3E). NfL is a structural neuronal protein which is released into peripheral blood upon neuronal degeneration, and is therefore considered a candidate biomarker in neurodegenerative diseases such as SMA(297). Overall, these results showed that ICV treatment better protected motor neurons from SMA-related degeneration up to 6 months after treatment. Interestingly, as neither treatment produced

a restoration of SMN protein in the SC at 6 months, it is possible that early restoration of SMN to the SC was sufficient to provide motor neuron protection.

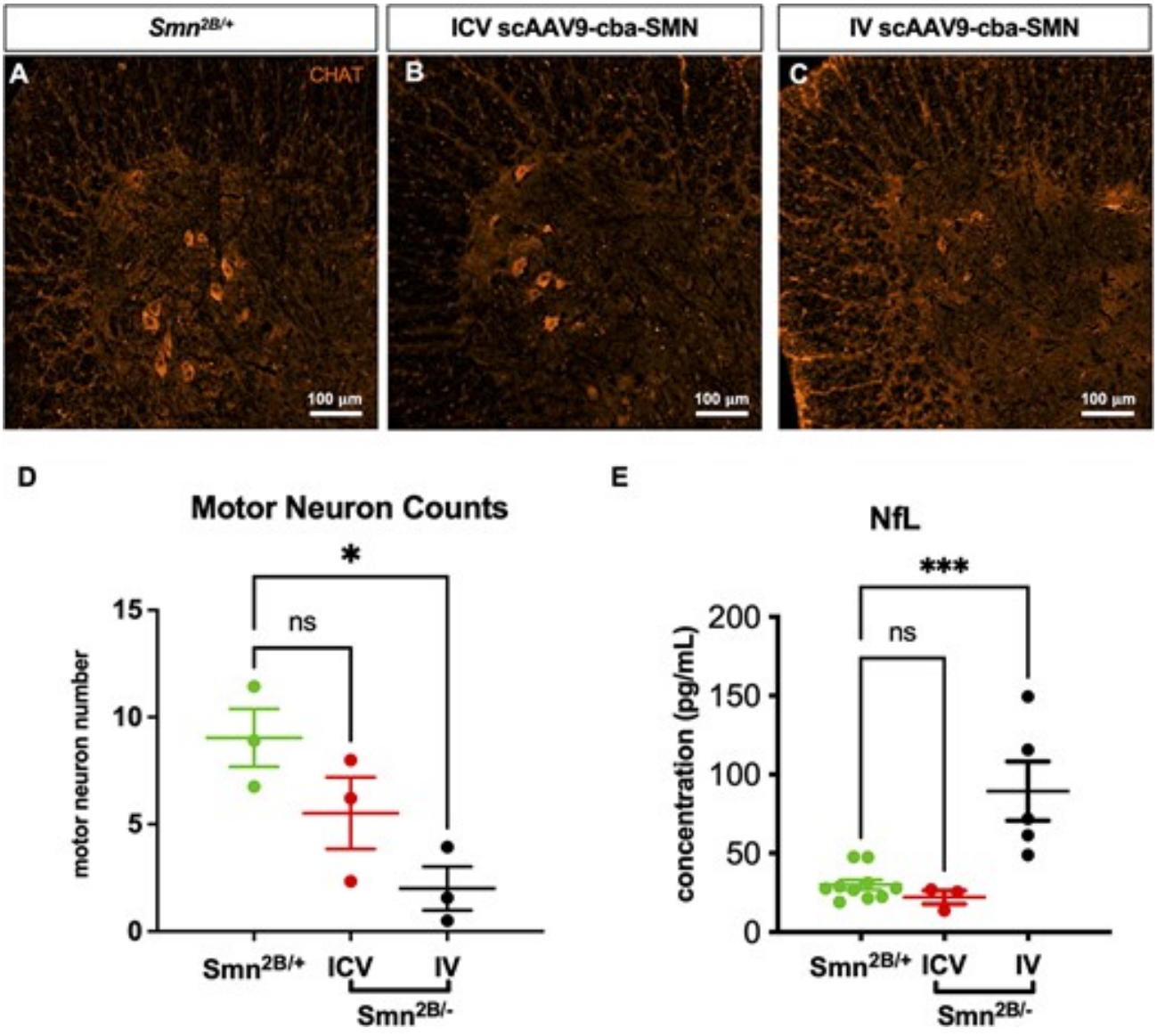


Figure 4.3. Impact of IV and ICV scAAV9-cba-SMN injection on motor neuron protection in *Smn$^{2B/-}$* mice. Representative immunofluorescent images of sections of lumbar spinal cord anterior horns stained for ChAT (red) from 6-month-old *Smn$^{2B/+}$* mice (A), ICV treated *Smn$^{2B/-}$* mice (B), and IV treated *Smn$^{2B/-}$* mice (C). (D) Quantification of motor neuron cell bodies. (E)

Plasma NfL levels were assessed using single molecule array (Simoa) technology (n=3, mean ± SEM, one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$ for *, $p \leq 0.01$ for **, $p \leq 0.001$ for ***, ns = not significant).

IV scAAV9-cba-SMN injection better rescues neuromuscular junction defects in $Smn^{2B/-}$ mice

Next, neuromuscular junction (NMJ) morphology was analyzed to further assess the health of motor neurons at 6 months after either IV or ICV delivery of scAAV9-cba-SMN in $Smn^{2B/-}$ mice. In SMA, NMJs are subject to morphological abnormalities, neurofilament accumulation, and denervation that impair function and lead to motor neuron loss. Neurofilament accumulation and endplate occupancy within NMJs of the transversus abdominis muscle were analyzed as a representation of NMJ pathology (Figure 4.4). NMJs from both treatments displayed no significant difference in the percentage of occupied endplates compared to $Smn^{2B/+}$ mice (Figure 4.4H). NMJs from ICV-treated mice displayed significantly more neurofilament accumulation than $Smn^{2B/+}$ mice, while neurofilament accumulation in IV-treated mice was not significantly different than $Smn^{2B/+}$ mice (Figure 4.4G). The slightly better rescue of neurofilament accumulation in IV-treated mice may be the result of higher expression of SMN in the muscle of these mice compared to ICV delivery (Figure 4.1).

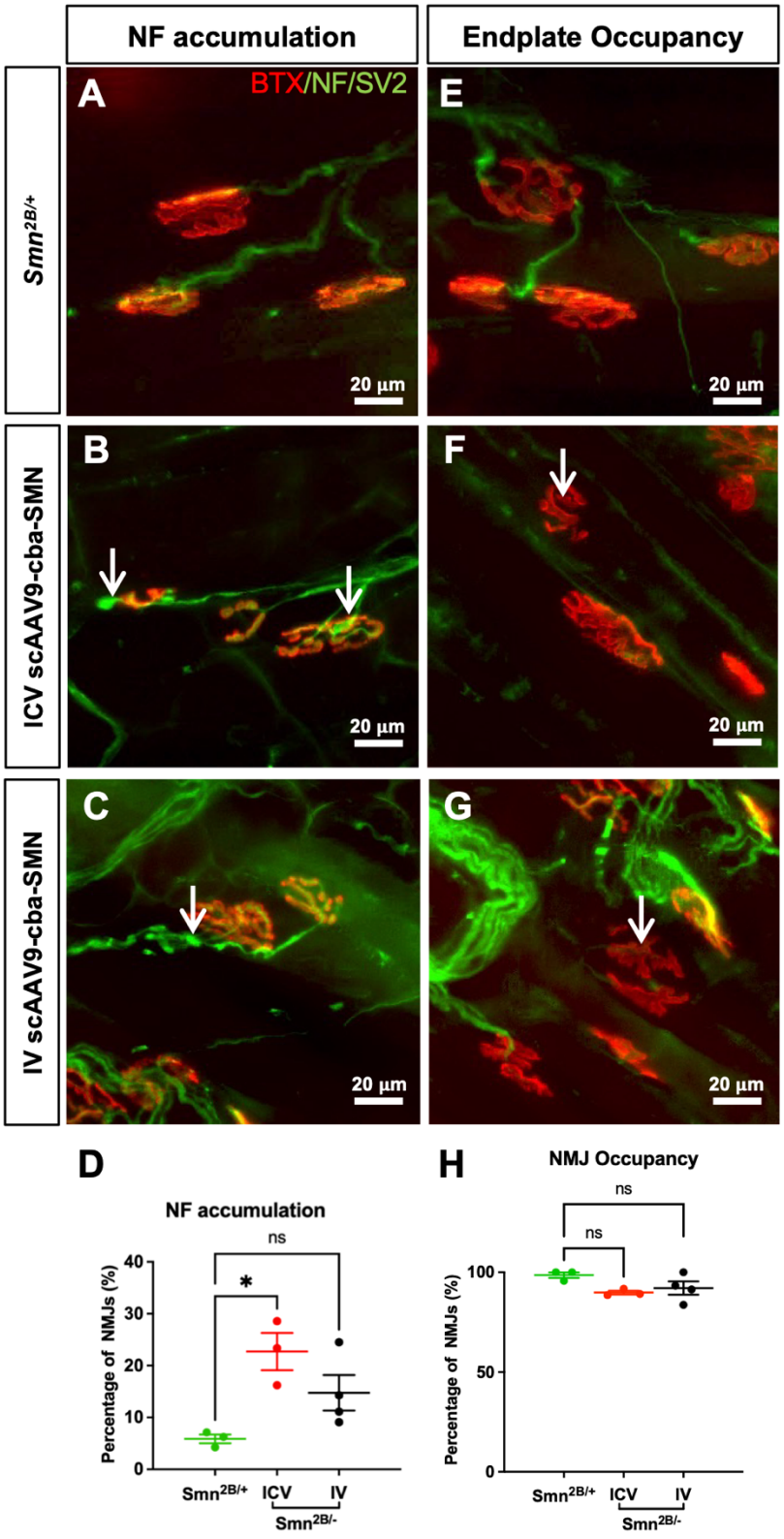

NF accumulation
Endplate Occupancy
Smn2B/+
ICV scAAV9-cba-SMN
IV scAAV9-cba-SMN
A
BTX/NF/SV2
E
B
F
C
G
20 µm
D
NF accumulation
ns
*
Percentage of NMJs (%)
40
30
20
10
0
Smn2B/+
ICV
IV
Smn2B/-
H
NMJ Occupancy
ns
ns
100
50
0

Figure 4.4. Impact of IV and ICV scAAV9-cba-SMN injection on neuromuscular junction pathology in *Smn$^{2B/-}$* mice. Representative immunofluorescent images of *transversus abdominis* (TVA) muscle stained with bungarotoxin (red, endplates), and for neurofilament (NF) (green, axons) and synaptic vesicle protein 2 (green, pre-synaptic nerve terminals) from 6-month-old *Smn$^{2B/+}$* mice (A, E), ICV treated *Smn$^{2B/-}$* mice (B, F), and IV treated *Smn$^{2B/-}$* mice (C, G). Quantification of NF accumulation (D) and endplate occupancy (H). (*Smn$^{2B/+}$*: n=*3, Smn$^{2B/-}$* IV: n=4, *Smn$^{2B/-}$* ICV n=3; B-C: arrow shows NF accumulation; F-G: arrow shows unoccupied endplate; D,H: mean ± SEM, one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$ for *, ns = not significant).

IV and ICV delivery of scAAV9-cba-SMN protect against liver and pancreatic defects in Smn$^{2B/-}$ mice

To evaluate the long-term rescue of non-neuronal organs, we assessed the impact of both treatments on peripheral organ defects that are present in *Smn$^{2B/-}$* mice. *Smn$^{2B/-}$* mice display pancreatic defects that produce cell fate imbalances of alpha and beta cells in pancreatic islets(34), and they display fatty acid metabolism defects leading to lipid accumulation in the liver(32). At 6 months after injection, both IV and ICV-treated mice displayed normal ratios of pancreatic alpha and beta cells (Figure 4.5A-D). Further, qualitative analysis of liver sections showed no evidence of microvesicular steatohepatitis and an absence of lipid accumulation (Figure 4.5E-G). Both treatments therefore appeared to equally rescue pancreas and liver pathology up to 6 months after treatment.

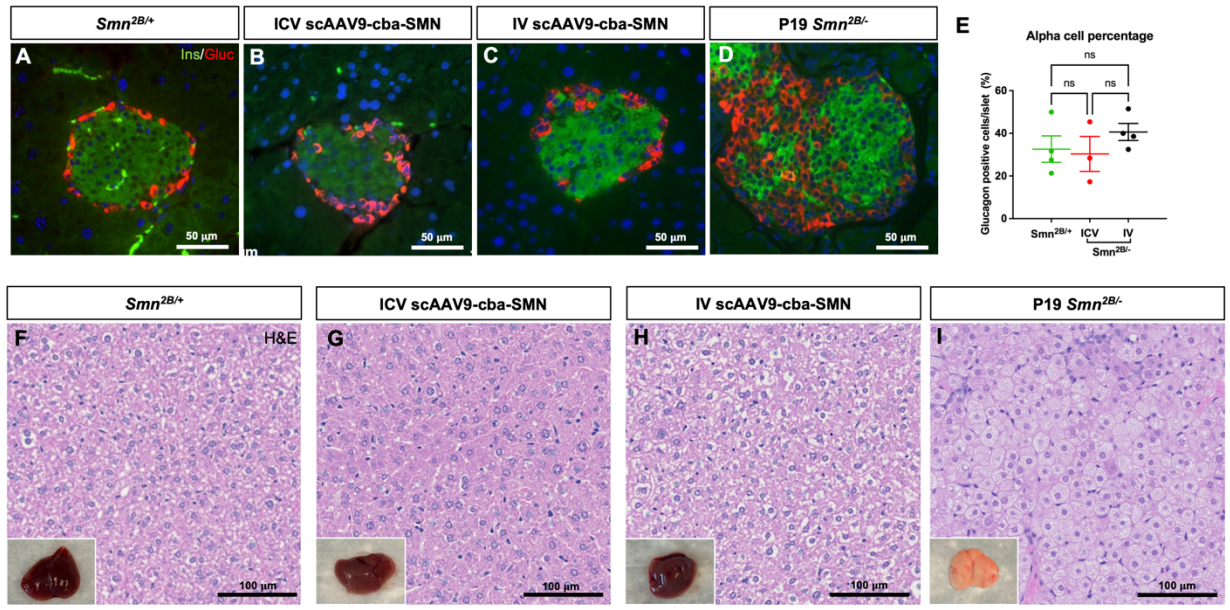


Figure 4.5. Impact of IV and ICV scAAV9-cba-SMN injection on peripheral organ defects in *Smn$^{2B/-}$* mice. Representative immunofluorescent images of sections of pancreatic islets stained for glucagon (red) and insulin (green) from 6-month-old *Smn$^{2B/+}$* mice (A), ICV treated *Smn$^{2B/-}$* mice (B), and IV treated *Smn$^{2B/-}$* mice (C). An image of a P19 *Smn$^{2B/-}$* mouse was included for reference (D). (E) Fraction of glucagon-positive alpha cells compared to total number of pancreatic islet cells. Representative images of H&E-stained liver sections from 6 month-old *Smn$^{2B/+}$* mice (F), ICV treated *Smn$^{2B/-}$* mice (F), and IV treated *Smn$^{2B/-}$* mice (H). An image of a P19 *Smn$^{2B/-}$* mouse was included for reference (I). (*Smn$^{2B/+}$*: n=*4, Smn$^{2B/-}$* IV: n=4, *Smn$^{2B/-}$* ICV n=3; mean ± SEM, one-way ANOVA with Tukey's post-hoc test, ns = not significant).

IV and ICV delivery of scAAV9-cba-SMN protects against muscle atrophy

Finally, tibialis anterior muscle was analyzed to determine the impact of both treatments on muscle fiber size at 6 months after either IV or ICV route of delivery of scAAV9-cba-SMN in

Smn$^{2B/-}$ mice. Muscle atrophy is a hallmark characteristic of SMA and is thought to be caused by both motor neuron degeneration and muscle intrinsic mechanisms. Both IV and ICV-treated mice showed comparable muscle fiber size to control *Smn*$^{2B/+}$ mice (Figure 4.6A-D). Though no statistically significant difference was observed, ICV-treated mice trended towards lower fiber size (Figure 4.6D). This trend is like that observed in NMJ pathology, suggesting that overexpression of SMN in the muscle in IV-treated mice may provide an additional protective benefit. Overall, these results also suggest that long-term restoration of SMN to the SC is not necessary to protect against muscle atrophy.

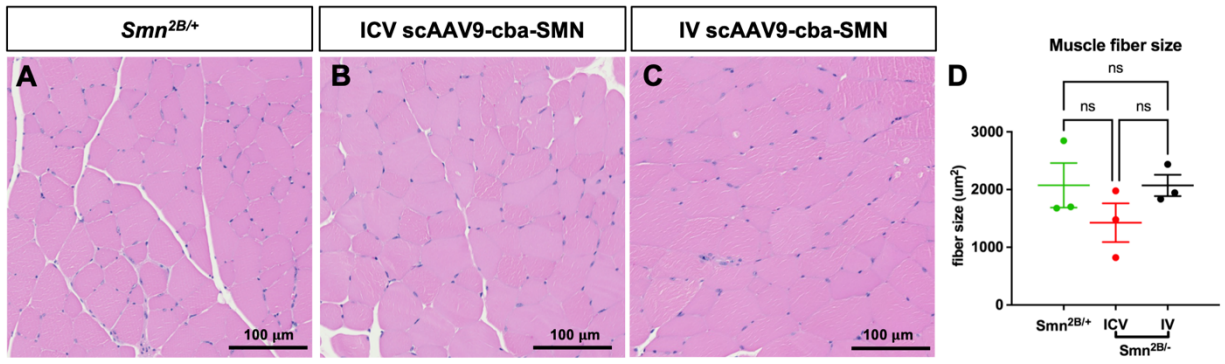


Figure 4.6. Impact of IV and ICV scAAV9-cba-SMN injection on muscle fiber size in *Smn*$^{2B/-}$ mice. Representative images of H&E-stained *tibialis anterior* muscle sections from 6-month-old *Smn*$^{2B/+}$ mice (A), ICV treated *Smn*$^{2B/-}$ mice (B), and IV treated *Smn*$^{2B/-}$ mice (C). (D) Quantification of muscle fiber size. (n=3, mean ± SEM, one-way ANOVA with Tukey's post-hoc test, ns = not significant).

Discussion

Our study compared the long-term effect of a peripherally directed IV AAV9-SMN treatment to a systemic ICV treatment in *Smn*$^{2B/-}$ mice. This was achieved through two different

routes of administration of the virus. Interestingly, at 6 months after injection, an increase in SMN protein was detected in the peripheral tissues but not in the SC of mice treated with both IV and ICV injection. However, based on our previous study, it is likely that a short-term increase of SMN protein in the SC was achieved by ICV injection(230). All mice from both treatments survived to 6 months, but ICV treated mice demonstrated better motor function than IV treated mice. This may have been a result of a greater motor neuron protection in ICV treated mice, which was demonstrated by a higher motor neuron count in the anterior horn and lower plasma NfL levels. In contrast, IV treated mice showed less neurofilament accumulation at motor endplates and trended towards larger muscle fiber size than ICV treated mice, highlighting the impact of muscle specific SMN restoration. Pancreas and liver pathology were also prevented by both routes of delivery of the virus. Overall, despite limited restoration of SMN to the SC, treated mice demonstrated an impressive rescue of the SMA-like phenotype, likely due to the rescue of peripheral organs. Further, it appears that a short-term rescue of SMN in the SC of ICV-treated mice may have been sufficient to protect motor neurons over the long-term, and that motor neuron protection is essential to maintaining motor function.

This study demonstrates that non-neuronal tissues continue to be important contributors to SMA disease throughout aging. These results agree with nusinersen preclinical trials that demonstrated a greater rescue when treatment was administered peripherally versus centrally(97), and demonstrated that a peripheral-specific SMN restoration provided a long-term rescue identical to that of a systemic SMN restoration(298). Here, we found similar results but with the use of an AAV9-SMN and in the *Smn*$^{2B/-}$ mouse model, supporting the idea that the rescue of the peripheral organs can compensate for low SMN levels in the SC. It is important to also note that our restoration of SMN may not have been entirely peripheral-specific. Though no

increase in SMN protein was detected, there remains a possibility that a low level of restoration to the motor neurons could have occurred but not been detected by western blot. This could also have contributed to the improved survival of motor neurons.

The role of the peripheral organs in SMA pathology is not completely clear. Here, IV and ICV treatment with AAV9-SMN equally rescued the liver and pancreatic defects usually observed in *Smn*$^{2B/-}$ mice. *Smn*$^{2B/-}$ mice demonstrate liver steatosis and functional defects including impaired protein production, iron hemostasis, and reduced insulin like growth factor (IGF1) levels(32). IGF1 is an important factor in growth and development, is implicated in neuronal protection and regeneration(299,300), and has been shown to protect motor neurons and extend survival when used as a treatment in ALS mouse models(301),(302). Protection of the liver and in turn the IGF1 pathway would therefore likely provide a protective effect to *Smn*$^{2B/-}$ mice. *Smn*$^{2B/-}$ mice also show glucose metabolism defects in the pancreas(34), the protection of which would extend survival as metabolic syndrome is associated with higher mortality and cardiovascular disease(303). Muscle atrophy is a primary component of SMA pathology, and skeletal muscle development and maturation are intrinsically affected by SMN protein loss(30,31,190). Thus, the restoration of SMN protein to muscle tissue was likely an important contributor to the rescue achieved in IV-treated mice. Further, restoration of SMN to skeletal muscle also could have protected again neuromuscular junction defects that lead to retraction of nerve terminals and denervation of muscle(304). Moreover, there are several other peripheral organs implicated in SMA that were not explored in this study, including the immune organs, heart, and bones, that may have contributed to the rescue achieved by the peripheral SMN restoration.

Onasemnogene abeparvovec gene therapy has only been in use for 6 years, making it difficult to predict the long-term safety and efficacy of the treatment. The treatment delivers *SMN1* cDNA into cells as an extrachromosomal episome, which can be lost over time in frequently dividing cells(305). The current study demonstrated the long-term efficacy of the treatment in $Smn^{2B/-}$ mice up to 6 months, but a longer time frame is needed to follow mice as they continue to age. SMN protein levels are highest during development and continue to decrease with age(306). The requirement for SMN is thus lower later in life, which may explain why a short-term increase of SMN protein in the SC was sufficient to protect motor neurons up to 6 months. This also emphasizes the well characterized importance of treating SMA patients early, when SMN requirements are highest. However, because SMN levels decrease with age, the effects of long-term overexpression could be harmful. Multiple studies have raised concerns about neurotoxicity in animal models(136,294), begging the question of whether over expression in non-neuronal tissues could also have negative effects.

Our results indicate that peripheral tissues are important contributors to the survival of $Smn^{2B/-}$ mice and should therefore be considered important factors in the treatment of SMA patients. Further studies should investigate peripheral organs in detail to determine their individual contributions to SMA pathology. Our findings argue against the sole use of CNS-directed treatments like nusinersen, as aging patients will likely experience peripheral organ impairments later in life. Onasemnogene abeparvovec presents as a promising alternative, but it is an irreversible treatment and thus the long-term effects need to be better understood. If the effects of treatment were to be lost in peripheral cells due to cell division over time, additional peripheral treatments such as risdiplam, or SMN-independent therapies that target peripheral organs, may be needed to maintain the effectiveness of the treatment over time. Follow-up

studies for onasemnogene abeparvovec in the clinic and longer-term animal studies are therefore needed to better inform patients and physicians about their treatment decisions.

Materials and Methods

Animals

Smn$^{2B/-}$ and *Smn*$^{2B/+}$ mice were bred in our laboratory by crossing *Smn*$^{+/-}$ mice and *Smn*$^{2B/2B}$ mice on a C57BL/6J background. Animals were housed at the University of Ottawa Animal Facility. All experimental protocols on mice were approved by the Animal Care Committee of the University of Ottawa. Care and use of experimental mice followed the guidelines of the Canadian Council on Animal Care, and the Animals for Research Act.

scAAV9-cba-SMN treatment

The scAAV9-cba-SMN vector was produced and delivered to P1 mice as previously described at 5 x 10^{10} vg/pup(230). Mice were injected intravenously through facial vein injection or by ICV injection. Mice were weighed every 2 weeks, then monthly after 3 months. Mice were monitored for survival up to 6 months.

Motor function tests

Mice were subject to three motor function tests: open field (Treat-NMD protocol DMD_M.2.1.002), rotarod (Jackson Laboratory protocol JAX-MNBF-ROT), and grip strength test (Treat-NMD protocol SMA_M.2.1.002). Tests were performed every 2 weeks, then monthly after 3 months. Tests were performed according to protocol with slight alterations. In brief, during the open field test mice were placed in the middle of the arena and recorded for 10 min. Testing was done in 100 lux light and four mice were tested at a time. Mice were rotarod tested accelerating from 4 to 45 rpm in 5 min for four trials, with a time between trials of 1 min. One to two mice were tested at any one time. For the grip strength test, each mouse was allowed to grip

the grate with both forepaws and was pulled gently away from the meter until it released the grate. Five pulls were completed, and the grams of force were recorded.

Blood collection and plasma analysis

Blood was collected from 6-month-old mice at the time of euthanasia as previously described(230). Plasma NfL was measured by the Eastern Ontario Regional Laboratory Association and The Ottawa Hospital using the Simoa NF-Light® assay (Quanterix, Billerica, MA).

Western blot

Upon euthanasia at 6 months, liver, *tibialis anterior* muscle, and SC were collected and flash frozen in liquid nitrogen for detection of SMN protein using western blot. Tissue processing and immunoblotting were performed as previously described(230). Antibodies are listed in Table 4.1.

Tissue processing and staining

Pancreas was collected and fixed in 4% paraformaldehyde (PFA) and immunohistochemistry was performed to visualize alpha and beta cells as previously described(230). Liver and *tibialis anterior* muscle were fixed in 1:10 dilution buffered formalin (Thermo Fisher Scientific, Waltham, MA) and stained with hematoxylin and eosin as previously described(230). SC was fixed in 4% PFA and immunohistochemistry was performed as previously described(230) to visualize motor neurons with choline acetyltransferase (ChAT).

Transversus abdominis muscle was fixed in 4% PFA and stained to visualize neuromuscular junctions as before (230). Antibodies are listed in Table 4.1.

Table 4.1. List of antibodies/reagents used for immunohistochemistry and western blot

Method	Antibody	Species	Dilution	Company (Catalog #)
IHC	ChAT	goat	1:100	Millipore (AB144P)
IHC	anti-goat Alexa Fluor 555	Donkey	1:200	Invitrogen (A21432)
IHC	TRITC conjugated bungarotoxin	Mouse	1:1 000	Invitrogen (T1175)
IHC	Neurofilament (NF-M)	Mouse	1:100	(Developmental Studies Hybridoma Bank, P12839)
IHC	synaptic vesicle glycoprotein 2A	Mouse	1:250	(Developmental Studies Hybridoma Bank, Q7L0J3)
IHC	Anti-mouse Alexa Fluor 488	Goat	1:250	Invitrogen (A11001)
Western Blot	SMN	Mouse	1:2 000	BD Transduction (610647)
Western Blot	Alpha-tubulin	Rabbit	1:10 000	Abcam (ab4074)
Western Blot	IRDye 680 anti-mouse IgG	Goat	1:10 000	Li-Cor (926-68070)
Western Blot	IRDye 800 anti-rabbit IgG	Goat	1:10 000	Li-Cor (926-32211)
Western Blot	Anti-mouse IgG HRP conjugate	Goat	1:3 000	Bio-Rad (1706516)
Western Blot	Anti-rabbit IgG HRP conjugate	Goat	1:3 000	Bio-Rad (1706515)

Statistical analysis

Data are presented as the mean ± standard error of the mean. One-way ANOVA with Tukey's post-test or Two-way ANOVA with Bonferroni post-test were performed using GraphPad Prism 9 to compare multiple means. Significance was indicated by * for $P \leq 0.05$, ** for $P \leq 0.01$, and *** for $P \leq 0.001$. Images were blinded prior to quantification.

Funding

This work was supported by Muscular Dystrophy Association (USA) [grant number 963652 to R.K.]; the Canadian Institutes of Health Research [grant number PJT-186300 to R.K.]; the Fredrick Banting and Charles Best Canadian Institutes of Health Research Doctoral Research Award to A.R.; and the University of Ottawa Brain and Mind Institute CNMD STAR Award to A.R.

Acknowledgements

The authors thank the University of Ottawa Animal Behaviour and Physiology core for performing motor function tests. We are grateful to the Kothary laboratory for helpful discussions. We thank Dr. Emma Sutton for critical reading of the manuscript.

Conflict of Interest Statement

None declared.

Chapter 5: General Discussion

Implications for SMA therapy

Systemic SMN restoration is essential for SMA treatment

Our work has demonstrated the independent contributions of peripheral tissues to SMA pathogenesis and has emphasized the importance of treating patients systemically. Upon treatment with an IV administered scAAV9-cba-SMN, *Smn*$^{2B/-}$ mice showed a surprisingly robust rescue of weight, survival, motor function, and metabolic defects despite no detectable restoration of SMN protein to the spinal cord and no motor neuron protection. This rescue was comparable to that achieved with an ICV injection, which produced systemic restoration of SMN protein and protected motor neuron degeneration. When treated mice were aged, we demonstrated that a rescue of the peripheral organs with IV administered scAAV9-cba-SMN was sufficient to protect *Smn*$^{2B/-}$ mice up to 6 months of age.

The importance of peripheral SMN restoration was clear in early pre-clinical nusinersen studies, where a systemic injection of ASO10-27 provided strikingly longer extension of survival than a CNS-directed administration (97). As ASOs are known to cross the blood-brain-barrier in neonatal mice, the group repeated the systemic injection using a decoy ASO that neutralizes the effects of ASO10-27 in the CNS. They found that peripherally restricted ASO10-27 treatment provided a 21-fold increase of survival to severe SMA mice, and protected tail length, motor function, motor neuron counts, and the neuromuscular junction (298). Based on these models and our experiments, it appears that peripheral restoration of SMN may be sufficient to protect against SMA. However, the finding that motor neuron-specific depletion of SMN is sufficient to cause an SMA-like phenotype in mice, albeit milder than that of a systemic depletion, suggests that all cell types contribute to SMA pathogenesis (219).

The field has debated the importance of targeting the peripheral tissues in SMN restoration therapies, with several studies of CNS-directed ASO and gene therapy treatments presenting impressive extensions of survival in mouse models of SMA. One study of a nusinersen-like ASO produced a 30-fold extension of survival in Delta 7 mice after ICV injection, which was equal to that achieved by systemic injection (307). An AAV-based SMN gene replacement therapy produced a 20-fold increase in the survival of Delta 7 mice when administered ICV, a 7-fold higher increase when compared to IV administration (308). However, as observed in our findings of ICV injection on neonatal mice, it is likely that a portion of the compounds crossed the blood-brain-barrier in these studies and was not limited to the CNS. Moreover, the most impressive rescue observed in the ASO study was from simultaneous central and peripheral administration (307). Similar results are seen in an AAV9-SMN pre-clinical study, where an ICV injection causing systemic distribution of the virus provides a greater rescue to Delta7 mice than an intravenous injection, and a virus administered with a neuron-specific promoter was not sufficient to rescue Delta7 mice (193).

Overall, these results emphasize the importance of targeting the entire body with SMN-restoring treatments. Caution should therefore be taken in choosing CNS-directed treatments like nusinersen, versus systemic treatments like risdiplam and onasemnogene abeparvovec. Further, while IT onasemnogene abeparvovec therapy is the only gene therapy treatment option for heavier and older patients, this route of administration may not provide the same benefits as intravenous treatment. In fact, twice as many patients from IT onasemnogene abeparvovec clinical trials sought additional therapies compared to those from IV trials, indicating that the treatment effect may be less powerful. In older patients that cannot receive IV onasemnogene abeparvovec, alternative systemic treatments like risdiplam could be considered instead.

Early treatment is important

As observed in our studies, SMN restoring treatments can only provide a limited rescue effect. This is also evident in the wide variation in treatment responses among SMA patients and the significant portion of non-responders. In fact, several onasemnogene abeparvovec patients have begun to supplement treatment with either risdiplam or nusinersen due to a limited treatment effect, and this is now being explored in clinical trials (154,155). One reason for the limitations of the available treatments is that SMN levels may not be restored early enough when patients are treated postnatally.

Pre-clinical data has demonstrated that the efficacy of a treatment is dependent on how early a drug is administered (309,310). In fact, a recent meta-analysis of 78 studies of SMN replacement therapies in mouse models identified the timing of a treatment to have a significant effect on survival, with prenatal treatment or treatment at birth producing the greatest effect (311). Similarly, early treatment is shown to produce the best outcomes in SMA patients treated with nusinersen and in onasemnogene abeparvovec clinical trials, with presymptomatic treatment producing the strongest effects (101,144,181,312). For these reasons, newborn screening programs have been implemented in several countries to allow for treatment of SMA patients before the onset of symptoms (313).

Our data emphasizes that early treatment allows for an impressive rescue of SMA-like features in *Smn*$^{2B/-}$ mice, with injection at P1 extending survival up to the 6-month follow-up period. However, a limited protection was seen in many features, emphasizing that even early treatment is not sufficient to completely protect against SMA-like pathology. Further, it is important to note that the development of mice is delayed compared to humans, and that a P1 mouse's CNS may be more comparable to that of a prenatal human (314). Our results thus

support the widespread sentiment by researchers to continue to expand the availability of newborn screening worldwide to ensure the best outcomes for SMA patients.

These strategies could also eventually be expanded to encompass prenatal screening and prenatal treatment approaches. Patients show greater improvement in motor function when treated earlier, but a prenatal SMN restoration is likely to produce the greatest effect. Autopsy data from SMA fetuses show pathological changes at the level of the motor neuron, neuromuscular junction, and muscle at as early as 12 gestational weeks (6). SMN expression is also highest in the CNS and skeletal muscle prenatally, suggesting that postnatal treatment may miss an important therapeutic window (315). In a study of the Taiwanese mouse model, embryos were smaller than littermate controls and proteomic changes were found in several organs as early as embryonic day 14 (316). These results all point towards a need to explore the potential of prenatal SMA therapy.

Long term effectiveness of gene therapy

Our 6-month follow up results have provided support for the long-term effectiveness and safety of scAAV9-SMN gene therapy treatment. However, there is still no long-term data to assess the true longevity of onasemnogene abeparvovec treatment in SMA patients. So far, the best data available is from a 7.5 year follow-up study, where all 10 children treated with the highest gene therapy dose had survived and maintained motor milestones (123). However, these results are confounded by the supplemental treatment of nusinersen in several patients. The long-term effectiveness of gene therapy could be limited by the dilution of episomal SMN cDNA due to cell division over time. Permanent long-term expression would thus be restricted to non-dividing cells such as neurons. Of note, the therapy could also not be readministered due to acquired AAV immunity by patients after treatment. Due to these concerns, it is important to

continue to monitor the long-term effectiveness of scAAV9-SMN gene replacement therapy in pre-clinical and clinical trials. A longer follow-up experiment in animal models would likely be beneficial, as the treatment effects did not appear to decline over time in our study.

Ongoing safety concerns of gene therapy

Despite the impressive results achieved by onasemnogene abeparvovec treatment to date, the safety risks associated with gene therapy pose an obstacle to treatment. Administering a viral vector at the high dose required for IV penetration of the blood-brain-barrier can produce a dangerous immune response, leading to potentially fatal liver failure (130,132). A recent gene therapy trial for X-linked myotubular myopathy (XLMTM) saw similar results, with three patients dying of liver failure after administration of an AAV8 vector (317). Of note, these patients may have been more susceptible to liver failure as liver defects are a clinically significant feature of XLMTM (318). However, this begs the question of whether the liver abnormalities present in some SMA patients may increase their susceptibility to liver toxicity following onasemnogene abeparvovec administration.

High AAV doses were also linked to DRG toxicity in preclinical trials (143). To avoid the use of high viral doses, especially in heavier patients, lower-dose IT administration is being explored. However, our work and others suggest that treatment of the peripheral organs is essential for SMN-restoration therapy, and that CNS-restricted administration does not provide the ideal treatment. Further, safety concerns have arisen around the use of CNS-restricted therapy from a pre-clinical mouse study that found that overexpression of SMN in the CNS lead to late-onset motor dysfunction and neurodegeneration (294). Overexpression of SMN was found to cause splicing dysregulation and transcriptomic abnormalities. This also raises concerns about attempting to combine SMN-restoration therapies when one treatment does not produce

sufficient effect, as too much SMN may result in toxic effects. To balance the risks and benefits associated with both routes of administration, research should prioritize the development of viral vectors that can achieve more efficient transduction at lower doses.

SMN-independent therapies

Although SMN-dependent therapies have proven beneficial to many patients, patient outcomes can be further improved and the safety concerns of SMN overexpression can be avoided with the addition of SMN-independent therapies. Further, as SMA appears to begin developing prenatally, SMN-independent therapies can attempt to regenerate tissue damage that cannot be reversed by SMN restoration. They can also provide a therapeutic option for older SMA patients who did not have access to SMN-restoring therapies during the early therapeutic window.

Motor neuron regeneration through stem cell-based therapy has been explored in mouse models. Isolated spinal cord neural stem cells and embryonic-derived neural stem cells grafted intrathecally into Delta 7 mice resulted in improvements in motor function and extended survival (319,320). SMA patient-derived induced pluripotent stem cells were also genetically modified to produce functional SMN protein and subsequent implantation into Delta7 mice improved the disease phenotype (321). This approach was proposed for human studies but has yet to be approved for use in clinical trials (322).

Therapies that aim to regenerate muscle have been explored with much more success. Reldesemtiv works as a fast skeletal muscle troponin activator, aiming to enhance muscle force and improve motor function (323). This treatment has proven effective in phase 2 clinical trials of Type 2-4 SMA patients at improving walking distance and breathing pressure (324). Another strategy to target muscles focuses on myostatin inhibition to increase muscle mass. Apitegromab

is a monoclonal antibody that inhibits activation of myostatin, a regulator of skeletal muscle growth and strength (325). This treatment has shown success in Phase 3 trials, producing improvements in motor function in ambulatory and nonambulatory Type 2 and 3 SMA patients, and will be submitted to the FDA for approval by Scholar Rock in 2025 (326).

Overall, SMN-independent therapies appear to be able to regenerate muscle that has been subject to SMN-irreversible damage. These therapies, as well as additional strategies that can aim to regenerate other tissues affected in SMA, may be the best way to target older patients, treat non-responders, and optimize the outcomes of current SMA restorative treatments. However, to produce these treatments, we must develop a better understanding of the multisystem nature of SMA and the mechanisms of disease in each affected tissue.

Implications for SMA as a multisystem disease

This work has compiled a collection of evidence to support the theory that SMA is a multisystem disease. Firstly, the *Smn*$^{2B/-}$ mouse, a mouse model of SMA that demonstrates peripheral organ defects similar to those increasingly reported by SMA patients, demonstrated hepatic, muscular, and metabolic defects prior to the loss of motor neuron cell bodies in the spinal cord. Secondly, IV administered scAAV9-cba-SMN provided a long-term rescue of motor function and survival without any apparent effects on the spinal cord. Systemic SMN restoration has also proven to be more effective than CNS-specific restoration at improving disease in several mouse models of SMA, as outlined above. Altogether, this evidence suggests that peripheral tissue defects precede motor neuron loss and may influence neurodegeneration.

The role of the liver in SMA pathogenesis

Reports from SMA patients and findings from animal models have described the involvement of several non-neuronal tissues in SMA including skeletal muscle (30,31), heart (197,200), vasculature (201), pancreas (34), liver (32,33), and immune organs (35). The *Smn*$^{2B/-}$ mouse model displays liver steatosis, damage, and dysfunction which develop before the onset of motor neuron degeneration. This was first observed at P13 in our study, prior to the onset of muscle atrophy, NMJ denervation, and pancreatic defects and was seen as early as P9 in other work (32). Additionally, higher proteomic differences were observed in the liver than the brain, SC, muscle, and heart of embryonic Taiwanese SMA mice (316).

The liver plays an important role in metabolism, detoxification, growth, and development, and the impacts of a potentially dysfunctional liver on the etiology of SMA disease should not be overlooked. The early emergence of liver pathology in mouse models, as well as the striking effectiveness of peripheral centred therapy, point towards an important role for the liver and metabolism in SMA pathogenesis. In fact, two recent studies from our laboratory have described the impact of the liver on other systems in *Smn*$^{2B/-}$ mice, revealing a connection between liver dysfunction and pancreatic pathology. A liver-directed scAAV9-SMN under the control of an albumin promoter rescued the liver phenotype in *Smn*$^{2B/-}$ mice and consequently protected against pancreatic defects (209). Further, a liver-specific SMN depletion in *Smn*$^{2B/-}$ mice produced a pancreatic phenotype similar to that of *Smn*$^{2B/-}$ mice, suggesting that pancreatic defects may be a result of liver steatosis (327).

The liver is also an important producer of growth factors and neurotrophic factors. For example, IGF1 has been found to be significantly decreased in serum of SMA patients and SMA mouse models (33,97,207). IGF1 is a neurotrophic factor produced by the liver that also plays an

important role in growth and development (328). Delayed growth is a hallmark symptom of SMA and is seen as early as the embryonic stage in SMA mice, indicating that early liver defects could be responsible. Treatments that increase IGF1 expression have shown to protect motor neuron degeneration and improve the phenotype of severe and Delta 7 mice (329,330). As IGF1 is also a neurotrophic factor, treatment with human neural progenitor cells over-expressing IGF1 has also been shown to protect neurons and restore motor function in a rat model of Parkinson's disease (331). This suggests that growth and neurodevelopment pathways may be disrupted early in SMA mice due to liver defects, contributing to the development of SMA.

Overall, there is a need for further investigation into the SMN-dependent molecular pathways contributing to liver defects and the impacts of liver pathology on other systems in SMA patients. Liver-directed treatment options should also be explored as a method of improving the outcomes of available therapies. IGF1 therapy presents as a possible SMN-independent treatment option, as it has been used to treat children with growth hormone deficiency and can penetrate the blood-brain-barrier (332). Further, the susceptibility of SMA patients to liver defects should be taken into account during treatment with onasemnogene abeparvovec, which is known to cause potentially life-threatening hepatotoxicity (130), and for patients taking risdiplam, which is metabolized by the liver (333). Nusinersen treatment may be a favourable option for patients with hepatic impairment, as it is primarily metabolized by the kidneys (334).

NfL as a biomarker for SMA

NfL as a prognostic biomarker and marker of disease progression

The above work outlined a potential role for NfL as a prognostic biomarker, as it was elevated in *Smn*$^{2B/-}$ mice several days prior to the onset of motor neuron degeneration. In patient

studies, NfL levels were found to be higher in the serum of patients with fewer SMN2 copy numbers, and have been found to be associated with earlier age of disease onset, indicating its potential for the prognosis of SMA (185,232,335). Currently, the best tool for determining the prognosis of an SMA patient is through genetic profiling of *SMN2* copy number. However, there is variability in disease presentation between copy numbers and this number does not change over time to reflect disease progression. Our work also showed a progressive increase in NfL levels as the disease phenotype worsened in $Smn^{2B/-}$ mice, indicating its potential as a marker of disease progression. Nevertheless, long-term assessments of NfL levels in SMA patients would be needed to determine its potential as a disease progression biomarker, but this is complicated by SMA patients being typically treated at the earliest possible stage of disease.

NfL as a pharmacodynamic biomarker

Nusinersen studies have found declines in plasma pNF-H and CSF NfL levels over time in treated patients (336,337). However, this should be interpreted with caution as NF levels are known to decline with age in infants. We observed this trend in our mouse study as well, where both heterozygous and $Smn^{2B/-}$ mice showed early elevations in NfL that declined quickly with age. One study using age-matched sham-control treated infants found that blood pNF-H levels declined more rapidly in nusinersen treated patients than placebo treated patients, indicating the utility of NFs as biomarkers of treatment response (179). However, NFs have not yet shown useful as biomarkers of other SMA treatments. In our work, $Smn^{2B/-}$ mice treated with IV scAAV9-SMN showed elevations in NfL equal to those of untreated $Smn^{2B/-}$ mice, while ICV treated mice had lower NfL levels than IV-treated and untreated $Smn^{2B/-}$ mice. These results were observed at P19 and again at 6 months after injection. This indicated that NfL is likely only representative of motor neuron degeneration, as this feature was only rescued in ICV-treated

mice while survival, motor function, and peripheral features were rescued by both treatments. These results further enforce the influence of the peripheral tissues on survival and motor function and suggest that caution should be taken when using NfL as a sole biomarker for SMA. Multiple biomarkers may instead be needed to monitor the full picture in an SMA patient, especially in those receiving systemic treatments. NfL may be more suitable for nusinersen-treated patients as this treatment mainly targets the CNS.

Alternative molecular biomarkers for SMA

As we are beginning to recognize the metabolic features of SMA, molecular biomarkers like IGF1, insulin-like growth factor binding protein 6 (IGFB6) and dipeptyl peptidase IV (DPPIV) have been found to be reduced in SMA patients and are being explored as potential biomarkers (208,338,339). Our lab recently demonstrated a restoration of IGF1 levels in *Smn*$^{2B/-}$ mice after a liver directed scAAV9-SMN treatment (209). Another group restored DPPIV levels in Delta 7 mice after ASO treatment (339). Serum creatinine and creatine kinase are biomarkers of muscle mass and muscle function that have been shown to correlate with disease severity and motor function in SMA patients (340,341). Overall, NfL is only representative of a small portion of SMA pathology, and it is not a useful biomarker in older patients. There is a need to implement additional biomarkers into clinical trials to allow for better monitoring of patients' responses to treatment. A better understanding of the multi-system nature of SMA is essential to identifying these biomarkers.

Conclusion

Our findings, along with the breadth of evidence continuing to emerge in the field, underscore the complexity of SMA as a multisystem disease. We have demonstrated the

importance of peripheral SMN restoration in the treatment of SMA and have suggested that a systemic restoration provides the most effective treatment. While early treatment is critical to SMN-dependent therapy, the limitations of these therapies highlight the need for further investigation into additional SMN-independent treatments. A more diverse set of biomarkers must also be developed to monitor all aspects of SMA, including neuronal, muscular, and metabolic features. All of this can be achieved with a deeper understanding of the mechanisms of the disease and the function of the SMN protein in each affected tissue.

References

1. Sugarman EA, Nagan N, Zhu H, Akmaev VR, Zhou Z, Rohlfs EM, et al. Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. Eur J Hum Genet EJHG. 2012 Jan;20(1):27–32.

2. Verhaart IEC, Robertson A, Wilson IJ, Aartsma-Rus A, Cameron S, Jones CC, et al. Prevalence, incidence and carrier frequency of 5q-linked spinal muscular atrophy - a literature review. Orphanet J Rare Dis. 2017 Jul 4;12(1):124.

3. Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell. 1995 Jan 13;80(1):155–65.

4. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci. 1999 May 25;96(11):6307–11.

5. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH, et al. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum Mol Genet. 1999 Jul;8(7):1177–83.

6. Sumner CJ, Paushkin S, Ko CP. Spinal Muscular Atrophy: Disease Mechanisms and Therapy. London (UK). Academic Press; 2017

7. Vitte J, Fassier C, Tiziano FD, Dalard C, Soave S, Roblot N, et al. Refined Characterization of the Expression and Stability of the SMN Gene Products. Am J Pathol. 2007 Oct;171(4):1269–80.

8. Curry M, Cruz R, Belter L, Schroth M, Lenz M, Jarecki J. Awareness screening and referral patterns among pediatricians in the United States related to early clinical features of spinal muscular atrophy (SMA). BMC Pediatr. 2021 May 17;21(1):236.

9. Pierzchlewicz K, Kępa I, Podogrodzki J, Kotulska K. Spinal Muscular Atrophy: The Use of Functional Motor Scales in the Era of Disease-Modifying Treatment. Child Neurol Open. 2021 Jan 1;8:2329048X211008725.

10. Sleutjes BTHM, Wijngaarde CA, Wadman RI, Otto LAM, Asselman FL, Cuppen I, et al. Assessment of motor unit loss in patients with spinal muscular atrophy. Clin Neurophysiol. 2020 Jun 1;131(6):1280–6.

11. Hoffmann J. Ueber chronische spinale Muskelatrophie im Kindesalter, auf familiiirer Basis. Deutsche Zeitschrift für Nervenheilkunde. 1893 May;3:427-70.

12. Harding BN, Kariya S, Monani UR, Chung WK, Benton M, Yum SW, et al. Spectrum of Neuropathophysiology in Spinal Muscular Atrophy Type I. J Neuropathol Exp Neurol. 2015 Jan 1;74(1):15–24.

13. Murayama S, Bouldin TW, Suzuki K. Immunocytochemical and ultrastructural studies of Werdnig-Hoffmann disease. Acta Neuropathol (Berl). 1991 Mar 1;81(4):408–17.

14. Chou SM, Nonaka I. Werdnig-Hoffmann disease: Proposal of a pathogenetic mechanism. Acta Neuropathol (Berl). 1978 Jan 1;41(1):45–54.

15. Ghatak NR. Spinal roots in Werdnig-Hoffmann disease. Acta Neuropathol (Berl). 1978 Jan 1;41(1):1–7.

16. Chou SM, Fakadej AV. Ultrastructure of chromatolytic motoneurons and anterior spinal roots in a case of Werdnig-Hoffmann disease. J Neuropathol Exp Neurol. 1971 Jul;30(3):368–79.

17. Dubowitz V, Sewry CA, Oldfors A. Muscle Biopsy, 5th Edition. Amsterdam, Netherlands. Elsevier Inc.; 2020

18. Mastaglia FL, Walton JN. Histological and histochemical changes in skeletal muscle from cases of chronic juvenile and early adult spinal muscular atrophy (the Kugelberg-Welander syndrome). J Neurol Sci. 1971 Jan 1;12(1):15–44.

19. Zalneraitis EL, Halperin JJ, Grunnet ML, Russman BS, Peress N. Muscle Biopsy and the Clinical Course of Infantile Spinal Muscular Atrophy. J Child Neurol. 1991 Oct 1;6(4):324–8.

20. Martínez-Hernández R, Bernal S, Also-Rallo E, Alías L, Barceló MJ, Hereu M, et al. Synaptic defects in type I spinal muscular atrophy in human development. J Pathol. 2013 Jan;229(1):49–61.

21. Rochette CF, Gilbert N, Simard LR. SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens. Hum Genet. 2001 Mar;108(3):255–66.

22. Monani UR, Sendtner M, Coovert DD, Parsons DW, Andreassi C, Le TT, et al. The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. Hum Mol Genet. 2000 Feb 12;9(3):333–9.

23. Schrank B, Götz R, Gunnersen JM, Ure JM, Toyka KV, Smith AG, et al. Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. Proc Natl Acad Sci U S A. 1997 Sep 2;94(18):9920–5.

24. Jablonka S, Schrank B, Kralewski M, Rossoll W, Sendtner M. Reduced survival motor neuron (Smn) gene dose in mice leads to motor neuron degeneration: an animal model for spinal muscular atrophy type III. Hum Mol Genet. 2000 Feb 12;9(3):341–6.

25. Hsieh-Li HM, Chang JG, Jong YJ, Wu MH, Wang NM, Tsai CH, et al. A mouse model for spinal muscular atrophy. Nat Genet. 2000 Jan;24(1):66–70.

26. Tsai LK, Tsai MS, Lin TB, Hwu WL, Li H. Establishing a standardized therapeutic testing protocol for spinal muscular atrophy. Neurobiol Dis. 2006 Nov 1;24(2):286–95.

27. Le TT, Pham LT, Butchbach MER, Zhang HL, Monani UR, Coovert DD, et al. SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. Hum Mol Genet. 2005 Mar 15;14(6):845–57.

28. DiDonato CJ, Lorson CL, De Repentigny Y, Simard L, Chartrand C, Androphy EJ, et al. Regulation of murine survival motor neuron (Smn) protein levels by modifying Smn exon 7 splicing. Hum Mol Genet. 2001 Nov 1;10(23):2727–36.

29. Eshraghi M, McFall E, Gibeault S, Kothary R. Effect of genetic background on the phenotype of the Smn2B/- mouse model of spinal muscular atrophy. Hum Mol Genet. 2016 Oct 15;25(20):4494–506.

30. Boyer JG, Murray LM, Scott K, De Repentigny Y, Renaud JM, Kothary R. Early onset muscle weakness and disruption of muscle proteins in mouse models of spinal muscular atrophy. Skelet Muscle. 2013 Oct 11;3(1):24.

31. Boyer JG, Deguise MO, Murray LM, Yazdani A, De Repentigny Y, Boudreau-Larivière C, et al. Myogenic program dysregulation is contributory to disease pathogenesis in spinal muscular atrophy. Hum Mol Genet. 2014 Aug 15;23(16):4249–59.

32. Deguise MO, Baranello G, Mastella C, Beauvais A, Michaud J, Leone A, et al. Abnormal fatty acid metabolism is a core component of spinal muscular atrophy. Ann Clin Transl Neurol. 2019;6(8):1519–32.

33. Deguise MO, Pileggi C, De Repentigny Y, Beauvais A, Tierney A, Chehade L, et al. SMN Depleted Mice Offer a Robust and Rapid Onset Model of Nonalcoholic Fatty Liver Disease. Cell Mol Gastroenterol Hepatol. 2021;12(1):354-377.e3.

34. Bowerman M, Swoboda KJ, Michalski JP, Wang GS, Reeks C, Beauvais A, et al. Glucose metabolism and pancreatic defects in spinal muscular atrophy. Ann Neurol. 2012 Aug;72(2):256–68.

35. Deguise MO, De Repentigny Y, McFall E, Auclair N, Sad S, Kothary R. Immune dysregulation may contribute to disease pathogenesis in spinal muscular atrophy mice. Hum Mol Genet. 2017 Feb 15;26(4):801–19.

36. Gombash SE, Cowley CJ, Fitzgerald JA, Iyer CC, Fried D, McGovern VL, et al. SMN deficiency disrupts gastrointestinal and enteric nervous system function in mice. Hum Mol Genet. 2015 Jul 1;24(13):3847–60.

37. Deguise MO, De Repentigny Y, Tierney A, Beauvais A, Michaud J, Chehade L, et al. Motor transmission defects with sex differences in a new mouse model of mild spinal muscular atrophy. EBioMedicine. 2020 Apr 24;55:102750.

38. Bowerman M, Murray LM, Beauvais A, Pinheiro B, Kothary R. A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. Neuromuscul Disord. 2012 Mar 1;22(3):263–76.

39. Oprea GE, Kröber S, McWhorter ML, Rossoll W, Müller S, Krawczak M, et al. Plastin 3 Is a Protective Modifier of Autosomal Recessive Spinal Muscular Atrophy. Science. 2008 Apr 25;320(5875):524–7.

40. Hosseinibarkooie S, Peters M, Torres-Benito L, Rastetter RH, Hupperich K, Hoffmann A, et al. The Power of Human Protective Modifiers: PLS3 and CORO1C Unravel Impaired Endocytosis in Spinal Muscular Atrophy and Rescue SMA Phenotype. Am J Hum Genet. 2016 Sep 1;99(3):647–65.

41. Helmken C, Hofmann Y, Schoenen F, Oprea G, Raschke H, Rudnik-Schöneborn S, et al. Evidence for a modifying pathway in SMA discordant families: reduced SMN level decreases the amount of its interacting partners and Htra2-beta1. Hum Genet. 2003 Dec 1;114(1):11–21.

42. Doran B, Gherbesi N, Hendricks G, Flavell RA, Davis RJ, Gangwani L. Deficiency of the zinc finger protein ZPR1 causes neurodegeneration. Proc Natl Acad Sci U S A. 2006 May 9;103(19):7471–5.

43. Ahmad S, Wang Y, Shaik GM, Burghes AH, Gangwani L. The zinc finger protein ZPR1 is a potential modifier of spinal muscular atrophy. Hum Mol Genet. 2012 Jun 15;21(12):2745–58.

44. Akutsu T, Nishio H, Sumino K, Takeshima Y, Tsuneishi S, Wada H, et al. Molecular Genetics of Spinal Muscular Atrophy: Contribution of the NAIP Gene to Clinical Severity. Kobe Journal of Medical Sciences. 2002 Feb 31;48(1/2):25-32.

45. Abd El Mutaleb ANH, Ibrahim FAR, Megahed FAK, Atta A, Ali BA, Omar TEI, et al. NAIP Gene Deletion and SMN2 Copy Number as Molecular Tools in Predicting the Severity of Spinal Muscular Atrophy. Biochem Genet. 2024 Feb 22:1-22.

46. Ahn EJ, Yum MS, Kim EH, Yoo HW, Lee BH, Kim GH, et al. Genotype-Phenotype Correlation of SMN1 and NAIP Deletions in Korean Patients with Spinal Muscular Atrophy. J Clin Neurol Seoul Korea. 2017 Jan;13(1):27–31.

47. Battaglia G, Princivalle A, Forti F, Lizier C, Zeviani M. Expression of the SMN Gene, the Spinal Muscular Atrophy Determining Gene, in the Mammalian Central Nervous System. Hum Mol Genet. 1997 Oct 1;6(11):1961–71.

48. Coovert DD, Le TT, McAndrew PE, Strasswimmer J, Crawford TO, Mendell JR, et al. The Survival Motor Neuron Protein in Spinal Muscular Atrophy. Hum Mol Genet. 1997 Aug 1;6(8):1205–14.

49. Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell. 1995 Jan;80(1):155–65.

50. Liu Q, Dreyfuss G. A novel nuclear structure containing the survival of motor neurons protein. EMBO J. 1996 Jul 15;15(14):3555–65.

51. Morris GE. The Cajal body. Biochim Biophys Acta BBA - Mol Cell Res. 2008 Nov 1;1783(11):2108–15.

52. Liu Q, Fischer U, Wang F, Dreyfuss G. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. Cell. 1997 Sep 19;90(6):1013–21.

53. Carissimi C, Saieva L, Baccon J, Chiarella P, Maiolica A, Sawyer A, et al. Gemin8 Is a Novel Component of the Survival Motor Neuron Complex and Functions in Small Nuclear Ribonucleoprotein Assembly *. J Biol Chem. 2006 Mar 24;281(12):8126–34.

54. Gubitz AK, Mourelatos Z, Abel L, Rappsilber J, Mann M, Dreyfuss G. Gemin5, a Novel WD Repeat Protein Component of the SMN Complex That Binds Sm Proteins *. J Biol Chem. 2002 Feb 15;277(7):5631–6.

55. Carissimi C, Baccon J, Straccia M, Chiarella P, Maiolica A, Sawyer A, et al. Unrip is a component of SMN complexes active in snRNP assembly. FEBS Lett. 2005 Apr 25;579(11):2348–54.

56. See K, Yadav P, Giegerich M, Cheong PS, Graf M, Vyas H, et al. SMN deficiency alters Nrxn2 expression and splicing in zebrafish and mouse models of spinal muscular atrophy. Hum Mol Genet. 2014 Apr 1;23(7):1754–70.

57. Sleigh JN, Barreiro-Iglesias A, Oliver PL, Biba A, Becker T, Davies KE, et al. Chondrolectin affects cell survival and neuronal outgrowth in in vitro and in vivo models of spinal muscular atrophy. Hum Mol Genet. 2014 Feb 15;23(4):855–69.

58. Zhang Z, Lotti F, Dittmar K, Younis I, Wan L, Kasim M, et al. SMN Deficiency Causes Tissue-Specific Perturbations in the Repertoire of snRNAs and Widespread Defects in Splicing. Cell. 2008 May 16;133(4):585–600.

59. Baumer D, Lee S, Nicholson G, Davies JL, Parkinson NJ, Murray L, et al. Alternative Splicing Events Are a Late Feature of Pathology in a Mouse Model of Spinal Muscular Atrophy. PLoS Genet. 2009 Dec;5(12):e1000773.

60. Akten B, Kye MJ, Hao LT, Wertz MH, Singh S, Nie D, et al. Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. Proc Natl Acad Sci. 2011 Jun 21;108(25):10337–42.

61. Fallini C, Rouanet JP, Donlin-Asp PG, Guo P, Zhang H, Singer RH, et al. Dynamics of survival of motor neuron (SMN) protein interaction with the mRNA-binding protein IMP1 facilitates its trafficking into motor neuron axons. Dev Neurobiol. 2014;74(3):319–32.

62. Lorson CL, Androphy EJ. The Domain Encoded by Exon 2 of the Survival Motor Neuron Protein Mediates Nucleic Acid Binding. Hum Mol Genet. 1998 Aug 1;7(8):1269–75.

63. Zhang HL, Pan F, Hong D, Shenoy SM, Singer RH, Bassell GJ. Active Transport of the Survival Motor Neuron Protein and the Role of Exon-7 in Cytoplasmic Localization. J Neurosci. 2003 Jul 23;23(16):6627–37.

64. Rossoll W, Jablonka S, Andreassi C, Kröning AK, Karle K, Monani UR, et al. Smn, the spinal muscular atrophy–determining gene product, modulates axon growth and localization of β-actin mRNA in growth cones of motoneurons. J Cell Biol. 2003 Nov 17;163(4):801–12.

65. Peter CJ, Evans M, Thayanithy V, Taniguchi-Ishigaki N, Bach I, Kolpak A, et al. The COPI vesicle complex binds and moves with survival motor neuron within axons. Hum Mol Genet. 2011 May 1;20(9):1701–11.

66. Donlin-Asp PG, Fallini C, Campos J, Chou CC, Merritt ME, Phan HC, et al. The Survival of Motor Neuron Protein Acts as a Molecular Chaperone for mRNP Assembly. Cell Rep. 2017 Feb 14;18(7):1660–73.

67. Saal L, Briese M, Kneitz S, Glinka M, Sendtner M. Subcellular transcriptome alterations in a cell culture model of spinal muscular atrophy point to widespread defects in axonal growth and presynaptic differentiation. RNA. 2014 Jan 11;20(11):1789–802.

68. Fallini C, Donlin-Asp PG, Rouanet JP, Bassell GJ, Rossoll W. Deficiency of the Survival of Motor Neuron Protein Impairs mRNA Localization and Local Translation in the Growth Cone of Motor Neurons. J Neurosci. 2016 Mar 30;36(13):3811–20.

69. Sanchez G, Dury AY, Murray LM, Biondi O, Tadesse H, El Fatimy R, et al. A novel function for the survival motoneuron protein as a translational regulator. Hum Mol Genet. 2013 Feb 15;22(4):668–84.

70. Kye MJ, Niederst ED, Wertz MH, Gonçalves I do CG, Akten B, Dover KZ, et al. SMN regulates axonal local translation via miR-183/mTOR pathway. Hum Mol Genet. 2014 Dec 1;23(23):6318–31.

71. Bernabò P, Tebaldi T, Groen EJN, Lane FM, Perenthaler E, Mattedi F, et al. In Vivo Translatome Profiling in Spinal Muscular Atrophy Reveals a Role for SMN Protein in Ribosome Biology. Cell Rep. 2017 Oct 24;21(4):953–65.

72. Wang CH, Finkel RS, Bertini ES, Schroth M, Simonds A, Wong B, et al. Consensus Statement for Standard of Care in Spinal Muscular Atrophy. J Child Neurol. 2007 Aug 1;22(8):1027–49.

73. Mercuri E, Finkel RS, Muntoni F, Wirth B, Montes J, Main M, et al. Diagnosis and management of spinal muscular atrophy: Part 1: Recommendations for diagnosis, rehabilitation, orthopedic and nutritional care. Neuromuscul Disord. 2018 Feb 1;28(2):103–15.

74. Finkel RS, Mercuri E, Meyer OH, Simonds AK, Schroth MK, Graham RJ, et al. Diagnosis and management of spinal muscular atrophy: Part 2: Pulmonary and acute care; medications, supplements and immunizations; other organ systems; and ethics. Neuromuscul Disord. 2018 Mar 1;28(3):197–207.

75. Wiederholt WC. Guanidine hydrochloride therapy in neuromuscular disorders. West J Med. 1975 Aug;123(2):132–3.

76. Angelini C, Micaglio GF, Trevisan C. Guanidine hydrochloride in infantile and juvenile spinal muscular atrophy. A double blind controlled study. Acta Neurol (Napoli). 1980 Dec;2(6):460–5.

77. Steinböck H, Binder H, Gerstenbrand F, Pilz E, Maida E. [The efficacy of guanidine hydrochloride in the treatment of Werdnig-Hoffmann disease (author's transl)]. Wien Klin Wochenschr. 1977 Apr 15;89(8):269–72.

78. Takeuchi Y, Miyanomae Y, Komatsu H, Oomizono Y, Nishimura A, Okano S, et al. Efficacy of Thyrotropin-Releasing Hormone in the Treatment of Spinal Muscular Atrophy. J Child Neurol. 1994 Jul 1;9(3):287–9.

79. Merlini L, Solari A, Vita G, Bertini E, Minetti C, Mongini T, et al. Role of gabapentin in spinal muscular atrophy: results of a multicenter, randomized Italian study. J Child Neurol. 2003 Aug;18(8):537–41.

80. Miller RG, Moore DH, Dronsky V, Bradley W, Barohn R, Bryan W, et al. A placebo-controlled trial of gabapentin in spinal muscular atrophy. J Neurol Sci. 2001 Oct 15;191(1):127–31.

81. Tzeng AC, Cheng J, Fryczynski H, Niranjan V, Stitik T, Sial A, et al. A Study of Thyrotropin-Releasing Hormone for the Treatment of Spinal Muscular Atrophy: A Preliminary Report. Am J Phys Med Rehabil. 2000 Oct;79(5):435.

82. Wong BL, Hynan LS, Iannaccone ST, Group and the ASMART (AmSMART). A Randomized, Placebo-Controlled Trial of Creatine in Children With Spinal Muscular Atrophy. J Clin Neuromuscul Dis. 2007 Mar;8(3):101.

83. Chang JG, Hsieh-Li HM, Jong YJ, Wang NM, Tsai CH, Li H. Treatment of spinal muscular atrophy by sodium butyrate. Proc Natl Acad Sci. 2001 Aug 14;98(17):9808–13.

84. Avila AM, Burnett BG, Taye AA, Gabanella F, Knight MA, Hartenstein P, et al. Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy. J Clin Invest. 2007 Mar;117(3):659–71.

85. Brichta L, Hofmann Y, Hahnen E, Siebzehnrubl FA, Raschke H, Blumcke I, et al. Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. Hum Mol Genet. 2003 Oct 1;12(19):2481–9.

86. Andreassi C, Angelozzi C, Tiziano FD, Vitali T, De Vincenzi E, Boninsegna A, et al. Phenylbutyrate increases SMN expression in vitro: relevance for treatment of spinal muscular atrophy. Eur J Hum Genet. 2004 Jan;12(1):59–65.

87. Swoboda KJ, Scott CB, Crawford TO, Simard LR, Reyna SP, Krosschell KJ, et al. SMA CARNI-VAL trial part I: double-blind, randomized, placebo-controlled trial of L-carnitine and valproic acid in spinal muscular atrophy. PloS One. 2010 Aug 19;5(8):e12140.

88. Kissel JT, Scott CB, Reyna SP, Crawford TO, Simard LR, Krosschell KJ, et al. SMA CARNIVAL TRIAL PART II: a prospective, single-armed trial of L-carnitine and valproic acid in ambulatory children with spinal muscular atrophy. PloS One. 2011;6(7):e21296.

89. Mercuri E, Bertini E, Messina S, Solari A, D'Amico A, Angelozzi C, et al. Randomized, double-blind, placebo-controlled trial of phenylbutyrate in spinal muscular atrophy. Neurology. 2007 Jan 2;68(1):51–5.

90. Grzeschik SM, Ganta M, Prior TW, Heavlin WD, Wang CH. Hydroxyurea enhances SMN2 gene expression in spinal muscular atrophy cells. Ann Neurol. 2005;58(2):194–202.

91. Liang WC, Yuo CY, Chang JG, Chen YC, Chang YF, Wang HY, et al. The effect of hydroxyurea in spinal muscular atrophy cells and patients. J Neurol Sci. 2008 May 15;268(1):87–94.

92. Angelozzi C, Borgo F, Tiziano FD, Martella A, Neri G, Brahe C. Salbutamol increases SMN mRNA and protein levels in spinal muscular atrophy cells. J Med Genet. 2008 Jan 1;45(1):29–31.

93. Kinali M, Mercuri E, Main M, De Biasia F, Karatza A, Higgins R, et al. Pilot trial of albuterol in spinal muscular atrophy. Neurology. 2002 Aug 27;59(4):609–10.

94. Hua Y, Vickers TA, Baker BF, Bennett CF, Krainer AR. Enhancement of SMN2 Exon 7 Inclusion by Antisense Oligonucleotides Targeting the Exon. PLOS Biol. 2007 Mar 13;5(4):e73.

95. Hua Y, Vickers TA, Okunola HL, Bennett CF, Krainer AR. Antisense Masking of an hnRNP A1/A2 Intronic Splicing Silencer Corrects SMN2 Splicing in Transgenic Mice. Am J Hum Genet. 2008 Apr 4;82(4):834.

96. Hua Y, Sahashi K, Hung G, Rigo F, Passini MA, Bennett CF, et al. Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. Genes Dev. 2010 Aug 1;24(15):1634–44.

97. Hua Y, Sahashi K, Rigo F, Hung G, Horev G, Bennett CF, et al. Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. Nature. 2011 Oct 5;478(7367):123–6.

98. Passini MA, Bu J, Richards AM, Kinnecom C, Sardi SP, Stanek LM, et al. Antisense Oligonucleotides Delivered to the Mouse CNS Ameliorate Symptoms of Severe Spinal Muscular Atrophy. Sci Transl Med. 2011 Mar 2;3(72):72ra18.

99. Darrow JJ, Sharma M, Shroff M, Wagner AK. Efficacy and costs of spinal muscular atrophy drugs. Sci Transl Med. 2020 Nov 11;12(569):eaay9648.

100. Zhong ZJ, Zheng PM, Dou HH, Wang JG. Adverse events in the treatment of spinal muscular atrophy in children and adolescents with nusinersen: A systematic review and meta-analysis. Front Pediatr. 2023 Apr 25;11:1152318.

101. Crawford TO, Swoboda KJ, De Vivo DC, Bertini E, Hwu WL, Finkel RS, et al. Continued benefit of nusinersen initiated in the presymptomatic stage of spinal muscular atrophy: 5-year update of the NURTURE study. Muscle Nerve. 2023 Aug;68(2):157–70.

102. Pane M, Coratti G, Sansone VA, Messina S, Catteruccia M, Bruno C, et al. Type I spinal muscular atrophy patients treated with nusinersen: 4-year follow-up of motor, respiratory and bulbar function. Eur J Neurol. 2023;30(6):1755–63.

103. Rogers S, Pfuderer P. Use of viruses as carriers of added genetic information. Nature. 1968 Aug 17;219(5155):749–51.

104. Puumalainen AM, Vapalahti M, Agrawal RS, Kossila M, Laukkanen J, Lehtolainen P, et al. Beta-galactosidase gene transfer to human malignant glioma in vivo using replication-deficient retroviruses and adenoviruses. Hum Gene Ther. 1998 Aug 10;9(12):1769–74.

105. Marshall E. Gene Therapy Death Prompts Review of Adenovirus Vector. Science. 1999 Dec 17;286(5448):2244–5.

106. Nienhuis AW, Dunbar CE, Sorrentino BP. Genotoxicity of Retroviral Integration In Hematopoietic Cells. Mol Ther. 2006 Jun 1;13(6):1031–49.

107. Azzouz M, Ralph GS, Storkebaum E, Walmsley LE, Mitrophanous KA, Kingsman SM, et al. VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. Nature. 2004 May;429(6990):413–7.

108. Azzouz M, Le T, Ralph GS, Walmsley L, Monani UR, Lee DCP, et al. Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. J Clin Invest. 2004 Dec 15;114(12):1726–31.

109. Schultz BR, Chamberlain JS. Recombinant Adeno-associated Virus Transduction and Integration. Mol Ther. 2008 Jul 1;16(7):1189–99.

110. Marks WJ, Ostrem JL, Verhagen L, Starr PA, Larson PS, Bakay RA, et al. Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2–neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. Lancet Neurol. 2008 May 1;7(5):400–8.

111. Kaplitt MG, Feigin A, Tang C, Fitzsimons HL, Mattis P, Lawlor PA, et al. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. The Lancet. 2007 Jun 23;369(9579):2097–105.

112. Inagaki K, Fuess S, Storm TA, Gibson GA, Mctiernan CF, Kay MA, et al. Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. Mol Ther. 2006 Jul 1;14(1):45–53.

113. Nakai H, Fuess S, Storm TA, Muramatsu S ichi, Nara Y, Kay MA. Unrestricted Hepatocyte Transduction with Adeno-Associated Virus Serotype 8 Vectors in Mice. J Virol. 2005 Jan;79(1):214–24.

114. Wang Z, Zhu T, Qiao C, Zhou L, Wang B, Zhang J, et al. Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. Nat Biotechnol. 2005 Mar;23(3):321–8.

115. Blankinship MJ, Gregorevic P, Allen JM, Harper SQ, Harper H, Halbert CL, et al. Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. Mol Ther. 2004 Oct 1;10(4):671–8.

116. Duque S, Joussemet B, Riviere C, Marais T, Dubreil L, Douar AM, et al. Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. Mol Ther J Am Soc Gene Ther. 2009 Jul;17(7):1187–96.

117. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. Nat Biotechnol. 2009 Jan;27(1):59–65.

118. Benkhelifa-Ziyyat S, Besse A, Roda M, Duque S, Astord S, Carcenac R, et al. Intramuscular scAAV9-SMN Injection Mediates Widespread Gene Delivery to the Spinal Cord and Decreases Disease Severity in SMA Mice. Mol Ther. 2013 Feb 1;21(2):282–90.

119. Dominguez E, Marais T, Chatauret N, Benkhelifa-Ziyyat S, Duque S, Ravassard P, et al. Intravenous scAAV9 delivery of a codon-optimized SMN1 sequence rescues SMA mice. Hum Mol Genet. 2011 Feb 15;20(4):681–93.

120. Glascock JJ, Shababi M, Wetz MJ, Krogman MM, Lorson CL. Direct central nervous system delivery provides enhanced protection following vector mediated gene replacement in a severe model of Spinal Muscular Atrophy. Biochem Biophys Res Commun. 2012 Jan 6;417(1):376–81.

121. Valori CF, Ning K, Wyles M, Mead RJ, Grierson AJ, Shaw PJ, et al. Systemic Delivery of scAAV9 Expressing SMN Prolongs Survival in a Model of Spinal Muscular Atrophy. Sci Transl Med. 2010 Jun 9;2(35):35ra42-35ra42.

122. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. N Engl J Med. 2017 Nov 2;377(18):1713–22.

123. Mendell JR, Al-Zaidy SA, Lehman KJ, McColly M, Lowes LP, Alfano LN, et al. Five-Year Extension Results of the Phase 1 START Trial of Onasemnogene Abeparvovec in Spinal Muscular Atrophy. JAMA Neurol. 2021 Jul 1;78(7):834–41.

124. Strauss KA, Farrar MA, Muntoni F, Saito K, Mendell JR, Servais L, et al. Onasemnogene abeparvovec for presymptomatic infants with two copies of SMN2 at risk for spinal muscular atrophy type 1: the Phase III SPR1NT trial. Nat Med. 2022 Jul;28(7):1381–9.

125. Strauss KA, Farrar MA, Muntoni F, Saito K, Mendell JR, Servais L, et al. Onasemnogene abeparvovec for presymptomatic infants with three copies of SMN2 at risk for spinal muscular atrophy: the Phase III SPR1NT trial. Nat Med. 2022 Jul;28(7):1390–7.

126. Mercuri E, Muntoni F, Baranello G, Masson R, Boespflug-Tanguy O, Bruno C, et al. Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy type 1 (STR1VE-EU): an open-label, single-arm, multicentre, phase 3 trial. Lancet Neurol. 2021 Oct;20(10):832–41.

127. Day JW, Finkel RS, Chiriboga CA, Connolly AM, Crawford TO, Darras BT, et al. Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy in patients with two copies of SMN2 (STR1VE): an open-label, single-arm, multicentre, phase 3 trial. Lancet Neurol. 2021 Apr;20(4):284–93.

128. Government of Canada. Regulatory Decision Summary for Zolgensma [Internet]. Canada: Health Canada Drug and Health Product Portal. [updated 2024, cited 2024 Sep 26]. Available from: https://dhpp.hpfb-dgpsa.ca/review-documents/resource/RDS00741

129. Commissioner O of the. FDA. FDA approves innovative gene therapy to treat pediatric patients with spinal muscular atrophy, a rare disease and leading genetic cause of infant mortality. United States: FDA; 2024 [updated 2019, cited 2024 Sep 26]. Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-innovative-gene-therapy-treat-pediatric-patients-spinal-muscular-atrophy-rare-disease

130. Chand D, Mohr F, McMillan H, Tukov FF, Montgomery K, Kleyn A, et al. Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy. J Hepatol. 2021 Mar 1;74(3):560–6.

131. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV Serotypes 1–9 Mediated Gene Expression and Tropism in Mice After Systemic Injection. Mol Ther. 2008 Jun 1;16(6):1073–80.

132. Philippidis A. Novartis Confirms Deaths of Two Patients Treated with Gene Therapy Zolgensma. Hum Gene Ther. 2022 Sep;33(17–18):842–4.

133. Chand DH, Zaidman C, Arya K, Millner R, Farrar MA, Mackie FE, et al. Thrombotic Microangiopathy Following Onasemnogene Abeparvovec for Spinal Muscular Atrophy: A Case Series. J Pediatr. 2021 Apr;231:265–8.

134. Guillou J, de Pellegars A, Porcheret F, Frémeaux-Bacchi V, Allain-Launay E, Debord C, et al. Fatal thrombotic microangiopathy case following adeno-associated viral SMN gene therapy. Blood Adv. 2022 Jul 22;6(14):4266–70.

135. Day JW, Mendell JR, Mercuri E, Finkel RS, Strauss KA, Kleyn A, et al. Clinical Trial and Postmarketing Safety of Onasemnogene Abeparvovec Therapy. Drug Saf. 2021 Oct 1;44(10):1109–19.

136. Hinderer C, Katz N, Buza EL, Dyer C, Goode T, Bell P, et al. Severe Toxicity in Nonhuman Primates and Piglets Following High-Dose Intravenous Administration of an Adeno-Associated Virus Vector Expressing Human SMN. Hum Gene Ther. 2018 Mar;29(3):285–98.

137. Hordeaux J, Wang Q, Katz N, Buza EL, Bell P, Wilson JM. The Neurotropic Properties of AAV-PHP.B Are Limited to C57BL/6J Mice. Mol Ther J Am Soc Gene Ther. 2018 Mar 7;26(3):664–8.

138. Thomsen G, Burghes AHM, Hsieh C, Do J, Chu BTT, Perry S, et al. Biodistribution of onasemnogene abeparvovec DNA, mRNA and SMN protein in human tissue. Nat Med. 2021 Oct;27(10):1701–11.

139. Gyngell C, Stark Z, Savulescu J. Drugs, genes and screens: The ethics of preventing and treating spinal muscular atrophy. Bioethics. 2020 Jun;34(5):493–501.

140. Meyer K, Ferraiuolo L, Schmelzer L, Braun L, McGovern V, Likhite S, et al. Improving Single Injection CSF Delivery of AAV9-mediated Gene Therapy for SMA: A Dose–response Study in Mice and Nonhuman Primates. Mol Ther. 2015 Mar 1;23(3):477–87.

141. Finkel RS, Darras BT, Mendell JR, Day JW, Kuntz NL, Connolly AM, et al. Intrathecal Onasemnogene Abeparvovec for Sitting, Nonambulatory Patients with Spinal Muscular Atrophy: Phase I Ascending-Dose Study (STRONG). J Neuromuscul Dis. 2023;10(3):389–404.

142. Novartis. Novartis announces AVXS-101 intrathecal study update. [Internet]. Basel, Switzerland: Novartis; 2019 [updated 2019; cited 2024 Sep 27]. Available from: https://www.novartis.com/news/media-releases/novartis-announces-avxs-101-intrathecal-study-update

143. Tukov FF, Mansfield K, Milton M, Meseck E, Penraat K, Chand D, et al. Single-Dose Intrathecal Dorsal Root Ganglia Toxicity of Onasemnogene Abeparvovec in Cynomolgus Monkeys. Hum Gene Ther. 2022 Jul 1;33(13-14):740-56.

144. Darras B, Mercuri E, Strauss K, Day J, Chien YH, Masson R, et al. Intravenous and Intrathecal Onasemnogene Abeparvovec Gene Therapy in Symptomatic and Presymptomatic Spinal Muscular Atrophy: Long-Term Follow-Up Study (S34.002). Neurology. 2023 Apr 25;100(17_supplement_2):2439.

145. Naryshkin NA, Weetall M, Dakka A, Narasimhan J, Zhao X, Feng Z, et al. SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy. Science. 2014 Aug 8;345(6197):688–93.

146. Cure SMA. Roche Announces New Clinical Trial of RG7916 - Cure SMA [Internet]. Elk Grove Village, USA: Cure SMA; 2019 [updated 2019; cited 2024 Sep 27]. Available from: https://www.curesma.org/roche-announces-new-clinical-trial-of-rg7916/

147. Ratni H, Ebeling M, Baird J, Bendels S, Bylund J, Chen KS, et al. Discovery of Risdiplam, a Selective Survival of Motor Neuron-2 (SMN2) Gene Splicing Modifier for the Treatment of Spinal Muscular Atrophy (SMA). J Med Chem. 2018 Aug 9;61(15):6501–17.

148. Baranello G, Darras BT, Day JW, Deconinck N, Klein A, Masson R, et al. Risdiplam in Type 1 Spinal Muscular Atrophy. N Engl J Med. 2021 Mar 11;384(10):915–23.

149. Darras BT, Masson R, Mazurkiewicz-Bełdzińska M, Rose K, Xiong H, Zanoteli E, et al. Risdiplam-Treated Infants with Type 1 Spinal Muscular Atrophy versus Historical Controls. N Engl J Med. 2021 Jul 29;385(5):427–35.

150. Mercuri E, Deconinck N, Mazzone ES, Nascimento A, Oskoui M, Saito K, et al. Safety and efficacy of once-daily risdiplam in type 2 and non-ambulant type 3 spinal muscular atrophy (SUNFISH part 2): a phase 3, double-blind, randomised, placebo-controlled trial. Lancet Neurol. 2022 Jan;21(1):42–52.

151. Commissioner O of the. FDA. FDA Approves Oral Treatment for Spinal Muscular Atrophy. [Internet]. USA: FDA; 2020 [updated 2020; cited 2024 Sep 27]. Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-oral-treatment-spinal-muscular-atrophy

152. Cure SMA Canada. Health Canada approves Risdiplam! [Internet]. Canada: Cure SMA Canada; 2023 [updated 2023; cited 2024 Sep 27]. Available from: https://curesma.ca/2021/04/16/health-canada-approves-risdiplam/

153. Walter MC, Wenninger S, Thiele S, Stauber J, Hiebeler M, Greckl E, et al. Safety and Treatment Effects of Nusinersen in Longstanding Adult 5q-SMA Type 3 – A Prospective Observational Study. J Neuromuscul Dis. 2019 Jan 1;6(4):453–65.

154. Oechsel KF, Cartwright MS. Combination therapy with onasemnogene and risdiplam in spinal muscular atrophy type 1. Muscle Nerve. 2021;64(4):487–90.

155. Chiriboga CA, Bruno C, Duong T, Fischer D, Mercuri E, Kirschner J, et al. Risdiplam in Patients Previously Treated with Other Therapies for Spinal Muscular Atrophy: An Interim Analysis from the JEWELFISH Study. Neurol Ther. 2023 Apr 1;12(2):543–57.

156. Mirea A, Shelby ES, Axente M, Badina M, Padure L, Leanca M, et al. Combination Therapy with Nusinersen and Onasemnogene Abeparvovec-xioi in Spinal Muscular Atrophy Type I. J Clin Med. 2021 Jan;10(23):5540.

157. Krosschell KJ, Bosch M, Nelson L, Duong T, Lowes LP, Alfano LN, et al. Motor Function Test Reliability During the NeuroNEXT Spinal Muscular Atrophy Infant Biomarker Study. J Neuromuscul Dis. 2018 Sep 14;5(4):509–21.

158. Glanzman AM, Mazzone E, Main M, Pelliccioni M, Wood J, Swoboda KJ, et al. The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND): Test development and reliability. Neuromuscul Disord. 2010 Mar 1;20(3):155–61.

159. Al-Zaidy SA, Kolb SJ, Lowes L, Alfano LN, Shell R, Church KR, et al. AVXS-101 (Onasemnogene Abeparvovec) for SMA1: Comparative Study with a Prospective Natural History Cohort. J Neuromuscul Dis. 2019;6(3):307–17.

160. Weng WC, Hsu YK, Chang FM, Lin CY, Hwu WL, Lee WT, et al. CMAP changes upon symptom onset and during treatment in spinal muscular atrophy patients: lessons learned from newborn screening. Genet Med. 2021 Feb 1;23(2):415–20.

161. Darras BT, Chiriboga CA, Iannaccone ST, Swoboda KJ, Montes J, Mignon L, et al. Nusinersen in later-onset spinal muscular atrophy. Neurology. 2019 May 21;92(21):e2492–506.

162. Bonati U, Holiga Š, Hellbach N, Risterucci C, Bergauer T, Tang W, et al. Longitudinal characterization of biomarkers for spinal muscular atrophy. Ann Clin Transl Neurol. 2017;4(5):292–304.

163. Wu JS, Darras BT, Rutkove SB. Assessing spinal muscular atrophy with quantitative ultrasound. Neurology. 2010 Aug 10;75(6):526–31.

164. Czech C, Tang W, Bugawan T, Mano C, Horn C, Iglesias VA, et al. Biomarker for Spinal Muscular Atrophy: Expression of SMN in Peripheral Blood of SMA Patients and Healthy Controls. Wishart TM, editor. PLOS ONE. 2015 Oct 15;10(10):e0139950.

165. Crawford TO, Paushkin SV, Kobayashi DT, Forrest SJ, Joyce CL, Finkel RS, et al. Evaluation of SMN Protein, Transcript, and Copy Number in the Biomarkers for Spinal Muscular Atrophy (BforSMA) Clinical Study. PLOS ONE. 2012 Apr 27;7(4):e33572.

166. Alves CRR, Zhang R, Johnstone AJ, Garner R, Eichelberger EJ, Lepez SDSD, et al. Whole blood survival motor neuron protein levels correlate with severity of denervation in spinal muscular atrophy. Muscle Nerve. 2020;62(3):351–7.

167. Chiriboga CA, Swoboda KJ, Darras BT, Iannaccone ST, Montes J, De Vivo DC, et al. Results from a phase 1 study of nusinersen (ISIS-SMNRx) in children with spinal muscular atrophy. Neurology. 2016 Mar 8;86(10):890–7.

168. Nash LA, McFall ER, Perozzo AM, Turner M, Poulin KL, De Repentigny Y, et al. Survival Motor Neuron Protein is Released from Cells in Exosomes: A Potential Biomarker for Spinal Muscular Atrophy. Sci Rep. 2017 Oct 24;7(1):13859.

169. Trifunov S, Natera-de Benito D, Carrera-García L, Codina A, Expósito-Escudero J, Ortez C, et al. Full-Length SMN Transcript in Extracellular Vesicles as Biomarker in Individuals with Spinal Muscular Atrophy Type 2 Treated with Nusinersen. J Neuromuscul Dis. 10(4):653–65.

170. Yuan A, Rao MV, Veeranna, Nixon RA. Neurofilaments and Neurofilament Proteins in Health and Disease. Cold Spring Harb Perspect Biol. 2017 Apr;9(4):a018309.

171. Petzold A. Neurofilament phosphoforms: Surrogate markers for axonal injury, degeneration and loss. J Neurol Sci. 2005 Jun 15;233(1):183–98.

172. Benatar M, Wuu J, Andersen PM, Lombardi V, Malaspina A. Neurofilament light: A candidate biomarker of presymptomatic amyotrophic lateral sclerosis and phenoconversion. Ann Neurol. 2018;84(1):130–9.

173. Benatar M, Wuu J, Lombardi V, Jeromin A, Bowser R, Andersen PM, et al. Neurofilaments in pre-symptomatic ALS and the impact of genotype. Amyotroph Lateral Scler Front Degener. 2019 Oct 2;20(7–8):538–48.

174. Weydt P, Oeckl P, Huss A, Müller K, Volk AE, Kuhle J, et al. Neurofilament levels as biomarkers in asymptomatic and symptomatic familial amyotrophic lateral sclerosis. Ann Neurol. 2016;79(1):152–8.

175. Thebault S, R. Tessier D, Lee H, Bowman M, Bar-Or A, Arnold DL, et al. High serum neurofilament light chain normalizes after hematopoietic stem cell transplantation for MS. Neurol Neuroimmunol Neuroinflammation. 2019 Sep;6(5):e598.

176. Siller N, Kuhle J, Muthuraman M, Barro C, Uphaus T, Groppa S, et al. Serum neurofilament light chain is a biomarker of acute and chronic neuronal damage in early multiple sclerosis. Mult Scler Houndmills Basingstoke Engl. 2019 Apr;25(5):678–86.

177. Lin YS, Lee WJ, Wang SJ, Fuh JL. Levels of plasma neurofilament light chain and cognitive function in patients with Alzheimer or Parkinson disease. Sci Rep. 2018 Nov 26;8(1):17368.

178. Lin CH, Li CH, Yang KC, Lin FJ, Wu CC, Chieh JJ, et al. Blood NfL. Neurology. 2019 Sep 10;93(11):e1104–11.

179. Darras BT, Crawford TO, Finkel RS, Mercuri E, De Vivo DC, Oskoui M, et al. Neurofilament as a potential biomarker for spinal muscular atrophy. Ann Clin Transl Neurol. 2019;6(5):932–44.

180. Darras B, Finkel R, Mercuri E, Sumner C, Oskoui M, Tizzano E, et al. NEW GENES, FUNCTIONS AND BIOMARKERS: O.5Association of phosphorylated neurofilament

heavy chain (pNF-H) with nusinersen treatment of SMA: analyses from the ENDEAR and CHERISH studies. Neuromuscul Disord. 2018 Oct 1;28:S31.

181. De Vivo DC, Bertini E, Swoboda KJ, Hwu WL, Crawford TO, Finkel RS, et al. Nusinersen initiated in infants during the presymptomatic stage of spinal muscular atrophy: Interim efficacy and safety results from the Phase 2 NURTURE study. Neuromuscul Disord. 2019 Nov;29(11):842–56.

182. Wurster CD, Steinacker P, Günther R, Koch JC, Lingor P, Uzelac Z, et al. Neurofilament light chain in serum of adolescent and adult SMA patients under treatment with nusinersen. J Neurol. 2020 Jan 1;267(1):36–44.

183. Rich KA, Fox A, Yalvac M, Heintzman S, Tellez M, Bartlett A, et al. Neurofilament Levels in CSF and Serum in an Adult SMA Cohort Treated with Nusinersen. J Neuromuscul Dis. 2022 Jan 1;9(1):111–9.

184. Andrés-Benito P, Vázquez-Costa JF, Ñungo Garzón NC, Colomina MJ, Marco C, González L, et al. Neurodegeneration Biomarkers in Adult Spinal Muscular Atrophy (SMA) Patients Treated with Nusinersen. Int J Mol Sci. 2024 Jan;25(7):3810.

185. Alves CRR, Petrillo M, Spellman R, Garner R, Zhang R, Kiefer M, et al. Implications of circulating neurofilaments for spinal muscular atrophy treatment early in life: A case series. Mol Ther Methods Clin Dev. 2021 Dec 10;23:524–38.

186. Flotats-Bastardas M, Bitzan L, Grell C, Martakis K, Winter B, Zemlin M, et al. Paradoxical increase of neurofilaments in SMA patients treated with onasemnogene abeparvovec-xioi. Front Neurol. 2023 Dec 13;14:1269406.

187. Martínez-Hernández R, Soler-Botija C, Also E, Alias L, Caselles L, Gich I, et al. The developmental pattern of myotubes in spinal muscular atrophy indicates prenatal delay of muscle maturation. J Neuropathol Exp Neurol. 2009 May;68(5):474–81.

188. Ripolone M, Ronchi D, Violano R, Vallejo D, Fagiolari G, Barca E, et al. Impaired Muscle Mitochondrial Biogenesis and Myogenesis in Spinal Muscular Atrophy. JAMA Neurol. 2015 Jun;72(6):666–75.

189. Shafey D, Côté PD, Kothary R. Hypomorphic Smn knockdown C2C12 myoblasts reveal intrinsic defects in myoblast fusion and myotube morphology. Exp Cell Res. 2005 Nov 15;311(1):49–61.

190. Mutsaers CA, Wishart TM, Lamont DJ, Riessland M, Schreml J, Comley LH, et al. Reversible molecular pathology of skeletal muscle in spinal muscular atrophy. Hum Mol Genet. 2011 Nov 15;20(22):4334–44.

191. Cifuentes-Diaz C, Frugier T, Tiziano FD, Lacène E, Roblot N, Joshi V, et al. Deletion of murine SMN exon 7 directed to skeletal muscle leads to severe muscular dystrophy. J Cell Biol. 2001 Mar 5;152(5):1107–14.

192. Gavrilina TO, McGovern VL, Workman E, Crawford TO, Gogliotti RG, DiDonato CJ, et al. Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. Hum Mol Genet. 2008 Apr 15;17(8):1063–75.

193. Besse A, Astord S, Marais T, Roda M, Giroux B, Lejeune FX, et al. AAV9-Mediated Expression of SMN Restricted to Neurons Does Not Rescue the Spinal Muscular Atrophy Phenotype in Mice. Mol Ther J Am Soc Gene Ther. 2020 Aug 5;28(8):1887–901.

194. Rudnik-Schöneborn S, Heller R, Berg C, Betzler C, Grimm T, Eggermann T, et al. Congenital heart disease is a feature of severe infantile spinal muscular atrophy. J Med Genet. 2008 Oct;45(10):635–8.

195. Menke LA, Poll-The BT, Clur SA, Bilardo CM, van der Wal AC, Lemmink HH, et al. Congenital heart defects in spinal muscular atrophy type I: a clinical report of two siblings and a review of the literature. Am J Med Genet A. 2008 Mar 15;146A(6):740–4.

196. Lipnick SL, Agniel DM, Aggarwal R, Makhortova NR, Finlayson SG, Brocato A, et al. Systemic nature of spinal muscular atrophy revealed by studying insurance claims. PloS One. 2019;14(3):e0213680.

197. Shababi M, Habibi J, Yang HT, Vale SM, Sewell WA, Lorson CL. Cardiac defects contribute to the pathology of spinal muscular atrophy models. Hum Mol Genet. 2010 Oct 15;19(20):4059–71.

198. Bevan AK, Hutchinson KR, Foust KD, Braun L, McGovern VL, Schmelzer L, et al. Early heart failure in the SMNDelta7 model of spinal muscular atrophy and correction by postnatal scAAV9-SMN delivery. Hum Mol Genet. 2010 Oct 15;19(20):3895–905.

199. Shababi M, Habibi J, Ma L, Glascock JJ, Sowers JR, Lorson CL. Partial restoration of cardio-vascular defects in a rescued severe model of spinal muscular atrophy. J Mol Cell Cardiol. 2012 May;52(5):1074–82.

200. Heier CR, Satta R, Lutz C, DiDonato CJ. Arrhythmia and cardiac defects are a feature of spinal muscular atrophy model mice. Hum Mol Genet. 2010 Oct 15;19(20):3906–18.

201. Somers E, Lees RD, Hoban K, Sleigh JN, Zhou H, Muntoni F, et al. Vascular Defects and Spinal Cord Hypoxia in Spinal Muscular Atrophy. Ann Neurol. 2016 Feb;79(2):217–30.

202. Zhou H, Ying H, Scoto M, Brogan P, Parson S, Muntoni F. Microvascular abnormality in spinal muscular atrophy and its response to antisense oligonucleotide therapy. Neuromuscul Disord. 2015 Oct 1;25:S193.

203. Araujo A prufer de QC, Araujo M, Swoboda KJ. Vascular perfusion abnormalities in infants with spinal muscular atrophy. J Pediatr. 2009 Aug;155(2):292–4.

204. Tein I, Sloane AE, Donner EJ, Lehotay DC, Millington DS, Kelley RI. Fatty acid oxidation abnormalities in childhood-onset spinal muscular atrophy: primary or secondary defect(s)? Pediatr Neurol. 1995 Jan;12(1):21–30.

205. Kelley RI, Sladky JT. Dicarboxylic aciduria in an infant with spinal muscular atrophy. Ann Neurol. 1986 Dec;20(6):734–6.

206. Zolkipli Z, Sherlock M, Biggar WD, Taylor G, Hutchison JS, Peliowski A, et al. Abnormal fatty acid metabolism in spinal muscular atrophy may predispose to perioperative risks. Eur J Paediatr Neurol EJPN Off J Eur Paediatr Neurol Soc. 2012 Sep;16(5):549–53.

207. Kaymaz AY, Bal A, Bora-Tatar G, Ozon A, Alikasifoglu A, Topaloglu H, et al. Serum IGF1 and IGFBP3 levels in SMA patients. Neuromuscul Disord. 2016 Oct 1;26:S105.

208. Kolb SJ, Coffey CS, Yankey JW, Krosschell K, Arnold WD, Rutkove SB, et al. Baseline results of the NeuroNEXT spinal muscular atrophy infant biomarker study. Ann Clin Transl Neurol. 2016 Feb;3(2):132–45.

209. Sutton ER, Beauvais A, Yaworski R, Repentigny YD, Reilly A, Almeida MMA de, et al. Liver SMN restoration rescues the Smn2B/- mouse model of spinal muscular atrophy. eBioMedicine. 2024 Dec 1;110.

210. Davis RH, Miller EA, Zhang RZ, Swoboda KJ. Responses to Fasting and Glucose Loading in a Cohort of Well Children with Spinal Muscular Atrophy Type II. J Pediatr. 2015 Dec;167(6):1362-1368.e1.

211. Bjelica B, Wohnrade C, Osmanovic A, Schreiber-Katz O, Schuppner R, Greten S, et al. Metabolic syndrome is common in adults with 5q-spinal muscular atrophy and impacts quality of life and fatigue. Muscle Nerve. 2024;70(2):257–64.

212. Sproule DM, Montes J, Montgomery M, Battista V, Koenigsberger D, Shen W, et al. Increased fat mass and high incidence of overweight despite low body mass index in patients with spinal muscular atrophy. Neuromuscul Disord. 2009 Jun 1;19(6):391–6.

213. Khairallah MT, Astroski J, Custer SK, Androphy EJ, Franklin CL, Lorson CL. SMN deficiency negatively impacts red pulp macrophages and spleen development in mouse models of spinal muscular atrophy. Hum Mol Genet. 2017 Mar 1;26(5):932–41.

214. Thomson AK, Somers E, Powis RA, Shorrock HK, Murphy K, Swoboda KJ, et al. Survival of motor neurone protein is required for normal postnatal development of the spleen. J Anat. 2017 Feb;230(2):337–46.

215. B R, M P. Preliminary immunological studies in spinal muscular atrophy. Eur J Pediatr. 1978 Mar;128:57-60.

216. Martinez TL, Kong L, Wang X, Osborne MA, Crowder ME, Meerbeke JPV, et al. Survival Motor Neuron Protein in Motor Neurons Determines Synaptic Integrity in Spinal Muscular Atrophy. J Neurosci. 2012 Jun 20;32(25):8703–15.

217. Gogliotti RG, Quinlan KA, Barlow CB, Heier CR, Heckman CJ, DiDonato CJ. Motor Neuron Rescue in Spinal Muscular Atrophy Mice Demonstrates That Sensory-Motor Defects Are a Consequence, Not a Cause, of Motor Neuron Dysfunction. J Neurosci. 2012 Mar 14;32(11):3818–29.

218. Lee AJH, Awano T, Park GH, Monani UR. Limited Phenotypic Effects of Selectively Augmenting the SMN Protein in the Neurons of a Mouse Model of Severe Spinal Muscular Atrophy. PLOS ONE. 2012 Sep 27;7(9):e46353.

219. Park GH, Maeno-Hikichi Y, Awano T, Landmesser LT, Monani UR. Reduced survival of motor neuron (SMN) protein in motor neuronal progenitors functions cell autonomously to cause spinal muscular atrophy in model mice expressing the human centromeric (SMN2) gene. J Neurosci Off J Soc Neurosci. 2010 Sep 8;30(36):12005–19.

220. Sugarman EA, Nagan N, Zhu H, Akmaev VR, Zhou Z, Rohlfs EM, et al. Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. Eur J Hum Genet EJHG. 2012 Jan;20(1):27–32.

221. Verhaart IEC, Robertson A, Wilson IJ, Aartsma-Rus A, Cameron S, Jones CC, et al. Prevalence, incidence and carrier frequency of 5q-linked spinal muscular atrophy - a literature review. Orphanet J Rare Dis. 2017 Jul 4;12(1):124.

222. Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell. 1995 Jan 13;80(1):155–65.

223. Lorson CL, Androphy EJ. An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. Hum Mol Genet. 2000 Jan 22;9(2):259–65.

224. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH, et al. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum Mol Genet. 1999 Jul;8(7):1177–83.

225. Feldkötter M, Schwarzer V, Wirth R, Wienker TF, Wirth B. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. Am J Hum Genet. 2002 Feb;70(2):358–68.

226. Hua Y, Sahashi K, Rigo F, Hung G, Horev G, Bennett CF, et al. Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. Nature. 2011 Oct 5;478(7367):123–6.

227. Hua Y, Liu YH, Sahashi K, Rigo F, Bennett CF, Krainer AR. Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models. Genes Dev. 2015 Feb 1;29(3):288–97.

228. Besse A, Astord S, Marais T, Roda M, Giroux B, Lejeune FX, et al. AAV9-Mediated Expression of SMN Restricted to Neurons Does Not Rescue the Spinal Muscular Atrophy Phenotype in Mice. Mol Ther. 2020 Aug 5;28(8):1887–901.

229. Eshraghi M, McFall E, Gibeault S, Kothary R. Effect of genetic background on the phenotype of the Smn2B/- mouse model of spinal muscular atrophy. Hum Mol Genet. 2016 Oct 15;25(20):4494–506.

230. Reilly A, Deguise MO, Beauvais A, Yaworski R, Thebault S, Tessier DR, et al. Central and peripheral delivered AAV9-SMN are both efficient but target different pathomechanisms in a mouse model of spinal muscular atrophy. Gene Ther. 2022 Sep;29(9):544–54.

231. Reilly A, Yaworski R, Beauvais A, Schneider BL, Kothary R. Long term peripheral AAV9-SMN gene therapy promotes survival in a mouse model of spinal muscular atrophy. Hum Mol Genet. 2024 Mar 15;33(6):510–9.

232. Nitz E, Smitka M, Schallner J, Akgün K, Ziemssen T, von der Hagen M, et al. Serum neurofilament light chain in pediatric spinal muscular atrophy patients and healthy children. Ann Clin Transl Neurol. 2021;8(10):2013–24.

233. Murray LM, Comley LH, Thomson D, Parkinson N, Talbot K, Gillingwater TH. Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy. Hum Mol Genet. 2008 Apr 1;17(7):949–62.

234. Hellbach N, Peterson S, Haehnke D, Shankar A, LaBarge S, Pivaroff C, et al. Impaired myogenic development, differentiation and function in hESC-derived SMA myoblasts and myotubes. PLoS ONE. 2018 Oct 10;13(10):e0205589.

235. Wali JA, Thomas HE. Pancreatic Alpha Cells Hold the Key to Survival. EBioMedicine. 2015 Apr 22;2(5):368–9.

236. Maruszczak K, Radzikowski K, Schütz S, Mangge H, Bergsten P, Forslund A, et al. Determinants of hyperglucagonemia in pediatric non-alcoholic fatty liver disease. Front Endocrinol. 2022 Sep 5;13:1004128.

237. Réthelyi JM, Vincze K, Schall D, Glennon J, Berkel S. The role of insulin/IGF1 signalling in neurodevelopmental and neuropsychiatric disorders – Evidence from human neuronal cell models. Neurosci Biobehav Rev. 2023 Oct 1;153:105330.

238. Deguise MO, Boyer JG, McFall ER, Yazdani A, De Repentigny Y, Kothary R. Differential induction of muscle atrophy pathways in two mouse models of spinal muscular atrophy. Sci Rep. 2016 Jun 28;6:28846.

239. Ling KKY, Gibbs RM, Feng Z, Ko CP. Severe neuromuscular denervation of clinically relevant muscles in a mouse model of spinal muscular atrophy. Hum Mol Genet. 2012 Jan 1;21(1):185–95.

240. McGivern JV, Patitucci TN, Nord JA, Barabas MEA, Stucky CL, Ebert AD. Spinal muscular atrophy astrocytes exhibit abnormal calcium regulation and reduced growth factor production. Glia. 2013 Sep;61(9):1418–28.

241. Tarabal O, Caraballo-Miralles V, Cardona-Rossinyol A, Correa FJ, Olmos G, Lladó J, et al. Mechanisms involved in spinal cord central synapse loss in a mouse model of spinal muscular atrophy. J Neuropathol Exp Neurol. 2014 Jun;73(6):519–35.

242. Jablonka S, Karle K, Sandner B, Andreassi C, von Au K, Sendtner M. Distinct and overlapping alterations in motor and sensory neurons in a mouse model of spinal muscular atrophy. Hum Mol Genet. 2006 Feb 1;15(3):511–8.

243. Lotti F, Imlach WL, Saieva L, Beck ES, Hao LT, Li DK, et al. An SMN-dependent U12 splicing event essential for motor circuit function. Cell. 2012 Oct 12;151(2):440–54.

244. Davis RH, Miller EA, Zhang RZ, Swoboda KJ. Responses to Fasting and Glucose Loading in a Cohort of Well Children with Spinal Muscular Atrophy Type II. J Pediatr. 2015 Dec;167(6):1362-1368.e1.

245. Kolb SJ, Kissel JT. Spinal Muscular Atrophy. Neurol Clin. 2015 Nov 1;33(4):831–46.

246. Feldkötter M, Schwarzer V, Wirth R, Wienker TF, Wirth B. Quantitative Analyses of SMN1 and SMN2 Based on Real-Time LightCycler PCR: Fast and Highly Reliable Carrier Testing and Prediction of Severity of Spinal Muscular Atrophy. Am J Hum Genet. 2002 Feb 1;70(2):358–68.

247. Sumner CJ, Paushkin S, Ko CP. Spinal Muscular Atrophy: Disease Mechanisms and Therapy. Academic Press; 2016. 508 p.

248. Boyer JG, Murray LM, Scott K, De Repentigny Y, Renaud JM, Kothary R. Early onset muscle weakness and disruption of muscle proteins in mouse models of spinal muscular atrophy. Skelet Muscle. 2013 Oct 11;3(1):24.

249. Boyer JG, Deguise MO, Murray LM, Yazdani A, De Repentigny Y, Boudreau-Larivière C, et al. Myogenic program dysregulation is contributory to disease pathogenesis in spinal muscular atrophy. Hum Mol Genet. 2014 Aug 15;23(16):4249–59.

250. Bricceno KV, Martinez T, Leikina E, Duguez S, Partridge TA, Chernomordik LV, et al. Survival motor neuron protein deficiency impairs myotube formation by altering myogenic gene expression and focal adhesion dynamics. Hum Mol Genet. 2014 Sep 15;23(18):4745–57.

251. Rudnik-Schöneborn S, Heller R, Berg C, Betzler C, Grimm T, Eggermann T, et al. Congenital heart disease is a feature of severe infantile spinal muscular atrophy. J Med Genet. 2008 Oct;45(10):635–8.

252. Shababi M, Habibi J, Yang HT, Vale SM, Sewell WA, Lorson CL. Cardiac defects contribute to the pathology of spinal muscular atrophy models. Hum Mol Genet. 2010 Oct 15;19(20):4059–71.

253. Deguise M, Baranello G, Mastella C, Beauvais A, Michaud J, Leone A, et al. Abnormal fatty acid metabolism is a core component of spinal muscular atrophy. Ann Clin Transl Neurol. 2019 Jul 26;6(8):1519–32.

254. Zolkipli Z, Sherlock M, Biggar WD, Taylor G, Hutchison JS, Peliowski A, et al. Abnormal fatty acid metabolism in spinal muscular atrophy may predispose to perioperative risks. Eur J Paediatr Neurol EJPN Off J Eur Paediatr Neurol Soc. 2012 Sep;16(5):549–53.

255. Bowerman M, Swoboda KJ, Michalski JP, Wang GS, Reeks C, Beauvais A, et al. Glucose metabolism and pancreatic defects in spinal muscular atrophy. Ann Neurol. 2012 Aug;72(2):256–68.

256. Davis RH, Miller EA, Zhang RZ, Swoboda KJ. Responses to Fasting and Glucose Loading in a Cohort of Well Children with Spinal Muscular Atrophy Type II. J Pediatr. 2015 Dec;167(6):1362-1368.e1.

257. Deguise MO, De Repentigny Y, McFall E, Auclair N, Sad S, Kothary R. Immune dysregulation may contribute to disease pathogenesis in spinal muscular atrophy mice. Hum Mol Genet. 2017 Feb 15;26(4):801–19.

258. Khairallah MT, Astroski J, Custer SK, Androphy EJ, Franklin CL, Lorson CL. SMN deficiency negatively impacts red pulp macrophages and spleen development in mouse models of spinal muscular atrophy. Hum Mol Genet. 2017 Mar 1;26(5):932–41.

259. Thomson AK, Somers E, Powis RA, Shorrock HK, Murphy K, Swoboda KJ, et al. Survival of motor neurone protein is required for normal postnatal development of the spleen. J Anat. 2017 Feb;230(2):337–46.

260. Gombash SE, Cowley CJ, Fitzgerald JA, Iyer CC, Fried D, McGovern VL, et al. SMN deficiency disrupts gastrointestinal and enteric nervous system function in mice. Hum Mol Genet. 2015 Oct 1;24(19):5665.

261. Wang CH, Finkel RS, Bertini ES, Schroth M, Simonds A, Wong B, et al. Consensus statement for standard of care in spinal muscular atrophy. J Child Neurol. 2007 Aug;22(8):1027–49.

262. Dominguez E, Marais T, Chatauret N, Benkhelifa-Ziyyat S, Duque S, Ravassard P, et al. Intravenous scAAV9 delivery of a codon-optimized SMN1 sequence rescues SMA mice. Hum Mol Genet. 2011 Feb 15;20(4):681–93.

263. Foust KD, Wang X, McGovern VL, Braun L, Bevan AK, Haidet AM, et al. Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. Nat Biotechnol. 2010 Mar;28(3):271–4.

264. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. N Engl J Med. 2017 Nov 2;377(18):1713–22.

265. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. Nat Biotechnol. 2009 Jan;27(1):59–65.

266. Novartis Gene Therapies. Phase I, Open-Label, Dose Comparison Study of AVXS-101 for Sitting But Non-ambulatory Patients With Spinal Muscular Atrophy [Internet]. clinicaltrials.gov; 2021 Jun [cited 2021 Jul 22]. Report No.: NCT03381729. Available from: https://clinicaltrials.gov/ct2/show/NCT03381729

267. Bowerman M, Murray LM, Beauvais A, Pinheiro B, Kothary R. A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. Neuromuscul Disord NMD. 2012 Mar;22(3):263–76.

268. Deguise MO, Pileggi C, De Repentigny Y, Beauvais A, Tierney A, Chehade L, et al. SMN Depleted Mice Offer a Robust and Rapid Onset Model of Nonalcoholic Fatty Liver Disease. Cell Mol Gastroenterol Hepatol. 2021;12(1):354-377.e3.

269. Deguise MO, De Repentigny Y, Tierney A, Beauvais A, Michaud J, Chehade L, et al. Motor transmission defects with sex differences in a new mouse model of mild spinal muscular atrophy. EBioMedicine. 2020 May 1;55:102750.

270. Murray L, Gillingwater TH, Kothary R. Dissection of the Transversus Abdominis Muscle for Whole-mount Neuromuscular Junction Analysis. JoVE J Vis Exp. 2014 Jan 11;(83):e51162.

271. Wurster CD, Steinacker P, Günther R, Koch JC, Lingor P, Uzelac Z, et al. Neurofilament light chain in serum of adolescent and adult SMA patients under treatment with nusinersen. J Neurol. 2020 Jan 1;267(1):36–44.

272. Murray LM, Comley LH, Thomson D, Parkinson N, Talbot K, Gillingwater TH. Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy. Hum Mol Genet. 2008 Apr 1;17(7):949–62.

273. Park GH, Maeno-Hikichi Y, Awano T, Landmesser LT, Monani UR. Reduced Survival of Motor Neuron (SMN) Protein in Motor Neuronal Progenitors Functions Cell Autonomously to Cause Spinal Muscular Atrophy in Model Mice Expressing the Human Centromeric (SMN2) Gene. J Neurosci. 2010 Sep 8;30(36):12005–19.

274. Meyer K, Ferraiuolo L, Schmelzer L, Braun L, McGovern V, Likhite S, et al. Improving Single Injection CSF Delivery of AAV9-mediated Gene Therapy for SMA: A Dose–response Study in Mice and Nonhuman Primates. Mol Ther. 2015 Mar;23(3):477–87.

275. Audic F, de la Banda MGG, Bernoux D, Ramirez-Garcia P, Durigneux J, Barnerias C, et al. Effects of nusinersen after one year of treatment in 123 children with SMA type 1 or 2: a French real-life observational study. Orphanet J Rare Dis. 2020 Jun 12;15:148.

276. Lavie M, Diamant N, Cahal M, Sadot E, Be'er M, Fattal-Valevski A, et al. Nusinersen for spinal muscular atrophy type 1: Real-world respiratory experience. Pediatr Pulmonol. 2021 Jan;56(1):291–8.

277. Valori CF, Ning K, Wyles M, Mead RJ, Grierson AJ, Shaw PJ, et al. Systemic delivery of scAAV9 expressing SMN prolongs survival in a model of spinal muscular atrophy. Sci Transl Med. 2010 Jun 9;2(35):35ra42.

278. Gavrilina TO, McGovern VL, Workman E, Crawford TO, Gogliotti RG, DiDonato CJ, et al. Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. Hum Mol Genet. 2008 Apr 15;17(8):1063–75.

279. Martinez TL, Kong L, Wang X, Osborne MA, Crowder ME, Van Meerbeke JP, et al. Survival motor neuron protein in motor neurons determines synaptic integrity in spinal muscular atrophy. J Neurosci Off J Soc Neurosci. 2012 Jun 20;32(25):8703–15.

280. Gogliotti RG, Quinlan KA, Barlow CB, Heier CR, Heckman CJ, DiDonato CJ. Motor Neuron Rescue in Spinal Muscular Atrophy Mice Demonstrates That Sensory-Motor Defects Are a Consequence, Not a Cause, of Motor Neuron Dysfunction. J Neurosci. 2012 Mar 14;32(11):3818–29.

281. Paez-Colasante X, Seaberg B, Martinez TL, Kong L, Sumner CJ, Rimer M. Improvement of Neuromuscular Synaptic Phenotypes without Enhanced Survival and Motor Function in Severe Spinal Muscular Atrophy Mice Selectively Rescued in Motor Neurons. PLoS ONE. 2013 Sep 23;8(9):e75866.

282. Didonna A, Opal P. The role of neurofilament aggregation in neurodegeneration: lessons from rare inherited neurological disorders. Mol Neurodegener. 2019 May 16;14(1):19.

283. de Waegh SM, Lee VM, Brady ST. Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating Schwann cells. Cell. 1992 Feb 7;68(3):451–63.

284. Brooks SP, Dunnett SB. Tests to assess motor phenotype in mice: a user's guide. Nat Rev Neurosci. 2009 Jul;10(7):519–29.

285. Hess PL, Al-Khalidi HR, Friedman DJ, Mulder H, Kucharska-Newton A, Rosamond WR, et al. The Metabolic Syndrome and Risk of Sudden Cardiac Death: The Atherosclerosis Risk in Communities Study. J Am Heart Assoc Cardiovasc Cerebrovasc Dis. 2017 Aug 23;6(8):e006103.

286. Van Alstyne M, Tattoli I, Delestrée N, Recinos Y, Workman E, Shihabuddin LS, et al. Gain of toxic function by long-term AAV9-mediated SMN overexpression in the sensorimotor circuit. Nat Neurosci. 2021 Jul;24(7):930–40.

287. Crawford TO, Pardo CA. The neurobiology of childhood spinal muscular atrophy. Neurobiol Dis. 1996 Apr;3(2):97–110.

288. Prior TW, Leach ME, Finanger E. Spinal Muscular Atrophy. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993 [cited 2023 Aug 14]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1352/

289. Chiriboga CA. Nusinersen for the treatment of spinal muscular atrophy. Expert Rev Neurother. 2017 Oct;17(10):955–62.

290. McMillan HJ, Proud CM, Farrar MA, Alexander IE, Muntoni F, Servais L. Onasemnogene abeparvovec for the treatment of spinal muscular atrophy. Expert Opin Biol Ther. 2022 Sep;22(9):1075–90.

291. Paik J. Risdiplam: A Review in Spinal Muscular Atrophy. CNS Drugs. 2022 Apr;36(4):401–10.

292. Feldman AG, Parsons JA, Dutmer CM, Veerapandiyan A, Hafberg E, Maloney N, et al. Subacute Liver Failure Following Gene Replacement Therapy for Spinal Muscular Atrophy Type 1. J Pediatr. 2020 Oct;225:252-258.e1.

293. Chand D, Mohr F, McMillan H, Tukov FF, Montgomery K, Kleyn A, et al. Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy. J Hepatol. 2021 Mar 1;74(3):560–6.

294. Van Alstyne M, Tattoli I, Delestrée N, Recinos Y, Workman E, Shihabuddin LS, et al. Gain of toxic function by long-term AAV9-mediated SMN overexpression in the sensorimotor circuit. Nat Neurosci. 2021 Jul;24(7):930–40.

295. Bricceno KV, Martinez T, Leikina E, Duguez S, Partridge TA, Chernomordik LV, et al. Survival motor neuron protein deficiency impairs myotube formation by altering myogenic gene expression and focal adhesion dynamics. Hum Mol Genet. 2014 Sep 15;23(18):4745–57.

296. Rudnik-Schöneborn S, Heller R, Berg C, Betzler C, Grimm T, Eggermann T, et al. Congenital heart disease is a feature of severe infantile spinal muscular atrophy. J Med Genet. 2008 Oct;45(10):635–8.

297. Nitz E, Smitka M, Schallner J, Akgün K, Ziemssen T, von der Hagen M, Tüngler V. Serum neurofilament light chain in pediatric spinal muscular atrophy patients and healthy children. Annals of Clinical and Translational Neurology. 2021 Oct;8(10):2013-24.

298. Hua Y, Liu YH, Sahashi K, Rigo F, Bennett CF, Krainer AR. Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models. Genes Dev. 2015 Feb 1;29(3):288–97.

299. Kizhakke Madathil S, Evans HN, Saatman KE. Temporal and Regional Changes in IGF-1/IGF-1R Signaling in the Mouse Brain after Traumatic Brain Injury. J Neurotrauma. 2010 Jan;27(1):95–107.

300. D'Ercole AJ, Ye P, Calikoglu AS, Gutierrez-Ospina G. The role of the insulin-like growth factors in the central nervous system. Mol Neurobiol. 1996 Dec;13(3):227–55.

301. Dobrowolny G, Giacinti C, Pelosi L, Nicoletti C, Winn N, Barberi L, et al. Muscle expression of a local Igf-1 isoform protects motor neurons in an ALS mouse model. J Cell Biol. 2005 Jan 17;168(2):193–9.

302. Dodge JC, Haidet AM, Yang W, Passini MA, Hester M, Clarke J, et al. Delivery of AAV-IGF-1 to the CNS Extends Survival in ALS Mice Through Modification of Aberrant Glial Cell Activity. Mol Ther. 2008 Jun 1;16(6):1056–64.

303. Hess PL, Al-Khalidi HR, Friedman DJ, Mulder H, Kucharska-Newton A, Rosamond WR, et al. The Metabolic Syndrome and Risk of Sudden Cardiac Death: The Atherosclerosis Risk in Communities Study. J Am Heart Assoc. 2017 Aug 23;6(8):e006103.

304. Feng Z, Lam S, Tenn EMS, Ghosh AS, Cantor S, Zhang W, et al. Activation of Muscle-Specific Kinase (MuSK) Reduces Neuromuscular Defects in the Delta7 Mouse Model of Spinal Muscular Atrophy (SMA). Int J Mol Sci. 2021 Jul 27;22(15):8015.

305. Lejman J, Panuciak K, Nowicka E, Mastalerczyk A, Wojciechowska K, Lejman M. Gene Therapy in ALS and SMA: Advances, Challenges and Perspectives. Int J Mol Sci. 2023 Jan 6;24(2):1130.

306. Groen EJN, Perenthaler E, Courtney NL, Jordan CY, Shorrock HK, van der Hoorn D, et al. Temporal and tissue-specific variability of SMN protein levels in mouse models of spinal muscular atrophy. Hum Mol Genet. 2018 Aug 15;27(16):2851–62.

307. Zhou H, Janghra N, Mitrpant C, Dickinson RL, Anthony K, Price L, et al. A novel morpholino oligomer targeting ISS-N1 improves rescue of severe spinal muscular atrophy transgenic mice. Hum Gene Ther. 2013 Mar;24(3):331–42.

308. Passini MA, Bu J, Roskelley EM, Richards AM, Sardi SP, O'Riordan CR, et al. CNS-targeted gene therapy improves survival and motor function in a mouse model of spinal muscular atrophy. The Journal of clinical investigation. 2010 Apr 1;120(4):1253-64.

309. Kariya S, Obis T, Garone C, Akay T, Sera F, Iwata S, et al. Requirement of enhanced Survival Motoneuron protein imposed during neuromuscular junction maturation. J Clin Invest. 2014 Feb 3;124(2):785–800.

310. Lutz CM, Kariya S, Patruni S, Osborne MA, Liu D, Henderson CE, et al. Postsymptomatic restoration of SMN rescues the disease phenotype in a mouse model of severe spinal muscular atrophy. J Clin Invest. 2011 Aug 1;121(8):3029–41.

311. Chaytow H, Motyl AAL, Huang YT, Wong C, Currie GL, Bahor Z, et al. Timing of SMN replacement therapies in mouse models of spinal muscular atrophy: a systematic review and meta-analysis. Brain Commun. 2024 Aug 1;6(4):fcae267.

312. Dangouloff T, Servais L. Clinical Evidence Supporting Early Treatment Of Patients With Spinal Muscular Atrophy: Current Perspectives. Ther Clin Risk Manag. 2019 Oct 2;15:1153–61.

313. Dangouloff T, Vrščaj E, Servais L, Osredkar D, Adoukonou T, Aryani O, et al. Newborn screening programs for spinal muscular atrophy worldwide: Where we stand and where to go. Neuromuscul Disord. 2021 Jun 1;31(6):574–82.

314. Zeiss CJ. Comparative Milestones in Rodent and Human Postnatal Central Nervous System Development. Toxicol Pathol. 2021 Dec 1;49(8):1368–73.

315. Ramos DM, d'Ydewalle C, Gabbeta V, Dakka A, Klein SK, Norris DA, et al. Age-dependent SMN expression in disease-relevant tissue and implications for SMA treatment. J Clin Invest. 2019 Nov 1;129(11):4817–31.

316. Motyl AAL, Faller KME, Groen EJN, Kline RA, Eaton SL, Ledahawsky LM, et al. Pre-natal manifestation of systemic developmental abnormalities in spinal muscular atrophy. Hum Mol Genet. 2020 Sep 29;29(16):2674–83.

317. Philippidis A. After Third Death, Audentes' AT132 Remains on Clinical Hold. Hum Gene Ther. 2020 Sep;31(17–18):908–10.

318. Molera C, Sarishvili T, Nascimento A, Rtskhiladze I, Muñoz Bartolo G, Fernández Cebrián S, et al. Intrahepatic Cholestasis Is a Clinically Significant Feature Associated with Natural History of X-Linked Myotubular Myopathy (XLMTM): A Case Series and Biopsy Report. J Neuromuscul Dis. 2022 Jan 1;9(1):73–82.

319. Corti S, Nizzardo M, Nardini M, Donadoni C, Salani S, Ronchi D, et al. Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. J Clin Invest. 2008 Oct 1;118(10):3316–30.

320. Corti S, Nizzardo M, Nardini M, Donadoni C, Salani S, Ronchi D, et al. Embryonic stem cell-derived neural stem cells improve spinal muscular atrophy phenotype in mice. Brain. 2010 Feb 1;133(2):465–81.

321. Corti S, Nizzardo M, Simone C, Falcone M, Nardini M, Ronchi D, et al. Genetic Correction of Human Induced Pluripotent Stem Cells from Patients with Spinal Muscular Atrophy. Sci Transl Med. 2012 Dec 19;4(165):165ra162-165ra162.

322. Zanetta C, Riboldi G, Nizzardo M, Simone C, Faravelli I, Bresolin N, et al. Molecular, genetic and stem cell-mediated therapeutic strategies for spinal muscular atrophy (SMA). J Cell Mol Med. 2014;18(2):187–96.

323. Andrews JA, Miller TM, Vijayakumar V, Stoltz R, James JK, Meng L, et al. CK-2127107 amplifies skeletal muscle response to nerve activation in humans. Muscle Nerve. 2018;57(5):729–34.

324. Rudnicki SA, Andrews JA, Duong T, Cockroft BM, Malik FI, Meng L, et al. *Reldesemtiv* in Patients with Spinal Muscular Atrophy: a Phase 2 Hypothesis-Generating Study. Neurotherapeutics. 2021 Apr 1;18(2):1127–36.

325. Welsh BT, Cote SM, Meshulam D, Jackson J, Pal A, Lansita J, et al. Preclinical Safety Assessment and Toxicokinetics of Apitegromab, an Antibody Targeting Proforms of Myostatin for the Treatment of Muscle-Atrophying Disease. Int J Toxicol. 2021 Jul 1;40(4):322–36.

326. Crawford TO, Day JW, De Vivo DC, Krueger JM, Mercuri E, Nascimento A, et al. Long-term efficacy, safety, and patient-reported outcomes of apitegromab in patients with spinal muscular atrophy: results from the 36-month TOPAZ study. Front Neurol. 2024 Jul 22;15:1419791.

327. Almeida MMA de, Repentigny YD, Gagnon S, Sutton ER, Kothary R. Impact of liver specific survival motor neuron (SMN) depletion on peripheral and central nervous system tissue pathology. eLife. 2024 Sep 24;13.

328. Laron Z. Insulin-like growth factor 1 (IGF-1): a growth hormone. Mol Pathol. 2001 Oct;54(5):311–6.

329. Bosch-Marcé M, Wee CD, Martinez TL, Lipkes CE, Choe DW, Kong L, et al. Increased IGF-1 in muscle modulates the phenotype of severe SMA mice. Hum Mol Genet. 2011 May 1;20(9):1844–53.

330. Tsai LK, Chen CL, Ting CH, Lin-Chao S, Hwu WL, Dodge JC, et al. Systemic Administration of a Recombinant AAV1 Vector Encoding IGF-1 Improves Disease Manifestations in SMA Mice. Mol Ther. 2014 Aug 1;22(8):1450–9.

331. Ebert AD, Beres AJ, Barber AE, Svendsen CN. Human neural progenitor cells over-expressing IGF-1 protect dopamine neurons and restore function in a rat model of Parkinson's disease. Exp Neurol. 2008 Jan 1;209(1):213–23.

332. Grimberg A, DiVall SA, Polychronakos C, Allen DB, Cohen LE, Quintos JB, et al. Guidelines for Growth Hormone and Insulin-Like Growth Factor-I Treatment in Children and Adolescents: Growth Hormone Deficiency, Idiopathic Short Stature, and Primary Insulin-Like Growth Factor-I Deficiency. Horm Res Paediatr. 2016 Nov 25;86(6):361–97.

333. Kletzl H, Ajmi H, Antys I, Heinig K, Jaber B, Marbury TC, et al. Effect of mild or moderate hepatic impairment on the pharmacokinetics of risdiplam. Br J Clin Pharmacol. 2022 Aug;88(8):3749–59.

334. Chen L, Liu F, Fang D, Li J. Study on the efficacy, safety, and biomarkers of nusinersen in type II and III spinal muscular atrophy in children. Front Pediatr. 2023 Dec 4;11:1294405.

335. Faravelli I, Meneri M, Saccomanno D, Velardo D, Abati E, Gagliardi D, et al. Nusinersen treatment and cerebrospinal fluid neurofilaments: An explorative study on Spinal Muscular Atrophy type 3 patients. J Cell Mol Med. 2020;24(5):3034–9.

336. Olsson B, Alberg L, Cullen NC, Michael E, Wahlgren L, Kroksmark AK, et al. NFL is a marker of treatment response in children with SMA treated with nusinersen. J Neurol. 2019 Sep 1;266(9):2129–36.

337. Darras BT, Sumner CJ, Muntoni F, Crawford TO, Finkel RS, Mercuri E, et al. Plasma Phosphorylated Neurofilament Heavy Chain Levels Over Time in Participants With Infantile-and Later-onset SMA: Data from the SHINE Study (1692). Neurology. 2020 Apr 14;94(15_supplement):1692.

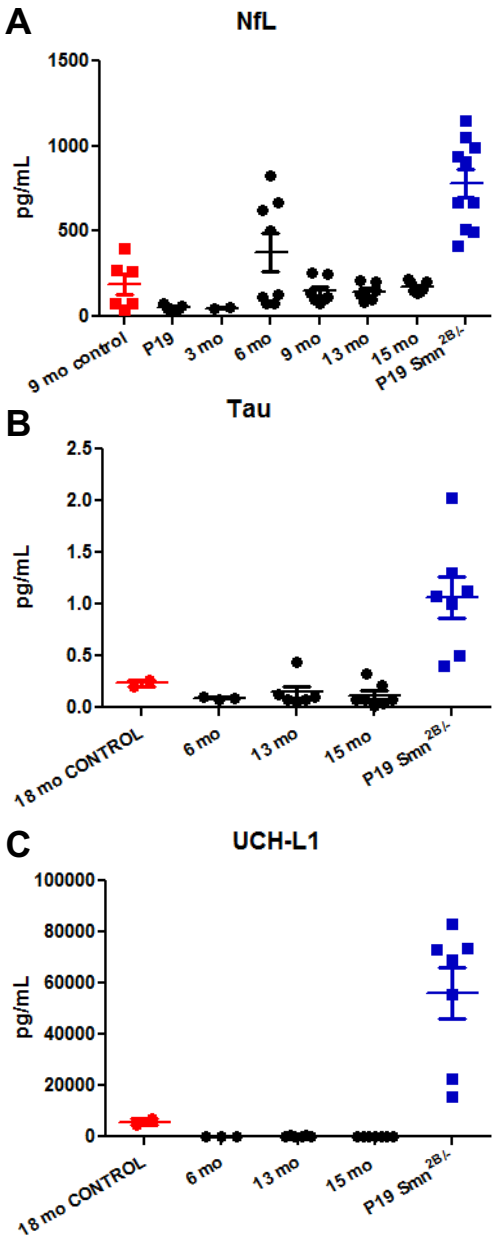
338. Kobayashi DT, Shi J, Stephen L, Ballard KL, Dewey R, Mapes J, et al. SMA-MAP: A Plasma Protein Panel for Spinal Muscular Atrophy. PLOS ONE. 2013 Apr 2;8(4):e60113.

339. Arnold WD, Duque S, Iyer CC, Zaworski P, McGovern VL, Taylor SJ, et al. Normalization of Patient-Identified Plasma Biomarkers in SMNΔ7 Mice following Postnatal SMN Restoration. PLoS ONE. 2016 Dec 1;11(12):e0167077.

340. Alves CRR, Zhang R, Johnstone AJ, Garner R, Nwe PH, Siranosian JJ, et al. Serum creatinine is a biomarker of progressive denervation in spinal muscular atrophy. Neurology. 2020 Mar 3;94(9):e921–31.

341. Freigang M, Wurster CD, Hagenacker T, Stolte B, Weiler M, Kamm C, et al. Serum creatine kinase and creatinine in adult spinal muscular atrophy under nusinersen treatment. Ann Clin Transl Neurol. 2021;8(5):1049–63.

Appendix



Appendix A. Plasma levels of NfL (A), Tau (B), and UCHL1 (C) in *Smn$^{2B/-}$;SMN2+/-* mice at various timepoints, compared to 18-month-old *Smn$^{2B/+}$;SMN2+/-* controls. P19 *Smn$^{2B/-}$* mice were included for comparison. (n= 2-7 for each time point)