Age-dependent SMN expression in disease-relevant tissue and implications for SMA treatment

Daniel M. Ramos, …, Thomas O. Crawford, Charlotte J. Sumner

*J Clin Invest.* 2019. [https://doi.org/10.1172/JCI124120](https://doi.org/10.1172/JCI124120).

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1Department of Neuroscience and 2Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 3PTC Therapeutics, South Plainfield, New Jersey, USA. 4Ionis Pharmaceuticals, Carlsbad, California, USA. 5PharmOptima, Portage, Michigan, USA. 6Center for Human Genetics, Case Western Reserve University, Cleveland, Ohio, USA. 7Department of Pathology, Ohio State University, Columbus, Ohio, USA. 8The Sidney Kimmel Comprehensive Cancer Center, Department of Oncology, and 9Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 10Center for Genomic Medicine, Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA. 11SMA Foundation, New York, New York, USA. 12Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

BACKGROUND. Spinal muscular atrophy (SMA) is caused by deficient expression of survival motor neuron (SMN) protein. New SMN-enhancing therapies are associated with variable clinical benefits. Limited knowledge of baseline and drug-induced SMN levels in disease-relevant tissues hinders efforts to optimize these treatments.

METHODS. SMN mRNA and protein levels were quantified in human tissues isolated during expedited autopsies.

RESULTS. SMN protein expression varied broadly among prenatal control spinal cord samples, but was restricted at relatively low levels in controls and SMA patients after 3 months of life. A 2.3-fold perinatal decrease in median SMN protein levels was not paralleled by comparable changes in SMN mRNA. In tissues isolated from nusinersen-treated SMA patients, antisense oligonucleotide (ASO) concentration and full-length (exon 7 including) SMN2 (SMN2-FL) mRNA level increases were highest in lumbar and thoracic spinal cord. An increased number of cells showed SMN immunolabeling in spinal cord of treated patients, but was not associated with an increase in whole-tissue SMN protein levels.

CONCLUSIONS. A normally occurring perinatal decrease in whole-tissue SMN protein levels supports efforts to initiate SMN-inducing therapies as soon after birth as possible. Limited ASO distribution to rostral spinal and brain regions in some patients likely limits clinical response of motor units in these regions for those patients. These results have important implications for optimizing treatment of SMA patients and warrant further investigations to enhance bioavailability of intrathecally administered ASOs.

FUNDING. SMA Foundation, SMART, NIH (R01-NS096770, R01-NS062869), Ionis Pharmaceuticals, and PTC Therapeutics. Biogen provided support for absolute real-time RT-PCR.

Introduction
The neuromuscular disease spinal muscular atrophy (SMA), affecting approximately 1 in 10,000 individuals, is the most common inherited cause of infant death (1). The most frequent and severe type I form causes profound muscle hypotrophy and weakness, particularly of bulbar, truncal, and proximal limb muscles. Without treatment, such infants never attain motor milestones, including the ability to sit or stand, and succumb to respiratory insufficiency in the first year or two of life. SMA is caused by recessive, loss-of-function mutations of the survival motor neuron 1 gene (SMN1) (2). All patients retain at least one copy of the paralog gene SMN2, but transcripts arising from SMN2 mostly lack exon 7 and make a truncated, rapidly degraded protein (3–6). A minority of SMN2 transcripts retain exon 7 and encode sufficient full-length SMN protein to enable viability, but insufficient levels to prevent motor neuron degeneration. SMN is ubiquitously expressed and best known for regulating assembly of small nuclear ribonucleoproteins (7). Why and how impairment of this or other putative functions of SMN predominately affects motor neurons are poorly understood. Variation in genomic SMN2 copy number correlates inversely with SMA disease severity (8, 9). Most of the severely affected infants inherit 2 copies of SMN2, while those with milder

Conflict of Interest: VC, AD, and NN are employees of PTC Therapeutics. SIKK, DAN, JM, FR, and CFB are employees of Ionis Pharmaceuticals. CFB holds a patent covering nusinersen licensed to Biogen (8980853). KJS receives grant support from Biogen and Cure SMA. CJS served as a paid advisor, consultant, and/or speaker to the SMA Foundation, Biogen, Ionis Pharmaceuticals, PTC Therapeutics, Roche/Genentech, AveXis, Cytokinetics, and Pfizer and is an associate editor for the JCI. This arrangement has been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies.

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Reference information: J Clin Invest. https://doi.org/10.1172/JCI124120.
forms have 3 or 4 copies; a few individuals with 5 copies of SMN2 have been asymptomatic (10).

The last decade has witnessed remarkable progress in the development of therapeutic strategies to increase SMN expression in the CNS of SMA patients (11), either by modifying the splicing of SMN2 pre-mRNAs using antisense oligonucleotides (ASOs) (12) or small molecules (13, 14) or by delivering exogenous SMN1 using viral vectors (15). Both nusinersen, a splice-switching ASO delivered by lumbar intrathecal injection 4 times in the first 2 months followed by chronic dosing every 4 months, and onasemnogene abeparvovec-xioi, a recombinant self-complementary adeno-associated virus 9 expressing SMN1 cDNA (scAAV9-SMN) delivered once intravenously, were recently shown to improve survival and motor function in infantile SMA patients (16, 17). Nusinersen also improves motor function in children and adolescents with milder forms of SMA (18). These results led to rapid FDA approval of nusinersen for SMA patients of all ages in December of 2016. The FDA approved onasemnogene abeparvovec-xioi in May 2019 for infants 2 years and younger. Importantly, however, the trial results demonstrate a spectrum of clinical efficacy. Treated infants manifest a range of benefit ranging from attainment of major motor milestones to little or no change in motor function. One factor that appears to play a key role in therapeutic efficacy is Earlier age at treatment initiation, but mechanisms that underlie this temporal dependency in humans have not been determined. While the goal of each of these therapies is to increase SMN expression in motor neurons, there are few data about baseline normal or disease-associated SMN levels in disease-relevant human tissues and there is even less understanding of either changes in SMN expression over the course of development or of changes with treatment because SMN levels cannot be measured in the CNS of living patients. Understanding the dynamics of SMN expression in the human CNS and non-CNS tissues of unaffected and SMA patients will enable further optimization of SMN-augmenting treatments.

**Results**

We implemented an expedited autopsy protocol to dissect and immediately freeze tissues isolated from 37 non-SMA control subjects and 42 SMA patients of different ages. Of these, 13 control and 27 SMA cases were analyzed, along with 115 control cases from the NIH NeuroBioBank (Figure 1); 38% of cases had a postmortem interval (PMI) of 6 or fewer hours, 68% of 12 or fewer hours, and 97% of 24 or fewer hours. All control cases had at least 1 copy of SMN1. All SMA cases, except the one fetal SMA sample, had 0 SMN1 copies and 2 SMN2 copies, consistent with their early age of disease onset (fetal sample: SMN2 copy number = 3) (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI124120DS1).

Spinal cord SMN protein levels are high during fetal development and decrease during the perinatal period. SMN protein levels were measured in 91 thoracic or lumbar spinal cord samples (75 control, 16 SMA; the one fetal SMA sample was from an unspecified spinal level) isolated from subjects ranging in age from 15 weeks gestation to 14 years by homogenous time-resolved fluorescence (HTRF) (Figure 2A), a fluorescence resonance energy transfer–based (FRET-based) technology often used in high-throughput screening (19). SMN protein levels varied widely, ranging in expression from $27 \Delta F/mg$ to $2288 \Delta F/mg$ in control samples, but median SMN protein was 2.3-fold higher in prenatal controls compared with postnatal controls younger than 3 months (defined as early postnatal) and 6.5-fold higher than in postnatal controls aged 3 months through 14 years (defined as late postnatal) (Figure 2B). The decrease in SMN expression was most evident in samples spanning the 3 months before and 3 months after birth (the perinatal period). Thereafter, SMN levels remained low in cases aged 3 months through 14 years. In some control cases, fetal death was caused by chromosomal abnormalities and/or CNS malformations (Figure 2A), and those samples had lower SMN levels (median: 1004 $\Delta F/mg$ in prenatal control samples without and 195 $\Delta F/mg$ with chromosomal abnormalities and/or CNS malformations, $P = 0.0014$). A sensitivity analysis indicated that inclusion of these samples in our statistical analyses did not change outcomes (data not shown).
In SMA spinal cord tissues, SMN protein levels were low in all postnatal cases assessed (range: 0.5 months–12 years). SMN protein expression was approximately 6-fold lower in SMA spinal cord samples compared with control samples aged 0–3 months, but only trended toward being decreased in samples greater than 3 months (Figure 2B), suggesting that differences in SMN levels between control and SMA diminish with increasing age. The single fetal SMA case (18 weeks gestational age [GA])...
threshold for significance ($\alpha$) was adjusted to 0.0167 and all associations remained significant.

To further validate the data obtained with HTRF, we quantified SMN protein levels using 2 other methods: electrochemiluminescence (ECL) (20) and Western blot. Very similar results were obtained with all 3 detection methods, and correlation coefficients between each of the methods were robust (Figure 2, D–F). A representative sample had the highest SMN expression of SMA cases (SMA prenatal: 198 $\Delta F$/mg vs. postnatal SMA median: 66 $\Delta F$/mg; Figure 2B), but was approximately 5-fold reduced compared with median SMN levels of prenatal control samples. No differences were seen in median SMN levels between early postnatal and late postnatal SMA samples. When Bonferroni’s correction for multiple comparisons between control cases in the 3 age groups was applied, threshold for significance ($\alpha$) was adjusted to 0.0167 and all associations remained significant.

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Overall, the range of SMN transcript levels was restricted compared with that of protein and the decreases in median SMN protein levels were modest compared with those of protein (79%). Interestingly, in both spinal cord and cortex samples, a significant drop of median SMN2-FL transcript occurred only between prenatal and early postnatal control samples (Figure 3, C and D, and Supplemental Figure 1B), whereas median SMN1-FL mRNA levels decreased significantly only between early and late postnatal samples (Figure 3, A and B, Supplemental Figure 1A, and Supplemental Tables 3 and 4). This may indicate an earlier developmental decrease in SMN2 expression relative to that of SMN1. SMN2-Δ7 mRNA levels and SMN2-FL/Δ7 ratios changed minimally between prenatal and postnatal controls, indicating little effect of age on exon 7-splicing patterns (Figure 3, E–H, Supplemental Figure 1, C and D, and Supplemental Tables 3 and 4). Either very weak or no correlation was observed between SMN transcript expression and PMI in spinal cord or cortex samples (Supplemental Figure 2). As some SMN-derived transcripts may lack exon 5 (22, 23), we also examined levels of SMN mRNA lacking exon 5 (SMN-Δ5) in a subset of 4 prenatal and 5 postnatal control samples. Although detectable in all samples, no differences were seen between prenatal and postnatal samples (data not shown).

To determine the relationships between SMN mRNA and protein levels, we examined case-by-case correlations (Figure 4). When including all control samples across the age spectrum, SMN protein only modestly correlated with SMN1-FL mRNA levels. Importantly, the correlation between SMN1 mRNA expression and SMN protein was driven by prenatal samples (Figure 4A). The sum of SMN1-FL and SMN2-FL levels showed similar results (Figure 4B). In SMA cases, SMN protein modestly correlated with SMN2-FL levels. Of note, the single prenatal SMA case expressed the highest SMN protein and SMN2-FL levels (Figure 4C). We applied a multivariable quantile regression analysis to determine whether changes in SMN1-FL and SMN2-FL expression could account for the decrease in SMN protein. Although SMN1-FL and SMN2-FL levels significantly contributed to SMN protein expression (SMN1-FL, \( P < 0.001 \); SMN2-FL, \( P = 0.03 \)), SMN protein independently decreased with age between prenatal and late postnatal samples (\( P = 0.003 \) after adjusting for SMN1-FL and SMN2-FL expression and PMI). This may indicate that additional, postranscriptional mechanisms contribute to the decrease in SMN protein levels during perinatal development. Comprehensive tables of protein
levels in control spinal cord samples, excluding cases with chromosomal abnormalities and CNS malformations (Figure 5) and cortex samples (Supplemental Figure 3). Modest correlations were observed between SMN1 copy number and SMN1 transcript levels (Figure 5D) as well as SMN2 copy number and SMN2-FL transcript levels (Figure 5E), but not between SMN2 copy number and SMN2-Δ7 transcript levels or SMN2-FL/Δ7 ratio (Figure 5, F and G). Neither SMN1 copy number, SMN2 copy number, nor the sum of SMN1 and SMN2 copy numbers significantly correlated with SMN protein levels in controls (Figure 5, A–C). When controlled for copy number, SMN2-FL, SMN2-Δ7, and SMN2-FL/Δ7 transcript expression did not significantly differ between postnatal control (2 SMN1 copies, 2 SMN2 copies) and postnatal SMA (0 SMN1 copies, 2 SMN2 copies) spinal cord or cortex samples (data not shown), suggesting that SMN2 transcript expression is unaltered by the loss of SMN1 in the CNS. Although not reaching statistical significance, when controlled for SMN2 copy number, postnatal control cases had median 2.1-fold more SMN protein than postnatal SMA cases (median control: 143 ΔF/mg vs. SMA: 67 ΔF/mg; P = 0.061).

**Similar developmental SMN expression patterns are observed in skeletal muscle.** While spinal cord is considered the principal site of pathology in SMA, SMN-deficient muscle may also play an important role in disease manifestations (24, 25). We therefore assessed SMN levels in clinically affected iliopsoas muscles isolated from 14 prenatal controls, 9 postnatal controls, and 10 postnatal SMA cases as well as in clinically resistant diaphragm muscles isolated from 16 prenatal controls, 6 postnatal controls, and 12 postnatal SMA cases (Supplemental Table 1). The median SMN protein levels measured by HTRF (Figure 6A), ECL, and Western blot (not shown) were similar in postnatal muscle and CNS tissues. Similarly to patterns found in the CNS, SMN protein levels decreased approximately 3-fold in postnatal compared with prenatal control iliopsoas samples. However, prenatal diaphragm SMN protein levels were 2-fold less than prenatal iliopsoas muscle samples (P = 0.003). SMN protein levels were reduced approximately 2-fold in postnatal SMA iliopsoas and diaphragm compared with postnatal control samples. SMN transcript levels were also similar across tissues (Figure 6, B–E), with the exception of the muscle tissues in which SMN1 was 2- to 3-fold higher relative to CNS tissues both prenatally and postnatally (Figure 6, B–E). Comprehensive comparisons between tissues are available in Supplemental Tables 5 and 6.

**Intrathecal nusinersen treatment results in variable SMN induction in the CNS.** To determine the impact of treatment on SMN mRNA and protein levels, we examined tissues isolated from 5 SMA patients who had been treated with nusinersen (number of doses received ranging from 1 to 11) as well as tissues isolated from 5 age-matched, untreated SMA patients (Supplemental Table 1). In treated cases, ASO drug concentrations ranged from 6.74 μg/g to 27.69 μg/g in lumbar and thoracic spinal cord regions; 2-fold lower levels were seen in cervical spinal cord, and variable drug levels were seen in brain tissue for the 3 patients who had available tissue (Figure 7, A and F, Supplemental Figures 4 and 5, and Supplemental Table 7). This pattern of drug concentration was associated with a 3-fold increase in SMN2-FL mRNA levels in cervical, thoracic, and lumbar/sacral spinal cord samples isolated from nusinersen-treated cases (Figure 7, B–F). Although fewer brain
samples were available (2 from cases who had received multiple doses of nusinersen), those that were assessed showed no change in SMN2-FL expression (Figure 7B). As expected, muscle and liver tissues also showed no increase of SMN2-FL expression. Decreases in SMN2-Δ7 levels were not robust (Figure 7C), but SMN2-FL/Δ7 ratios highlighted a caudal-to-rostral gradient in the CNS that paralleled drug levels (Figure 7D and Supplemental Figure 4A). Changes of SMN2 transcript levels measured by absolute real-time RT-PCR (26) in a subset of samples were highly correlated with those measured by relative RT-qPCR (Supplemental Figure 4, B and C). The nusinersen-treated case with the least robust increase of the SMN2-FL/Δ7 ratio had received a single dose of nusinersen just 5 days before death, whereas the 2 cases with the highest ratios had received multiple doses, with the last given 2 months prior to death (Figure 7F). No increases in whole-tissue SMN protein levels were observed (Figure 7, E and F). In one additional 11-month-old child who received 5 doses of nusinersen (the last 5 days prior to death), drug concentrations in a detailed set of CNS tissues showed a caudal-to-rostral gradient, but little induction of SMN transcript or protein was observed (Supplemental Figure 4, D and E). Importantly, the final dosing was complicated by the presence of blood in the CSF sample, raising doubt about drug delivery, and thus this case was excluded from group analyses.

To further understand the effects of nusinersen, we next assessed ASO and SMN immunostaining in spinal cord sections from the nusinersen-treated patients. Immunohistochemistry using an antibody targeting the nusinersen ASO backbone revealed ASO uptake in a subset of cell types in the spinal cord,
with staining particularly evident in ventral horn neurons. ASO staining intensity was highest in lumbar/sacral and thoracic spinal cord, less in cervical spinal cord (Figure 8, A–C), and modest in upper brain regions (Supplemental Figure 5). SMN immunostaining was observed in a small percentage of total spinal cord cells in both control and SMA subjects, and this staining was evident in ventral horn neurons, macroglia, and ependymal cells. To quantify SMN protein expression, all cells were identified, counted, and staining particularly evident in ventral horn neurons. ASO concentration in each tissue indicated with a yellow diamond. Data are represented as median ± SEM. ***P < 0.001; ****P < 0.0001. Statistical analysis was performed using 2-way ANOVA (B, C, D, and E) and corrected for multiple comparisons for tissues with n ≥ 3. Rx, nusinersen treatment; CSC, cervical spinal cord; TSC, thoracic spinal cord; L/SSC, lumbar/sacral spinal cord.
and SMN expression quantified using Neuron ID 2-RBD software with a user-trained paradigm. Cells were classified as having low, medium, or high SMN staining or undetectable SMN staining (see Methods). In lumbar spinal cord, both the total percentage of SMN-positive cells and the percentage of high-SMN-positive cells in treated patients were significantly increased compared with that in untreated patients (Figure 8, J–L). In cervical spinal cord, the total percentage of SMN-positive cells and the percentage of low-SMN-positive cells were significantly increased in treated compared with untreated SMA spinal cords (Figure 8, D, G, and M), while thoracic cord showed a trend (Figure 8, E, H, and N). At all spinal levels assessed, the percentages of SMN-positive cells in treated patients were either not significantly different or were higher relative to those in unaffected controls (Figure 8, J–O). Some SMN-positive cells were detected in frontal and temporal cortex in 2 treated cases (Supplemental Figure 5).

Discussion
In order to assess SMN expression in human tissues, we enacted a multistate and decade-long program of expedited autopsy tis-
SMN protein levels decrease during development. Our data reveal a substantial, 6.5-fold decline of normal SMN protein expression in the human spinal cord between fetal and postnatal stages. Measured by 3 different methods, SMN protein levels show a broad range in spinal cord, cortex, and muscle tissues isolated during the second and third trimester, but become restricted at low levels postnatally, particularly after 3 months, in both controls and SMA subjects. These data are consistent with a limited number of prior studies describing perinatal decline of SMN protein expression in human (36, 37) and mouse (20, 38–42) spinal cord and muscle via Western blot and immunohistochemistry. However, quantitative assessment of SMN in these studies, particularly in human CNS tissues in patients with other neurological diseases being targeted with novel gene targeting therapeutics (34, 35).

Mechanisms regulating SMN expression during development. Developmental changes in SMN protein levels were accompanied by more modest reductions of SMN1 and SMN2 transcript levels, but no change in SMN2 mRNA exon 7 splicing patterns. Prior studies suggest that SMN gene promoter activity may decrease during development and neuronal differentiation (4, 50, 51), and we have previously shown that epigenetic changes at the SMN gene promoters, including those mediated by histone acetylation (41) and a long noncoding RNA, SMN-ASI (21), repress the SMN promoter during development. Because of the high-sequence similarity between the SMN1 and SMN2 promoters, it has been difficult to determine whether or not the genes undergo parallel regulation in humans, but luciferase reporter assays identify an approximately 2-fold higher promoter activity of SMN1 compared with SMN2 (4). Interestingly, while SMN2-FL decreased perinatally, SMN1-FL did not significantly decrease until late postnatal ages. This earlier decrease of SMN2 transcriptional activity could further contribute to earlier developmental reductions in SMN protein in SMA patients compared with adult motor neurons in this study.

Implications for timing of treatment. Recent clinical trials of SMN-inducing drugs nusinersen and scAAV9-SMN demonstrate a
range of clinical efficacy, with the time of treatment initiation playing a critical role in the magnitude of clinical response (16, 17, 52). In a phase III trial of nusinersen, symptomatic infants with 2 copies of SMN2 dosed starting at an average age of 5.4 months showed reduced mortality, and 51% demonstrated improvements in motor function compared with 0% of infants in the control group, with 8% sitting independently at study completion (16). In a phase I trial of onasemnogene abeparvovec-xioi, 12 SMA type I infants were dosed with high doses at an average age of 3.4 months, with 92% achieving sitting and 16% walking (17). In data from presymptomatic SMA infants (15 SMN2 copy number = 2, 10 SMN2 copy number = 3), treatment with nusinersen was initiated at less than 6 weeks of life, and after a median of 2.9 years of treatment, 100% of children sat and 88% walked independently (52, 53). Together, these trials powerfully illustrate that a delay of SMN induction of weeks or months can substantially reduce achievement of motor milestones. The data reported here provide a possible explanation for both the general benefit of earlier treatment and the individual differences in response. SMN levels were variably low in control subjects in the third trimester, but consistently low by 3 months of age. If a normal developmental decrease in SMN expression is a factor in the initiation of motor neuron dysfunction in those with SMA, earlier initiation of SMN enhancement during this period could thus be more effective. Recently, the American Advisory Committee on Heritable Disorders in Newborns and Children in the US recommended SMA as a condition meeting the criteria for nationwide newborn screening. As of March 2019, 18 states have begun to develop and implement screening programs. The data presented here emphasize the urgency of this effort as well as the importance of mobilizing infrastructure to provide SMN induction treatment as soon after birth as possible (54). Moreover, these results raise the possibility that SMN induction prior to birth will be required for optimal patient outcomes at least in some patients.

**Topographical variations of SMN induction with nusinersen treatment.** SMA is characterized by a stereotypical pattern of muscle weakness, with proximal muscles more affected than distal, limbs more affected than face, and chest wall more affected than diaphragm. We did not identify striking regional differences in baseline SMN protein expression between spinal cord segments that might account for these regional variations. Interestingly, prenatal diaphragm samples expressed approximately 2-fold lower SMN protein compared with prenatal iliopsoas or spinal cord. This could indicate that resistance to disease in tissues such as the diaphragm is conferred by an overall lower requirement for SMN protein during gestation. Alternatively, intrinsic differences in the rate of development of different tissues might determine the timing of SMN decrements. Of note, the diaphragm is known to mature more quickly than other muscle groups because of the need to breathe immediately upon birth (55) and consequently might experience an earlier decrease in SMN expression.

Nusinersen does not cross the blood-brain barrier and thus must be delivered by intermittent lumbar intrathecal injection. Limited data obtained during autopsies of 3 severely affected infants (ages 12.8, 5.2, and 11.4 months old) dosed with nusinersen by lumbar injection in an early clinical trial (26) demonstrated higher drug concentrations in caudal compared with rostral tissues (lumbar cord: 26.6, 31.8, and 19.4 μg/g vs. brainstem: 13.8, 8.13, and 3.68 μg/g). A previous study of an ASO targeting huntingtin mRNA following lumbar intrathecal injection in rhesus monkeys showed a similar rostral-to-caudal gradient of drug concentrations (56). Although our autopsy cases may be biased toward those with poor clinical outcomes, our data also demonstrate regional variation in ASO distribution. In SMA patients receiving more than 1 dose of nusinersen, higher ASO concentrations in lumbar/sacral, thoracic, and cervical spinal levels were also associated with an increase in SMN2-FL transcript levels. Importantly, the current recommended dosage of nusinersen is 12 mg in a volume of 5 mL, regardless of patient age and size. CSF volume increases from 8 mL in neonates to 150 mL by 5 years of age (57, 58). Results from a study modeling nusinersen pharmacokinetics in CSF from treated SMA patients suggest that patients over 2 years of age have reduced ASO distribution in the CSF relative to patients under 2 years of age (59). The 2 treated patients with the most robust SMN induction in our cohort were 1 and 2 years old at the time of death. Two patients that were treated with more than one dose of nusinersen and demonstrated less SMN induction were 4 years and 13.5 years old at time of death. Whether larger volumes or higher amounts of nusinersen would improve ASO distribution in older patients is an important area for future investigation.

Despite increases in SMN2-FL transcript levels, particularly in caudal spinal cord regions, no increase in SMN protein levels in whole spinal cord tissue was observed in any treated case. Our IHC revealed cellular uptake of ASO by a small percentage of cells in spinal cord and cortex tissues, with this uptake most evident in neurons, in part due to their larger cell bodies. Quantification of SMN IHC staining intensity in treated versus untreated samples was technically challenging, but an increase in SMN expression in motor neurons from treated subjects was observed, as reported previously (26). Limited increase in SMN protein staining was observed in the majority of cells in the spinal cord. Given that motor neurons make up a very small percentage of the total cells in the spinal cord, the lack of whole-tissue protein increase is not surprising given these IHC results. Additional work is needed to fully characterize nusinersen effects in different cell populations, but it is possible that the apparent limited targeting of nusinersen to different CNS cell types also limits its efficacy in some patients, particularly as an increasing body of literature suggests potential contributions of cell types other than motor neurons in disease pathogenesis (60).

The results from this study have important implications beyond the treatment of SMA patients. ASOs are being developed or are in clinical trials for numerous neurological diseases, including Huntington’s disease and amyotrophic lateral sclerosis. Efficacy will require efficient delivery to neurons in spinal and brain regions as well as other cell types, such as glia, implicated in disease pathogenesis. Our data from a limited number of subjects highlight the difficulty in achieving high ASO levels in rostral CNS regions after intrathecal delivery and the cell-type variability in ASO uptake. Further work to optimize both ASO delivery and distribution will likely ultimately benefit patients with different neurological diseases.

**Methods**

**Human samples.** Tissue samples were dissected at autopsy and immediately flash-frozen in liquid nitrogen or fixed in 4% paraformaldehyde for 24 hours at 4°C, washed 3 × 10 minutes in PBS, and stored in
either PBS or 30% sucrose. Tissues used for histology were cut using a human brain protocol on a Sakura Tissue Tek tissue processor. After embedding, slides were cut at 4 microns, air dried overnight, then dried at 60°C for 1 hour.

**HTRF assay.** Tissue samples were homogenized on the TissueLyzer II (QIAGEN) in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP-40 [v/v]; 0.5% sodium deoxycholate [w/v]; 0.1% SDS [w/v]) containing a cocktail of protease inhibitors (Roche) at a tissue weight to RIPA buffer volume of 50 mg/mL. The samples were then centrifuged for 20 minutes at 14,000 g at 4°C. The homogenates were transferred to a 96-well plate and were diluted in RIPA buffer to approximately 1 mg/mL for SMN HTRF assay (Cisbio) and 0.5 mg/mL for total soluble protein measurement using the BCA Protein Assay (Pierce). Samples were run in duplicate and averaged. For the SMN HTRF assay, 35 μL of tissue homogenate was transferred to a 384-well plate containing 5 μL of the antibody solution (1:100 dilution of anti-SMN d2 [acceptor] and anti-SMN cryptate [donor]). The plate was incubated for 20 hours in the dark at room temperature. Fluorescence was measured at 665 nm and 620 nm on an EnVision Plate Reader (PerkinElmer). The data in the dark at room temperature. Fluorescence was measured at 665 nm and 620 nm on an EnVision Plate Reader (PerkinElmer). The data were processed according to the Cisbio protocol (ref. 19 and https://www.cisbio.com/content/signaltreatment-and-analysis). SMN protein signal ($\Delta F = \frac{[665\text{ nm}/620\text{ nm}]_{\text{sample}} - [665\text{ nm}/620\text{ nm}]_{\text{background}}}{[665\text{ nm}/620\text{ nm}]_{\text{background}}}$) was normalized to total soluble protein.

**SMN immunohistochemistry.** Slides were stained with rabbit monoclonal anti-SMN (Abcam, catalog ab108424) antibody on a Ventana Ultra staining system. SMN slides received Ventana’s CCI-heat induced antigen retrieval (Ventana, 950-500) for 92 minutes. The primary antibody was diluted with Discovery Antibody Diluent (Ventana, 760-108) and incubated for 1 hour at 37°C. The antibodies were detected with biotin-labeled goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, catalog 005-000-121). The primary antibody was diluted with Discovery Antibody Diluent (Ventana, 760-108) and incubated for 1 hour at 37°C. The antibodies were detected with biotin-labeled goat anti-rabbit secondary antibody (Jackson Immunoresearch, catalog 111-005-003). The secondary antibody was labeled with the Chromomap DAB Kit (Ventana, 760-124). Images were scanned on a Hamamatsu S360 scanner at x40 resolution.

**SMN IHC quantification.** Tissues showing morphological signs of autolysis or unspecific dark background were excluded from further quantification. SMN expression in glial and neuronal cells of the CNS was quantified using a customer-developed App with Visiopharm image analysis software (VIS 2019.02.1.6005; visiopharm.com). CNS regions of interest (ROI) in digitalized images were manually applied and, for the spinal cord, color coded to separate lumbar, thoracic, and cervical cord. Tissue folds, artificially darkened tissue edges, and blood vessels were excluded from quantification. The app detected all cells within the ROI, quantified their area and intensity, and binned them into 4 categories based on staining intensity (either negative for SMN staining or low, medium, or high SMN staining) based on user-taught parameters. A ratio of negative-, low-, medium-, or high-expressing SMN cells per total cells in the ROI was calculated.

**ASO immunohistochemistry.** Slides were stained with rabbit polyclonal ASO (Ionis Pharmaceuticals) (56) antibody on a Ventana Ultra Staining System. ASO slides were treated enzymatically with Trypsin (MilliporeSigma, T8003). The slides were then blocked with Endogenous Biotin Blocking Kit (Ventana, 760-050) and normal goat serum (Jackson ImmunoResearch, 005-000-121). The primary antibody was diluted with Discovery Antibody Diluent (Ventana, 760-108) and incubated for 1 hour at 37°C. The antibodies were detected with biotin-labeled goat anti-rabbit secondary antibody (Jackson Immunoresearch, catalog 111-005-003). The secondary antibody was labeled with the DABMap Kit (Ventana, 760-124). Images were scanned on a Hamamatsu S360 scanner at x40 resolution.

**ASO IHC quantification.** Tissues showing morphological signs of autolysis were excluded from further quantification. ASO uptake in glial, neuronal, and endothelial cells of CNS was scored semiquantitatively by a board-certified pathologist. Scores ranged from 0 to 3, with 0 = no uptake, 1 = minimal uptake, 2 = good uptake, and 3 = strong uptake.

**ASO concentration measurement.** Human autopsy samples were analyzed using a hybridization ELISA method. Briefly, human samples and monkey brain for a calibration curve and monkey brains for quality control (QC) were minced, weighed, homogenized in buffer, and extracted by liquid-liquid extraction using phenol chloroform. The aqueous was then dried down under vacuum and reconstituted in 500 μL of control human plasma. Samples were then diluted into range with control human plasma and run with a hybridization-based ELISA method. A Molecular Devices Gemini XPS Instrument was used for the final hybridization ELISA (HELISA) analysis. The calibration curve range of the assay for parent was 0.0200–1.50 μg/g, with the low end of the range defining the lower limit of quantification (LLOQ) in 50 mg of control cynomolgus monkey brain (for all brain and spinal cord sections). All samples were quantified within the quantitative range of the assay (0.0200 to 1.50 μg/g). The percentage of coefficient of variation (CV) between duplicate wells on the plate was less than 20% for all curves, QCs, and samples. All samples were stored at -70°C to 10°C upon receipt. Human lumbar, thoracic, and cervical spinal cord samples as well as frontal or temporal cortex, cerebellum, pons, or midbrain were all analyzed, as available for each patient, under the same conditions used for monkey brain tissue for calibrators and QC samples.

**RNA isolation and RT-qPCR analysis.** Tissues were homogenized in TRizol reagent (Thermo Fisher) using Lysing Matrix D Tubes (MP Biomedicals) in a Fastprep-24 instrument (MP Biomedicals) using 3 rounds of homogenization at a velocity of 6 m/s with 5-minute intervals on ice to avoid heating of the samples. Samples were subsequently mixed with 70% (v/v) ethanol and transferred into RNase columns (QIAGEN). Total RNA was cleaned up and eluted in nuclease-free water using the manufacturer’s protocol. Total RNA concentration was determined using a Nanodrop-2000 spectrophotometer (Thermo Fisher) with minimal required 260/280 absorbance ratios of 1.8. Equal amounts of RNA were converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) per the manufacturer’s recommendations. RT-qPCR was performed with the 7900HT Real-Time PCR System (Thermo Fisher) using TaqMan Universal PCR Master Mix (Thermo Fisher) and custom FAM-based TaqMan assays (Thermo Fisher; see Supplemental Table 2 for sequences) using the manufacturer’s recommendations. The expression levels of SMN-d5 and 8 different housekeeping genes were evaluated using custom RT-qPCR SYBR Green primers (Supplemental Table 2) and SYBR Green Universal PCR Master Mix (Thermo Fisher) per the manufacturer’s protocol. The stability of the housekeeping genes was evaluated by GeNorm analysis using qbase+ software (Biogazelle), and SMN levels were normalized to the levels of the 2 or 3 most stable housekeeping genes (61). Each sample was run in triplicate, and the average normalized SMN data were calibrated to a unique sample set to 1. All data are expressed as calibrated normalized relative quantities, as previously described (61).

**Absolute real-time RT-PCR.** Real-time RT-PCR was performed following the manufacturer’s instructions for the Express One-Step Superscript qRT-PCR Kit (Thermo Fisher Scientific) by Asuragen, as previous-
ly described (26). The final concentrations of primers and probes were 125 and 62.5 nM, respectively. RT-PCR reactions of calibration standards and autopsy RNA samples were performed using an ABI 7900HT Real-Time PCR Instrument. The thermal profile was as follows: 50°C for 15 minutes, 90°C for 2 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Optical data from all runs were analyzed using the automatic baseline setting and automatic threshold. The threshold cycle value for each sample was converted to a copy number by extrapolation to the calibration curve. For each sample, measurements were performed in triplicate, and the average is reported. The percentage of full-length SMN2 transcript was calculated as a percentage of the sum of SMN2 transcripts that include or exclude exon 7. The absolute real-time RT-PCR method was evaluated by Asuragen for upper and lower limits of detection and quantification, inter- and intraassay precision and accuracy, dilution linearity, and specificity.

Statistics. Statistical analyses for analysis of SMN RNA and protein analysis based on age were performed in STATA 14.2. Median values and IQR for each age group of each tissue were generated. P values of pairwise comparison of medians by age group were calculated using Wilcoxon’s rank-sum test. For comparisons between tissues, a Kruskal-Wallis test was used to determine whether differences in RNA or protein expression existed between tissues in each age group. P values were generated for pair-wise comparisons of medians using Wilcoxon’s rank-sum test. Bonferroni’s correction was applied to adjust for multiple comparisons between control age groups. Three pairwise comparisons were made in each analysis resulting in an adjusted α level of 0.0167 (0.05/3). P values before multiple comparisons are represented in figures to highlight biologically significant differences that may be lost after adjustment for multiple comparisons due to relatively small sample size. Comparisons between control and SMA within each age group were independent and thus not adjusted for multiple comparisons. Univariable quantile regression analysis was used to test for effects of PMI on SMN protein and RNA as well as the effects of SMN1-FL and SMN2-FL RNA on SMN protein. Based on the scatterplot of PMI versus SMN protein expression, PMI was modeled using a quadratic term to best model the distribution of the data. Multivariable quantile regression analysis was used to adjust for effects of PMI, SMN1-FL, and/or SMN2-FL on SMN protein changes with age.

For nusinersen-treated cases (Figures 7 and 8), statistical analyses were performed using Prism version 7.03 (GraphPad). The level of significance was set to 0.05. For 2-way ANOVA, variation between groups was assumed equal (standard in GraphPad Prism), followed by correction for multiple comparisons using Tukey’s multiple comparisons test for all experimental groups compared. Data related to nusinersen treatment (Figures 7 and 8) are represented as mean ± SEM, except with individual data points overlaid onto the charts. Simple linear regression was performed using Prism version 7.03 (GraphPad). Additional methods for electrochemiluminescence assay (20), Western blot (21), and SMN1 and SMN2 gene copy number analyses (62) are available in the Supplemental Methods.

Study approval. Study protocols were approved by the Office of Human Subjects Research Institutional Review Board at Johns Hopkins University (protocol IRB NA_00035399) and the Institutional Review Board at the University of Utah School of Medicine. Written, informed consent for autopsy was received from patients or their parents prior to autopsy. Other samples were obtained from the NIH NeuroBioBank. Some cases were collected in coordination with the Living Legacy Foundation. Deidentified samples were stored with only age, sex, SMA status, postmortem time, and tissue type.

Author contributions

DMR, CDY, NN, SP, TOC, and CJS designed the research studies. DMR, CDY, VG, AD, SKK, DAN, JM, SJT, PGZ, PJS, and IW conducted experiments. DMR, CDY, VG, AD, SKK, DAN, JM, SJT, PGZ, PJS, and IW acquired data. DMR, CDY, SKK, and NG analyzed data. DV, CLH, TWP, and NN provided reagents and samples for the study. DMR, CDY, and CJS wrote the manuscript. DMR, CDY, VG, AD, SKK, DAN, JM, SJT, PGZ, TWP, PJS, DV, CLH, IW, NG, KJS, FR, CFB, NN, SP, TOC, and CJS critically revised the manuscript.

Acknowledgments

We would like to express our profound gratitude for the generosity and altruism of donors and their families, whose gifts of organs and tissues serve an integral role in advancing medical research and education, as well as our colleagues who referred patients, including Kathryn J. Swoboda, John Brandsema, Gihan Tennekoon, Samiah Al-Zaidy, Richard Shell, Nancy Kunz, and Christine DiDonato, among others. We also thank the numerous pathologists with whom we have worked, including Jody Hooper, who leads the rapid autopsy program at Johns Hopkins, and Brian Harding at Children’s Hospital of Philadelphia. Additionally, we would like to thank Kathleen Smart at Children’s National Hospital for aid in program coordination. We also acknowledge the support of The Living Legacy Foundation of Maryland, the organization that works with donor families to honor their loved ones through the donation of organs and tissue for transplantation, education, and research. Some human tissues were received from the NIH NeuroBioBank at the University of Maryland (Baltimore, Maryland, USA). This work was supported by funding to CJS from the SMA Foundation, SMART, the NIH (R01-NS096770, R01-NS062869), Ionis, and PTC Therapeutics. KJS receives grant support from the NIH. Biogen provided support for absolute SMN2 mRNA quantitation and absolute real-time RT-PCR.

Address correspondence to: Charlotte J. Sumner, Departments of Neurology and Neuroscience, 855 North Wolfe Street, John G. Rangos Sr. Building, Room 248, Baltimore, Maryland 21205, USA. Phone: 410.502.6085; Email: csumner1@jhmi.edu.


