Make today a breakthrough.

February 28, 2017

Dr. Joseph A. Bocchini, Jr., M.D., *Chairman*
Advisory Committee on Heritable Disorders in Newborns and Children
5600 Fishers Lane, Room 18W68
Rockville, MD 20857

Dear Dr. Bocchini:

On behalf of all children born with spinal muscular atrophy (SMA), Cure SMA and the Muscular Dystrophy Foundation (MDA) are submitting this application for the Advisory Committee's consideration to nominate SMA as a condition to be listed on the Recommended Uniform Screening Panel (RUSP). The timing of this submission follows the December 23, 2016 FDA approval of the first-ever disease modifying therapy approved to treat SMA patients.

SMA is the number one genetic cause of death for infants, and affects approximately 1 in 11,000 babies. SMA is a progressive neurodegenerative disease that robs people of physical strength by affecting the motor nerve cells in the spinal cord, taking away the ability to walk, eat, and breathe. SMA can affect any race or gender. The disease is an autosomal recessive genetic disease caused by a mutation in the survival motor neuron gene 1 (*SMN1*). In a healthy person, this gene produces a protein that is critical to the function of the nerves that control muscles. Without it, those nerve cells cannot function properly and eventually die, leading to debilitating and often fatal muscle weakness.

As many on the committee know, Cure SMA is the largest organization in the United States dedicated to the treatment and cure of spinal muscular atrophy, and to supporting families affected by the disease. Cure SMA's reach includes 14,000 households in the SMA community, representing all four types of SMA as well as researchers and healthcare providers working in the neuromuscular fields—plus over 115,000 additional supporters. These stakeholders represent all 50 states as well as dozens of countries. In addition, Cure SMA has funded just over $62 million in research, with another $3 million pledged for the next 12 months.

MDA is a national 501(c)(3) organization dedicated to improving and saving the lives of people living with neuromuscular diseases, including SMA, muscular dystrophy, and ALS. All of the disorders under MDA's umbrella are classified as rare diseases and are progressive in nature, with life expectancy varying by disease. For more than 65 years, MDA has been committed to helping bring safe and effective treatments and cures to families as quickly as possible. To that end, MDA has funded over $1 billion in research grants and hosts a scientific conference in alternating years that brings together the leaders in neuromuscular disease from around the world. MDA is also committed to ensuring expert clinical care, and to that end, MDA supports more than 150 clinics (MDA Care Centers) nationwide that provide coordinated care to people with over 40 different kinds of neuromuscular disease, including SMA.

Cure SMA and MDA have joined together in this important effort—to ensure that all babies born in the United States are tested for SMA—as both organizations are committed to ensuring that those living with SMA are identified and treated as early as possible. In our combined effort to see NBS for SMA, we are pleased to be joined by the experts engaged in the SMA Newborn Screening Working Group (list enclosed). Together we have welcomed the opportunity to work with and testify before this Committee in advance of our submission and we appreciate the interest and attention of this

body over the months. As we submit this nomination, we offer our continued support throughout the committee's review process, and request and encourage you to reach out to us in any way we can be helpful.

The materials included in this application have been assembled and reviewed by the aforementioned Working Group, comprised of experts in the SMA field. The materials clearly demonstrate the importance of newborn screening for SMA as evidenced by (1) the substantial need for and benefit of pre-symptomatic treatment for SMA patients; (2) the availability of validated laboratory tests used to screen for SMA; (3) the availability of an FDA-approved treatment for SMA; and (4) the ability to treat successfully, once diagnosed, positive patients detected through newborn screening pilot studies.

An examination of the natural history, pathology, and continuing clinical trials for treatment development has shown that time has a clear and profound effect on the outcome for SMA patients. Just a few weeks can mean the difference between life and death for an infant with type I SMA, where the median survival is between 8 and 11 months in recent natural history studies. Preliminary results of the continuing NURTURE study conducted during the development and approval of SPINRAZA™, the first disease-modifying drug approved by the FDA, demonstrated a clear benefit for the pre-symptomatic treatment of SMA patients. Infants who were diagnosed before the onset of symptoms – either through the successful newborn screening pilots or because of a previously affected sibling – and who received SPINRAZA™ have achieved unprecedented motor milestones of sitting, standing, and walking, never before seen in Type I infants with SMA.

It is with great urgency and also excitement that we submit this application to the Committee for its consideration. To assist the Committee in its review enclosed please find: 1) member list of the SMA Newborn Screening Working Group and 2) table of contents setting out the content of the application and additional resource materials included.

We welcome all inquiries from the committee and look forward to working with your members, staff, and external advisory review group throughout the review process. Thank you for your time and dedication to these efforts and a special thank you to Debi Sarkar for her guidance and support as we worked to bring forth this application to the committee.

Sincerely,

Kenneth Hobby
President
Cure SMA

Valerie Cwik MD
Executive Vice President
Chief Medical & Scientific Officer
Muscular Dystrophy Association

CC: Ms. Debi Sarkar, MPH – ACHDNC Designated Federal Official

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Newborn Screening Multidisciplinary Working Group

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Nomination Form Table of Contents

DACHDNC Form for Nomination of a Condition for Inclusion in the Uniform Screening Panel	
DATE	
NAME OF NOMINATOR AND ORGANIZATION (include professional degrees)	**INDICATE AFFILIATION (i.e., Health Professional, Subject Matter Expert, Researcher, Clinician, Advocate, etc.)**
Cure SMA	Advocacy Organization
CO-SPONSORING ORGANIZATIONS (include professional degrees)	**INDICATE AFFILIATION (i.e., Health Professional, Subject Matter Expert, Researcher, Clinician, Advocate, etc.)**
Muscular Dystrophy Association	Advocacy Organization
SMA NBS Working Group (see cover letter)	Subject Matter Experts

Note: Please reference each statement/answer with the corresponding reference number listed in Section III – Key References.

SECTION I – CONDITION INFORMATION AND TREATMENT

SECTION I, PART A

CONDITION	STATEMENT
Nominated Condition	Spinal Muscular Atrophy
Type of Disorder	Autosomal recessive neuromuscular disease
Screening Method	Newborn blood spot screening test using multiplexed real-time PCR.[1]
Gene	Survival of Motor Neuron T (SMN1)
Locus	Include ClinVar link if applicable. 5q12.2-13.3, https://www.ncbi.nlm.nih.gov/clinvar/?term=SMN1[all]
OMIM or other names for condition	Include Genetics Home Reference link if applicable. 253300 (SMA1), 253550 (SMA2), 253400 (SMA3), 271150 (SMA 4) http://www.omim.org/entry/600354#0007
Case Definition	NA
Incidence	Determined by what method(s): pilot screening or clinical identification? In the United States, the pan-ethnic disease incidence of SMA, calculated using the measured carrier frequency of SMA of 1/54 and a detection rate of 91.2%, is calculated to be 1/11,000.[2]
Timing of Clinical Onset	Relevance of the timing of newborn screening to onset of clinical manifestations. There are four main clinical subtypes of SMA caused by mutations in the SMN1 gene. While the same disease with the same genetic cause, each subtype has a different

	timing of clinical onset.[3] Infants with the severe variant called SMA Type I, which accounts for 50 to 60% of all cases, are normal at birth. They manifest onset of weakness and respiratory or bulbar insufficiency within the first few months of life. A very small subset of infants are already weak from birth, or are born with congenital arthrogryposis (SMA Type 0). SMA Type II, comprising 30-40% of all cases, has onset of symptoms typically between 6-18 months. SMA Type III patients, comprising about 10% of cases, typically present after 18 months of age through the teen years. Another very small subgroup present in adulthood, and this is called SMA Type IV.[3]
Severity of Disease	Morbidity, disability, mortality, spectrum of severity. As summarized above, according to the consensus care guidelines for SMA, four main clinical sub-types are distinguished.[3] These include the acute infantile type, or Werdnig-Hoffmann disease (SMA Type I; affected infants are never able to sit independently), the intermediate type (SMA Type II; affected children are able to sit but never walk), the mild type (SMA Type III; affected individuals are ambulatory and typically manifest weakness after 18 months of age), and the adult onset form (SMA Type IV; affected individuals are ambulatory and typically manifest weakness as adults). The most severe form, SMA Type I, occurs during infancy and accounts for 50-60% of all cases; these children never sit, and 100% suffer bulbar and respiratory insufficiency with early mortality.[3] Two recent natural history studies in infants with SMA Type I have shown that the median age to reach the combined endpoint of death or requiring at least 16 hours/day of ventilation support is 13.5 and 8 months, respectively.[4,5] In these natural history studies, requirements for nutritional support preceded ventilation support, and the mean rate of decline in motor function as measured by The Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders scale was 1.27 points/year.[4] SMA Type II and III are slowly progressive with little change in motor function observed in most patients over a twelve-month period. Functional declines are observed over periods exceeding one-year.[6] Survival probabilities at 2, 4, 10, and 20 years of age have been reported to be 100%, 100%, 98%, and 77% in children with SMA Type II. For patients with SMA Type III, life expectancy has not been reported to be significantly less than in the unaffected population, although a significant portion lose the ability to walk by 40 years of age.[7,8] A very small subgroup of individuals present in adulthood, and this is called SMA Type IV. Regardless of clinical severity, 95% of all SMA patients have the same homozygous SMN1 gene deletion, and detection of the SMN1 gene deletion is used as the primary diagnostic assay. All patients possess a low-functioning analog to the SMN1 gene called SMN2. The SMN2 copy number is predictive of clinical severity. Humans have a variable copy number of the *SMN2* gene (0-8 copies), which correlates with SMA disease severity. Importantly, in the context of NBS, 80% of patients with SMA Type I carry one or two SMN2 copies, and 82% of patients with SMA Type II carry three SMN2 copies, whereas 96% of patients with Type III SMA carry three or four SMN2 copies.[9] *SMN2* is a key determinant of disease phenotype and is routinely determined after initial diagnosis to help predict the clinical phenotype. Thus, it is highly likely an infant identified by NBS with subsequent testing showing 3 or fewer copies of SMN2 will present with Type I or Type II SMA, which are associated with substantial early morbidity and/or mortality. Therefore, the identification of homozygous SMN1 deletion and determination of SMN2 copy number allows confident prediction that an infant will develop SMA.

SECTION I, PART B

TREATMENT	STATEMENT
Modality	Drug(s), diet, replacement therapy, transplant, other. Include information regarding regulatory status of treatment. **On December 23, 2016, the FDA approved the first disease-modifying therapy for SMA called Spinraza (nusinersen) for the treatment of SMA patients of all types and ages.** Spinraza, marketed by Biogen, is an antisense oligonucleotide drug that alters splicing of the SMN2 pre-mRNA to increase the amount of full-length SMN2 mRNA. Full-length SMN2 mRNA is translated into mRNA to increase the amount of functional SMN protein. (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm534611.htm). In addition to Spinraza, a number of clinical care approaches have been shown to improve survival and quality of life in SMA Type I, including: 1) nutritional support and careful monitoring of nutritional intake and swallow function, typically resulting in additional supplementation orally at first and then placement of nasogastric, nasojejunal or gastrostomy tube as needed, and prevention of fasting/catabolic state given their severe sarcopenia, and 2) respiratory support including techniques to mobilize and clear lower airway secretions such as chest physiotherapy devices, cough assist devices and pulse oximetry monitoring, and also the use of respiratory support devices including bi-level positive airway pressure via face/nose mask or tracheostomy tube to treat sleep disordered breathing.[3,10] There are also five additional therapies in development for the treatment of SMA, including SMN1 gene replacement therapy, small molecules designed to alter SMN2 mRNA splicing, and additional small molecule approaches aimed at motor neuron protection and muscle enhancement. These include Olesoxime sponsored by F. Hoffman - La Roche, which is a small molecule designed to prevent neuronal cell death (clinical trial identifiers: NCT02628743, NCT01302600, contact: Sangeeta Jethwa Schnetzler MD at sangeeta.jethwa@roche.com). There is also AVXS-101 sponsored by AveXis, which is a gene therapy to replace the SMN1 gene (clinical trial identifier: NCT02122952, contact: Douglas M. Sproule MD MSc at dsproule@avexis.com). LMI070 is sponsored by Novartis Pharmaceutical and is a small molecule designed to alter splicing of SMN2 mRNA and increase the amount of functional SMN protein (clinical trial identifier: NCT02268552, contact: Lawrence Charnas MD PhD at lawrence.charnas@novartis.com). RO7034067 and RO6885247 are sponsored by F. Hoffmann – La Roche and are small molecules designed to alter splicing of SMN2 mRNA and increase functional SMN protein (clinical trial identifiers: NCT02633709, NCT02240355, NCT02908685, NCT02913482, contact: Sangeeta Jethwa Schnetzler MD at sangeeta.jethwa@roche.com). Finally, there is CK-2127107, which is sponsored by Cytokinetics and is a small molecule to enhance muscle contraction (clinical trial identifier: NCT02644668, contact: Stacy A. Rudnicki MD at srudnicki@cytokinetics.com).
Urgency	How soon after birth must treatment be initiated to be effective? Both human natural history data and animal model data suggest that early drug intervention is required for greatest efficacy in the most common and severe form of SMA Type I. **In fact, in human SMA Type I, there is strong evidence that the irreplaceable loss of motor neurons begins early in the perinatal period, with severe denervation in the first 3 months of life and loss of more than 90% of motor units within 6 months of age.**[1] Moreover, a recent multi-center natural history study conducted by the NINDS NeuroNEXT clinical trial network in infants under six

	months of age with genetically confirmed SMA has shown significant differences between the SMA and control infants at the baseline visit in motor function tests, ulnar compound muscle action potential, and electrical impedance myography (EIM).[5] Moreover, studies looking at the timing of drug delivery in mouse models of SMA Type I have strongly suggested that early administration of SMN-based drug therapies is more effective than post-symptomatic delivery. **The results have been remarkably consistent across modalities including genetic means, gene therapy vectors, and antisense oligonucleotides to increase SMN levels. All have demonstrated the best results when the drugs are given as early as possible before significant motor weakness or loss in severe mouse models of SMA.[12, 13]** In addition, supportive treatment in the first few weeks to months of life prolongs survival and improves quality of life. In fact, the increases in survival of the type I infants over the past decade have been documented to correlate specifically to proactive respiratory and nutritional care.[10] However, in the current environment in the absence of newborn screening, these interventions remain predominantly reactive to medical crises. Many SMA Type I infants' initial presentation is in crisis with acute respiratory failure or bulbar insufficiency with aspiration prior to diagnosis and often associated with common viral respiratory infections. **In fact, diagnostic delay is very common in SMA. A recent systematic literature search conducted from 21 reports in PubMed and Web of Science databases for studies published between 2000 and 2014 showed that the mean ages of onset were 2.5, 8.3, and 39.0 months for SMA Types I, II, and II, respectively, while the weighted mean ages of confirmed spinal muscular atrophy genetic diagnosis were 6.3, 20.7, and 50.3 months, respectively, for Types I, II, and III.[14]** Better clinical outcomes are possible simply with the use of the currently available proactive care options, such as gastrostomy tube surgery prior to an aspiration event, and proactive respiratory care including use of the cough assist device to mobilize respiratory secretions and nocturnal bi-level positive airway pressure support via mask or nasal interface.[3] **A comprehensive rationale for the urgency for SMA newborn screening has been delineated in the review article, "Newborn screening for spinal muscular atrophy: Anticipating an imminent need".[12]**
Efficacy (Benefits)	Extent of prevention of mortality, morbidity, disability. Treatment limitations, such as Difficulty with acceptance or adherence. *Spinraza in Symptomatic Infants:* The efficacy of Spinraza was demonstrated in the ENDEAR Phase III randomized, double-blinded, sham-controlled clinical trial in 121 patients with infantile-onset SMA with two copies of SMN2 who were diagnosed before 6 months of age and who were less than 7 months old at the time of their first dose. Results were reported at the 2016 International Congress of the World Muscle and at the 43rd Annual Congress of the British Paediatric Neurology Association Meeting (see both slide decks appended to the references and at http://media.corporate-ir.net/media_files/IROL/22/222170/Kuntz_ENDEAR_study_design_WMS_LB_podium_Draft2v2_PIPE14989.pdf; http://newsroom.biogen.com/press-release/rare-and-genetic-diseases/new-data-show-spinraza-nusinersen-significantly-reduces-risk). Patients were randomized to receive an injection of Spinraza, into the fluid surrounding the spinal cord, or undergo a mock procedure without drug injection (sham). The trial assessed two primary endpoints: 1) percentage of patients with improvement in motor milestones, such as head control, sitting, ability to kick in supine position, rolling, crawling, standing and walking by measuring the proportion of motor milestone responders with the Hammersmith Infant Neurological Examination (HINE) and 2)

percentage of patients reaching the combined endpoint of death or greater than 16 hours per day of ventilatory support.

At a pre-specified interim analysis, 78 of 121 patients had the opportunity to be on treatment/sham for at least 6 months and were eligible for analysis (data available in the appended slides). Forty-one percent of patients treated with Spinraza (n=51) achieved improvement in motor milestones, whereas none of the control patients did (n=27, p<0.0001). Spinraza met the pre-specified primary endpoint for event-free survival, demonstrating a statistically significant 47% reduction in the risk of death or permanent ventilation (p<0.01). In the analysis, a greater percentage of untreated infants (68%) died or required permanent ventilation compared to infants treated with Spinraza (39%). Spinraza also demonstrated a favorable safety profile. The commonly reported adverse events include respiratory events and constipation, consistent with those expected in the general population of infants with SMA. The interim analysis represents 44.89 patient years of exposure to Spinraza treatment.

Open label trial results of Spinraza in both infants and children have been recently published.[15,16,17]

Spinraza in Pre-symptomatic Infants:
Biogen is currently conducting a Phase 2, open-label, multicenter study in 10 countries for pre-symptomatic infants with SMA termed NURTURE. The study objective is to evaluate the efficacy and safety profile of Spinraza in infants with genetically diagnosed and pre-symptomatic SMA. The planned enrollment is up to 25 infants, with key inclusion criteria of 1) less than 6 weeks of age at first dose, 2) pre-symptomatic, 3) genetic diagnosis of 5q SMA gene deletion or mutation, 4) 2 or 3 SMN2 copies, and 5) Ulnar CMAP amplitude ≥1 mV at baseline. The primary study endpoints are time to respiratory intervention (invasive or non-invasive ventilation for ≥ 16 hours/day continuously for ≥7 days or tracheostomy) or death. The secondary endpoints include: safety, tolerability, pharmacokinetics, motor function milestones, survival (proportion of patients alive), and growth parameters.

The results of an interim analysis were presented at the 2016 International Congress of the World Muscle Society (see slides at appended here and link to media.corporate-ir.net/media_files/IROL/22/222170/Bertini_NURTURE_interim_WMS_LB_podium_draft%202_PIPE-14990_3Oct16.pdf) and at the 43rd Annual Congress of the British Paediatric Neurology Association (BPNA) Meeting (see appended slides). At the interim analysis on June 8, 2016, 13 infants had reached their first efficacy assessment. All were still alive and did not require invasive ventilation at all or non-invasive ventilation for greater than 6 hours per day continuously for more than 7 days. 10 of 13 infants demonstrated increased motor milestones from baseline to last evaluation as measured by HINE and CHOP INTEND tests of motor function. In infants with 2 copies of SMN2 (likely to present with SMA Type I and having the same genotype of those enrolled in the ENDEAR Phase III trial) motor milestone development based on HINE Motor Milestone Achievements were as follows: 1) head control in 55% (5 of 9 infants), 2) sitting independently in 44% (4 of 9 infants), 3) standing in 22% (2 of 9 infants), and walking in 11% (1 of 9 infants). This data can be seen in slide 9 of the appended slide deck on NURTURE at from the WMS meeting. In comparison, a recent natural history study of developmental milestone achievement in 33 Type 1 SMA infants confirmed that no infants in the study achieved a major milestone such as rolling over, or sitting independently.[18] **More importantly, comparing the NURTURE trial data to the ENDEAR trial data of symptomatic infants clearly demonstrates greater attainment of milestones with pre-**

symptomatic treatment. In the interim analysis of the ENDEAR trial, the following age appropriate motor milestone development based on HINE Motor Milestone Achievements were met: 1) head control in 18% infants with treatment (n=51) and 0% in the sham (n=27), 2) sitting independently in 10% of infants with treatment and 0% in the sham, 3) standing in 2% of infants with treatment and 0% in the sham.

Table 1. Summary of HINE motor milestone achievements of infants receiving Spinraza in NURTURE[a] versus infants receiving Spinraza in ENDEAR.[b, c]

MILESTONE	Total no. of infants achieving milestone (%)	
	NUTURE (open-label, n=9)	ENDEAR (treated infants, n=51)
Head control (Full)	5/9 (55%)	9/51 (18%)
Sitting (Independent: stable, pivot)	4/9 (44%)	5/51 (10%)
Standing (Stands with support, unaided)	2/9 (22%)	1/51 (2%)
Walking (Cruising, walking)	1/9 (11%)	0/51 (0%)

[a]Only infants with 2 copies of SMN2 were included in this table (no 3 copy SMN2 patients were included from the NUTURE trial). All infants who enrolled in ENDEAR had 2 copies of SMN2.

[b]The ENDEAR interim was performed when 51 subjects who received Spinraza had the opportunity to be treated and observed for at least 183 days and up to 394 days.

[c]The data included in this chart are taken from a June 8, 2016 interim analysis of NUTURE and a June 15th, 2016 interim analysis of ENDEAR. An updated data set for NUTURE is expected to be presented at the American Academy of Neurology Annual Meeting April 22-28, 2017.

The greater attainment of motor milestones in NUTURE versus ENDEAR is also demonstrated by the mean total HINE score. This data can be seen in slide 11 of appended slide deck from BPNA meeting. The following was observed around 300 days of treatment: ~12 point total mean improvement in NURTURE (n=5), ~4 point mean total improvement in the treatment group of ENDEAR (n=51), and less than a 2 point total mean improvement in the ENDEAR sham group (n=27). This data can be seen on slide 12 of an appended presentation from the recent BPNA meeting. **Thus, the total mean HINE score improvement was substantially higher in the pre-symptomatically-treated infants.**

Spinraza in Children and Teens:
In addition, a placebo controlled Phase III trial in children called CHERISH has been ongoing at over 30 sites worldwide. CHERISH was a fifteen-month study investigating Spinraza in 126 non-ambulatory patients with later-onset SMA (consistent with Type 2), including patients with the onset of signs and symptoms at greater than 6 months and an age of 2 to 12 years at screening. The trial has been recently stopped due to positive results from an interim analysis. Results from the primary endpoint of the pre-specified interim analysis demonstrated a difference of 5.9 points (p= 0.0000002) at 15 months between the treatment (n=84) and sham-controlled (n=42) study arms, as measured by the Hammersmith Functional Motor Scale Expanded (HFMSE). From baseline to 15 months of treatment, patients who received Spinraza achieved a mean improvement of 4.0 points in the HFMSE, while patients who were not on treatment declined by a mean of 1.9 points. See http://media.biogen.com/press-

	release/corporate/biogen-and-ionis-pharmaceuticals-announce-spinraza-nusinersen-meets-primary- for more details. *Phase 1 Gene Therapy Trial of AVXS-101 in Symptomatic Infants:* At the 2016 International Congress of the World Muscle Society, AveXis presented information on their Phase 1/2 trial of AVXS-101 for SMA (see slides at http://investors.avexis.com/phoenix.zhtml?c=254285&p=irol-calendar). The open-label study is designed to evaluate safety and efficacy of AVXS-101 in infants with two copies of SMN2 less than nine months of age. The primary outcome in the study is safety and tolerability. The secondary outcome measure is an efficacy measure as defined by the time from birth to an "event." Exploratory outcome measures include the CHOP-INTEND score, a motor function scale used in infants with SMA. There were two dosing cohorts, consisting of three patients in a low-dose cohort (6.7 $X10^{13}$ vg/kg) and six patients in a mid-dose cohort (2.0 $X10^{14}$ vg/kg). As of September 15, 2016, AVXS-101 appeared to have a favorable safety profile and to be generally well tolerated. Four patients experienced treatment related elevation in serum transaminase levels, which were clinically asymptomatic and resolved with prednisolone treatment. Observed increases in motor function appear to be dose dependent, with the low dose cohort increasing an average of 9.0 points from an average baseline CHOP INTEND score of 16.3 points and the high dose cohort increasing an average of 24.8 points from an average baseline CHOP INTEND score of 28.2 points. Comparative natural history data for similar patients with SMA Type I has shown that none have been observed scoring above 40 points by 6 months of age, with one transient exception. In this study, 11 out of 12 patients in the high dose cohort reached a CHOP INTEND score ≥40 points, 9 out of 12 patients reached a CHOP INTEND score ≥50 points, and 3 out of 12 patients reached a CHOP INTEND score ≥60 points. In addition, all but one patient in the high dose cohort gained a milestone: -11 out of 12 patients could sit with assistance -8 out of 12 could sit unassisted, including one patient who achieved the milestone after September 15, 2016 -7 of 12 patients could roll -2 of 12 were walking independently; these two patients each achieved earlier and important developmental milestones such as crawling, standing with support, standing alone and walking with support. In contrast, data from a recently reported natural history study in 33 Type I SMA infants, shows that none achieved a major milestone such as rolling over, or sitting independently.[18] All patients in Cohort 2 (proposed therapeutic dose) were alive and event free. The median age at last follow-up for Cohort 2 is 17.3 months, with the oldest patient at 27.4 months. Natural history data shows a 25% survival rate at 13.6 months.[4] AveXis announced recently that it plans a pivotal study of AVXS-101 in SMA Type I infants starting in the second quarter of 2017. It will use a single-arm design with natural history of the disease as a comparator and is expected to enroll 20 patients.
Availability	Limits of availability? Spinraza, which is marketed by Biogen, is FDA approved for the treatment of SMA patients of all ages and types. The FDA approved Spinraza on December 23rd, 2016. (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm534611.htm).

SECTION I, PART B

	In addition to Spinraza, interventions including bi-level pressure support (BIPAP) and placement of a gastrostomy feeding tube are widely available at major medical centers. They are currently recommended as the standard care options of choice for infants with SMA Type I in the "Consensus Statement for Standard of Care in Spinal Muscular Atrophy".[3]
Potential Harms of Treatment	Potential medical or other ill effects from treatment Spinraza is a therapy administered into the intrathecal space and in some cases, anesthesia is required for administration. Therefore, the established risks of routine lumbar puncture procedure exist, which include headache, nausea, bleeding and CSF leak. In addition, there are standard risks of anesthesia, which depend upon the anesthetic used. Specifically in the SMA population, there is the respiratory risk for general anesthesia, but for whom local anesthesia would not be sufficient. Also, SMA patients who have undergone scoliosis surgery may have complicated intrathecal access, potentially requiring fluoroscopic guidance, which would include cumulative radiation risk. Finally, children responding to therapy may have improving, but still weak motor skills, and may roll or fall resulting in respiratory compromise or injury. In regard to Spinraza, the most common side effects found in participants in the clinical trials were upper respiratory infection, lower respiratory infection and constipation. Warnings and precautions include low blood platelet count and toxicity to the kidneys (renal toxicity). In the randomized Phase III ENDEAR clinical trial, no patient had a platelet count less than 50,000 cells per microliter and no patient developed a sustained low platelet count despite continued drug exposure. Toxicity in the nervous system (neurotoxicity) was observed in animal studies. The FDA USPI for Spinraza can be accessed at: http://www.accessdata.fda.gov/drugsatfda_docs/label/2016/209531lbl.pdf

SECTION II – EVIDENCE-BASED INFORMATION

For a nominated condition to be considered there are 3 core requirements:

1. Validation of the laboratory test (see Section II, Part A)
2. Widely available confirmatory testing with a sensitive and specific diagnostic test (see Section II, Part B)
3. A prospective population based pilot study (see Section II, Part C)

SECTION II, PART A

TEST	STATEMENT
Screening test(s) to be used	Description of the high volume method, instrumentation and if available as part of multi-analyte platform. There have been two pilots conducted using the assays described below. They are referred to by the location in which they took place, Taiwan and New York state. Also listed is an assay in development by PerkinElmer for which R&D studies are currently being conducted and pilot studies are being planned. It is being adapted from the assay described in reference number 1. TAIWAN: Real-time PCR TaqMan® single nucleotide polymorphism (SNP)

SECTION I, PART B

	genotyping assay on a StepOnePlus™ RT-PCR 96-well System (Applied Biosystems). The assay targets a SNP in SMN1 intron 7 to distinguish SMN1 from SMN2 using a Taqman probe.[1] NY State: A custom TaqMan real-time polymerase chain reaction (PCR) assay targeting the SMN1 exon 7 deletion and a fragment of RNaseP (used as an internal control gene) are run on a real-time PCR platform such as an ABI 7900 or QuantStudio™ 12K Flex Real-Time PCR System (ThermoFisher™ Scientific). Testing can be conducted in 96-well or 384-well format. The screen can be conducted using the same instrumentation and platform as used for molecular SCID screening, allowing the tests to be multiplexed as described previously.[1] PERKINELMER: Real-time PCR assay targeting SMN1 and SMN2 SNPs in exon 7 using dual-labeled lock nucleic acid Taqman® probes. The 5-plex assay detects SMN1, SMN2, TREC (to detect SCID), KREC (to detect XLA), and RNaseP (internal control). Preliminary testing has been conducted using a 96-well or 384-well format on a ThermoFisher™ QuantStudio 5 or a QuantStudio™ DX real time PCR platform.
Modality of Screening	(Dried blood spot, physical or physiologic assessment, other) TAIWAN: DNA extracted from 3-mm dried blood spot punch to detect homozygous deletions in SMN1 intron 7. NY State: DNA extracted from 3-mm dried blood spot punch to detect homozygous and heterozygous deletions in SMN1 exon 7. PERKINELMER: DNA extracted from 3-mm dried blood spot punch to detect homozygous and heterozygous (if desired) deletions in SMN1 exon 7 and SMN2 copy number.
Does the screening algorithm include a second tier test? If so, what type of test and availability?	(Dried blood spot, physical or physiologic assessment, other) TAIWAN: A feasibility assessment for a second tier test has been completed. The proposed second tier test is a digital droplet PCR (ddPCR) to exclude false positives and to detect SMN2 copy number. NY State: Second tier testing includes: 1. Targeted sequencing in infants positive for SMN1 deletion for quality assurance, to rule out allelic dropout due to polymorphisms in the SMN1 assay primer and probe binding sites. 2. SMN2 copy number testing can be performed in affected infants using a custom TaqMan assay and run on the same platform as the SMN1 assay. Alternatively, a digital droplet PCR kit (ddPCR SMN2 Copy Number Determination Kit) is commercially available from BioRad and can be run on the BioRad platform (for example, QX200 Auto DG Digital Droplet PCR system). PERKINELMER: No second tier test is needed as the primary assay detects both SMN1 and SMN2 copy number.

TEST	STATEMENT
Clinical Validation	Location, duration, size, preliminary results of past/ongoing pilot study for clinical validation, positive predictive value, false positive rate, analytical specificity, sensitivity. TAIWAN: The screening method was validated by testing the DBS samples of 2,937 anonymous newborns and 9 DNA samples with known SMN1 and SMN2 copy numbers. From November 2014 to September 2016, 120,267 infants have been tested in a consented pilot study at the National Taiwan University Hospital with: PPV=100% FPR=0% (First tier screen identified 15 positives out of 120,000 screened. Eight of the 15 positives were ruled false positives during second tier testing.) Analytical SP=100% Analytical SS=100% NY State: From January 2016 to December 2016, 3,269 infants have been tested in an ongoing consented pilot study at three hospitals in New York City with: PPV= 100% FPR= 0% Analytical SP= 100% Analytical SS= 100% PERKINELMER: An R&D study is being conducted to screen over 3,000 DBS samples. Thus far, 1,080 DBS samples, along with characterized reference samples and controls, have been screened. From those: PPV=100% FPR=0% Analytical SP=100% Analytical SS=100%
Analytical Validation	Limit of detection/quantitation, detection rate, reportable range of test results, reference range. Include regulatory status of test, information about reference samples and controls required for testing and availability of or potential for external quality assurance system, e.g., QC and PT for both screening and confirmatory tests. TAIWAN: LOD/LOQ= Provided that DNA can be extracted from a given blood spot, the screen is valid. The presence of DNA is assessed by ensuring amplification of the internal control gene. Detection rate=100%. All specimens with homozygous deletions of SMN1 have screened positive with this method. Reportable range: Results are reported as: No SMN1 homozygous deletion; SMN1 homozygous deletion. This screening method does not detect point mutations in the SMN1 gene, which are present in around 5% of SMA. It does detect a hybrid SMN1 allele present in the Taiwanese population resulting in identification of some false positives. Quality controls in each 96-well plate included a water blank, a filter paper blank, and 3 DNA samples with known SMN1:SMN2 copy numbers, 0:2 (affected), 1:2 (carrier), and 2:2 (normal). This test is intended as a LDT (laboratory developed test) and is not FDA approved. NY State: LOD/LOQ= Provided that DNA can be extracted from a given blood spot, the screen is valid. The presence of DNA is assessed by ensuring amplification of the internal control RNaseP gene, where amplification at Ct

	<=37 indicates the presence of DNA. Detection rate= 100%. All specimens with known homozygous deletions of SMN1 exon 7 have screened positive using the screening assay. This screening method does not detect point mutations in the SMN1 gene. Reportable range= Results are reported as: No SMN1 exon 7 deletion; Heterozygous SMN1 exon 7 deletion; Homozygous SMN1 exon 7 deletion. The NYS newborn SMA screening tests are LDTs and are not FDA-cleared/approved. The SMN1 screen underwent NYS regulatory approval and is an approved clinical test. Specimens from infants with known SMA genotypes (including affected and carriers) obtained from outside collaborators are used as positive controls and for proficiency testing. PERKINELMER: LOD/LOQ= Provided that DNA can be extracted from a given blood spot, the screen is valid. The presence of DNA is assessed by ensuring amplification of the internal control RNaseP gene. Detection rate= 100%. All specimens with known homozygous deletions of SMN1 exon 7 have screened positive using the screening assay. Reportable range= Results are reported as: No SMN1 exon 7 deletion; Heterozygous SMN1 exon 7 deletion; Homozygous deletion. SMN2 copy number is also reported. PerkinElmer offers a Customer Contracted Manufacturing Service (CCMS) where laboratories with an existing LDT may source their raw materials from, achieving a greater level of control over the quality.
Considerations of Screening and Diagnostic Testing	False positives, carrier detection, invasiveness of method, other. TAIWAN: False positives: A hybrid allele of SMN1 present in the Taiwanese population gives rise to a false positive result. 8 false positives have been reported. Carrier detection: Carriers are not detected. Invasiveness of method: Minimal, results obtained from routine newborn DBS acquired via heel stick. NY State: False positives: None reported. Carrier detection: Carriers are detected and reported (heterozygous SMN1 exon 7 deletion). 48 carriers have been detected thus far. Invasiveness of method: Minimal, results obtained from routine newborn DBS acquired via heel stick. PERKINELMER: False positives: None reported. Carrier detection: Carriers are detected (SMN1 heterozygous). SMN2 copy number (a determinant of disease severity) is detected. Invasiveness of method: Minimal, results obtained from routine newborn DBS acquired via heel stick.
Potential Secondary Findings	Detection or suggestion of other disorders. TAIWAN: None NY State: None PERKINELMER: None

SECTION II, PART B

CONFIRMATORY	

TESTING	STATEMENT
Clinical and Analytical Validity	Quantitative or qualitative? Include sensitivity, specificity, etc. The diagnostic test for SMA detects the presence or absence of the SMN1 gene, due to deletion or gene conversion of the SMN1 gene to the SMN2 gene, using DNA analysis of both SMN1 alleles. It is an allele specific real-time PCR assay. It is estimated to be 99% analytically sensitive for the presence of the SMN1 gene and approximately 95% clinically sensitive for patients with features of SMA. Approximately 5% of affected individuals with SMA have compound heterozygosity for a rare intragenic point mutation within the SMN1 gene on one chromosome and a deletion/gene conversion of SMN1 exon 7 on the other chromosome. The standard molecular diagnostic test is unable to detect those individuals.[19] However, there are clinical tests available, although not widely utilized, which can detect SMN1 point mutations in cases in which a patient shows clinical signs of SMA but the carrier genotype: www.ncbi.nlm.nih.gov/gtr/all/tests/?term=C0026847. The standard diagnostic test does not identify patients who are heterozygous carriers for the SMN1 deletion, and a distinct diagnostic test is routinely utilized to detect carriers.[19, 20]
Type of test and/or sample matrix (blood, radiology, urine, tissue sample, biophysical test)	The SMA diagnostic test is conducted from a whole blood sample.[18] It can also be conducted prenatally from amniotic fluid or amniocytes or chorionic villus (CVS) culture.[18]
Is test FDA cleared/approved	Include availability information, sole source manufacturer, etc. No
List all CLIA certified labs offering testing in the US	Link to GeneTests and Genetic Test Reference if applicable. The SMA diagnostic test is available at CLIA certified labs throughout the United States. A comprehensive list can be found at: https://www.genetests.org/search/tests.php?locations[]=USA&user_submitted=1&search=SPINAL+MUSCULAR+ATROPHY&filter_status=1

SECTION II, PART C

POPULATION-BASED PILOT STUDY	STATEMENT
Location of Prospective Pilot	Two prospective pilots have been ongoing in SMA. They are described below. TAIWAN: National Taiwan University Hospital newborn screening center, Taiwan (manuscript under review at time of submission, Dr. Yin-Hsiu Chien personal communication). Contact: Dr. Yin-Hsiu (Nancy) Chien, National Taiwan University: chienyh@ntu.edu.tw NY STATE: New York, New York (unpublished, Dr. Wendy Chung personal communication). Contact: Dr. Wendy Chung, Columbia University (New York): wkc15@cumc.columbia.edu PERKINELMER: No prospective pilots had begun using this assay at the time of submission, but plans are in development for such a study in Wisconsin, once the retrospective study on de-identified dried blood spots described in the section above is completed. Dr. Mei Baker, Wisconsin Laboratory of Health: mei.baker@slh.wisc.edu Additional pilots are proposed in Massachusetts and North Carolina. -MA Contact: Dr. Anne Comeau: New England Newborn Screening Lab (Massachusetts): Anne.Comeau@umassmed.edu -NC Contact: Dr. Don Baily at RTI: dbailey@rti.org Plan to begin Spring 2018. Legislation to add SMA to statewide NBS in Missouri (HB 66) has been proposed and is currently undergoing the approval process. As of 2/16/17, the bill had passed the Missouri House of Representatives and will be moved to the Senate shortly.
Number of Newborns Screened	TAIWAN: From November 2014 to September 2016, 120,267 newborns were screened. NY STATE: From January 2016 to December 2016, 3,269 newborns have been screened (pilot ongoing at the time of submission).
Number of Screen Positive Results	Positive by primary test vs. 2nd tier test if applicable. TAIWAN: 15 by primary test, 7 by 2nd tier test, with a measured incidence of 1 in 17,181 infants screened. NY STATE: One infant screened positive in both the primary and secondary test.
False Positive Rate; False Negative Rate (if known)	False positive by primary test vs. 2nd tier test if applicable. TAIWAN: 8 false positives by primary test, zero false positives by 2nd tier test (false negatives unknown) NY STATE: False positive rate = 0% False negative rate = unknown (none have been reported to the newborn screening program). *The qPCR test targeting the SMN1 exon 7 deletion will only

SECTION II, PART C

	identify infants with the exon 7 deletion. Infants with SMA who are compound heterozygous for the deletion and a different mutation could be incorrectly classified as carriers, and infants with SMA and two mutations not tested could be incorrectly classified as screen negative
Number of Infants Confirmed with Diagnosis	How is diagnosis confirmed [clinical, biochemical, molecular]? TAIWAN: 7 confirmed by molecular confirmation of homozygous SMN1 exon 7 deletion. For these 7 cases, the following is known: Patient #1- Normal at age 25 months Patient #2- Sibling has SMA, refused further contact Patient #3- Clinically followed, SMA onset at 13 months of age Patient #4- Respiratory failure at birth, death at 3 months of age Patient #5- SMA onset at age 2 months Patient #6- Enrolled in the Biogen NURTURE Trial. Patient #7- Enrolled in the Biogen NURTURE Trial. NY STATE: One infant with molecular confirmation of homozygous SMN1 exon 7 deletion. The patient was enrolled in Biogen's NURTURE trial.

SECTION III – KEY REFERENCES

LIST OF REFERENCES **Limited to 20 references from scientific journals to support statements in Sections I-IV. For sources based on un/non-published data, references may be written statements from clinicians, researchers, and/or investigators.**	
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SUBMISSION CHECK LIST		SUBMIT NOMINATIONS ELECTRONICALLY TO:
☐	Cover letter by Nominator	Deboshree Sarkar, MPH DACHDNC Designated Federal Officer Genetics Services Branch Division of Services for Children with Special Health Needs, Maternal and Child Health Bureau, HRSA 5600 Fishers Lane, Room 18A-19, Rockville, MD 20857 Email: Screening@hrsa.hhs.gov Fax: 301-480-1312 Phone: 301-443-1080
☐	Nomination form	
☐	Conflict of Interest Forms filled out by Nominator and all Co-Sponsoring Organizations	
☐	Copies of publications/articles used as references	

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Newborn Blood Spot Screening Test Using Multiplexed Real-Time PCR to Simultaneously Screen for Spinal Muscular Atrophy and Severe Combined Immunodeficiency

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BACKGROUND: Spinal muscular atrophy (SMA) is a motor neuron disorder caused by the absence of a functional survival of motor neuron 1, telomeric (*SMN1*) gene. Type I SMA, a lethal disease of infancy, accounts for the majority of cases. Newborn blood spot screening (NBS) to detect severe combined immunodeficiency (SCID) has been implemented in public health laboratories in the last 5 years. SCID detection is based on real-time PCR assays to measure T-cell receptor excision circles (TREC), a byproduct of T-cell development. We modified a multiplexed real-time PCR TREC assay to simultaneously determine the presence or absence of the *SMN1* gene from a dried blood spot (DBS) punch in a single reaction well.

METHOD: An *SMN1* assay using a locked nucleic acid probe was initially developed with cell culture and umbilical cord blood (UCB) DNA extracts, and then integrated into the TREC assay. DBS punches were placed in 96-well arrays, washed, and amplified directly using reagents specific for TREC, a reference gene [ribonuclease P/MRP 30kDa subunit (*RPP30*)], and the *SMN1* gene. The assay was tested on DBS made from UCB units and from peripheral blood samples of SMA-affected individuals and their family members.

RESULTS: DBS made from SMA-affected individuals showed no *SMN1*-specific amplification, whereas DBS made from all unaffected carriers and UCB showed *SMN1* amplification above a well-defined threshold. TREC and *RPP30* content in all DBS were within the age-adjusted expected range.

CONCLUSIONS: SMA caused by the absence of *SMN1* can be detected from the same DBS punch used to screen newborns for SCID.

Spinal muscular atrophy (SMA),[5] the most common genetic cause of death in infancy *(1)*, is an α-motor neuron disorder caused by insufficient concentrations of the survival of motor neuron (SMN) protein. In about 95% of SMA cases, the reduction in SMN concentrations is due to deletions involving the survival of motor neuron 1, telomeric (*SMN1*)[6] gene *(2)*. The nearly identical survival of motor neuron 2, centromeric (*SMN2*) gene, a paralog of *SMN1*, also produces SMN protein, but at much lower concentrations. *SMN2* copy numbers vary widely between individuals, ranging from complete absence to 5 or more copies per genome *(3)*. Because some SMN protein is essential for fetal development, all babies born with SMA have at least 1 *SMN2* gene. Higher *SMN2* copy numbers in SMA patients are associated with later onset and milder disease, including juvenile onset (type III; OMIM 253400) and adult onset (type IV; OMIM 271150). However, the majority of SMA newborns become symptomatic as infants (type I; OMIM 253300) or toddlers (type II; OMIM 253550). Type 1 children will never sit unsupported, often require

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[†] Jennifer L. Taylor and Francis K. Lee contributed equally to the work, and both should be considered as first authors.

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[5] Nonstandard abbreviations: SMA, spinal muscular atrophy; SMN, survival of motor neuron; DBS, dried blood spot; NBS, newborn blood spot screening; SCID, severe combined immunodeficiency; TREC, T-cell receptor excision circles; UCB, umbilical cord blood; Cq, quantification cycle value; LNA, locked nuclei acids.

[6] Human genes: *SMN1,* survival of motor neuron 1, telomeric; *SMN2,* survival of motor neuron 2, centromeric; *RPP30,* ribonuclease P/MRP 30kDa subunit.

ventilatory support in the first year of life, and usually die before 2 years of age. Type II children can survive to early adulthood but will never walk. Cognitive abilities are not impaired by SMA and affected children are generally bright and sociable.

Several potential therapies for SMA are in development *(1, 4)*, 2 of which are undergoing clinical trials in symptomatic children with some encouraging results *(5, 6)*. Because of the early onset and rapid progression of infantile SMA, evaluation of these therapies in presymptomatic infants will require prompt detection. Such early detection would be possible in about 95% of SMA cases by screening newborn dried blood spots (DBS) for the homozygous absence of *SMN1* sequences around exon 7 *(7, 8)*.

The most recent condition added to the US Recommended Uniform Screening Panel for newborn blood spot screening (NBS) is severe combined immunodeficiency (SCID) *(9)*, a congenital disorder with severe impairment of cellular and humoral immune function due to a profound deficiency in T cells *(10)*. The assay most commonly used for SCID-NBS is real-time PCR to measure T-cell receptor excision circles (TREC), extrachromosomal DNA byproducts of somatic recombination in T cells *(10)*. SCID is the first NBS condition for which DNA analysis is the primary (first-tier) screening method. Since the initial pilot experiences in 2 US state public health laboratories *(11, 12)*, SCID-NBS has expanded to many other state programs *(13, 14)* and now covers the majority of newborns in the US as well as many newborns globally *(15)*. SCID-NBS prevents infant death through early medical intervention and is highly cost-effective *(16, 17)*.

Because of similarities in methods, we reasoned that both SCID and SMA could be detected in the same real-time PCR reaction-well by modifying existing high-throughput TREC PCR assays to include *SMN1* genotyping. The combined assay had to be specific for *SMN1* to avoid cross-reactivity with the *SMN2* gene. Here we show that homozygous *SMN1* absence can be reliably detected from the same DBS punch used to measure TREC at minimal incremental cost.

Materials and Methods

SOURCES OF SAMPLES

DBS and DNA extracts with TREC values in the expected range for typical term newborns were made from residual excluded umbilical cord blood (UCB) units collected at the Duke University Stem Cell Laboratory. SCID-like DBS were prepared from peripheral blood containing no measurable TREC obtained from adults above age 50 years. Before spotting, the blood was depleted of mononuclear cells by layering on Histopaque (Sigma-Aldrich), centrifuging 30 min at 2100*g*, aspirating the fluid above the buffy coat, and reconstituting to 50% hematocrit with pooled serum. Immortalized B lymphocyte and fibroblast cell lines from patients with SMA (GM 23689, GM 10684, GM 03813, GM 00232, and GM 09677) and carriers (GM 23688, GM 23687, GM 03814, and GM 03815) were obtained from the Coriell Institute for Medical Research. DBS with SMA-affected or -carrier genotypes were made from residual peripheral blood samples obtained with informed consent *(3)* from 11 SMA-affected individuals (age range, 1–50 years) and from 15 unaffected parents (age range, 25–57 years). DBS were stored in low-permeability ziplock bags with silica gel desiccant packs (Poly Lam Products) up to one month at room temperature, up to 6 months at 4 °C, and up to 2 years at −20 °C. The CDC laboratory staff was blinded to sample status and had no access to personal identifiers. The study was therefore classified as human subjects research for which the CDC was not engaged.

REAL-TIME PCR ASSAYS

All primers and probes (Table 1) were custom synthesized by Integrated DNA Technologies. Real-time PCR assays were conducted in PCR plates (96-well formats; Agilent Technologies) using a scanning photofluorometric thermal cycler (Stratagene MxPro 3000p). Cycle thresholds were initially determined by inspection of amplification curves and then retained in fixed positions. Quantification cycles (Cq) were reported by instrument software.

TREC QUANTIFICATION IN DBS

The real-time PCR assay was performed in situ on 2-mm discs punched from DBS samples directly into 96-well PCR arrays. After 125 μL wash buffer was added (DNA elution solution, Qiagen) to each well, the PCR array was incubated at room temperature for 15 min on a microtiter plate shaker set at 1200 rpm. The wash buffer was then removed, and 15 μL of the complete real-time PCR master mix (PerfecTa Toughmix, Quanta Biosciences) containing primers and probes (Table 1) was added to each sample well. The PCR plate was sealed with optical film and processed using the following amplification conditions: 45 °C for 3 min and 95 °C for 20 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. A reagent blank and a blank filter paper punch (no-template control) were included in the analytical runs.

SMN1 ANALYSIS IN DNA EXTRACTS

DNA was extracted from cell lines or UCB samples using the Qiagen QlAamp® Mini DNA kit according to the manufacturer's protocol. DNA extracts were analyzed immediately or stored at −20 °C for up to 6 months. Extracts from tissue culture cells contained 3–50 ng/μL DNA and extracts from UCB contained 100–150 ng/uL

Table 1. PCR primers and probes for the triplex real-time PCR assay to amplify sequences in the δRec-ΨJα signal joint (TREC) and in *RPP30* and *SMN1*.

Target/reagent	Sequence	Concentration (nmol/L)[a]
TREC		
Forward primer	5`-TTT GTA AAG GTG CCC ACT CCT-3`	800
Reverse primer	5`-TAT TGC AAC TCG TGA GAA CGG TGA AT-3`	800
Probe	5-FAM/CGGTGATGCATAGGCACCT/Iowa Black quencher-3`	120
RPP30		
Forward primer	5`-TTT GGA CCT GCG AGC G –3`	60
Reverse primer	5`-GAG CGG CTG TCT CCA CAA GT-3`	150
Probe	5`-HEX/TTCTGACCTGAAGGCTCTGCGCG/Iowa Black quencher-3`	200
SMN1		
Forward primer	5`-GTGGAAAACAAATGTTTTTGAACATT-3`	900
Reverse primer	5`-GTAGGGATGTAGATTAACCTTTTATCTAATAGTTT-3`	900
LNA probe[b]	5`-Cy5/CAA***C***TT***TT***TAA***C***AT***C***T/3IAbRQSp-3`	100

[a] Final concentration in PCR reaction.
[b] Bases in bold italic font denote LNA nucleotides; the underlined nucleotide indicates the position of the A>G transition in intron 7 that distinguishes *SMN1* from *SMN2*.

DNA. Real-time PCR was conducted in 20-μL reaction volumes containing 5 μL of the DNA extract, a commercial real-time PCR premix (PerfecTa, Toughmix, Quanta), the *SMN1* forward primer and reverse primer, the *SMN1* locked nucleic acid (LNA) probe overlying the A>G transition at position 100 of intron 7 to distinguish *SMN1* from *SMN2*, and the *RPP30* forward primer, reverse primer, and probe (Table 1). Primers and probe sequences for *SMN1* were designed using Primer Express (Life Technologies) and confirmed for in silico specificity. Probe modification with LNA bases was designed with software from IDT Biophysics. To determine the optimal annealing temperature, reactions were carried out in a Bio-Rad Laboratories CFX96 real-time PCR instrument with the following amplification conditions: 45 °C for 3 min and 95 °C for 10 min, followed by 45 cycles of melting at 95 °C for 15 s and annealing/extension between 60 °C and 67 °C for 1 min.

Results

SMN1 ASSAY DEVELOPMENT

Prototype assays were initially explored using extracted DNA from cell lines of patients with SMA, cell lines of SMA carriers, and UCB samples. We first used a temperature gradient to determine the optimal PCR annealing temperature for maximal specificity to discriminate *SMN1* and *SMN2* amplification. The results (Fig. 1) showed increasing discrimination between the patients and unaffected controls with increased annealing temperature. At 65 °C, no *SMN1* amplicon was detected by the LNA probe in patients with SMA, whereas healthy individuals and carriers showed clear amplification. This annealing temperature of 65 °C provided the highest analytical specificity and was chosen for all subsequent experiments.

The working parameters for a duplex assay that included *SMN1* and a genomic sequence of *RPP30* as an internal control reference for PCR amplification were then optimized. This duplex assay was applied to DNA extracts prepared from commercially available cell lines from 5 SMA patients and 4 parental carriers as well as cellular DNA from 5 UCB samples. Results (Fig. 2) demonstrated a 100% concordance with the clinical status of the donors. No amplification was seen with DNA extracted from any of the SMA patient cell lines, indicating no detectable cross-reactivity with *SMN2*. In contrast, DNA extracted from cell lines from the unaffected carriers and UCB produced robust amplification, with Cq values ranging from 18 to 25 cycles. *RPP30* in all samples showed amplification in the expected Cq range.

Having established the working conditions for the *SMN1* assay, we incorporated it into the in situ DBS assay for TREC with the annealing temperature raised to 65 °C. Reference DBS samples from a SCID-like positive control with no TREC, a positive control from an SMA infant with no *SMN1*, and a normal control from a UCB sample showed clear discrimination between the amplification patterns of the corresponding targets (Figs. 3). Varying the SMN primer concentration from 50 nmol/L to 900 nmol/L exerted no effect on the other multiplexed targets (TREC and *RPP30*; see Fig. 1 in Data Supple-

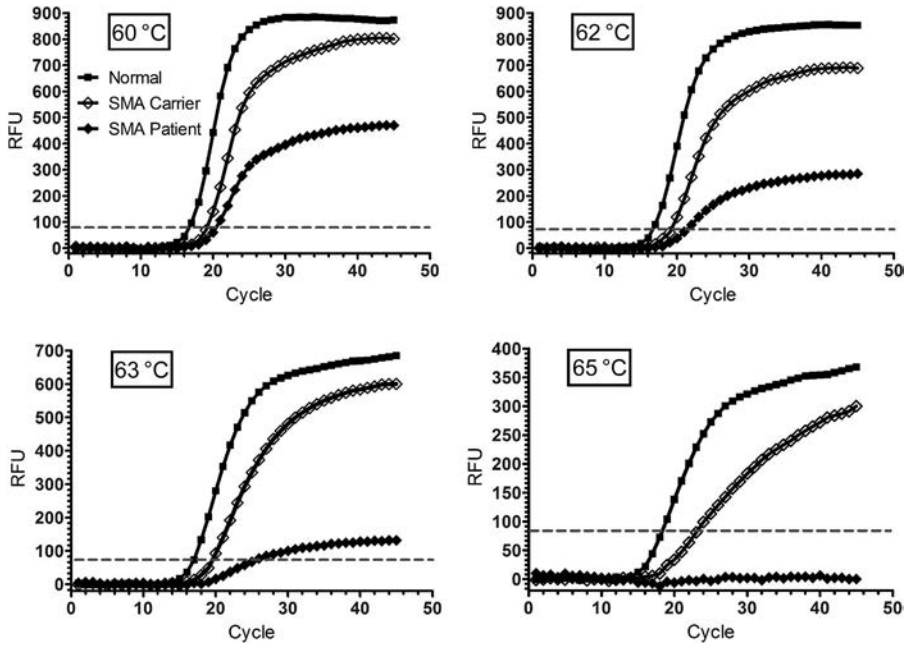

Fig. 1. Seven temperatures (60 °C to 67 °C) were tested to determine the appropriate annealing temperature for the *SMN1* gene; results are shown for 4 selected temperatures. The dashed grey horizontal line denotes the fluorescence threshold for positive results and Cq determination. RFU, relative fluorescence units.

ment that accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue2). Therefore primer limitation was not necessary, and the maximum primer concentration (900 nmol/L) in the range tested was used for both forward and reverse *SMN1* primers in all subsequent experiments.

To determine whether the inclusion of the *SMN1* target and the higher (65 °C) annealing temperature would alter the TREC assay, we first compared results on DNA extracts (see online Supplemental Fig. 2). The TREC Cq values were highly correlated ($r^2 = 0.87$) and showed no significant difference between the original duplex (TREC and *RPP30* at 60 °C annealing temperature) and the modified triplex assay ($P > 0.14$ by paired *t*-test). Next we tested 150 DBS made from UCB with both the original duplex assay and the modified triplex assay. All samples showed the typical amplification curves expected for TREC, *SMN1,* and *RPP30*. TREC Cq values obtained by the in situ DBS triplex assay showed a slight decrease (mean 0.4 Cq) compared to those from the duplex assay but were consistent with the overall ranges for term newborns reported by public health newborn screening programs *(11–14)*. The Cq values for *SMN1* showed a near-gaussian distribution (Fig. 4A). Cq values for *SMN1* and *RPP30* (Fig. 4B) were significantly correlated ($r^2 = 0.66$, $Sy/x = 0.47$, $P < 0.01$), suggesting that the variation between samples was due mostly to differences in leukocyte content and thus in the total amount of genomic DNA.

VALIDATION IN CLINICAL SAMPLES

The clinical validity of the TREC-*RPP30*-*SMN1* triplex assay was examined in a double-blind testing of DBS made from 26 blood samples from SMA patients and their carrier parents. This sample set was analyzed independently at the 2 collaborating laboratories (CDC and Biogen Idec) using the triplex in situ DBS method. The *SMN1* categorical genotypes obtained by both laboratories were identical and showed 100% concordance with the clinical status of the patient (Table 2). All samples showed typical amplification of the internal reference control *RPP30*, and the TREC Cq values were within the expected range for the age of the donor. Reagent blank and blank filter paper punches (no template controls) showed no amplification for any of the 3 targets.

Discussion

The early onset and precipitous clinical course of infantile SMA make it a prime target for early detection through NBS *(7)*. Compared with other conditions currently recommended for NBS in the US *(18)*, SMA has a high birth prevalence, ranking just below the top 3 of

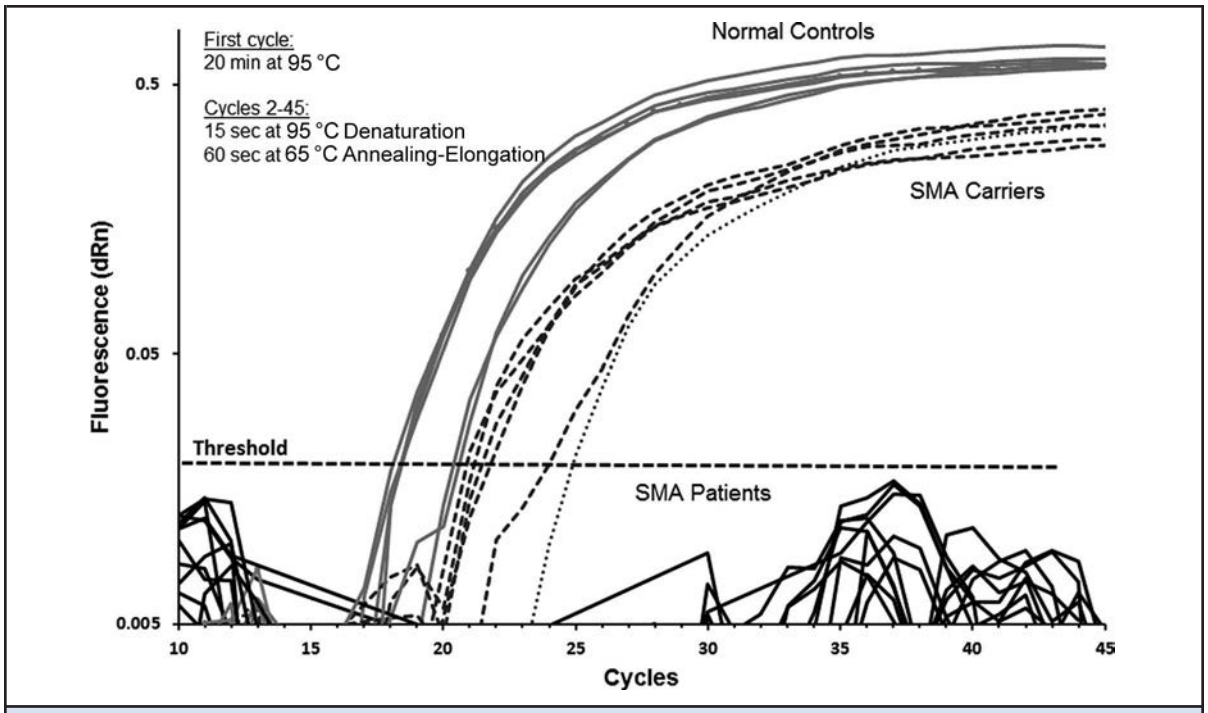

Fig. 2. PCR amplification curves from DNA extracts of 5 randomly selected umbilical cord blood samples (normal controls), 6 cell lines from SMA parental carriers, and 5 cell lines from SMA patients with homozygous *SMN1* deletion. dRn, Δ normalized reporter.

29 NBS primary target conditions presently on the Recommended Uniform Screening Panel (congenital hypothyroidism, cystic fibrosis, and hemoglobinopathy). As with SCID, early detection of SMA is likely to provide the best opportunity for effective treatment. With multiple therapeutics currently in clinical trials, and more in development *(1, 3, 19, 20)*, NBS laboratories are in an ideal position to facilitate presymptom-

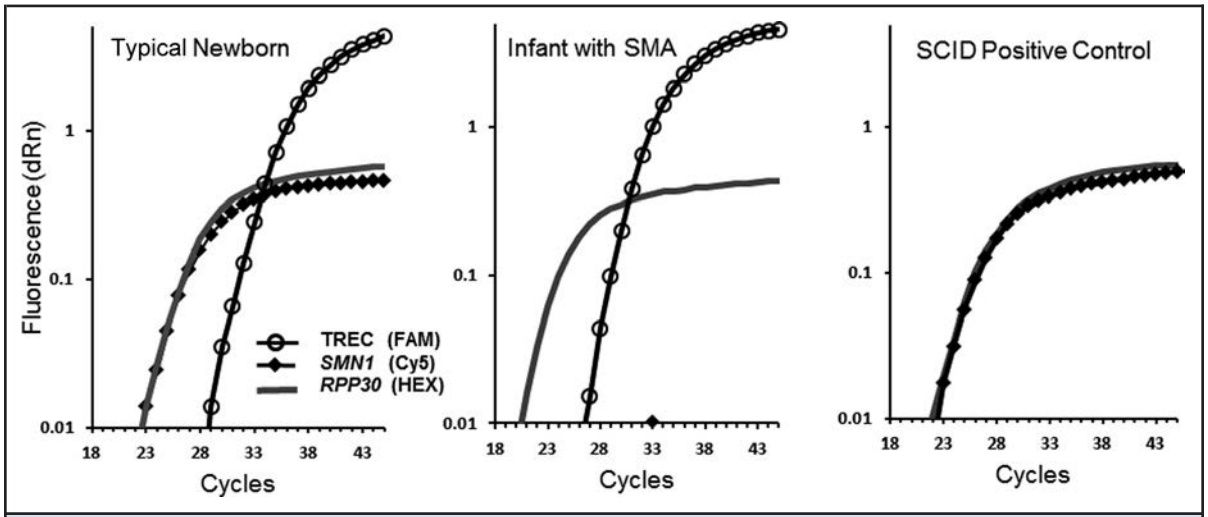

Fig. 3. Triplex TREC-SMN1-RPP30 DBS real-time PCR amplification curves.

DNA targets were amplified on each DBS punch without DNA extraction. *RPP30* (present in all samples); *SMN1* (absent in SMA infant); TREC (absent in SCID-positive control). dRn, Δ normalized reporter; FAM, 6-carboxyfluorescein; HEX, β-hexosaminidase; Cy5, cyanine 5.

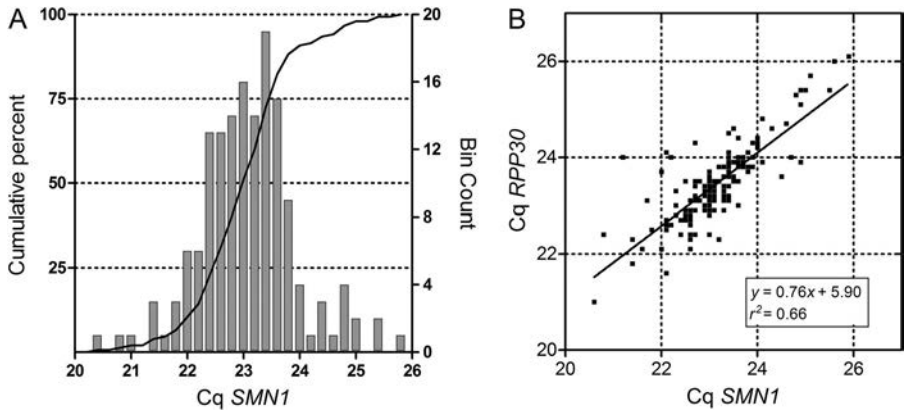

Fig. 4. (A) Distribution of *SMN1* Cq results on dried blood spots made from 150 umbilical cord blood samples analyzed at the CDC laboratory. (B) Correlation between Cq results for *SMN1* and *RRP30* ($r = 0.66$, $P < 0.01$).

atic intervention that could maximize the benefits of potential therapies.

Over the last 2 decades, the complex biology and natural history of SMA have been largely delineated *(2)*. Although several factors influence pathogenesis, about 95% of diagnosed SMA cases are caused by the homozygous absence of a functional *SMN1* gene through either deletion or gene conversion. Various approaches to detecting the *SMN1* null genotype have been developed, and some have been validated in DBS samples, including real-time PCR *(8)*, high-resolution melting *(21)*, and microbead suspension arrays *(22)*.

Two major considerations determined our approach to developing an NBS assay for SMA. First, it would be preferable to use an assay platform that is well established in public health newborn screening. Although genotype-based sickle cell disease screening had been performed previously *(23)*, until recently none of the primary screening assays used in public health NBS programs used molecular DNA methods. The incorporation of a DNA-based assay has been prompted by the recommended inclusion of SCID-NBS, which is based on measuring TREC *(24)*. The preferred platform for measuring TREC is real-time PCR, and this technology is now routinely used in screening the majority of newborns in the US for SCID. Second, it would be advantageous to multiplex the *SMN1* assay with an existing routine NBS assay, thereby assuring its robustness and minimizing the incremental cost for detecting SMA. We therefore explored the possibility that *SMN1* genotyping could be multiplexed with the TREC assay developed at CDC, which is used to characterize DBS reference materials for global distribution to participants in the CDC Newborn Screening Quality Assurance Program *(25)*.

The main challenge in developing a PCR assay for *SMN1* gene absence is caused by the near identity of the *SMN1* and *SMN2* gene sequences. Conventional real-time PCR probes to any targeted *SMN1* sequence could cross-react with the corresponding *SMN2* sequence and produce false amplification signals when the *SMN1* sequence is absent, as in an affected SMA sample. Other investigators have previously reported successful reduction of cross-reacting fluorescent signals by using unlabeled *SMN2* probes as a blocker, in combination with a labeled *SMN1* probe with a minor grove binder group at the 3′ end. We opted for an alternative approach to achieve the required analytical specificity by increasing the stringency of probe hybridization using a novel LNA probe. LNA oligonucleotides increase assay specificity through restriction of the ribose conformation in the oligonucleotide backbone, allowing the use of higher annealing temperatures. These shorter probes are known to improve the ability to discern single-nucleotide polymorphisms *(26)*, such as those that distinguish *SMN1* from *SMN2*. LNA probes therefore simultaneously increase both the specificity and sensitivity of the assay.

Another important concern in multiplexing PCR is the potential for different amplicons to compete for PCR reagents. Since TREC is present in a much lower concentration than *SMN1*, the effect of competition would be a decreased amplification for TREC. A serial dilution series for the *SMN1* primers was analyzed to explore this possibility and showed no effect on the other multiplexed targets (TREC and *RPP30*). However, with DNA extracts of normal UCB samples and carrier cell lines, higher primer concentrations did increase the plateau fluorescence for *SMN1*, increasing visual discrimination from the baseline fluorescence of *SMN1*-absent SMA patients.

In addition to familiar instrumentation and multiplexing capability, another factor in developing an approach to SMA-NBS was the high prevalence of SMA carriers. In our assay, both normal and carrier *SMN1* genotypes showed

Table 2. Results of blinded testing of DBS samples prepared from peripheral blood samples of 11 SMA patients and 15 parental carriers by the multiplex TREC-*SMN1*-*RPP30* DBS real-time PCR assay.

SMA status	SMA type	Age, years	*SMN1* result[a]	*SMN1*, Cq[b]	*RPP30*, Cq[b]	TREC, Cq[b]
Patient	III	4	Absent	No Cq	23.8	30.2
Patient	III	2	Absent	No Cq	24.2	31.7
Patient	II	50	Absent	No Cq	25.1	34.6
Patient	III	3	Absent	No Cq	23.5	29.6
Patient	II	1	Absent	No Cq	24.5	30.0
Patient	II	22	Absent	No Cq	23.7	30.4
Patient	III	13	Absent	No Cq	23.2	31.3
Patient	III	3	Absent	No Cq	23.2	29.5
Patient	II	1	Absent	No Cq	21.6	28.7
Patient	III	4	Absent	No Cq	22.6	28.5
Patient	III	2	Absent	No Cq	22.5	29.1
Carrier[c]	NA[d]	45	Present	24.2	23.3	34.3
Carrier	NA	33	Present	26.9	25.0	34.3
Carrier	NA	34	Present	25.6	24.8	33.9
Carrier	NA	29	Present	25.0	23.8	34.0
Carrier	NA	32	Present	24.2	23.2	34.4
Carrier	NA	43	Present	23.3	22.2	34.8
Carrier	NA	43	Present	22.7	21.9	33.0
Carrier	NA	41	Present	23.0	22.4	35.0
Carrier	NA	57	Present	25.5	24.9	34.4
Carrier	NA	48	Present	22.7	22.3	35.3
Carrier	NA	48	Present	22.5	22.8	34.4
Carrier	NA	44	Present	25.8	25.1	36.7
Carrier	NA	35	Present	21.1	21.1	31.3
Carrier	NA	33	Present	22.6	22.6	No Cq
Carrier	NA	25	Present	21.8	22.0	31.8

[a] Concordant categorical results from CDC and Biogen-Idec laboratories.
[b] Cq results from CDC laboratory.
[c] Unaffected parent of SMA patient.
[d] NA, not applicable.

robust *SMN1* amplification, and the difference in Cq was not sufficiently precise for reliable carrier identification. In contrast, all of the homozygous *SMN1*-negative samples failed to cross the cycle threshold, and they were clearly discriminated from samples with one or more *SMN1* copies.

The determination of *SMN2* copy number is an essential second-tier assay for following up screen-positive samples. It is the most informative prognostic marker and will guide selection criteria for clinical trials. *SMN2* copy number may be determined by real-time qPCR *(27)* as well as other methods *(28)*. Ideally, public health NBS laboratories could perform a single second-tier assay that would both confirm the absence of *SMN1* and quantify the *SMN2* copy number, thereby identifying newborns at high risk for infantile onset. Preliminary studies in our laboratories suggest that digital droplet PCR *(29)* will also be a useful platform for independent confirmation and characterization of SMA screen-positive samples.

The rapid and increasingly widespread implementation of SCID-NBS has ushered real-time PCR technology into the repertoire of NBS laboratories. Since its inception in 2008, SCID-NBS has been implemented in 25 US public health NBS programs, collectively identifying SCID at twice the previously estimated birth prevalence and achieving high survival rates in treated infants *(11)*. This experience strongly suggests that SMA-NBS will be technically feasible and cost-efficient.

In conclusion, we developed a multiplexed real-time PCR assay to simultaneously measure TREC and screen for the absence of *SMN1* in a single reaction-well. The

addition of *SMN1* genotyping to the TREC assay does not require new equipment or any changes in sample processing or the overall testing procedure. The additional reagent and supply costs beyond those of the current TREC assay are limited to the *SMN1* primers and probe, which amount to less than 5 cents per test. The assay allows clear identification of the *SMN1* null genotype without quantifying copy number, thereby avoiding carrier detection. NBS programs screening for SCID will require evidence of effective presymptomatic intervention in newborns with SMA before combining the 2 tests into routine screening. However, as soon as an effective therapy becomes available, SMA-NBS could be readily implemented alone or in combination with TREC measurements by public health programs already using real-time PCR to screen for SCID.

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ARTICLE

Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72 400 specimens

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Spinal muscular atrophy (SMA) is a leading inherited cause of infant death with a reported incidence of ~1 in 10 000 live births and is second to cystic fibrosis as a common, life-shortening autosomal recessive disorder. The American College of Medical Genetics has recommended population carrier screening for SMA, regardless of race or ethnicity, to facilitate informed reproductive options, although other organizations have cited the need for additional large-scale studies before widespread implementation. We report our data from carrier testing (*n*=72 453) and prenatal diagnosis (*n*=121) for this condition. Our analysis of large-scale population carrier screening data (*n*=68 471) demonstrates the technical feasibility of high throughput testing and provides mutation carrier and allele frequencies at a level of accuracy afforded by large data sets. In our United States pan-ethnic population, the calculated *a priori* carrier frequency of SMA is 1/54 with a detection rate of 91.2%, and the pan-ethnic disease incidence is calculated to be 1/11 000. Carrier frequency and detection rates provided for six major ethnic groups in the United States range from 1/47 and 94.8% in the Caucasian population to 1/72 and 70.5% in the African American population, respectively. This collective experience can be utilized to facilitate accurate pre- and post-test counseling in the settings of carrier screening and prenatal diagnosis for SMA.
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Keywords: spinal muscular atrophy (SMA); pan-ethnic; carrier screening; *SMN1*

INTRODUCTION

Spinal muscular atrophy (SMA) is a severe neuromuscular disease characterized by degeneration of the anterior motor neurons, leading to progressive muscle weakness and paralysis. SMA is the leading inherited cause of infant death[1] with an incidence of ~1/10 000.[2] Childhood SMA is subdivided based on age of onset and clinical severity into three types.[3] Approximately 60% of SMA patients have type I (Werdnig–Hoffman) disease,[4] with severe generalized muscle weakness and hypotonia presenting at birth or within the first few months of life and respiratory failure leading to death or permanent ventilator support by 2 years of age. Type II accounts for ~27% of SMA,[4] and is variable, with some children having severe respiratory insufficiency and transient ability to sit, whereas others have milder respiratory involvement and are mobile with mechanical support. Individuals with type III SMA (Kugelberg–Welander) experience delayed motor milestones, mild muscle weakness and fatigue.

SMA is caused by mutations in the survival motor neuron 1 (*SMN1*) gene.[4] The *SMN1* gene is located in a complex region of 5q13 containing *SMN2*, a homologous pseudogene of *SMN1*. *SMN1* and *SMN2* differ by five nucleotides, one of which is in the coding region, in exon 7. This sequence change affects splicing resulting in reduced expression of full-length functional protein from the *SMN2* gene.[5] The homozygous absence of *SMN1*, due to deletion or gene conversion (of *SMN1* to *SMN2*) is responsible for ~95% of SMA. Most of the remaining patients are compound heterozygotes with a deletion/gene conversion of the *SMN1* gene paired with an intragenic mutation. Affected individuals lacking functional *SMN1* retain at least 1 copy of *SMN2*. An inverse relationship between disease severity and *SMN2* copy number in affected individuals has been observed.[6] Other modifying factors, including *SMN2* sequence variants, may also influence phenotypic variability.[7]

Among normal alleles, 1-copy and 2-copy chromosomes are designated as '1' (*b*) and '2' (*c*), respectively.[4,8] Chromosomes resulting from a deletion or gene conversion are referred to as a '0' (*a*), whereas those with subtle *SMN1* intragenic mutations are referred to as '1^{d}' (*d*).[4]

Determination of *SMN1* copy number in a general carrier screening population permits identification of the majority of SMA carriers before the birth of an affected child. Initial reports of *SMN1* copy number quantification focused on diagnostic testing for affected individuals without a homozygous deletion and carrier testing for individuals with a family history of SMA.[9] In 2002, Ogino *et al*[2] reported testing 663 asymptomatic individuals by *SMN1* copy number analysis, the majority (71%) of whom had a family history of confirmed or suspected SMA. Subsequently,[10–12] studies from around the world including Australia,[13] Korea,[14] Taiwan,[11] Israel,[15,16] China[17] and the United States[18] have called for screening individuals without a family history of SMA for carrier status, citing disease severity and a high pan-ethnic carrier frequency. In 2008, the American College of

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Medical Genetics (ACMG) issued practice guidelines recommending all couples be offered SMA carrier screening, regardless of race or ethnicity, with the goal of allowing identified carriers to make informed reproductive choices.[19] The guidelines identified SMA as meeting generally accepted criteria for a successful screening program including clinical severity, a high frequency of carriers in the screened population, reliable testing with high sensitivity and specificity, available prenatal diagnosis and access to genetic counseling. In 2009, a conference at the US National Institutes of Health reviewed the scientific basis of SMA carrier screening and concluded that pan-ethnic carrier screening for SMA is technically feasible.[20] Recently, the Association for Molecular Pathology (AMP) issued a statement recognizing the utility and feasibility of population-based SMA carrier screening.[21] Among the specific recommendations identified were the needs to offer pilot screening programs and determine SMA carrier frequency among different ethnic groups so as to improve risk assessment and post-test counseling. In 2009, the American College of Obstetricians and Gynecologists (ACOG) recommended restricting carrier screening to individuals with a family history of SMA. ACOG asserted that assessment of pilot programs, educational materials, cost effectiveness of screening and absence of laboratory standards and guidelines should be considered before widespread implementation of a carrier screening program for SMA.[22] Several recent reports address these issues.[18,20]

We report our SMA carrier screening data for >68 400 individuals without a family history of SMA. Our findings indicate a rapid test uptake beginning before the ACMG guidelines, and further support patient interest in the availability of SMA carrier screening. Furthermore, our data permit refinement of carrier frequency and detection rate information for six major ethnic groups and the general, pan-ethnic population and address the call for a large-scale population screening study.

MATERIALS AND METHODS

Patient's samples

Clinical laboratory data were reviewed for 72 453 individuals and 121 fetal samples referred for *SMN1* copy number analysis over a 12-month time period beginning in May 2008. Relevant information including the clinical indication for testing, family history and ethnicity was obtained by review of the test requisition forms. All individuals referred for testing were reportedly asymptomatic. For clinical testing, it is standard for the referring physician to obtain informed consent, therefore an ethics approval was not required. Individuals referred for testing spanned 44 US states, the District of Columbia and Puerto Rico.

Quantitative real time PCR analysis and sequencing

DNA was isolated from blood specimens, using a modification of the Qiagen QIAmp 96 DNA Blood Kit (Qiagen GmbH, Hilden, Germany, http://www.qiagen. com). DNA from prenatal cultured AF or CVS specimens was isolated using a modified salting-out method. Multiplex amplifications and qPCR measurements were performed on the ABI Prism 7900HT Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Each reaction utilized standard Taqman PCR reagents in total volume of 20 µl containing 180 ng of DNA, 0.9 μM of *SMN1* primers (SMN1FP-5′-ATAGCTATTTTTTTTAACTTCCTTTATTTTCC-3′ and SMN1RP-5′-CTTACTCCTTAATTTAAGGAATGTGAGCA-3′) and each of two internal standard reference gene primers (SMARCC1FP-5′-AGGTACCACTGGAATTGGTTGAA-3′, SMARCC1RP-5′-CATATATTAACCCTGTCCCTTAAAAGCA-3′, SUPT5HFP-5′-CACGTGAAGGTGATTGCTGG-3′, SUPT5HRP-5′-CGACCCTTCTATCCACCTACCTC-3′), 0.2 μM each of the *SMN1*-specific FAM-TAMRA hydrolysis probe (5′-AGGGTTTCAGACAAAATCAAAAAGAAGGAAG-3′), reference gene-specific VIC-TAMRA hydrolysis probes (SMARCC1-5′-AGTACAAGAAGCAGCACGAGCCTCTG-3′ and SUPT5-5′-CGTTATCCTGTTCTCTGACCTCACCATG-3′) and a competitive, non-fluorescent, non-hydrolysable probe specific for *SMN2* (5′-AGGGTTTTAGACAAAATCAAAAAGAAGGAAGG-3′). Thermal cycling conditions included an initial denaturation of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Sequencing of primer and probe binding sites was performed on all prenatal specimens, and blood specimens with an *SMN1* copy number of <2, by bidirectional sequence analysis using BigDye Terminator Cycle Sequencing Kit (version 3.1) followed by capillary electrophoresis (Applied Biosystems, Carlsbad, CA, USA).

Data evaluation was performed using the C_q (quantification cycle) data exported from SDS 2.2 software (Applied Biosystems, Foster City, CA, USA). The formula (2).$(2)^{\Delta\Delta C_q}$, where $\Delta\Delta C_q$=(*SMN1* C_q–reference gene C_q)–average ΔC_q of 2-copy calibrators, was utilized to estimate the *SMN1* copy number. Each control and test sample included a copy number CV (coefficient of variation) cutoff of 0.15 between replicate measurements. All results could be assigned to validated, non-overlapping genotype groups of 0, 1, 2 or ≥3 copies of *SMN1*.

Statistical analysis

The 95% confidence intervals around genotype frequency estimates (Table 1) were calculated based on the exact beta distribution model. The derived allele frequencies (Table 2) are maximum likelihood estimates calculated from observed genotype data under assumption of Hardy–Weinberg equilibrium. An EM algorithm was employed to account for missing observations of 0-copy *SMN1* genotype in the screening population. The algorithm converged to six significant digits in estimating allele frequencies after two iterations. The 95% confidence interval around allele frequency estimates and the previous risk estimates (Tables 2 and 3) were calculated as the corresponding percentiles of simulated populations of allele frequencies and risk estimates. These Monte Carlo simulations were based on 10 000 random genotype observations generated from the posterior beta distribution followed by maximum

Table 1 Observed *SMN1* genotype frequencies among carrier screening referrals (*n*=68 471)

	1-copy			*2-copy*			*≥3-copy*			
Ethnicity	n	*%*	*95% CI*	n	*%*	*95% CI*	n	*%*	*95% CI*	*Total*
Pan-ethnic[a]	1162	1.7	(1.6–1.8)	58 094	84.84	(84.6–85.1)	9215	13.46	(13.2–13.7)	68 471
Caucasian	494	2.02	(1.9–2.2)	22 252	90.93	(90.6–91.3)	1725	7.05	(6.7–7.4)	24 471
Ashkenazi Jewish	78	1.34	(1.1–1.7)	4913	84.62	(83.7–85.5)	815	14.04	(13.2–15.0)	5806
Asian	73	1.57	(1.3–2.0)	4148	89.26	(88.4–90.1)	426	9.17	(8.4–10.0)	4647
Hispanic	101	1.32	(1.1–1.6)	6406	83.68	(82.9–84.5)	1148	15.0	(14.2–15.8)	7655
Asian Indian	17	1.74	(1.1–2.8)	808	82.79	(80.4–85.1)	151	15.47	(13.4–17.9)	976
African American	48	0.98	(0.7–1.3)	2536	51.94	(50.5–53.3)	2299	47.08	(45.7–48.5)	4883
Not provided	290	1.68	(1.5–1.9)	14 645	84.97	(84.4–85.5)	2300	13.35	(12.8–13.9)	17 235

Abbreviation: CI, confidence interval.
[a]2798 individuals of mixed or other ethnicity are included in the pan-ethnic total of 68 471.

Table 2 Derived *SMN1* allele frequencies

Ethnicity (number of individuals)[a]	*0-copy (%) (a)*	*95% CI (%)*	*1-copy (%) (b)*	*95% CI (%)*	*2-copy (%) (c)*	*95% CI (%)*	*1[d] (%) (d)*
Pan-ethnic (*N*=68 471)	0.92	(0.87–0.98)	92.02	(91.86–92.17)	7.04	(6.90–7.18)	0.02
Caucasian (*N*=24 471)	1.06	(0.97–1.16)	95.3	(95.09–95.48)	3.63	(3.47–3.80)	0.02
Ashkenazi Jewish (*N*=5806)	0.73	(0.59–0.91)	91.91	(91.36–92.39)	7.34	(6.88–7.84)	0.02
Asian (*N*=4647)	0.83	(0.66–1.05)	94.42	(93.90–94.87)	4.74	(4.31–5.19)	0.02
Hispanic (*N*=7655)	0.72	(0.59–0.88)	91.4	(90.92–91.86)	7.86	(7.43–8.31)	0.02
Asian Indian (*N*=976)	0.96	(0.60–1.54)	90.88	(89.36–92.09)	8.15	(6.98–9.48)	0.02
African American (*N*=4883)	0.68	(0.52–0.91)	71.79	(70.73–72.76)	27.51	(26.57–28.51)	0.02
Not provided (*N*=17 235)	0.91	(0.81–1.03)	92.09	(91.78–92.38)	6.98	(6.71–7.26)	0.02

Abbreviation: CI, confidence interval.
[a]Number of individuals with no family history of SMA referred for *SMN1* copy number analysis by ethnic background.

Table 3 Carrier frequency and risk reduction by ethnicity

Ethnicity	*Detection rate (%)*	*a priori risk[a] (95% CI)*	*Reduced risk for 2-copy result*	*Reduced risk for ≥3-copy result*
Pan-ethnic	91.2	1:54 (1:51–1:57)	1:527	1:5400
Caucasian	94.8	1:47 (1:43–1:51)	1:834	1:5600
Ashkenazi Jewish	90.5	1:67 (1:54–1:83)	1:611	1:5400
Asian	93.3	1:59 (1:47–1:74)	1:806	1:5600
Hispanic	90.0	1:68 (1:57–1:83)	1:579	1:5400
Asian Indian	90.2	1:52 (1:33–1:82)	1:443	1:5400
African American	70.5	1:72 (1:54–1:94)	1:130	1:4200
Not provided	91.3	1:54 (1:48–1:161)	1:536	1:5450

[a]*a priori* risk includes the [1+0], [1+1[d]], [2+0] and [2+1[d]] allele pairings for individuals with no family history of spinal muscular atrophy.

likelihood estimation of the allele frequencies under Hardy–Weinberg assumption. The χ^2-test with Yates correction was used for comparison of 1-copy genotype frequencies between populations.

RESULTS

Population-based carrier screening

Approximately 95% of the 72 453 individuals referred for carrier testing had no family history of SMA. This ethnically diverse carrier screening population was comprised of 68 471 individuals, 94% of whom were female. For analysis purposes, our study population was categorized by ethnicity according to information provided on the test requisition. Ethnicity-specific risk calculations were generated for six ethnic groups (Caucasian, Ashkenazi Jewish, Hispanic, African American, Asian, Asian Indian) and for individuals for whom ethnicity was 'Not Provided' resulting in a total of seven categories. Pan-ethnic calculations included individuals from these categories as well as those reporting multiple or other ethnicities (*n*=2798). The *SMN1* allele frequencies (*a,b,c*) derived from the observed genotype frequencies (Table 1) are listed in Table 2. For the purpose of this study, the allele frequency of intragenic *SMN1* point mutations (*d*), was presumed to be constant across ethnic groups based upon an earlier report describing *SMN1* deletion/gene conversion and point mutation distribution among 501 affected patients.[23] The calculated SMA carrier frequency and *SMN1* deletion detection rate for the pan-ethnic population and each of seven categories are listed in Table 3. The calculated carrier frequency is the *a priori* risk for an individual to be a carrier and includes the probability of the most likely allele pairings expected to occur among carriers in the general population, namely [1+0], [1+1[d]], [2+0] and [2+1[d]]. The *SMN1* deletion detection rate (or sensitivity of the carrier test result) is the percentage of carriers with the [1+0] genotype (who will be identified by a 1-copy result), among all carriers of SMA (as determined by the carrier frequency). The reduced carrier risk following a 2- or 3-copy result in an individual with no family history of SMA is listed in Table 3.

The calculated pan-ethnic carrier frequency of 1/54 (Table 3) is consistent with the frequency of 1/40–1/60 frequently cited in the literature.[13] The carrier frequencies among each of the ethnic groups ranged from 1/47 in the Caucasian population to 1/72 in the African-American population. The carrier detection rate in the pan-ethnic screening population is 91.2%. Among the seven categories for whom prior carrier risk and carrier detection rate is calculated (Table 2), all but African Americans have a detection rate exceeding 90% and an *a priori* risk in the range of 1/47–1/68.

The carrier detection rate in the African American population is 70.5%. This finding from our large scale clinical experience confirms our earlier observation[24] of a decreased carrier detection rate (Table 3) due to an associated increased 2-copy (*c*) allele frequency (Table 1) in the African American population. Furthermore, the estimated negative predictive value of the carrier test as calculated by the derived allele pairings, was >99% for both the pan-ethnic group as well as each of the six major ethnic subgroups.

Other carrier test indications

Among 72 453 individuals referred for carrier testing, greater than 95% (*n*=68 945), had an indication for testing provided by the referring physician. The 4.8% (*n*=3508) of referrals for whom no indication was provided, were excluded from further analysis, although they were most likely referred for carrier screening, as 95% of these samples were from women of reproductive age. Fewer than 1% (*n*=329) of individuals referred for carrier testing had indications of a family history of SMA (including obligate carriers) or abnormal fetal ultrasound findings. Of the 71 individuals referred for an indication of abnormal fetal ultrasound findings, the most frequent findings were increased nuchal translucency (*n*=17) and cystic hygroma (*n*=4) in the first trimester and joint contractures (*n*=18) and increased nuchal fold (*n*=10) in the second trimester. Three individuals were identified as carriers; all others, including the partners of these carriers, had a 2- or 3-copy result. Among the three carriers, only one had fetal SMA testing in our laboratory and the fetus had 2 *SMN1* copies. These results indicate that SMA may be included as a differential diagnosis following identification of suggestive abnormal fetal ultrasound findings.

Prenatal diagnosis

A total of 121 fetal samples (51 cultured chorionic villi and 70 amniocyte) were received for *SMN1* copy number analysis. Of the 54 fetuses at a 25% risk to be affected with SMA, 47 were based on a previous affected child, four on identification of parental carrier status following carrier screening and three had both parents identified as carriers at other laboratories. Fifteen of the 54 (27.8%) were predicted to be affected with SMA, based on a 0-copy result from testing. This result is consistent with the expected 25% frequency given autosomal recessive inheritance (P=0.26).

In all, 59 fetuses were tested after identification of carrier status in one parent, with the other parent having a 2- or 3-copy result determined concurrently or before fetal testing. Of these, 27 fetuses had 1 copy and 32 had 2 copies of *SMN1*. At least 53 of the 59 had an indication for CVS or amniocentesis that was unrelated to the parental SMA carrier testing (eg, advanced maternal age, abnormal maternal serum screen). The remaining six samples were received as cultured cells from outside laboratories. The indication for the prenatal diagnostic procedure in these cases was not readily available, although the average age of these mothers was 37 years. Seven of the 121 fetal samples were referred based on abnormal ultrasound findings such as joint contractures suggestive of SMA, and all had 2-copy fetal results. Lastly, one sample was tested due to a reported family history of SMA, although neither parent was identified as a 1-copy carrier and the fetus had 2 copies of *SMN1*.

Sequence variants that interfere with *SMN1* copy number determination

To rule out false positive results due to the presence of sequence variants under the primer and probe-binding sites used in our analysis, follow-up sequencing restricted to these sites was performed on all samples with 1 copy of *SMN1* and all prenatal specimens regardless of *SMN1* copy number. This led to the identification of a recurrent variant, NT_006713.14:c.865T>A (g.29631T>A; p. C289S), in a heterozygous state among 35 individuals. This variant was most frequent among Hispanic individuals (n=19) but was also identified in Ashkenazi Jewish and Caucasian (n=9 combined) individuals and in individuals for whom ethnicity information was not provided to the laboratory (n=7). Another variant, NT_006713.14:c.867C>T (g.29633C>T; p.C289C) was identified in a heterozygous state in one Hispanic individual. Both these variants resulted in a 1-copy result by our real time PCR methodology and were subsequently identified by sequence analysis to be present in a heterozygous state within the *SMN1* gene as evidenced by presence of the nucleotide 'C' at the critical position, c.840 that distinguishes *SMN1* from *SMN2*. Neither of these variants was observed in a homozygous state among individuals analyzed. Our practice of restricting follow-up sequence analysis to individuals identified to have 1 copy of *SMN1* precludes an accurate estimation of the general population allele frequency of these variants.

DISCUSSION

SMA affects individuals of all ethnicities. The estimated pan-ethnic disease frequency, $(a+d)$,[2] derived from our carrier frequency data (1/11 000, Table 2) is consistent with the reported prevalence estimate of 1/10 000 reported for clinically typical SMA derived from larger population studies.[4] Previous reports on SMA carrier frequencies in limited populations have identified a discrepancy between the estimated disease prevalence (1/10 000) and that extrapolated from observed carrier frequency estimates (1/6000) under Hardy–Weinberg equilibrium. Although possible explanations for the earlier discrepancies have been proposed,[4] our present study is the first to report a pan-ethnic carrier frequency that is most consistent with the reported incidence of SMA.

For purposes of genetic counseling within the setting of population-based carrier screening, pan-ethnic carrier frequency (1/54) and carrier detection rate (91%) data are particularly useful when ethnicity is unknown or reflects admixture. When ethnicity is known, however, it may be possible to further refine the carrier frequency or detection rate provided in pre-test education and counseling, as well as provide more accurate estimates of residual risk following identification of a 2- or 3-copy *SMN1* result. For instance, a Caucasian individual with a 2-copy result following carrier screening would have an ~1/800 residual risk to be a carrier, whereas an African American individual with the same result would have a 1/130 risk to be a carrier.

Testing 4883 African American individuals with no family history of SMA confirmed earlier observations[24] of a higher frequency of the 2-copy allele (c) in this population as compared with other populations. This suggests a higher frequency of [2+0] carriers who would not be identified by an assay designed to detect deletion carriers who have 1 copy of *SMN1*. This is accurately reflected by the lower detection rate of 71% in this population. The *a priori* risk for an African American individual to be a carrier (1/72) is, however, comparable to other populations and supports the inclusion of African Americans among those being offered carrier screening for SMA, with appropriate counseling regarding the limitations of testing and associated residual risks.

In this study of 7655 Hispanic individuals, the observed *SMN1* 1-copy frequency did not differ significantly from that observed in our earlier study[24] of 1030 individuals (P=0.1869). In the Asian carrier-screening population, pair-wise comparison of observed *SMN1* 1-copy frequency between our current data set (n=4647) and those of our earlier study[24] (n=1027), as well as studies in native Chinese[17] (n=1712) and Korean[14] (n=326) populations, also did not yield significant differences at 1% (P-values=0.7776, 0.0366, 0.5674, respectively). Among individuals identified as Asian Indian (n=976), the 1-copy genotype frequency was 1.74% with a calculated *a priori* carrier frequency of 1/52. We are not aware of previous studies of SMA carrier frequency in the general Asian Indian population. Similarly, the *SMN1* 1-copy genotype frequency distribution in our Ashkenazi Jewish population was not significantly different from that reported in the Israeli population[16] (P=0.6575). Finally, an evaluation of genotype frequencies for individuals whose ethnic background was 'Not Provided' on the ordering test requisition supports the conclusion that the ethnicity distribution of this group does not differ from that of the pan-ethnic population as a whole as the 1-copy genotype frequencies are similar (P=0.9217).

At least seven couples with no family history of SMA in our carrier-screening population were identified as carriers with a 25% risk to have an affected child. As a reference laboratory we do not have complete ascertainment of family samples, and it is possible that some partners of individuals identified as carriers via screening were tested at other laboratories. Additionally, we could have incomplete ascertainment if partners were not identified as such on ordering paperwork or if a fetal sample from carrier parents was tested at another laboratory. Of the seven couples, four had fetal testing in our laboratory and no affected fetuses were identified in this small group.

Among individuals with a family history of SMA, there have been case reports of first-degree relatives with an *SMN1* copy number of 0 who are asymptomatic or mildly affected.[25] This raises the question of the frequency of individuals with 0 copies of *SMN1* in the general population. Among the 72 453 samples tested, we did not identify any individuals referred for carrier testing with an apparent 0-copy *SMN1* result.

The identification of 36 individuals with sequence variants in the primer/probe region underscores the importance of this additional quality assurance measure to identify potential false positive results. These individuals could be misclassified as being carriers of a deletion/gene conversion within the *SMN1* gene. At present, there is insufficient evidence to classify these variants as either disease causing or benign. Therefore, although the *SMN1* copy number of individuals identified with these variants can be reclassified, their SMA carrier status and associated residual risk cannot be accurately determined at present. Follow-up options, such as carrier testing of the partner can be explored. In the absence of follow-up sequencing, the estimated pan-ethnic-positive predictive value of our carrier analysis (TP (1162)/TP (1162)+FP (36)) would have been 97%.

In our prenatal testing cohort, as expected, ~25% of the at-risk fetuses were found to have 0 copies of *SMN1* and were predicted to be affected with SMA. Among 54 fetal samples referred for testing due to a 25% risk to be affected, 47 were from obligate carrier parents with a previous affected child. Of these, eight obligate carriers (8.5%) had 2 copies of *SMN1* and a partner with 1 copy of *SMN1*. This frequency is consistent with the frequency of 7/117 (6.0%) obligate carrier parents identified to have 2 copies of *SMN1* as reported by Smith *et al*[18] (P=0.79). In our study, additional studies to distinguish the [2+0] *versus* [1+1] status of these 2-copy obligate carrier parents were not performed. Therefore, the exact frequency of 2-copy chromosomes in our obligate carrier parent cohort cannot be determined.

A lack of agreement exists among the limited number of studies investigating a relationship between abnormal ultrasound findings and SMA. Some reports suggest an association between increased nuchal translucency and SMA,[26] although this association has not been supported by all studies.[27] The inclusion of abnormal ultrasound findings among indications for carrier testing and prenatal diagnosis within our study suggests possible physician interest in including SMA among the differential diagnoses for select ultrasound abnormalities, although we are unable to determine the frequency with which parents or fetuses with these findings are referred for SMA testing.

The most frequent indication for fetal testing (49%) was having one carrier parent identified during screening. In these circumstances, the other parent was identified as having 2 or 3 copies of *SMN1* or was tested for carrier status concurrently with the fetal *SMN1* copy number analysis. In all but six samples for which clinical indication could not be confirmed, invasive prenatal diagnosis was performed for a reason unrelated to the carrier parent's *SMN1* status. It is presumed that fetal testing was pursued in the interest of time in these cases, as was the case for individuals in whom the fetal sample and the untested parent were analyzed at the same time, or for additional reassurance.

The *a priori* risk for a fetus to be affected with SMA, when one parent is identified as a 1-copy carrier and the other parent has an *SMN1* copy number of 2, can vary by ethnic background. In a Caucasian couple, the risk to have an affected fetus is 1/2528. In contrast, the risk for an African American couple with the same parental results is 1/264. *SMN1* copy number analysis for a fetus in this circumstance could reveal a 0-copy *SMN1* result, consistent with a prediction for the fetus to be affected. In addition, such a result would set the parental phase for the 2-copy parent as a [2+0] carrier. Alternatively, a 1-copy fetal result in this situation would be associated with an ~1/4000 risk for the fetus to be affected with SMA, due to compound heterozygosity for a 1^d (*d*) allele.

In a pilot study of general population carrier screening in the United States, Prior *et al*[18] reported ~60% of individuals seeking prenatal genetic counseling accepted carrier testing for SMA. After result disclosure, 98.7% of patients were glad they pursued testing. In Israel, among women electing carrier screening for cystic fibrosis and fragile X syndrome, a large-scale population screening study found 93% requested SMA testing as well.[15] Our clinical laboratory analysis of >68 000 individuals without a family history of SMA, starting before the 2008 ACMG guideline, demonstrates (1) rapid test uptake by physicians and further supports patient interest in the availability of carrier screening for this disorder; (2) the feasibility of high throughput carrier testing for SMA; and (3) new and valuable information regarding *SMN1* copy number in the general United States population to permit more accurate residual carrier risk calculations based on ethnicity-specific carrier frequencies and detection rates.

Furthermore, these data address specific recommendations set forth by professional organizations such as the AMP and ACOG and fully support the ACMG recommendations to offer SMA carrier screening to all, regardless of race or ethnicity.

CONFLICT OF INTEREST

At the time this study was conducted, all authors were employed by the Genzyme Genetics unit of Genzyme Corporation, and held stock of and/or options with Genzyme Corp. Currently all authors, with the exception of Viatcheslav Akmaev, are employed by Esoterix Genetics Laboratories, LLC, a wholly owned subsidiary of Laboratory Corporation of America Holdings, and may hold stock of and/or stock options with LabCorp.

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Consensus Statement for Standard of Care in Spinal Muscular Atrophy

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Spinal muscular atrophy is a neurodegenerative disease that requires multidisciplinary medical care. Recent progress in the understanding of molecular pathogenesis of spinal muscular atrophy and advances in medical technology have not been matched by similar developments in the care for spinal muscular atrophy patients. Variations in medical practice coupled with differences in family resources and values have resulted in variable clinical outcomes that are likely to compromise valid measure of treatment effects during clinical trials. The International Standard of Care Committee for Spinal Muscular Atrophy was formed in 2005, with a goal of establishing practice guidelines for clinical care of these patients. The 12 core committee members worked with more than 60 spinal muscular atrophy experts in the field through conference calls, e-mail communications, a Delphi survey, and 2 in-person meetings to achieve consensus on 5 care areas: diagnostic/new interventions, pulmonary, gastrointestinal/nutrition, orthopedics/rehabilitation, and palliative care. Consensus was achieved on several topics related to common medical problems in spinal muscular atrophy, diagnostic strategies, recommendations for assessment and monitoring, and therapeutic interventions in each care area. A consensus statement was drafted to address the 5 care areas according to 3 functional levels of the patients: nonsitter, sitter, and walker. The committee also identified several medical practices lacking consensus and warranting further investigation. It is the authors' intention that this document be used as a guideline, not as a practice standard for their care. A practice standard for spinal muscular atrophy is urgently needed to help with the multidisciplinary care of these patients.

Keywords: spinal muscular atrophy; standard of care; consensus statement

Current Problems in the Medical Care of Patients With Spinal Muscular Atrophy

Spinal muscular atrophy is a recessively inherited neuromuscular disease characterized by degeneration of spinal cord motor neurons, resulting in progressive muscular atrophy and weakness. The clinical spectrum of spinal muscular atrophy ranges from early infant death to normal adult life with only mild weakness. These patients often require comprehensive medical care involving multiple disciplines. There is, however, no published practice standard for the care of these patients. Disparity in family resources, medical practitioners' knowledge, and regional and cultural standards produces wide variation in care and clinical outcome. Spinal muscular atrophy, as a field, has recently seen major advances in molecular diagnosis and clinical therapeutics that have not been matched by wide understanding and application. Parents of children newly diagnosed with spinal muscular atrophy often seek care over the Internet or outside of their geographic area to obtain expert care needed for their children, albeit in a costly and inefficient manner. This also undermines trust in local practitioners and their potential to render good clinical care during acute illness. Another consequence of these variations in practice is loss of trust in traditional medicine and increase in the attractiveness of untested and potentially harmful unconventional therapies. The large variation of clinical care in spinal muscular atrophy also

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results in challenges for future therapeutic trials. For all these reasons, we have identified an urgent need to establish a practice guideline, both to improve patient care and to provide a baseline standard for future clinical trials in spinal muscular atrophy.

The International Standard of Care Committee for Spinal Muscular Atrophy

Committee Formation

In September 2004, the National Institute of Neurological Diseases and Stroke sponsored an International Spinal Muscular Atrophy Conference in Bethesda, Maryland, with the goal of formulating strategies to coordinate future clinical trials in spinal muscular atrophy.[1] During the conference, it became clear that the wide variation of medical care received by spinal muscular atrophy patients likely increases the variability of outcomes in clinical trials. Thus, this Spinal Muscular Atrophy Standard of Care Committee was formed in January 2005, as a standing committee of the International Coordinating Committee for Spinal Muscular Atrophy clinical trials, to investigate the current state of science in clinical practice in spinal muscular atrophy and to attempt to achieve consensus on the standard of care for these patients.

Committee Structure

The Standard of Care Committee for Spinal Muscular Atrophy is cochaired by a US and a European neurologist. There are 12 core members and 3 consultants on the committee. Eleven of the core members are currently practicing pediatric neurologists, and 1 is a pediatric pulmonologist. The 3 consultants consist of 1 National Institute of Neurological Diseases and Stroke liaison and 2 representatives from patient advocacy groups. The committee is subdivided into 4 working groups: diagnostics/new interventions, pulmonary, gastrointestinal/nutrition, and orthopedics/rehabilitation. Each group is headed by a leader from the United States and a coleader from Europe. All committee members participated voluntarily, without pay. They were either nominated by their peers or have volunteered themselves for this task force. Each working group is composed of 6 to 11 experts in the field for that particular care issue (please see the Web site http://smascc.stanford.edu for a current roster of committee members).

Committee Missions

The committee has identified the following goals for all 4 working groups: (1) to identify current care issues in spinal muscular atrophy clinical practice, (2) to search for existing practices in spinal muscular atrophy clinical care and the rationale or data supporting such practices, (3) to achieve consensus of the most appropriate medical practice in caring for patients with spinal muscular atrophy, (4) to use this standard of care consensus to establish clinical care guidelines for future spinal muscular atrophy clinical trials, (5) to identify future research directions in the care of patients with spinal muscular atrophy, and (6) to publish the consensus as guidelines for clinical care of patients with spinal muscular atrophy.

Methods of Achieving Consensus on Standard of Care for Spinal Muscular Atrophy

Standards of medical practice are ideally established upon evidence-based clinical trial data. Unfortunately, committee core members found little data from well-designed clinical studies upon which evidence-based practice parameters in spinal muscular atrophy could be drafted (please see literature reviews in each care topic in the following sections). The absence of well-designed clinical trial data requires substitution of widely held opinion drawn from a survey of experts in the field. It is hoped that this consensus statement will serve both as an initial practice guideline for the care of spinal muscular atrophy and an outline of areas where needed clinical investigation may be best focused. We describe here the process leading to the drafting of this consensus statement.

Periodic Conference Calls and Literature Review

Since the inception of the committee in early 2005, the members have held periodic conference calls to discuss the ways to establish practice guidelines for spinal muscular atrophy. Group leaders were tasked with conducting literature reviews in their particular care areas. A password-protected Web site was established during this time. References of literature reviews were uploaded to the Web site. Having concluded that there were not enough published data to allow drafting of an evidence-based practice parameter, the group explored the possibility of using a Delphi survey to achieve consensus among experts in the field.

The Delphi Survey

The Delphi technique[2] was initially used to explore consensus expert opinion in government and education. More recently, it has been used in medicine, notably in rheumatology and neurology.[3-5] The goal of the Delphi technique is to identify if in aggregation there is a rank-ordered cluster of answers from respondents that reflects group consensus on that particular question. It also serves to identify if no consensus is present and where topics need further study. It presents group opinions anonymously, avoids domination by a few strong voices in the group, and can be completed by

electronic communications within a few weeks. Exploratory use of the Delphi technique was performed during an initial committee meeting in Philadelphia in June 2005. This served to familiarize the attending committee members with the mechanics of the Delphi technique and of its strengths and limitations. Having completed 2 rounds of pilot surveys among the committee members, the group concluded that the Delphi technique was suitable for establishing a consensus opinion among experts in spinal muscular atrophy. Group leaders then met by conference calls and e-mail communications to construct a formal Delphi survey questionnaire. During the first round of the Delphi survey, a set of open-ended questions was constructed for each of the 5 spinal muscular atrophy care topics (diagnostic/new interventions, pulmonary, gastrointestinal/nutrition, orthopedics/ rehabilitation, and palliative care). Each topic is divided into 3 parts: presenting signs and symptoms, diagnostic testing, and intervention options. The intervention part is then divided into acute management and health maintenance. These open-ended questions are named Question #1 (Q#1, available on the Web site http://smascc.stanford.edu). The Q#1 was distributed by ane-mail attachment to survey participants. A total of 86 spinal muscular atrophy experts were invited to participate in the survey. They were invited from 4 medical disciplines: 18 from the gastrointestinal/nutrition group, 21 from the pulmonary group, 25 neurologists from the diagnostic/new interventions group, and 22 from the orthopedics/rehabilitation group. Thirty-four of them were from Europe, and 52 were from the United States and Canada. All invited participants were recommended by committee members. The participants were allowed 3 weeks to respond to the questionnaire. Neurologists were encouraged to answer all 5 care topics. Respondents in the other 3 working groups generally limited their responses to respective areas of expertise. Fifty-six of the original 86 invited participants (65%) completed this Q#1. Twenty-two of them were from Europe, and 34 were from the United States and Canada. To ensure the anonymity of the process, a numeric code was assigned to each respondent by the survey coordinator upon receipt of answers to Q#1. Analysis and presentation of the data were performed by the survey coordinator using these numeric codes. The answers from Q#1 were collected and analyzed. The most frequent occurring answers to these Q#1 questions were chosen to construct the Question #2 (Q#2, available on the Web site http://smascc.stanford .edu) during the second round of the Delphi survey. In this second round, the questions were the same as those in Q#1 except that respondents were asked to rank order from the highest to the lowest importance among a list of choices. Forty-four (79%) respondents who answered the Q#1 also completed Q#2. These responses were summarized and presented to committee participants at the Standard of Care Conference described in the following section.

The International Conference on the Standard of Care for Spinal Muscular Atrophy

This conference was held May 5-6, 2006, at Stanford University Medical Center, Palo Alto, California. Thirty-five members of the committee and Delphi survey participants gathered to work on a consensus statement for spinal muscular atrophy standard of care. First, leaders and designated members of each working group presented a critical review of the literature. The individual working group then reviewed the results of the Delphi survey in their care areas during breakout sessions. The final consensus within each working group was achieved by using the Delphi data as a guideline, incorporating the available data in the literature and the opinions of group members. These results were presented by group leaders to all conference participants for comments. The group leaders and coleaders then worked with each working group to draft the consensus statement on each care area. The summaries of these statements are listed in the following sections.

Diagnostic Testing and Care of New Spinal Muscular Atrophy Patients

Clinical Diagnosis and Classification of Spinal Muscular Atrophy

Physicians encountering children with hypotonia and weakness should maintain a high index of suspicion for the diagnosis of spinal muscular atrophy. Certain physical characteristics are readily identifiable. The weakness is usually symmetrical and more proximal than distal. Sensation is preserved. Tendon reflexes are absent or diminished. Weakness in the legs is greater than in the arms. The severity of weakness generally correlates with the age of onset. The most severe type presents in infancy. The infant may appear normal at birth. Weakness evolves within the first few months of life. Occasionally, decreased intrauterine movements suggest prenatal onset of the disease and present with severe weakness and joint contractures at birth.[6] Milder types of spinal muscular atrophy present with later onset, and the course is more insidious. Some children sit but never walk, whereas others show delayed walking but may be able to maintain walking until adult years. For the purpose of clinical care and discussion, individuals manifesting different levels of weakness due to spinal muscular atrophy have been divided into 4 groups defined by functional ability. We list typical clinical features of spinal muscular atrophy in Table 1. The first 3 types are classified according to criteria established by the International Spinal Muscular Atrophy Consortium.[7,8] Type 4 spinal muscular atrophy is a mild form that presents in adulthood. It can be expected that some patients will manifest features that are at the margins between groups.

Table 1. Clinical Classification of Spinal Muscular Atrophy

SMA Type	Age of Onset	Highest Function	Natural Age of Death
Type 1 (severe)	0-6 mo	Never sits	<2 y
Type 2 (intermediate)	7-18 mo	Never stands	>2 y
Type 3 (mild)	>18 mo	Stands and walks	Adult
Type 4 (adult)	Second or third decade	Walks during adult years	Adult

NOTE: SMA = spinal muscular atrophy.

In addition to these defining criteria, unique clinical features of each spinal muscular atrophy type include the following: (1) Type 1 spinal muscular atrophy. This type is also called Werdnig-Hoffmann disease. Children with this disease have impaired head control, with a weak cry and cough. Swallowing, feeding, and handling of oral secretion are affected before 1 year of age. The tongue may show atrophy and fasciculation. Weakness and hypotonia in the limbs and trunks are eventually accompanied by intercostal muscle weakness. Combining intercostal weakness with initial sparing of the diaphragm, the infants exhibit characteristic paradoxical breathing and a bell-shaped trunk with chest wall collapse and abdominal protrusion. Early morbidity and mortality are most commonly associated with bulbar dysfunction and pulmonary complications. (2) Type 2 spinal muscular atrophy. These children have delayed motor milestones. Some learned to achieve independent sitting, whereas others need help to sit up. The defining characteristic is an ability to maintain a sitting position unsupported. At the strongest end of this category are those who can stand with a standing frame or long leg braces but are not able to walk independently. Bulbar weakness with swallowing difficulties may lead to poor weight gain in some children. Intercostal muscles are weak, and some are also diaphragmatic breathers. They have difficulty coughing and clearing tracheal secretion. They have fine tremors with extended fingers or when attempting hand grips. Kyphoscoliosis eventually develops, and bracing or spinal surgery is needed. Joint contractures commonly evolve over years. (3) Type 3 spinal muscular atrophy. This type is also called Kugelberg-Welander disease or juvenile spinal muscular atrophy. These patients have later but variable age of onset. All achieve independent walking. Some patients lose the ability to walk in childhood, yet others maintain walking until adolescence or adulthood. Scoliosis can develop in these patients. Swallowing, cough, and nocturnal hypoventilation are less common than in type 2 spinal muscular atrophy but may occur. Muscle aching and joint overuse symptoms are common. (4) Type 4 spinal muscular atrophy. The onset of weakness is usually in the second or third decade of life. Motor impairment is mild without respiratory or gastrointestinal problems.

Within each spinal muscular atrophy type, subclassifications have been proposed and can add to prognostic significance. For example, only 22% of patients with type 3a, with onset of symptoms before age 3 years, were still ambulatory at age 40 years, whereas 58.7% of the patients with type 3b, with onset after age 3 years, were still walking by age 40 years.[9] Type 1 patients have also been subclassified into types 1a (neonatal or antenatal onset), 1b (typical Werdnig-Hoffmann disease with onset after neonatal period), and 1c (later onset, better head control in supported sitting, mild feeding or respiratory difficulties during the first 6 months of life).[10,11] However, these subclassifications have not been widely used among clinicians.

During the preparation of the Delphi survey, the committee decided that the most appropriate care for patients with spinal muscular atrophy should be tailored according to their current functional status rather than the original classification of disease types because these represent the best level of function rather than the present status. Therefore, the committee decided to use the classification of current functional level in the form of nonsitters, sitters, and walkers. The nonsitters include the group of children who currently are not able to sit independently. The sitters include those who can sit independently but cannot walk independently. The walkers can walk independently.

Other Forms of Spinal Muscular Atrophy

There are other inherited motor neuron disorders, not caused by mutation of the *SMN* gene (non-5q spinal muscular atrophy), that present with early denervation weakness but different clinical symptoms than those stated above.[12] These atypical symptoms include joint contractures, distal rather than proximal weakness, diaphragmatic paralysis with early respiratory failure, and pontocerebellar degeneration. DNA testing has become available for some but not all of these disorders. If a child with clinical features of spinal muscular atrophy is found not to have an *SMN* deletion on either chromosome 5, the child should be reexamined and receive additional diagnostic testing. (Please see the next section for the diagnostic strategies for these patients.) Table 2 lists some spinal muscular atrophy variants that exhibit early symptoms overlapping with 5q spinal muscular atrophy. Several later-onset motor neuron diseases overlap with milder 5q spinal muscular atrophy. These are beyond the scope of this document and are not listed here.

Table 2. Other Forms of Severe Spinal Muscular Atrophy Not Linked to *SMN* Gene

SMA Variants	Inheritance/Linkage/Gene	Clinical Presentation	Reference
Scapuloperoneal spinal muscular atrophy	Autosomal dominant 12q24.1-q24.31	Congenital absence of muscles, progressive weakness of scapuloperoneal and laryngeal muscles	(13, 14)
Pontocerebellar hypoplasia with spinal muscular atrophy	Autosomal recessive	Onset 0-6 mo, cerebellar and brainstem hypoplasia, absent dentate nucleus, neuronal loss in basal ganglia, cortical atrophy	(15-19)
X-linked infantile spinal muscular atrophy with arthrogryposis	X-linked Xp11.3-q11.2	Onset at birth or infancy, contractures, death less than 2 y	(20, 21)
Spinal muscular atrophy with respiratory distress type 1	Autosomal recessive 11q13.2-q13.4 IGHMBP2	Onset within the first 3 mo of life, eventration of the right or both hemidiaphragms, finger contractures, pes equines foot deformities	(22, 23)

NOTE: SMA = spinal muscular atrophy.

Diagnostic Procedures

The stepwise algorithm of the diagnostic procedure is summarized in Figure 1. Briefly, the first diagnostic test for a patient suspected to have spinal muscular atrophy should be the *SMN* gene deletion test. This test is currently performed by several diagnostic laboratories, and the result can be obtained within 2 to 4 weeks. The test achieves up to 95% sensitivity and nearly 100% specificity.[24,25] A homozygous deletion of *SMN1* exon 7 (with or without deletion of exon 8) confirms the diagnosis of *SMN*-associated spinal muscular atrophy (5q spinal muscular atrophy). The next group of tests following a negative *SMN* test result includes repeat clinical examination of the patient for atypical clinical features as listed in Table 2. Laboratory tests should include muscle enzyme creatine kinase, electrophysiological testing such as electromyography (EMG), and nerve conduction study with repetitive stimulation. This will help to identify muscle diseases, motor neuropathies, and disorders of neuromuscular junctions. If EMG suggests a motor neuron disease, then further testing for *SMN* mutations should be pursued. Some laboratories are currently offering *SMN1* gene copy number testing. If the patient possesses only a single copy of *SMN1* (missing 1 copy), then it is possible that the remaining copy contains subtle mutations, including point mutations, insertions, and deletions, rendering homozygous dysfunction of the gene. Sequencing of the coding region of the remaining *SMN1* copy may identify the mutation on the remaining copy and confirm the diagnosis of 5q spinal muscular atrophy. Unfortunately, sequencing the coding region of *SMN* is currently not widely available and is usually performed only in a few diagnostic or research laboratories. If the patient possesses 2 copies of *SMN1*, then other motor neuron disorders such as spinal muscular atrophy with respiratory distress, X-linked spinal muscular atrophy, distal spinal muscular atrophy, and juvenile amyotrophic lateral sclerosis should be considered. If EMG, nerve conduction study, and repetitive stimulation reveal characteristic patterns associated with diseases in muscle, nerve, or neuromuscular junction, then further diagnostic tests, including muscle or nerve biopsy and edrophonium test, may be performed. When disease of the neuromuscular system is ruled out, then one should pursue diagnostic tests to identify spinal cord or brain anomalies by imaging studies such as magnetic resonance imaging or computed tomography scans. Other diagnostic tests should then be performed to identify systemic diseases, such as metabolic disorders or other genetic disorders.

Clinical Management of Newly Diagnosed Spinal Muscular Atrophy Patients

Many care issues arise when a patient is newly diagnosed with spinal muscular atrophy. Clinicians need to address the various aspects of care issues as soon as possible.

Family Education and Counseling

Because of the complexity of medical problems associated with the diagnosis of spinal muscular atrophy, the committee suggests that medical providers designate a person to meet with the family. This person is usually a pediatric neurologist or a geneticist. The primary care physician (pediatrician or family physician) should be well informed of the multidisciplinary needs of these patients and play a central role in coordinating follow-up care. During the first meeting with parents, it is important to explain the disease process, pathogenesis, phenotype classification, and the patient's prognosis. The physician should also formulate a plan of multidisciplinary intervention with the family. This usually includes referral to a pediatric neuromuscular clinic and/or pediatric subspecialties such as genetics, pulmonary, gastroenterology/nutrition, and orthopedic/rehabilitation. The families will appreciate online resources for further information regarding spinal muscular atrophy. Providing information on spinal muscular atrophy patient advocacy groups has proved to be the most useful to help families cope with the diagnosis (please see the acknowledgments section for links to some patient support group Web sites). Several clinical trials are currently in progress both in the

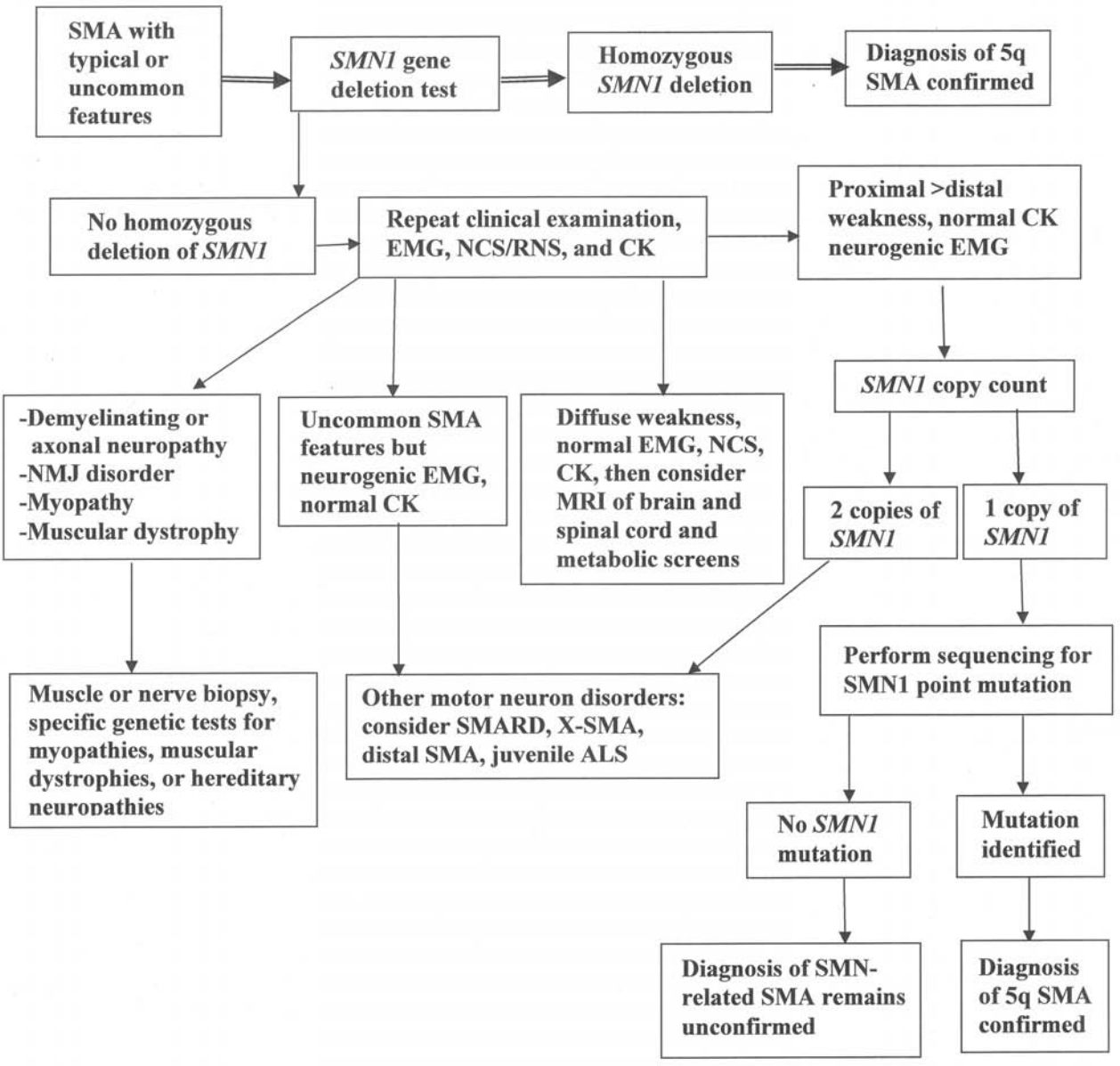

Figure 1. Diagnostic evaluation for spinal muscular atrophy. A diagnostic algorithm for spinal muscular atrophy and other neuromuscular disorders. The Standard of Care Committee recommends the stepwise diagnostic procedure outlined in this flow chart when encountering patients with clinical symptoms of spinal muscular atrophy (SMA). Please see text for detailed explanation of this diagram. EMG = electromyography; NCS = nerve conduction study; RNS = repetitive nerve stimulation; CK = creatine kinase; NMJ = neuromuscular junction; MRI = magnetic resonance imaging; SMARD = spinal muscular atrophy with respiratory distress; X-SMA = X-link SMA; ALS = amyotrophic lateral sclerosis.

United States and in Europe. Physicians should provide information regarding these trials or refer families to clinical trial Web sites (www.clinicaltrials.gov provides a current listing of open clinical trials). Many factors can influence the families' choice to participate in a clinical trial. The families should be encouraged to contact as many study sites as possible before they decide to participate in any trial.

Genetic Topics

Several genetic topics should be addressed with the diagnosis of spinal muscular atrophy. This is often done by a neurologist or geneticist. Topics related to the genetics of spinal muscular atrophy, such as autosomal recessive inheritance and genomic structure of *SMN—SMN1* and *SMN2*

copies—should be explained to the family. The current literature suggests *SMN2* copy numbers correlate with spinal muscular atrophy clinical phenotypes.[24-27] However, although a higher copy number of *SMN2* is correlated with milder phenotype, phenotypes can vary substantially given *SMN2* copy number. Therefore, predicting clinical phenotype using *SMN2* copy number can be risky and is not currently recommended. Other important genetic topics include sibling recurrence risk, carrier testing, and information that may help with reproductive planning (prenatal diagnosis or preimplantational diagnosis). Presymptomatic diagnosis of unaffected siblings is controversial. According to American Society of Human Genetics guidelines, presymptomatic diagnosis in children should be considered only if early intervention can delay the onset or slow the progression of the disease.[28] The committee agrees that presymptomatic diagnosis of at-risk siblings of spinal muscular atrophy patients may lead to early intervention and improve clinical outcome. Therefore, parental request of testing unaffected siblings of the spinal muscular atrophy patient should be granted. The current *SMN1* deletion test will detect the *SMN1* copy number and provide the information of whether the sibling is affected (0 copy) or is a carrier (1 copy). The topic of neonatal screening is also controversial. Although there is currently no proven therapy in spinal muscular atrophy, the committee recognizes the utility of neonatal screening as a tool for identifying effective treatments. Furthermore, in view of recent therapeutic advances, it is possible that in the future, spinal muscular atrophy may be treated more effectively if presymptomatic patients are detected through neonatal screening and treatment is started prior to weakness becoming apparent.

Consensus on Pulmonary Care

Overview of Pulmonary Problems in Spinal Muscular Atrophy

The key respiratory problems in spinal muscular atrophy are as follows:

1. impaired cough resulting in poor clearance of lower airway secretions;
2. hypoventilation during sleep;
3. chest wall and lung underdevelopment; and
4. recurrent infections that exacerbate muscle weakness.

Pulmonary disease is the major cause of morbidity and mortality in spinal muscular atrophy types 1 and 2 and may occur in a small proportion of patients with spinal muscular atrophy type 3. Without respiratory support, infants who are unable to sit usually die before the age of 2 years.[8] Pulmonary compromise is caused by a combination of inspiratory and expiratory muscle weakness, with greater involvement of expiratory and intercostal muscles. The diaphragm is relatively spared. In nonsitters, the result is a bell-shaped chest with sternal depression. In older sitters and walkers, respiratory function may be compromised further by scoliosis. Swallowing dysfunction and reflux are important contributors to pulmonary morbidity. Individuals tend to progress to daytime respiratory failure via a sequence of recurrent chest infections, nocturnal oxygen desaturation, nocturnal hypoventilation, and then daytime hypercarbia.[29-31] In contrast to Duchenne muscular dystrophy, there is no strong correlation between pulmonary functional score and need for mechanical ventilation in spinal muscular atrophy.[32] However, baseline assessment and longitudinal monitoring can identify those at risk for sleep-disordered breathing and ineffective clearance of secretions. There are several case series of the natural history of severe to mildly affected spinal muscular atrophy patients but no large prospective study of treatment intervention.[9,33] The evidence base is limited by heterogeneous groups of patients with a mixture of neuromuscular disorders in natural history and intervention studies, variable classification of spinal muscular atrophy subtypes, and different respiratory support protocols employing a range of ventilators and cough assistance techniques.[30,33-36] Case series[29,30,36,37] and consensus conference[38,39] evidence demonstrate that ventilatory support should be added at night if sleep-disordered breathing is present and cough assistance provided if cough efficiency is reduced. Figure 2 shows a flow chart for pulmonary natural history, assessment, and intervention in spinal muscular atrophy.

Assessment and Monitoring

There is no formal study evaluating any protocol for routine pulmonary assessment of patients with spinal muscular atrophy. However, consensus was achieved within the pulmonary working group on current standard of care for spinal muscular atrophy. The following assessments should be used during baseline and subsequent evaluations of respiratory status and are listed by order of importance as identified by the Delphi survey. Assessment frequency depends on the clinical status and rate of progression of disease for each individual. Suggested frequency of evaluation is every 3 to 6 months, less often in stable walkers, and more frequently in clinically unstable nonsitters.

Nonsitters

Recommendations for respiratory assessment include evaluation of cough effectiveness, observation of breathing, and monitoring gas exchange. Respiratory muscle function tests are indirect measures of cough effectiveness and include peak cough flow, maximal inspiratory pressure, and maximal expiratory pressure. The majority of nonsitters with spinal muscular atrophy may be too weak or too young to perform

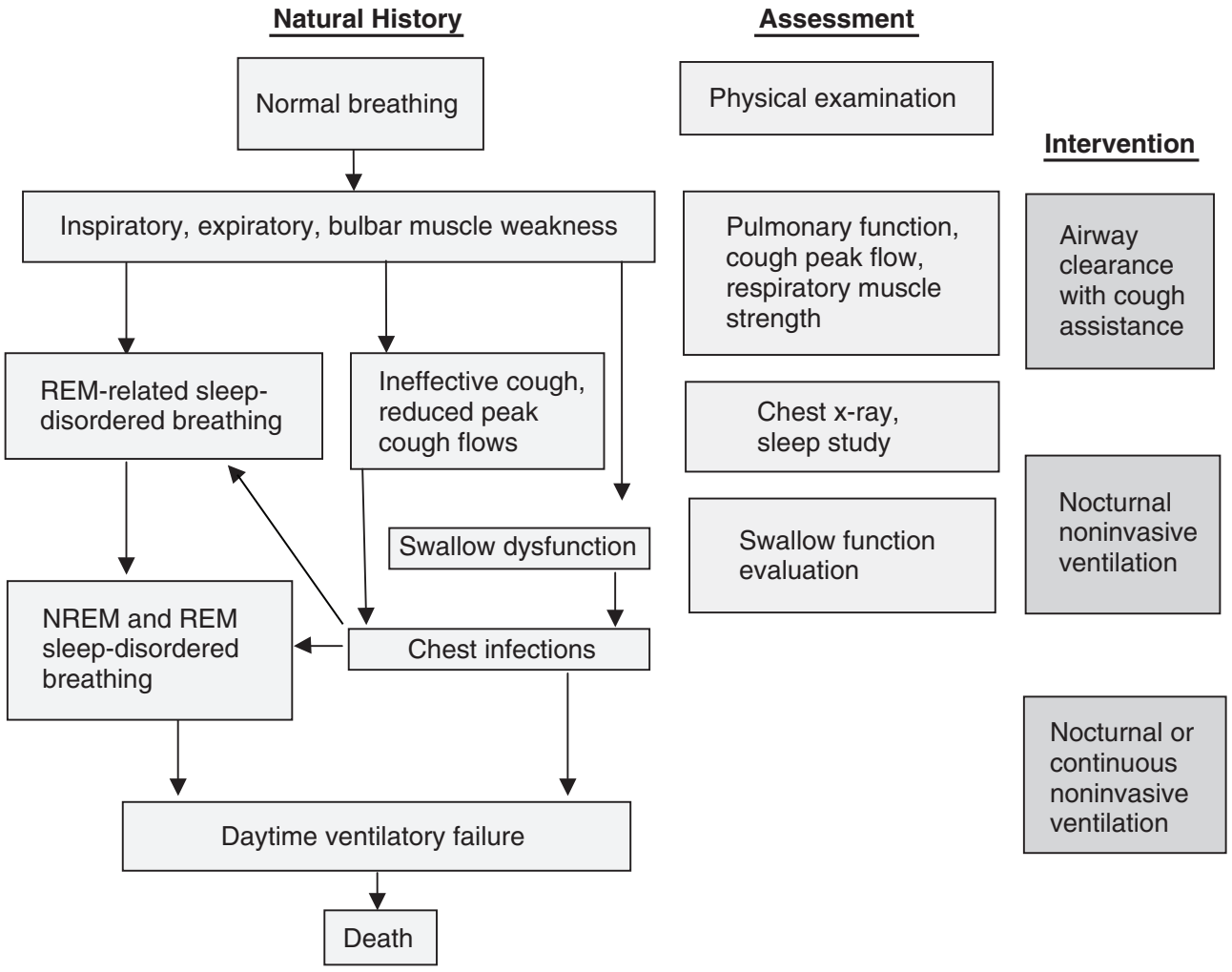

Figure 2. Summary of the natural history of pulmonary problems, assessment, and intervention in spinal muscular atrophy. The progression of pulmonary problems is accompanied by appropriate assessment and intervention strategies. REM = rapid eye movement; NREM = non–rapid eye movement.

pulmonary function testing. Therefore, the most useful evaluation of respiratory muscle function may be observation of cough ability. The physical examination also provides an important assessment of respiratory status including respiratory rate, work of breathing, presence of paradoxical breathing, chest wall shape, and skin color (cyanosis or pallor). Gas exchange monitoring, including pulse oximetry, can be used as a spot check during the day for hypoxemia and as a guide to direct airway clearance. For example, if oxygen saturation is less than 94%, airway clearance techniques should be used. Overnight pulse oximetry with chart recording can be used to screen for nocturnal hypoxemia. Routine overnight monitoring using pulse oximetry may help identify unsuspected hypoxic events but is usually very disruptive to the family due to frequent false alarms. Currently there are no data to support routine continuous oximetry monitoring. Further research is needed before recommending this as part of routine clinical care.

End-tidal carbon dioxide, transcutaneous CO_2, and serum bicarbonate measurement were also identified as important assessment tools. However, serum bicarbonate may give a false sense of reassurance, as normal values may exist despite significant respiratory compromise during sleep. End-tidal carbon dioxide and transcutaneous CO_2 are frequently difficult to obtain and not available routinely. If available, these measurements can be used to assess for sleep-related hypoventilation. The onset of hypoventilation is insidious, and patients may be clinically asymptomatic. Initially hypoventilation will occur in sleep (particularly rapid eye movement sleep), but as deterioration progresses, daytime respiratory function will be impacted.[29,31] Polysomnography is a diagnostic tool during which respiration and sleep state are continuously monitored,[40] and thus it identifies the presence and severity of sleep-disordered breathing.[41] Polysomnography is useful in nonsitters, even in children without obvious symptoms, and can be used to initiate and

titrate respiratory support. When polysomnography is not available, an alternative is to use a 4-channel sleep study that records end-tidal carbon dioxide or transcutaneous CO_2, oxygen saturation, heart rate, nasal airflow, and chest wall movement during sleep. In cases where neither polysomnography nor 4-channel study is available, overnight pulse oximetry with continuous CO_2 monitoring may provide useful information about nighttime gas exchange. However, this will not detect sleep-disordered breathing not associated with oxygen desaturation or CO_2 retention. Further study to better identify the optimal methods for evaluation and monitoring is recommended. Additional screening tests include a baseline chest x-ray to provide an initial reference point and for comparison during respiratory deterioration or unexplained hypoxemia due to unsuspected atelectasis. Although formal radiologic evaluation of swallowing was not ranked very highly for routine evaluation during this Delphi survey, the risk for dysphagia and aspiration is high in nonsitters. Therefore, formal evaluation of swallowing is indicated in cases of acute unexplained respiratory deterioration and recurring pneumonia. Arterial blood gases for routine monitoring of respiratory function are not recommended because the discomfort could result in apnea or spurious hyperventilation.

Sitters

Recommendations of respiratory assessment for sitters are similar to nonsitters and include physical examination and evaluation of cough effectiveness with respiratory muscle function tests (maximal inspiratory pressure, maximal expiratory pressure, and peak cough flow) as described above. In addition, sitters should be evaluated for presence and severity of scoliosis and consider further evaluation with radiographs. Additional recommended assessments include forced vital capacity and lung volume measurements during pulmonary function tests, assessment of sleep-disordered breathing, and pulse oximetry monitoring. Less important assessments identified for sitters include blood gas, CO_2 monitoring, and chest x-ray. Routine swallow study was not recommended for sitters unless clinically indicated.

Walkers

In general, spinal muscular atrophy walkers have relatively preserved pulmonary function until late into their disease course. Recommendations for routine assessment include complete pulmonary function tests, including spirometry, lung volumes, and respiratory muscle function tests. In addition, cough effectiveness and the physical examination are important routine assessments. Further evaluation should be directed by clinical symptoms and indications.

Anticipatory Respiratory Care

Providing families with information about options for care and anticipating future needs are crucial to respiratory management of spinal muscular atrophy. Nonsitters are the most fragile group, and early discussions should include the option of noninvasive ventilation and secretion management because of the rapid progression of the disease. Ongoing discussion of the family's desires for support should occur, and the result should be a negotiated care plan with maximums and minimums outlined.[42]

In addition, anticipatory guidance and education for chronic care, illness management, and perioperative care should be provided. Day-to-day management should include understanding the child's baseline and deviations from his or her baseline, routine cough and secretion management techniques, understanding hypoventilation, and intervention. Illness management includes rapid access to specialty medical care providers, airway clearance and secretion management techniques, respiratory support (including noninvasive ventilation), nutrition and hydration management, and a low threshold to start antibiotics. Routine immunizations, including influenza vaccine, pneumococcus vaccine, and respiratory syncytial virus prophylaxis (palivizumab), are recommended.

Chronic Management

Essential to chronic management is discussion of the family's goals, which includes balancing caring for the child at home for as long as possible, long-term survival, quality of life and comfort, and the availability of resources. Goals of chronic management are to normalize gas exchange, improve sleep quality, facilitate home care, reduce hospitalizations and intensive care unit care, and reduce the burden of illness on the family. There is insufficient evidence, but based on experience and consensus, early aggressive and proactive intervention may prolong life without compromising quality of life.

Airway Clearance

Airway clearance is very important in both acute and chronic management of all patients with spinal muscular atrophy. Caregivers of these patients should learn to assist coughing in all patients with ineffective cough. These techniques include manually and mechanically assisted cough.[43-46] Availability of mechanically assisted cough devices (mechanical insufflation-exsufflation) varies by country but is now widely accepted in management of neuromuscular disease in the United States.[47] Daily assisted cough is recommended in more severely affected patients. Secretion mobilization techniques are also helpful and include chest physiotherapy and postural drainage. Oximetry should be used to provide feedback to guide therapy. Oral suctioning can assist in secretion management after assisted coughing. There is no evidence to support specific secretion mobilization devices such as high-frequency chest wall oscillation and intrapulmonary percussive ventilation in the spinal muscular atrophy population for chronic management.

Respiratory Support

In patients with daytime hypercapnia, respiratory support is clearly indicated. In children with sleep-disordered breathing, nocturnal noninvasive ventilation reduces symptoms of sleep disturbance, nocturnal sweating, and morning headaches and improves appetite and concentration.[29] Objectively, noninvasive ventilation reduces respiratory disturbance index, improves sleep stage distribution,[30] and enhances quality of life. In a randomized controlled trial using mixed groups of patients with neuromuscular disease who showed nocturnal hypoventilation and daytime normocapnia, noninvasive ventilation significantly improved nocturnal blood gas tensions.[48] Noninvasive ventilation with bilevel positive pressure support has been studied most frequently, although there is no evidence to suggest any 1 type of ventilator interface is superior. In addition, the optimal settings for noninvasive ventilation have not been established. In general, noninvasive ventilation settings are individualized to achieve adequate inspiratory chest wall expansion and air entry and normalization of oxygen saturation and end-tidal carbon dioxide or transcutaneous CO_2 measurements. Noninvasive ventilation should be combined with airway clearance techniques.

In nonsitters, care without ventilation support is an option if the burden of treatment outweighs benefit. Noninvasive ventilation can be used palliatively to facilitate discharge to home from a hospital and reduce work of breathing. Continuous positive airway pressure may be an option in a very young nonsitter infant who is not synchronous with bilevel positive airway pressure and can be used with the goal of transitioning to bilevel positive airway pressure. Use of noninvasive ventilation with high-span bilevel positive airway pressure, even for short daytime periods, may improve chest wall and lung development and reduce ribcage and sternal deformity in nonsitters and sitters,[49] resulting in potential beneficial effects on pulmonary function. Uncommonly, adult walkers may develop sleep-disordered breathing or acute ventilatory failure at the time of a chest infection or intercurrent event (eg, surgery). Noninvasive ventilation is an appropriate intervention and may be required during sleep chronically.

Tracheotomy for chronic ventilation is a decision that needs to be carefully discussed if requested by parents. In nonsitters, this is controversial and an ethical dilemma. There is a large spectrum of options that can be provided, ranging from no respiratory support to noninvasive ventilation to tracheotomy and mechanical ventilation. Our recommendation is to explore options with the family regarding the child's potential, quality-of-life issues, and family's desires.[50] Palliative care is an option for nonsitters. It should be noted that noninvasive ventilation can be used as a routine therapy or as a palliative tool. A key goal is to prevent pediatric intensive care unit stays and avoid tracheotomy if possible. If supportive ventilation is chosen by the family, noninvasive ventilation is recommended.

Additional Management

Recommended additional therapies are routine vaccinations, appropriate nutritional support orally or via a feeding tube, hydration management, and medical or surgical gastroesophageal reflux disease management. In addition, medical management for saliva control may be considered. Inhaled bronchodilators should be considered in children with asthma or bronchial hyperresponsiveness. Use of these agents in other situations requires further evaluation. There is no evidence to support the use of mucolytics on a chronic basis.

Perioperative Care

Patients with spinal muscular atrophy are at high risk for postanesthesia complications, which may lead to prolonged intubation, nosocomial infections, tracheotomy, and death. Perioperative complications include upper airway obstruction, hypoventilation, and atelectasis from impaired cough and impaired mucociliary clearance due to anesthetic agents. Postoperative pain may exacerbate respiratory compromise. Noninvasive ventilation associated with aggressive airway clearance techniques can successfully treat hypoventilation and airway secretion retention.

It is crucial that the patient's respiratory status be optimized before surgery. Preoperative evaluation, including pulmonary consultation, is strongly recommended. The assessment of respiratory function should include a physical examination, measurements of respiratory function and cough effectiveness, chest x-ray, and, if at risk, an evaluation for sleep-disordered breathing. In addition, complicating factors should be considered, including oropharyngeal aspiration, gastroesophageal reflux, and asthma. If measurements of respiratory function and/or sleep study are abnormal, nocturnal noninvasive ventilation and assisted coughing techniques may be indicated before surgery. The patient should become familiar with these techniques prior to surgery. The anesthesiology preoperative evaluation should include assessment for possible difficult intubation due to jaw ankylosis. If present, intubation should be performed by fiberoptic bronchoscopy.

Postoperative management should be determined by preoperative respiratory function and the type of surgery performed. Patients with normal cough clearance and relatively preserved muscle function are not at an increased risk for postoperative complications. Patients with decreased respiratory muscle strength require close monitoring and aggressive respiratory management. Any patient who requires respiratory support during sleep will require similar respiratory support in the immediate postoperative course. Extubation in the recovery room to noninvasive ventilation should be planned as a bridge to weaning to the patient's baseline respiratory support. Careful planning and coordination with the hospital respiratory therapists are crucial for success in this setting. Patients with continuous ventilator

support requirements (either via noninvasive interface or via tracheotomy tube) or patients who receive muscular blocking agents during surgery are best transferred directly from the operating room to the intensive care unit. Patients are encouraged to bring their personal devices, such as noninvasive ventilation and mechanical insufflation-exsufflation-E machines, to use in the postoperative period because the availability of these devices in hospitals may be limited. Although oxygen is used frequently in the postoperative setting, it must be applied with caution in the patient with spinal muscular atrophy. Hypoxemia secondary to hypoventilation may be mistaken with hypoxemia due to other causes, such as mucus plugging and atelectasis. End-tidal carbon dioxide or transcutaneous CO_2 monitoring or arterial blood gas analysis will facilitate appropriate oxygen use. Adequate pain control will aid in preventing hypoventilation secondary to splinting. Postoperative pain management should be titrated to promote airway clearance and minimize respiratory suppression. Transient increased respiratory support may be needed while controlling postoperative pain.

Acute Care Management

The goal of acute management is to normalize gas exchange by reducing atelectasis and enhancing airway clearance where possible by noninvasive respiratory support. Blood gas monitoring may be of benefit.

Airway Clearance

For nonsitters, sitters, and walkers experiencing acute illness, airway clearance with manual cough assist or mechanical insufflation-exsufflation, together with oral or airway suctioning, chest physiotherapy, oximetry feedback to guide airway clearance, and postural drainage, are important and recommended. Assisted cough techniques are preferred over deep suctioning and bronchoscopy.

Respiratory Support

Nonsitters. In acute illness, a vicious cycle of added ventilatory load, increased respiratory muscle weakness, and ineffective secretion clearance leads to ventilatory decompensation. Acute use of noninvasive ventilation reverses these features. Continuous positive airway pressure is not indicated in this situation because it does not reduce the ventilatory load. In nonsitters and sitters already using nocturnal noninvasive ventilation, daytime use may be required during acute illness, and airway clearance techniques can be carried out during noninvasive ventilation. Noninvasive ventilation in combination with airway clearance techniques may reduce the need for intubation. Oxygen therapy entrained into the noninvasive ventilation circuit should be used to correct oxygen desaturation, after inspiratory and expiratory positive pressure settings are optimized and airway clearance techniques are optimally utilized. If a noninvasive approach fails, nonsitters can be intubated and mechanically ventilated as a short-term measure. After recovery from the acute illness and arterial oxygen saturation on room air has normalized, they should be extubated back to noninvasive ventilation. Decision making about escalation to intubation should be carried out in advance as part of anticipatory care planning. In nonsitters with increasingly frequent acute pulmonary infections, tracheotomy and ventilation can be considered but may not improve quality of life or reduce hospitalizations. A tracheotomy is not an acute intervention. A noninvasive approach is preferred where feasible. In some nonsitters, with deteriorating function, it may be appropriate to redirect care to a palliative approach.

Sitters. For those already using nocturnal noninvasive ventilation, daytime noninvasive ventilation may be needed during acute illness. Noninvasive ventilation in combination with airway clearance techniques may reduce the need for intubation. Oxygen therapy and need for transient intubation should be carried out as outlined above for nonsitters. Continuous positive airway pressure and/or a tracheotomy are not appropriate interventions in sitters.

Walkers. Walkers may need noninvasive ventilation during an acute illness. Noninvasive ventilation in combination with airway clearance techniques may reduce the need for intubation. Oxygen therapy and need for transient intubation should be carried out as outlined above for nonsitters. If noninvasive ventilation was needed during an acute illness, noninvasive ventilation should be considered for home use. Continuous positive airway pressure and/or a tracheotomy are not appropriate interventions in walkers.

Additional Management

For nonsitters, sitters, and walkers, recommended additional therapies are antibiotics, adequate nutritional support via nasogastric or nasojejunal or gastrostomy tube, hydration, and gastroesophageal reflux management (see more details in the next section). In patients with bronchial hyperresponsiveness or asthma, bronchodilator therapy and inhaled steroids may be indicated. Uses of inhaled mucolytics, bronchodilators, and corticosteroids are areas in need of further research.

Conclusion

Pulmonary disease is the major cause of morbidity and mortality in spinal muscular atrophy types 1 and 2. Respiratory muscle weakness results in impaired cough and ability to clear lower airway secretions, lung and chest wall underdevelopment, and hypoventilation. Respiratory care of patients with spinal muscular atrophy is essential to their survival and quality of life. The pulmonary working group has achieved the following consensus recommendations:

1. Referral for respiratory care evaluation and discussion of options should occur shortly after diagnosis. Key components of the respiratory assessment include evaluation

of cough effectiveness, observation of breathing, and monitoring gas exchange.
2. Chronic respiratory management includes providing methods for airway clearance, including mechanical insufflation-exsufflation or manual cough assist and noninvasive ventilatory support. Routine immunizations are also recommended.
3. Discussion with families about the options for respiratory care and identifying the goals for chronic and acute respiratory care should occur early in the disease course and continue in an ongoing dialogue.
4. Acute respiratory illness management requires increased airway clearance and secretion management techniques using mechanical insufflation-exsufflation or manual cough assist, increased respiratory support (including noninvasive ventilation), nutrition and hydration management, and a low threshold to start antibiotics.
5. Perioperative care includes a thorough preoperative evaluation of respiratory status, ideally by a pulmonologist, and anticipatory guidance of the surgical team and postoperative management team regarding optimal care.

Future Research Directions

The following topics were identified as areas in which no consensus could be achieved because research data are lacking. These areas are in need of further study:

1. Optimal methods for evaluation and monitoring of hypoventilation.
2. Use of pulse oximetry in the home.
3. Optimal secretion mobilization techniques (eg, chest physiotherapy, postural drainage, high-frequency chest wall oscillation, and intrapulmonary percussive ventilation).
4. Optimal ventilatory support settings.
5. Effectiveness of inhaled and nebulized medications, including bronchodilators, mucolytics, and corticosteroids.

Consensus on Gastrointestinal and Nutritional Care

Overview of Gastrointestinal and Nutritional Complications in Spinal Muscular Atrophy

The key clinical problems associated with gastrointestinal and nutritional complications in spinal muscular atrophy are as follows:

1. *Feeding and swallowing problems*. Bulbar dysfunction is universal in spinal muscular atrophy patients with severe weakness and can result in feeding and swallowing difficulties and aspiration pneumonia, which often results in death. The severity of bulbar dysfunction is variable in patients with spinal muscular atrophy of intermediate severity and rare in those who are mildly affected.
2. *Gastrointestinal dysfunction*. Gastroesophageal dysmotility problems include constipation, delayed gastric emptying, and potentially life-threatening gastroesophageal reflux.
3. *Growth and undernutrition/overnutrition problems*. Without optimal management, growth failure is universal in nonsitters, whereas excessive weight gain is more common in sitters and walkers.
4. *Respiratory problems*. The presence of respiratory complications (weak cough, increased work of breathing, dyspnea, pneumonias, and cyanosis or desaturation with feeds) raises concern for gastrointestinal problems of aspiration and gastroesophageal reflux, which can be serious and life-threatening. Increased work of breathing may also result in increased energy expenditure.

In the following sections, we will take a problem-oriented approach to discussing the evaluation and management of these problems during chronic care and acute illness.

Feeding and Swallowing Problems

Feeding and swallowing difficulties are common in nonsitters and sitters but are rarely a concern in walkers. Key symptoms of feeding difficulties include prolonged mealtime, fatigue with oral feeding, and evident choking or coughing during or after swallowing. The presence of recurrent pneumonias is a potential indicator of aspiration, which may be silent (ie, without evident choking or coughing). Articles in the literature addressing the role of oral motor structures and specific chewing and swallowing impairments that impact oral feeding performance in spinal muscular atrophy are limited to class III and IV evidence. One review found a 36% prevalence of at least 1 feeding-related issue in children with spinal muscular atrophy.[51] Several other population studies and case series report swallowing problems in patients with spinal muscular atrophy.[51-53] Difficulties in the preoral phase included limited mouth opening and difficulties in getting food to the mouth for self-feeding.[51] In the oral phase, difficulties included weak bite force, reduced range of mandibular motion limiting mouth opening, and increased fatigue of masticatory muscles.[54] This affects biting and chewing abilities and can lead to prolonged mealtimes and fatigue, precluding sufficient intake. Masticatory and facial muscle weakness affects oral bolus control, chewing, and bolus propulsion, all of which contribute to reduced feeding efficiency. Difficulties with strength and efficiency are reported in the oral and pharyngeal phase of the swallow. Poor coordination of the swallow with airway closure can lead to penetration and aspiration of the airway. Poor head control may also be a factor in the development of feeding difficulties, precluding neck tuck or other compensatory postures to enhance the safety of swallowing.[55] The psychosocial impact of feeding difficulties on these children and their family should not be underestimated. Prolonged mealtimes can put time pressure on other activities. In addition, their inability to feed themselves can make these children seem more dependent

than their peers and lead to a sense of loss of control. In the weakest children, tube feeding can limit the nurturing role parents perceive from being able to orally feed their child.[53]

Evaluation of Feeding and Swallowing Problems

Assessment of feeding problems should be performed by a feeding specialist, most commonly a speech or occupational therapist. Routine clinical evaluation of feeding and swallowing difficulties should include a feeding assessment. Videofluoroscopic swallow studies should be performed when indicated. A feeding case history with mealtime observation is desirable. Examination of oral structures that influence feeding efficiency and consideration of the effect of positioning and head control on feeding and swallowing are essential. Videofluoroscopic swallow studies should be carried out after initial assessment if there are concerns about swallow safety.[51,53,56,57] Laryngeal aspiration requires specific assessment, as it is sometimes silent (ie, no clearing cough is triggered).[56] In severely affected children, vocal fold paralysis and consequent inability to protect the airway may be a diagnostic sign.[57,58] A videofluoroscopic swallow study is not simply a diagnostic test of aspiration but is an opportunity to evaluate therapeutic strategies, such as adapted food texture and positioning, to assess impact on swallow function. As position and consistency can affect swallow physiology, it is important that the videofluoroscopic swallow studies procedure is as representative of the child's usual meal and feeding position as possible.

Management of Feeding and Swallowing Difficulties

Treatment should aim at reducing the risk of aspiration during swallow and optimizing efficiency of feeding and promote enjoyable mealtimes. A Cochrane review[59] of treatment of swallowing difficulties in chronic muscle disease concluded it was not possible to determine the benefit or otherwise of dietary and feeding advice, surgical intervention (cricopharyngeal myotomy or upper esophageal dilatation), and enteral feeding. Changing food consistency and optimizing oral intake are appropriate treatment strategies. The literature suggests there is currently widespread use of consistency modification in helping to optimize oral intake.[51,53] A semisolid diet can be used to compensate for poor chewing and reduce length of mealtimes. Thickened liquids may protect against aspiration of thin fluids. Preferably, this intervention would be evaluated objectively on videofluoroscopic swallow studies. In 1 study, complete restriction orally to eliminate risk of aspiration during swallowing was not found to significantly affect the clinical course in severe spinal muscular atrophy.[60] This study failed to consider the risk of aspiration due to concomitant gastroesophageal reflux. Positioning and seating alterations and orthotic devices (eg, Neater Eater, elbow support, valved straw) to enhance self-feeding ability may improve swallow safety and efficiency.[51] Such interventions should be planned in liaison with an occupational therapist and/or physiotherapist as required. There is currently no supporting evidence that oral motor treatment programs impact safety or efficiency of oral feeding.

The gastrointestinal/nutrition working group did not reach consensus regarding when to refer a patient with spinal muscular atrophy for consideration for gastrostomy tube placement and whether one should supplement or replace oral feeding with tube feeding in a nonsymptomatic patient. Some practitioners prefer a proactive approach, particularly in the nonsitters, whereas others believe that exposing such patients to the risk of surgery is inappropriate prior to the onset of symptoms. However, 1 clear consensus is that optimal management requires proactive nutritional supplementation as soon as inadequate oral intake is recognized. Whether a gastrostomy tube is placed in a particular child often requires extensive discussion with multiple caregivers. It usually takes time to schedule a surgical procedure like gastrostomy tube placement. In the interim, nutritional supplementation via nasogastric or nasojejunal feeding is desirable. Nasojejunal feeding may be preferable in circumstances when gastroesophageal reflux with aspiration is a concern, especially when the patient is on ventilatory support. However, technical difficulty may prevent its feasibility. Gastrostomy tube feeding is the optimal method of feeding when insufficient caloric intake or unsafe oral feeding is of concern. It prevents the potential morbidity associated with prolonged use of either nasogastric or nasojejunal tubes. The presence of a nasojejunal or nasogastric tube may also result in a less-than-ideal mask fit when there is a need for the use of noninvasive ventilation such as bilevel positive airway pressure.

There are several options for gastrostomy tube placement, including insertion via percutaneous methods with endoscopic guidance, or placement via open or laparoscopic surgical techniques[61] together with an antireflux procedure such as Nissen fundoplication. The open surgical technique is associated with a relatively large upper abdominal incision, increased postsurgical pain, and risk for respiratory complications due to diaphragmatic splinting. A laparoscopic surgical technique provides the best possible setting for immediate or early postoperative extubation.[62] Such procedures are typically performed with general anesthesia, although placement using percutaneous methods with endoscopic guidance is performed in some centers with conscious sedation and local anesthesia. Care should be taken to minimize the amount of fasting preoperatively and to resume full nutritional support as quickly as possible following the procedure. Possible pulmonary complications of sedation should be anticipated and may require treatment with noninvasive ventilation (see "Pulmonary Care").

Gastrointestinal Dysfunction

Children with spinal muscular atrophy suffer from the following gastrointestinal problems: gastroesophageal

reflux, constipation, and abdominal distension and bloating. Gastroesophageal reflux is an important determinant of mortality and morbidity in patients with spinal muscular atrophy. It can be associated with silent aspiration and results in pneumonias and, at times, life-threatening events.[60] Frequent "spitting up" or vomiting after meals, complaints of chest or abdominal discomfort, bad breath, or obvious regurgitation of feeds may indicate gastroesophageal reflux. Some children may refuse feeds when they develop discomfort with swallowing, placing them at risk for undernutrition. High-fat foods delay gastric emptying and increase the risk of gastroesophageal reflux. Constipation is a frequently reported problem and is likely multifactorial in origin (ie, abnormal gastrointestinal motility, reduced intake of dietary fiber, inadequate fluid intake, low muscle tone of the abdominal wall). Infrequent bowel movements can lead to abdominal distention and bloating. In children dependent on their abdominal muscles to assist with respiration, desaturation or respiratory distress in association with attempted bowel movements may occur.

Evaluation of Gastrointestinal Dysfunction

The symptoms of gastroesophageal reflux (emesis, regurgitation, gurgling after feeds) should be sought early. A routine upper gastrointestinal series is recommended for presurgical evaluation for gastrostomy tube placement to primarily rule out anatomic anomalies and secondarily to document reflux. In rare cases, esophageal stricture, foreign body, or other abnormality may contribute to swallowing difficulties or gastrointestinal dysmotility. Motility studies, including scintigraphy, can be helpful in documenting delayed gastric emptying, which may contribute to gastroesophageal reflux and early satiety. There are no data to support the routine diagnostic use of pH probe studies in documenting reflux.

Management of Gastroesophageal Reflux

Medical management of gastroesophageal reflux typically involves the use of acid neutralizers (eg, magnesium or calcium carbonate) and/or inhibitors of acid secretion. This latter category includes both histamine blockers and proton pump inhibitors (eg, famotidine, ranitidine, omeprazole). Short-term use of these agents is reasonable for symptomatic management. However, increasing evidence suggests that prolonged use of these agents may be associated with a greater risk for gastroenteritis and pneumonia.[63,64] When delayed gastric emptying or diminished motility is present, prokinetic agents may be useful (eg, metaclopramide, erythromycin). Use of probiotics such as acidophilus or lactobacillus to help maintain a healthy gastrointestinal flora, particularly after antibiotic treatment or in the setting of prolonged use of acid inhibitors, is an area deserving further study.

Gastrostomy tube feeding does not ameliorate gastroesophageal reflux. This is of particular concern in nonsitters who are the least able to protect their airway via a triggered cough. Determining whether aspiration has occurred during swallow or as a result of gastroesophageal reflux is often difficult, and sometimes both may contribute. A laparoscopic antireflux procedure (eg, Nissen fundoplication) is commonly performed as a combined procedure during the same general anesthesia for gastrostomy tube insertion. Although some physicians support a proactive combined laparoscopic Nissen and gastrostomy tube procedure in those children with spinal muscular atrophy who are deemed at greatest risk for aspiration, there is as yet no published data nor consensus to support this strategy. However, in the spinal muscular atrophy patient with medically refractory gastroesophageal reflux, and in whom the benefit is deemed to outweigh the associated surgical and anesthetic risks, laparoscopic Nissen fundoplication during gastrostomy tube placement is supported as an appropriate intervention.

Growth and Undernutrition/Overnutrition Problems

Children with spinal muscular atrophy are at risk for growth failure or excessive weight gain. Growth failure is commonly seen in nonsitters and some sitters, whereas obesity is a problem of the stronger sitters and walkers. There are no articles in the literature that specifically address body composition and growth expectations or typical anthropometric measures in children with spinal muscular atrophy. However, data can be extrapolated from literature on patients with spinal cord injury. Individuals with spinal cord injury have been shown to have lower lean tissue and higher percentage body fat than controls. Body mass index significantly underestimates body fat in these patients.[65,66] Children with spinal muscular atrophy may have acceptable fat mass but may plot as underweight based on weight/height criteria due to the decrease in lean body mass.[53] Hence, normal body mass indexes may not represent the ideal weights for children with spinal muscular atrophy. Decreased activity and lean body mass will lead to reduced resting energy expenditure and increased risk of obesity.

Management of Growth and Undernutrition or Overnutrition Problems

Routine history, physical examination, and monitoring of growth velocity measures (growth charts) form the evaluation process to detect signs and symptoms of growth failure or excess. This will influence decisions regarding when and how to intervene. The goal is to maintain each child on his or her own growth velocity. Growth velocity curves (weight, height/length, weight/height) followed over a period of time are, for the most part, the most accurate indicator of nutritional status. Difficulty in obtaining accurate standing height measurements due to contractures or inability to

stand may complicate growth monitoring in these children. Recumbent length, segmental measurements, or arm span may be useful surrogate markers for linear growth in these children. Other methods for monitoring body composition include skinfold measurements, muscle circumference, or bioelectric impedance analysis.[67] Assessment of nutritional intake by a dietitian or other health care provider proficient in nutrition is recommended at each visit. A 3-day dietary record is a simple and accurate tool that can help assess whether nutritional intake is adequate.[53] A 24-hour food recall is a practical method to highlight major nutritional concerns and to obtain information regarding use of any special supplements. Analysis should target adequacy of macronutrient (including fiber intake) as well as micronutrient intake. Currently, there is no indication for increasing or decreasing specific nutrients (ie, protein, fat, or selected vitamins or minerals). Until more specific data are available, nutrient intake should meet the daily recommended intakes for age. Supplements to provide more than the dietary recommended intake for vitamin, mineral, protein, or fat should be discouraged. Although anecdotal benefit with the use of elemental or semi-elemental formulas has been reported by some families and care providers (satisfactory growth and decreased secretions), there is currently insufficient data to make specific recommendations regarding their use. If an elemental formula is used, a dietitian should be involved to help ensure the child does not receive insufficient or excessive amounts of nutrients, to perform laboratory assessments as needed, and to monitor adequate growth.[68] As previously mentioned, with a reduction in lean body mass, calculated body mass index will significantly underestimate body fat.[66] Children with spinal muscular atrophy may have acceptable fat mass but may be perceived as underweight based on weight/height criteria because of their decreased lean body mass. This will result in inappropriate dietary recommendations that could lead to relative obesity.[53] The spinal muscular atrophy patients at risk for obesity should have growth parameters in the lower percentiles for weight/height and body mass index. In any case, each child should be evaluated individually on a routine basis, with the goal of following their established growth curves and avoiding inadequate or excessive intake. There is some evidence that decreased bone mineral density may occur in nonsitters and sitters, resulting in recurrent fractures in a subset of patients.[69] There is preliminary evidence that dual energy x-ray absorptiometry could be a useful technique for estimating lean versus fat mass in spinal muscular atrophy patients.[70-73] However, insufficient data are available at this time to recommend the routine use of dual energy x-ray absorptiometry scans for monitoring bone mineral density or body composition. Instead, the importance of documenting appropriate intake of calcium and vitamin D was emphasized. There is no consensus regarding performing biochemical tests to monitor nutritional status for patients with spinal muscular atrophy. However, consideration should be given to checking prealbumin levels to help assess adequate protein status.[53]

Management of Nutrition in the Acutely Sick Spinal Muscular Atrophy Patient

Spinal muscular atrophy patients are particularly vulnerable to catabolic and fasting states. Patients with severe muscle wasting from any disorder, including spinal muscular atrophy, are more likely to develop hypoglycemia in the setting of fasting.[74,75] A number of case series and individual case reports have documented secondary mitochondrial dysfunction and abnormalities of mitochondrial fatty acid oxidation in spinal muscular atrophy patients.[76-83] Significant abnormalities are most likely in nonsitters and sitters, increasing their vulnerability for metabolic decompensation in the setting of a catabolic state. Thus, it is necessary to avoid prolonged fasting, particularly in the setting of acute illness, in all spinal muscular atrophy patients. Nutritional intake should be optimized to meet full caloric needs within 4 to 6 hours after an admission for acute illness, via enteral feeding, parenteral feeding, or a combined approach as necessary. Prompt postoperative caloric supplementation is recommended to avoid muscle catabolism, particularly in a child with reduced fat store. If enteral intake is not imminent, then intravenous caloric feeding should be considered.

Conclusion and Future Directions

Because nutritional problems associated with spinal muscular atrophy influence the patient's pulmonary status and general well-being, optimal management of these problems by a multidisciplinary or interdisciplinary team of physicians, speech therapists or occupational therapists, dietitians, and pediatric surgeons should greatly improve survival and quality of life.[62]

The following topics were identified as areas in need of further study:

1. Use of elemental formulas to support/refute perceived benefits of optimal growth and decreased oral and airway secretions.
2. Need for a reduced fat intake, in view of the concern for mitochondrial fatty acid oxidation abnormalities.
3. Need for protein supplementation beyond dietary recommended intake, in view of the problem of muscle wasting/atrophy.
4. Need for checking biochemical tests for metabolic/mitochondrial fatty acid abnormalities, in view of the concern for mitochondrial fatty acid oxidation abnormalities.
5. Need to determine body composition and establish growth charts for the population of patients with spinal muscular atrophy to enable optimal growth monitoring in these patients.

Consensus on Orthopedic Care and Rehabilitation

Overview of Orthopedic Care and Rehabilitation Strategies in Spinal Muscular Atrophy

Key Problems

Muscle weakness of varying severity limits motor function of trunk and upper and lower extremities, resulting in contracture formation, spinal deformity, limited mobility and activities of daily living, and increased risk of pain, osteopenia, and fractures.

Key Evaluation Procedures

These include evaluating range of motion, strength, function, seating and mobility, orthotics, radiographs (spine and other joints), and dual energy x-ray absorptiometry. The value of these procedures varies by degree of functional impairment.

Key Interventions

In nonsitters, nutritional support, posture management, seating, contracture and pain management, therapy for activities of daily living and assistive equipment, wheelchairs for mobility, limb orthotics, and developmental therapies are important. In sitters, wheelchair mobility, contracture management, physical therapy, and occupational therapy are of highest value, with strong considerations for spine and limb orthotics and spine surgery. In walkers, the highest emphasis is on provision of physical therapy, occupational therapy, and wheelchair/mobility, although orthotics, scoliosis surgery, and pain management figured prominently. In the United Kingdom and some other European countries, chest physiotherapy is often done by physical therapists.

Literature Review

Rehabilitation and Orthopedic Problems in Spinal Muscular Atrophy

Literature review pertinent to these rehabilitation and orthopedic concerns reflects similar musculoskeletal and functional problems to those presented in "Key Problems".[1,84-86] Hip subluxation is a common comorbidity in patients with spinal muscular atrophy.[87,88] As patients with spinal muscular atrophy age, there is a significantly higher prevalence of kyphoscoliosis, difficulty coughing, joint contractures, and voice/speech problems in types 1 and 2. In type 3, there is also a significantly higher prevalence of fatigue and hypermobility of the hand.[89] Scoliosis develops in more than 50% of children with spinal muscular atrophy, most commonly in nonambulatory children or in those who lose the ability to walk.[90]

Evaluation

Traditional measurements of strength are not possible in severely affected infants and children; thus, emphasis is on observation of function. Evaluation procedures that address rehabilitation/orthopedic concerns include the CHOP-INTEND,[91] the Hammersmith Functional Motor Scale for Spinal Muscular Atrophy,[92,93] the Modified Hammersmith Functional Motor Scale for Spinal Muscular Atrophy,[94] the Gross Motor Function Measure,[95] and the Motor Function Measurement scale for neuromuscular disease.[86,93] Most children with spinal muscular atrophy require help or supervision with bathing and dressing and assistance with mobility. Stairs present a major obstacle.[96] Early and generalized joint contractures and scoliosis correlate with level of motor function and walking with support, rolling by 5 years of age correlates with eventual walking, and inability to roll correlates with severe disease (greater weakness).[97] Muscle strength can be quantified using myometers, videotaped movements,[98] handheld dynamometers,[99] and quantitative muscle testing in children with the type 2 or 3 forms of the disease.[100-102] Flexion contractures, which affect almost half of spinal muscular atrophy patients, are often noted during periods of inactivity and are considered intractable if greater than 45° Activities of daily living are hampered, and contractures are perceived to be associated with disability in about half. Pain increases in frequency and severity over time and correlates with decreased scores on quality-of-life indicators.[103] In all studies of scoliosis, spine radiographs were routinely used for diagnosis. A retrospective review of spinal full-length radiographs revealed a predominance of right-sided thoracic and thoracolumbar curves and left-sided lumbar curves.[104]

Interventions

No studies directly address physical therapy and occupational therapy as general therapies, although a case report documented the ability of a 20-month-old girl with spinal muscular atrophy to learn to operate a power wheelchair independently in 6 weeks and demonstrated developmental gains in all domains of the Batelle Developmental Inventory over the ensuing 6 months.[65] Regarding other interventions, 3 case series discussed the use of knee-ankle-foot orthoses in patients with spinal muscular atrophy. Evans[105] presented 5 cases (3 who had lost the ability to walk) who were treated with serial casting and bracing and were still ambulatory 2 to 5 years later. Granata presented 7 cases and later 12 cases of patients with type 2 spinal muscular atrophy. All were able to stand independently with knee-ankle-foot orthoses, and 7 achieved assisted ambulation. When compared with a historical control group, the treatment group had less scoliosis. There may have been a trend toward greater hip subluxation in the treatment group.[106]

Consensus Recommendations on Evaluation and Treatment by Functional Levels

The natural history of the disorder should be considered along with the results of the examination and the goals of the patient and family in planning treatment. Intervention should address the problems that were identified through a thorough history and examination. On the basis of the literature review, the results of the Delphi survey, and our group conferences, we list recommendations in this care area by the functional levels of these patients.

Nonsitters

These patients present in early infancy to rehabilitation providers with impairments in respiratory function and profound weakness. Limited range of motion, head control, postural control and alignment, and progressive scoliosis are found. There is significant fatigue during and after medical care and with therapies. Weakness leads to varying functional deficits that interfere with caretakers' abilities to perform activities of daily living and that also limit participation in developmental activities and later in school. A multidisciplinary approach to evaluation and management includes a strong partnership between therapists, patients and families, and physicians. Assessments include physical and occupational therapy and speech therapy if swallowing is impaired or if speech production is affected by jaw contractures and inadequate ventilatory support of voice. Play and occupational support should include lightweight toys and assistive technology with variable controls and a myriad of activation systems. Consideration of the patient's primary posture should direct choice of equipment and devices that support function. Upper extremity orthotics to aid in function include the use of mobile arm supports or slings that augment active range of motion and functional abilities. Use of linear elastic elements to balance out the effects of gravity in multiple dimensions can aid those with proximal weakness and improve control of distal function. Upper extremity or hand orthoses should be considered with caution because attempts to correct postural deviations and compensations with an orthosis may result in reduced function. Compensations due to hypermobility and lack of power should not always be discouraged. Splinting to preserve range of motion and prevent pain may be indicated.

Sitters

Weakness, contractures, respiratory dysfunction, and scoliosis characterize the main problems of this group. These impairments contribute to limitations in mobility, endurance, and activities of daily living. Evaluations by physical therapists, occupational therapists, and orthopedic surgeons include measurement of contractures and strength by goniometry, manual muscle testing, or myometry, with judicious use of spine and hip radiographs. Equipment evaluation includes seating and mobility, positioning, and equipment for self-care. The need for assistive technology and adaptive aids should be determined in the context of improved function. Pulmonary evaluation should be conducted, as it pertains to exercise tolerance and endurance. Evaluations for manual and power mobility may be conducted as early as 18 to 24 months of age. Contracture management and exercise are a major focus of treatment, with implementation of a regular stretching and bracing program to preserve flexibility. Serial casting for contractures may improve participation in a standing program and improve tolerance of bracing. Regular exercise should be encouraged to maintain fitness and endurance and might include swimming and adaptive sports. Lightweight ischial weight-bearing knee-ankle-foot orthoses or reciprocal gait orthoses should be considered for standing or assisted ambulation with a walker for patients with sufficient strength. Where this is not possible, a standing frame or mobile stander with ankle-foot orthoses should be considered. Upper extremity orthotics with mobile arm supports or slings augment active range of motion and functional abilities. Assistive technology and other adaptive equipment to enhance independent work and play should be considered.

Walkers

The combination of proximal weakness and impaired balance results in frequent falls. Limitations are found in transitions between the floor, sitting and standing, distance ambulation, changes in terrain, and stair climbing. There are consistent complaints of fatigue with activity. Musculoskeletal deformities and pain are most commonly reported in late childhood and early adolescence, and with their onset, functional limitations become more pronounced. Patients may present acutely for management of fractures or other musculoskeletal injury. Balance and ambulation evaluations include a specific survey of environmental adaptability and access. Evaluation of joint range of motion and spinal alignment as they affect function, comfort, and balance guides more specific orthotic and spinal assessment and x-rays. Physical and occupational therapy assessments to determine appropriate mobility aids, adaptive equipment, assistive technology, and environmental access will allow patients to maintain independence and mobility and to conserve energy. Activities of daily living assessment for equipment and adaptation may improve independence and access to home and community environment. Nonspine x-rays and dual energy x-ray absorptiometry are considered in the event of acute musculoskeletal injuries as a result of overuse, an accident, or a fall. Treatment and interventions should consider goals of the family and/or caretakers and should be problem-driven. Physical therapy consultation helps to maximize safety, endurance, and independence or to prolong ambulation. Orthotics also support functional walking. Wheelchair

mobility for longer distance transportation adds mobility and independence. Walkers appear less likely to develop scoliosis; thus, continued walking should be encouraged. Contracture management and education to maximize joint protection should be a part of any treatment program. Maximum functional activity includes access to leisure, adaptive sport, and play activities. Regular exercise to maintain fitness and stamina should be encouraged and may include swimming, aquatic therapy, horseback riding, and adaptive sports. Weight management with attention to fitness and education about nutrition are necessary. Equipment needs related to activities of daily living and assistive technology and other adaptive equipment may be useful to enhance abilities for independent work and play. Environmental controls and home modifications to allow for safe accessibility and optimal independence should be explored. Driver's education alternatives and consideration of customized driving controls should be part of the overall rehabilitation management of the adult with spinal muscular atrophy.

Orthotics

In selecting and fabricating an orthosis for patients with spinal muscular atrophy, it is important that the orthotist, therapist, and family work together to ensure that the appropriate orthosis is fabricated and allows wearers to meet their functional goal. For patients with spinal muscular atrophy, it is particularly important that the orthotist has a good background and experience in working with patients with neuromuscular disorders. Familiarity with patterns of weakness and compensations allows the orthotist to choose proper materials and to make adaptations that allow for "best" fit and function.

Spinal orthoses may be used for postural support, but there is insufficient evidence to support delayed curve progression. When used, spinal orthoses should be fabricated with an abdominal cutout to allow appropriate diaphragmatic excursion and access to gastrostomy tubes where present.

Orthopedic Surgery

Surgical correction of scoliosis should be considered based on the patient's curve progression, pulmonary function, and bony maturity. Scoliosis surgery in children with prolonged survival provides benefits in sitting balance, endurance, and cosmesis.[105,107-111] Evidence suggests that earlier surgery results in better outcome. Beneficial effects on pulmonary function remain controversial, but the rate of pulmonary decline may be slowed.[112] Intraoperatively, excessive bleeding may occur. Postoperatively, complications include loss of correction, pseudarthrosis, a requirement for prolonged ventilatory support, and chest and wound infections. Careful consideration is warranted for the spinal muscular atrophy patient who is still ambulatory because altered function, balance, and respiration may result in loss of independent walking. Pelvic obliquity may require surgical fixation.[113] Intraoperative neurophysiologic monitoring may detect temporary abnormalities during scoliosis surgery.[114]A survey of patient/parent satisfaction and clinical/functional outcome was sent to 21 patients with spinal muscular atrophy who underwent operations for scoliosis. Of those who returned the surveys, all found benefit from scoliosis surgery regarding cosmesis, quality of life, and overall satisfaction.[115] Although there is a higher rate of hip subluxation in spinal muscular atrophy, few are painful. Surgical reduction and osteotomy are frequently followed by redislocation.[116,117] In most circumstances, this surgery is avoidable. Ankle and foot deformities make conventional shoes difficult to wear, and orthopedic surgeons may consider soft tissue releases at the child and family's request. In walkers, if soft tissue releases are performed, rapid and aggressive physical therapy may improve outcome.

Perioperative Management in Spinal Muscular Atrophy

Perioperative management and the role of rehabilitation should be customized according to the specifications and needs of the patient and family, therapist, and surgeon. In general, preoperative management includes appropriate modification of the individual's environment, a plan for orthotic intervention, and confirmation of timing and modification of orthoses. New wheelchairs or wheelchair modifications of the seat, back, arm, leg, or headrests are likely to be required. One may anticipate increased sitting height after scoliosis surgery, resulting in the need for van modifications. Families need instruction in transfers, including arrangements for a mechanical lift, if necessary. Arrangements for bathing, toileting, and dressing equipment and potential modifications to clothes for ease in donning and doffing over, under, and/or around casts or orthoses are necessary. Two small studies found that noninvasive positive pressure ventilation, 1 with mechanical-assisted cough training prior to surgery, resulted in successful extubation.[118,119] Incentive spirometry practice may be initiated and coordinated with preoperative noninvasive pulmonary supports, such as bilevel positive airway pressure and exsufflator (cough-assist) devices.

Postoperatively, one must confirm timing of appropriate casting and fitting of orthoses, allowed range of motion, and activity and that appropriate adaptive equipment is available. Therapists can ensure appropriate use of incentive spirometry and instruction of nursing staff and family on bed mobility, transfers, dressing, bathing, and toileting. The individual should be mobilized as soon as possible, as allowed by the procedure and surgeon.

Conclusion

Infants and children with spinal muscular atrophy should have appropriate evaluation for their presenting musculoskeletal and functional deficits. Goals of therapy and surgery depend on functional level and the family's wishes. Even young children should be offered independent mobility and activities of daily living, which includes play. Whenever possible, walking should be encouraged with appropriate assistive devices and orthotics. Hip subluxation is rarely painful, and there is a high risk of recurrence despite surgical correction. Spinal orthoses may provide postural support but do not prevent curve progression and may impair respiratory effort. Scoliosis surgery appears to benefit patients who survive beyond 2 years of age when curves are severe and progressive and should be performed while pulmonary function is adequate. Preliminary studies show the benefit of preoperative training with noninvasive ventilation and cough-assist devices. Intraoperative neurophysiologic monitoring detects early neurologic compromise in some and may improve outcome.

Recommendations for Future Direction

The optimal evaluation procedure to assess motor function in very weak infants is evolving, with ongoing research efforts under way. Many questions remain regarding best practices in therapeutic interventions. There is a need for the development of creative technology to improve independent function. Further research is suggested to evaluate the effects of spinal bracing and surgery on function, balance, and respiratory function. The role of bone density evaluation and treatment of osteopenia must be further examined.

Palliative Care Issues

In most circumstances in the course of medical practice, the goal of therapy—to further quality and extent of life—is straightforward. In the case of patients with spinal muscular atrophy, however, the appropriate goal of therapy may not be clear. Some therapies may be perceived as placing quality of life in conflict with duration of life, prolonging suffering rather than relieving the burden of disease. Thus, there is little national or international consensus about the appropriate level of care, and local experience, training, habit, and resource availability appear to have a large effect upon recommendations and ultimately family decisions about interventional support.[120-122] Although not surveyed formally, the committee is aware of a similar broad range of practice regarding appropriate pulmonary, nutritional, orthopedic, and other forms of therapy.

Optimal clinical care for these patients should be mindful of potential conflict of therapeutic goals. This conflict is made more difficult by the need for surrogate decision makers for a dependent infant and the fact that many—including parents, siblings, other relatives, caregivers, payers, and the wider community—will be affected by and thus have some valid interest in care decisions. The committee reached consensus that these conflicts are real and that there is no moral imperative to any therapy. There is, however, a deep responsibility to present care options in an open, fair, and balanced manner.

A choice for or against interventional supportive care is not a single binary choice, nor must it be unchanging with circumstance. There are, however, some interventions that are better done early so as not to constrain later potential assistance. For example, placement of a gastrostomy tube is better done relatively early, when associated risks are lower, to provide more stable and comfortable nutritional support later when feeding is more tenuous. Similarly, it is important to discuss and determine the appropriate response to potential life-threatening respiratory insufficiency, as emergency resuscitation and endotracheal intubation during times of crisis without prior respiratory support are associated with many more problems in care than when decisions are made in advance. If appropriate, other forms of noninvasive respiratory device that might reduce the potential for emergent respiratory support should be introduced according to increasing need. Whenever possible, caregivers should ideally permit sufficient time after diagnosis prior to discussing these difficult issues; in all cases, sufficient time, honest appraisal of the choices, openness to revisiting decisions made, and personal rapport are essential to these discussions. If appropriate, other family members or trusted friends or spiritual advisors should be invited. End-of-life care decisions need to be defined and neither delayed nor aggressively foisted upon unsuspecting, grieving, and stunned parents.

Care for patients with spinal muscular atrophy is often best accomplished with a multispecialty team approach, when possible. Successful teams have a point person who is mindful of the many needs and can obtain appropriate medical, social, and spiritual assistance as appropriate. In addition, hospice referral or other provision for the specific issues regarding terminal care, grief, and bereavement support is important. In the circumstance of a choice against mechanical ventilatory support, appropriate provision for management of terminal dyspnea can be of comfort to the patient and family alike. Use of nebulized narcotics can avoid much of the concern that overdosing contributes to death and provide comfort to the patient.

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Observational study of spinal muscular atrophy type I and implications for clinical trials

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ABSTRACT

Objectives: Prospective cohort study to characterize the clinical features and course of spinal muscular atrophy type I (SMA-I).

Methods: Patients were enrolled at 3 study sites and followed for up to 36 months with serial clinical, motor function, laboratory, and electrophysiologic outcome assessments. Intervention was determined by published standard of care guidelines. Palliative care options were offered.

Results: Thirty-four of 54 eligible subjects with SMA-I (63%) enrolled and 50% of these completed at least 12 months of follow-up. The median age at reaching the combined endpoint of death or requiring at least 16 hours/day of ventilation support was 13.5 months (interquartile range 8.1–22.0 months). Requirement for nutritional support preceded that for ventilation support. The distribution of age at reaching the combined endpoint was similar for subjects with SMA-I who had symptom onset before 3 months and after 3 months of age ($p = 0.58$). Having 2 *SMN2* copies was associated with greater morbidity and mortality than having 3 copies. Baseline electrophysiologic measures indicated substantial motor neuron loss. By comparison, subjects with SMA-II who lost sitting ability (n = 10) had higher motor function, motor unit number estimate and compound motor action potential, longer survival, and later age when feeding or ventilation support was required. The mean rate of decline in The Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders motor function scale was 1.27 points/year (95% confidence interval 0.21–2.33, $p = 0.02$).

Conclusions: Infants with SMA-I can be effectively enrolled and retained in a 12-month natural history study until a majority reach the combined endpoint. These outcome data can be used for clinical trial design. ***Neurology*® 2014;83:810–817**

GLOSSARY

CHOP INTEND = The Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders; **CI** = confidence interval; **CMAP** = compound motor action potential; **IQR** = interquartile range; **MUNE** = motor unit number estimate; **SMA** = spinal muscular atrophy; ***SMN1*** = survival of motor neuron 1, telomeric.

Proximal spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disorder with an incidence of 1:11,000 live births.[1] Mutations in the survival of motor neuron 1, telomeric (*SMN1*) gene cause SMA.[2] A nearly identical gene, *SMN2*, harbors an exonic splicing enhancer mutation that limits inclusion of exon 7.[3] This results in a reduced amount of functional full-length SMN protein. The *SMN2* copy number is inversely related to clinical severity.[4] SMN protein functions within a spliceosomal complex and is important in RNA processing.[5] SMA is a clinical continuum, divided into 4 phenotypes based on maximal motor function achieved: type I (SMA-I, onset by 6 months, nonsitter), type II (SMA-II, onset 6–18 months, sitter),

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ambulatory type III children, and type IV adults.[6,7] Prior natural history studies have demonstrated shortened lifespan for SMA-I, with 68% succumbing within 2 years and 82% by 4 years of age.[6,7] SMA-I has been further subdivided into 3 groups: type IA (or zero, in some reports[8]), presentation at birth with joint contractures and respiratory compromise; type IB, onset of symptoms before age 3 months; and type IC, onset after 3 months of age.[9,10] Recent utilization of nutritional and respiratory support has altered the natural history of SMA-I, reducing mortality to approximately 30% at age 2 years, with approximately half of these survivors fully reliant on noninvasive ventilation.[11]

This prospective study was designed to describe the current natural history of SMA-I and guide planning of clinical trials for SMA-I.

METHODS This study was performed by the Pediatric Neuromuscular Clinical Research Network for SMA, with clinical sites at Columbia University Medical Center (New York), University of Pennsylvania (The Children's Hospital of Philadelphia), and Harvard University (Boston Children's Hospital). Data management and statistical analysis were performed by the Biostatistics Center of the Muscle Study Group at the University of Rochester (New York).

The overall study design and data on subjects with SMA types II and III have been reported separately.[12,13] The diagnosis of SMA and subtype classification were made by the principal investigator at each site (R.F., P.K., B.D.). Confirmation of the *SMN1* exon 7/8 common deletion was performed by PCR amplification and restriction digest of DNA using primers flanking *SMN1* and *SMN2* exon 7, and *SMN2* copy number was determined as previously described.[12] Only patients with homozygous deletions were included.

Previously identified patients followed in our clinics and newly diagnosed patients were enrolled. All eligible patients were offered participation. Study visits were scheduled at baseline and at 2, 4, 6, 9, and 12 months and every 6 months thereafter. The SMA standard of care guidelines published in 2007 were used as a basis for providing uniform care among the study sites.[14] For purposes of this study, sitting (for SMA-II) was defined as being able to sit independently for >10 seconds. To permit an analysis on the basis of function, rather than SMA type, subjects with type II ($n = 45$) were included and divided into type IIA, having lost the ability to sit ($n = 10$), and type IIB, having maintained the ability to sit ($n = 35$).

Demographic information included age, sex, and ethnicity. SMA history included age at symptom onset (by parental recollection and by verification of medical records when available), age at clinical diagnosis, means of diagnosis (clinical impression, molecular genetic confirmation), history of motor developmental milestones gained and/or lost, and family history of SMA. Other relevant medical and surgical history was captured, with attention to feeding/nutrition and respiratory function, hospitalizations, therapy received, and medication/supplements taken (none were disallowed). The primary outcomes of interest were age at death and age at reaching the combined endpoint of either death or requiring at least 16 hours/day of noninvasive ventilation support for at least 14 days in the absence of an acute reversible illness or perioperatively (as a surrogate for death).[10] Physical examination findings included weight, length/height, head and chest circumference, vital signs, motor function, scoliosis, and joint contractures. Measurement of the serum comprehensive metabolic panel and complete blood count was performed at each study visit. Laboratory abnormalities were determined based on each hospital laboratory's normal values for age.

Motor function testing utilized The Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders (CHOP INTEND), a validated 16-item, 64-point scale shown to be reliable and sensitive to change over time for SMA-I.[15,16] The CHOP INTEND was performed on all subjects with SMA-I and, for comparison, 5 subjects with SMA-II. Motor function testing was performed by clinical evaluators after a training and reliability session and reliability was re-established annually.[12]

Electrophysiologic measurements, the compound motor action potential (CMAP) and motor unit number estimate (MUNE, multipoint method), were obtained as previously described.[12]

Standard protocol approvals, registrations, and patient consents. All guardians of participants provided written informed consent approved by the individual institutional review boards at the participating sites.

Statistical analysis. For purposes of analysis, subjects with SMA-I were subdivided into types IB and IC, and classified as "recent" if enrolled within 3 months of diagnosis or "chronic" if beyond 3 months. The distributions of age at death and age at reaching the combined endpoint of either death or requiring at least 16 hours/day of noninvasive ventilation support for at least 2 weeks were described using Kaplan-Meier curves. The distributions of age at reaching the combined endpoint were compared between subjects with type IB and type IC, boys and girls, and those with 2 and 3 *SMN2* copies using log-rank tests. The mean rate of change over time (slope) in CHOP INTEND score was estimated using a mixed-effects linear regression model with random slopes and intercepts. The mean slopes were compared between subjects who were recently diagnosed and those with chronic SMA-I, as well as between subjects with type IB and type IC, by adding the subgroup variable and its interaction with time to the mixed-effects model and testing for significance of the interaction term. The distributions of electrophysiologic variables (CMAP and MUNE) at baseline were compared between various subgroups (type IB vs type IC; recently diagnosed vs chronic SMA-I) using Wilcoxon rank sum tests. SMA-I subjects with 2 vs 3 copies of *SMN2* were compared regarding baseline variables using Wilcoxon rank sum tests or χ^2 tests, as appropriate. Fisher exact tests were used to compare SMA-I and SMA-II subgroups regarding the percentage of subjects with abnormal laboratory values.

RESULTS All ages are listed in months. Descriptive results for several variables are expressed as median and interquartile range (IQR). Seventy-nine subjects (34 SMA-I and 45 SMA-II) were enrolled between May 2005 and April 2009. The baseline subject characteristics are summarized in table 1, and longitudinal data are summarized in table 2. Figure e-1 (available on the *Neurology*® Web site at Neurology.org) depicts the relationship between age at symptom onset and time between symptom onset and enrollment for subjects with SMA-I, and indicates

Table 1 Baseline demographic and clinical characteristics of study participants

	Type IB		Type IC		Type II	
	Recent	Chronic	Recent	Chronic	Type IIA	Type IIB
No.	8	10	6	10	10	35
Sex, M/F, n	2/6	4/6	5/1	8/2	2/8	16/19
Age at symptom onset, mo, median (IQR)	1.5 (1-2.5)	3.0 (2-3)	4.0 (4-5)	4.0 (4-6)	11.0 (7-12)	8.5 (6-12)
Age at clinical diagnosis, mo, median (IQR)	3.5 (1.8-6)	6.0 (4-7)	5.5 (5-7)	6.0 (6-8)	11.5 (9-14)	13.0 (10-18)
Age at enrollment, mo, median (IQR)	5.0 (2.5-6.5)	59 (17-184)	6.5 (6-8)	30.5 (22-78)	200 (137-298)	64 (38-90)
Screen failures,[a] n (%)	20/54 (37)				32/77 (42)	
SMN2 copy number, n						
2	7	6	3	7	1	1
3	1	3	3	2	9	34
Percent receiving nutritional support, no. by nasogastric tube/ gastrostomy tube	50 (4/8)	100 (10/10)	17 (1/6)	90 (9/10)	50 (5/10)	14 (5/35)
Percent receiving ventilation support, no. by noninvasive ventilation/ tracheostomy and ventilator	0 (0/8)	80 (8/10)	0 (0/6)	80 (8/10)	60 (6/10)	23 (8/35)
Percent receiving both nutritional and noninvasive ventilation support	0 (0/8)	80 (8/10)	0 (0/6)	80 (8/10)	40 (4/10)	11 (4/35)
CHOP INTEND score, median (IQR), n	32.5 (31-33), n = 6	11.0 (6.5-18), n = 8	28.5 (26.5-31), n = 4	28.0 (11-30), n = 9	33, (n = 1)	45.5 (42.5-53), n = 4
Maximum CMAP (negative peak amplitude, μV), median (IQR), n	329 (250-429), n = 4	238 (200-400), n = 9	300 (300-800), n = 3	400 (300-600), n = 6	1,200 (659.5-1,550), n = 8	1,200 (767-1,600), n = 29
MUNE, (IQR), n	3.8 (2.3-6.9), n = 4	3.4 (2-5.1), n = 9	6.4 (5.2-7.4), n = 3	7.5 (5.6-9.9), n = 6	9.2 (7.9-13.9), n = 8	11.7 (1.6-79.9), n = 29

Abbreviations: CHOP INTEND = The Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders; CMAP = compound motor action potential; IQR = interquartile range; MUNE = motor unit number estimate.
Type IB = symptom onset younger than 3 months of age; type IC = symptom onset older than 3 months of age; type IIA = lost independent sitting; type IIB = retained independent sitting.
[a] Reasons given by caregivers for screen failure/nonparticipation for patients with spinal muscular atrophy type I (1 or more/subject): declined (n = 7), too weak/ill to participate (n = 3), sought palliative care (n = 1), distance (n = 1), expense (n = 1), wanted a treatment trial (n = 1), and undecided/no reply (n = 10).

the type of supportive care received at baseline. Nutritional support (nasogastric tube or gastrostomy tube; median 8.0, IQR 6–13) was initiated approximately 3 months earlier than ventilation support (noninvasive ventilation or intubation leading to tracheostomy; median 11.0, IQR 5–19). At baseline, all subjects with SMA-I older than 12 months required feeding or combined feeding and ventilation support, and 14 required 16+ hours/day of ventilation support (11 with bilevel positive airway pressure by mask, 3 with tracheostomy). For the entire SMA-I cohort, the age at symptom onset (median 3.0, IQR 2–4) was approximately 3 months before the age at diagnosis (median 6.0, IQR 4–7) and the interval from diagnosis to enrollment (median 9.0, range 0.3–252) reflects that many chronic patients were included.

Mortality and combined endpoint data are presented in figure 1 and ventilation support data are detailed in table 1. Nine subjects with SMA-I died during follow-up: 6 with type IB and 3 with type IC. Causes of death were acute pulmonary infection (n = 6), airway obstruction (n = 2), and bradycardic arrest (n = 1). The median age at reaching the combined endpoint in subjects with SMA-I was 13.5 (IQR 8.1–22.0). This distribution did not differ significantly by sex ($p = 0.19$) or SMA-I subtype (figure 1B, $p = 0.58$): the median age at the combined endpoint for subjects with type IB was 11.9 (IQR 7.0–22.0) and for subjects with type IC was 13.6 (IQR 8.8–20.1). *SMN*2 copy number data were obtained in 31 of 34 subjects and are summarized in table 1. Nine subjects had an *SMN*2 copy number of 3; none died and 4 required 16+ hours of ventilation for at least 2 weeks during the course of this study. The distribution of the age at reaching the combined endpoint differed by *SMN*2 copy number (figure 1C, $p = 0.002$): the median age at the combined endpoint for subjects with 2 *SMN*2 copies was 10.5 (IQR 8.1–13.6), but the 25th percentile was 22.0 for those with 3 *SMN*2 copies (not enough events occurred to permit estimation of the median age).

Motor function as measured by the CHOP INTEND is summarized in table 1. CHOP

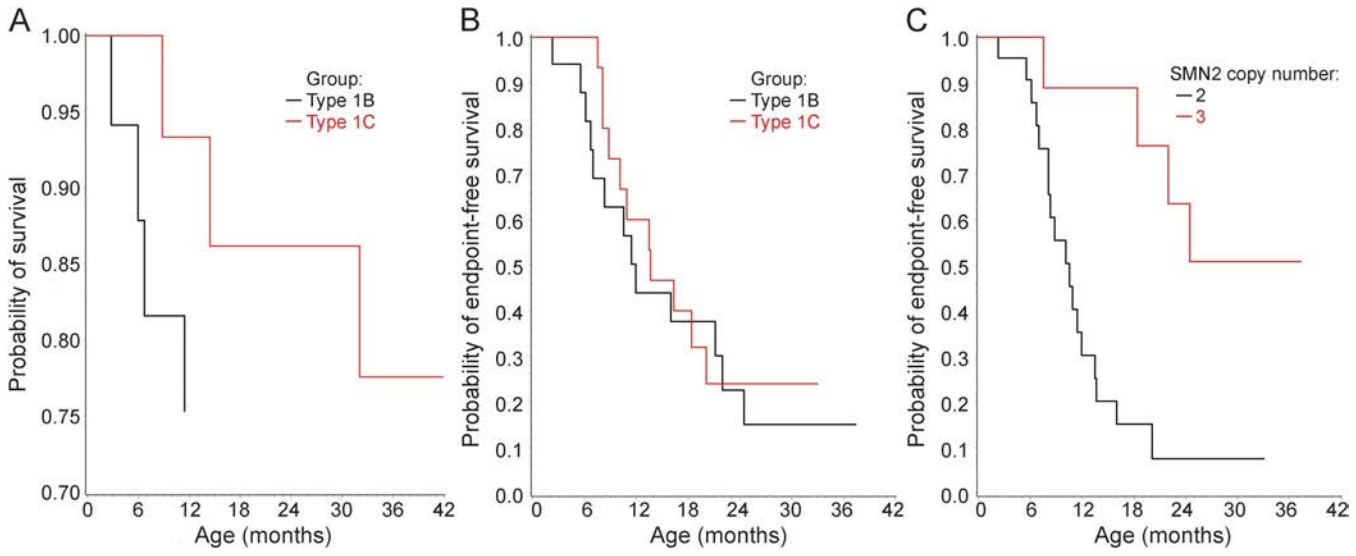
Table 2 Follow-up data for subjects with SMA-I by subtype (IB and IC) and by recent/chronic status (see text)

	Type IB		Type IC		Type II	
	Recent	Chronic	Recent	Chronic	Type IIA	Type IIB
Percentage of scheduled study visits completed (maximum 12 mo), up to time of censoring, death, or withdrawal, median (IQR)[a]	100 (92-100)	75 (50-83)	45 (17-83)	75 (67-100)	92 (67-100)	83 (67-100)
Subjects withdrawn from the study (excluding deaths), n (%)	1 (12.5)	3 (30)	1 (16.7)	2 (20)	4 (40)	4 (11.4)
Off-label "SMA medication"[b] use, n (%)	1 (12.5)	5 (50)	3 (50)	1 (10)	3 (30)	8 (22.9)
Gastrostomy tube placement, %	62.5	90	50	90	30	26
Age when gastrostomy tube was placed	6.0 (4-7)	9.0 (8-14)	6.0 (6-15)	8.0 (7-13)	30.0 (15-36)	55 (42-58)
Receipt of NIV, intubation, or ventilation at any time, %	12.5	100	50	100	100	57.1
Age when NIV started median (range)	6	59 (12-184)	11 (9-24)	18.5 (12-22)	170 (68-279)	62 (42-80)
Bi-PAP or intubation for 16+ h/d for 14+ d, %	25	80	50	70	10	3
Age when Bi-PAP or intubation for 16+ h/d for 14+ d was attained	3.5 (2-5)	13.5 (8-21.5)	10 (8-18)	13 (8-21)	28	16
Mortality, %	62.5	10	33.3	10	0	2.9
Age at death for individual subjects (median)	2, 6, 6, 11, 12 (6.0)	177	9, 14 (11.5)	32	NA	46

Abbreviations: Bi-PAP = bilevel positive airway pressure; IQR = interquartile range; NA = not applicable; NIV = noninvasive ventilation; SMA = spinal muscular atrophy.
The subjects with recent and chronic SMA-II are combined. Ages are reported as median (IQR) in months, unless otherwise specified.
[a] Duration of follow-up (months) to the point of withdrawal (including death) or end of the study was shorter for subjects with SMA-I (median 6.5, IQR 1–28) than for those with SMA-II (median 33, IQR 18–37).
[b] Medications and supplements taken (number of subjects, SMA-I/SMA-II, 7 subjects with SMA-II were on 2 medications): albuterol (3/4), carnitine (2/4), creatine (1/4), hydroxyurea (3/2), sodium phenylbutyrate (0/1), valproic acid (1/3).

INTEND scores did not differ significantly at baseline whether or not nutritional support was required, while the age was older and the scores were notably lower when both nutritional and ventilation support were required (table e-1). Seventeen subjects with SMA-I (4 recent, 13 chronic) were evaluated on at least 2 occasions using the CHOP INTEND (figure 2 by study visit and figure e-2 by age). The mean rate of

Figure 1 Time-to-event curves for SMA-I



Kaplan-Meier curves for SMA-I. (A) Probability of survival with advancing age by SMA-I subtype (type IB, n = 18; type IC, n = 16). (B) Probability of not reaching the combined endpoint of death or the need for a minimum of 16 hours/day of noninvasive ventilation support for a minimum of 14 continuous days, in the absence of an acute reversible illness or perioperatively, with advancing age by SMA-I subtype. (C) Probability of not reaching the combined endpoint with advancing age by *SMN2* copy number (2 copies, n = 23; 3 copies, n = 9). SMA-I = spinal muscular atrophy type I.

change over time in the CHOP INTEND score overall was −1.27 points/year (95% confidence interval [CI] −2.33 to −0.21, $p = 0.02$). The mean rate of change was similar ($p = 0.90$) for the recently diagnosed subgroup (−1.24 points/year, 95% CI −2.39 to −0.09, $p = 0.03$) and the chronic subgroup (−1.07 points/year, 95% CI −3.53 to 1.39, $p = 0.39$). The mean decline was slightly worse among type IB subjects (−1.83 points/year, 95% CI −3.35 to −0.32, $p = 0.02$) than among type IC subjects (−0.83 points/year, 95% CI −2.18 to 0.52, $p = 0.22$), but the group difference in mean rate of change was not significant ($p = 0.32$).

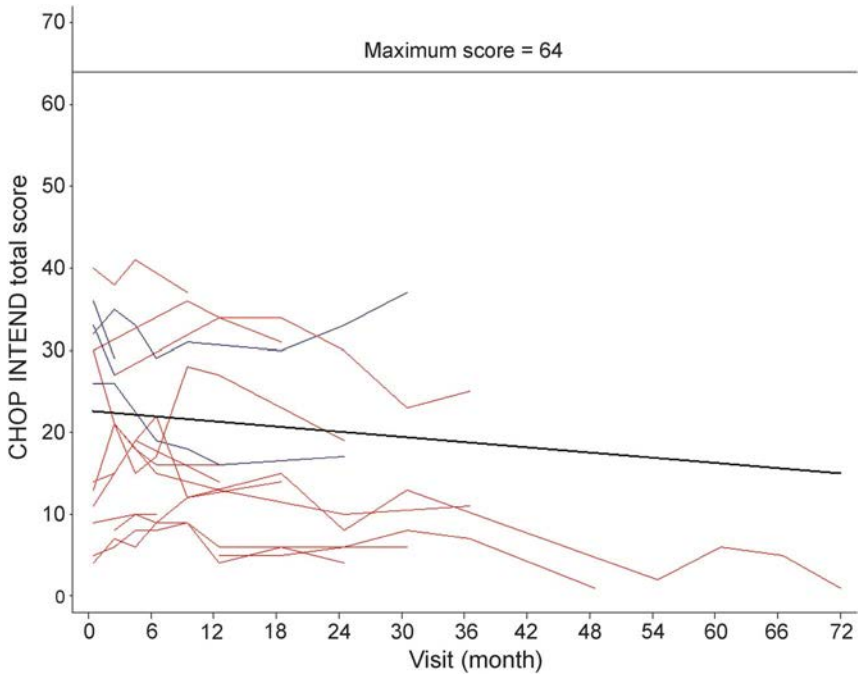
Of the 54 eligible subjects with SMA-I screened, 34 (63%) were enrolled, with an average of 1.20 subjects with SMA-I screened/month and 0.74 subjects enrolled/month among the 3 study sites. Overall, 50% of subjects with SMA-I and 84% of subjects with SMA-II completed at least 12 months of follow-up. Of those who lived for at least 12 months after enrollment, 5 of 25 (20%) completed all 6 scheduled study visits and 15 of 25 (60%) completed at least 4 of the 6 visits for the initial 12 months. Missed visits were attributable to illness, surgery, and travel difficulties. Early withdrawal occurred in 16 of the 34 subjects (47%): 9 because of death (all within the first 12 months) and 7 for other reasons (1 illness, 2 noncompliant, 2 enrolled in a treatment study, 1 time constraints for the caregivers, 1 withdrew consent).

Study procedures were well tolerated. Of the 34 adverse events that were reported, 65% were related to the pulmonary system (respiratory syncytial virus in 3), 21% to gastrointestinal issues, and 6% were perioperative in nature. All were determined to be related to progression of disease or to scheduled hospital admissions for surgery or testing; none were attributed to the study assessments or travel to the study site. No primary cardiac events were identified. The duration of study visits was approximately 4 hours, including breaks.

Most laboratory values were within normal ranges or were slightly outside the range of normal (alanine aminotransferase, hemoglobin, creatinine, glucose; table e-2).

CMAP and MUNE responses were obtained at the baseline visit in 20 of 34 subjects with SMA-I (59%) and were omitted otherwise upon parental request. The ulnar CMAP amplitude (median = 300 μV, range 41–1,100 μV) and MUNE (median 5, range 1–18) responses were substantially reduced relative to reference data for the lower limit of normal in infants from neonate to 2 years of age (CMAP: 1,800–5,000 μV; MUNE: 100–250). Normative CMAP values rise with development before plateauing at adult levels by the end of the first decade. Cross-sectional MUNE norms have been reported in infants and some age groups, although the trajectory of MUNE values during normal development has not been as well charted.[17] There were no

Figure 2 **CHOP INTEND motor function in SMA-I: Longitudinal data**



The CHOP INTEND longitudinal data are represented for each subject with 2 or more assessments. Those subjects enrolled within 3 months of diagnosis ("recent") are shown with a blue line and those enrolled more than 3 months after diagnosis ("chronic") are shown with a red line. CHOP INTEND = The Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders; SMA-I = spinal muscular atrophy type I.

significant differences in CMAP and MUNE between the recently diagnosed and chronic SMA-I subgroups (CMAP: $p = 0.80$; MUNE: $p = 0.97$), nor was there a significant difference in CMAP between the type IB and type IC subgroups ($p = 0.35$), but the MUNE values tended to be higher in the type IC subgroup than in the type IB subgroup ($p = 0.04$, table 1).

DISCUSSION We have demonstrated that a prospective, longitudinal natural history study of SMA-I can be performed effectively among 3 clinical sites. We found that subjects with type IB and type IC fare similarly regarding time to the combined endpoint. The need for gastrostomy tube placement and noninvasive ventilation was not significantly different in these SMA-I subgroups. Thus, it would be sensible to include both subgroups in a clinical trial that used time to reach the combined endpoint as the primary outcome variable, and there would appear to be no advantage of stratifying by subgroup in the randomization or statistical analysis. In a different study, time to the combined endpoint in patients with SMA-I did not differ between those receiving supportive nutritional care only vs proactive respiratory support.[18]

For purposes of clinical trial planning, our data suggest that the probability of reaching the combined endpoint at age 12 months (i.e., after 6–9 months of trial participation) is approximately 50%. A sample size of 63 subjects per group (126 total) would be required in a 2-group trial to provide 80% power to detect a relatively large group difference of 50% vs 75% regarding event-free survival (not reaching the combined endpoint), corresponding to a hazard ratio of 0.42, using a log-rank test and a 5% significance level (2-tailed), assuming a 10% dropout rate. An additional 6 months of follow-up would increase the probability of reaching the combined endpoint to approximately 65%, possibly allowing for detection of a smaller treatment effect, but one would have to consider the increased risk of dropout with longer-term follow-up in sample-size planning.

Motor status, as measured by the CHOP INTEND total score, declined over time in subjects with SMA-I but the mean rate of decline was not significantly different between the type IB and type IC subjects or between the recently diagnosed and chronic subgroups.

Despite advanced denervating disease, CMAP and MUNE could be obtained at baseline in the majority of subjects in this cohort. CMAP amplitudes, however, were similar across groups in patients with SMA-I and therefore less informative, likely because they were very low at the time of baseline evaluation. The baseline MUNE, however, while still quite low, was relatively higher in the type IC than in the type IB subgroup. CMAP amplitude and MUNE from the distal ulnar nerve may serve as useful markers in early-phase trials if an intervention causes some rescue of motor neurons, hastens deterioration, or promotes collateral reinnervation. However, the absence of change in these measures does not necessarily indicate a lack of benefit because they do not reflect motor neuron pools that control bulbar or respiratory function and may be limited because of very advanced denervation.

SMN2 copy number was associated with severity of disease and with the time to reach the combined endpoint in our cohort. Limiting a clinical trial to subjects with a copy number of 2 will yield a higher number of participants who reach the combined endpoint; however, this could make recruitment more difficult. It would seem useful, however, to stratify by *SMN2* copy number in the randomization of participants in future trials.

The elevated alanine aminotransferase findings may reflect muscle atrophy (creatine kinase levels were not evaluated here, but have been reported to be elevated in SMA[19]). Reduced serum bicarbonate may reflect an underlying metabolic acidosis, as has been described previously.[20] Reduced serum creatinine values reflect reduced muscle mass; these tended to occur more frequently in subjects with SMA-II than in subjects with SMA-I ($p = 0.08$). The use of serum cystatin C may be better than serum creatinine for evaluation of renal function. Random blood glucose levels were abnormally reduced or elevated in 8 of 26 subjects with SMA-I (30.8%) and in 23 of 43 subjects with SMA-II (53.5%). Both hypo- and hyperglycemia warrant close monitoring, especially in time of illness or perioperatively, as has been demonstrated.[21]

Limitations to the interpretation of these data include the relatively small sample size, incomplete retention, and having a mixture of recently diagnosed and more chronic subjects. The latter group (20 of 34 subjects, 59%) is more likely to have milder disease and to have longer-term survival. While all subjects with SMA were included, focusing on the recently diagnosed group may give a clearer picture of the natural history of SMA-I using contemporary support options.

Patients with SMA-I can be effectively enrolled in a longitudinal study. Subjects should be enrolled in trials shortly after diagnosis, before age 8 months, by which time about half of the subjects already need feeding support. Enrollment within 1 month of diagnosis is feasible but will be a challenge. The subjects tolerated extensive testing with appropriate rest periods. Retention was variable for the subjects with SMA-I, who missed many study visits because of illness. The frequency of study visits was challenging for many subjects with SMA-I and their parents. To maximize retention, study visits should be minimized; the use of

remote assessments in lieu of some study visits should be considered. Multiple pulmonary-related adverse events and minor laboratory abnormalities should be anticipated in an intervention study. *SMN*2 copy number serves as a predictive biomarker potentially useful for stratification, in contrast to SMA-I subtype (IB and IC). The composite endpoint of death or the need for at least 16 hours/day of noninvasive ventilation support for at least 2 weeks accurately captures the milestone of sustained respiratory failure, a surrogate for death. The CHOP INTEND may serve as a useful secondary outcome measure.

AUTHOR CONTRIBUTIONS

Dr. Finkel's contributions include drafting/revising the manuscript for content, study concept and design, analysis and interpretation of data, acquisition of data, study supervision or coordination, and obtaining funding. Dr. McDermott's contributions include drafting/revising the manuscript for content and analysis or interpretation of data. Dr. Kaufmann's contributions include drafting/revising the manuscript for content, study concept or design, analysis or interpretation of data, acquisition of data, study supervision or coordination, and obtaining funding. Dr. Darras' contributions include drafting/revising the manuscript for content, study concept or design, acquisition of data, study supervision or coordination, and obtaining funding. Dr. Chung's contributions include revising the manuscript for content and data acquisition. Dr. Sproule's contributions include drafting/revising the manuscript for content, study concept or design, analysis, interpretation of data, acquisition of data, and study supervision or coordination. Dr. Kang's contributions include data acquisition and revision of the manuscript for content. Dr. Foley's contributions include review/revision of the manuscript and data acquisition. Dr. Yang's contributions include review/revision of the manuscript and data acquisition. William Martens' contributions include revising the manuscript for content, analysis or interpretation of data, and data acquisition. Dr. Oskoui's contributions include revising the manuscript for content and data acquisition. Allan Glanzman's contributions include review/revision of the manuscript and data acquisition. Jean Flickinger's contributions include drafting/revising the manuscript for content and data acquisition. Jacqueline Montes' contributions include drafting/revising the manuscript for content, study concept or design, analysis or interpretation of data, acquisition of data, and study supervision or coordination. Sally Dunaway's contributions include revising the manuscript for content and data acquisition. Jessica O'Hagen's contributions include revising the manuscript for content, data acquisition, and study supervision or coordination. Janet Quigley's contributions include review/revision of the manuscript and data acquisition. Susan Riley's contributions include review/revision of the manuscript and data acquisition. Maryjane Benton's contributions include review/revision of the manuscript, analysis or interpretation of data, data acquisition, and study supervision or coordination. Patricia Ryan's contributions include revising the manuscript for content and data acquisition. Megan Montgomery's contributions include review/revision of the manuscript for content, data acquisition, and study supervision or coordination. Jonathan Marra's contributions include review/revision of the manuscript and data acquisition. Dr. Gooch's contributions include drafting/revising the manuscript for content, study concept or design, acquisition of data, and study supervision or coordination. Dr. De Vivo's contributions include drafting/revising the manuscript for content, study concept or design, analysis or interpretation of data, acquisition of data, study supervision or coordination, and obtaining funding.

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DISCLOSURE

R. Finkel served as a Data and Safety Monitoring Board (DSMB) member for a trial sponsored by Sarepta Therapeutics and now serves on a DSMB for an scAAV9 gene therapy clinical trial at Nationwide Children's Hospital, serves on advisory boards for DuchenneConnect, Families of SMA, SMA-REACH, PTC Therapeutics, Inc., Isis Pharmaceuticals, Inc; has received honoraria from Novartis and Roche, has received travel expenses for lectures not funded by industry; serves on the editorial board of *Neuromuscular Disorders* and *Journal of Neuromuscular Disorders*; receives research support from Isis Pharmaceuticals, Inc., PTC Therapeutics, Inc. (PTC124-016 study, site PI), Santhera Pharmaceuticals (DELOS Study in Duchenne MD, site PI), the NIH (5R21-NS058926 [PI], U54 AR0526446-03 [Co-I], 1U54 NS065712-01 [Co-I]), RO1-AR056973 [Co-I]), the SMA Foundation (PNCR network for SMA, site PI), the Muscular Dystrophy Association, Genzyme Corporation, and the Charcot-Marie-Tooth Association; and his spouse serves on the editorial board of *Arthritis Research and Therapy*, holds and has received license fees for numerous patents related to T-cell activation and HIV, and receives research support from the Gates Foundation, Merck Serono, and the NIH in the field of T-cell activation, HIV, and genomics of juvenile arthritis. M. McDermott serves as a DSMB member for trials sponsored by Isis Pharmaceuticals, Biogen Idec, Inc., The ALS Association/FDA, and the Muscular Dystrophy Association. He serves on the editorial board for *Movement Disorders* and has been a consultant for the New York State Department of Health, Teva Pharmaceutical Industries, Ltd., Synosia, Inc., Smith & Nephew, Inc., Impax Pharmaceuticals, Bioness, Inc., and Asubio Pharmaceuticals, Inc. He receives research support from the Michael J. Fox Foundation, Spinal Muscular Atrophy Foundation, Muscular Dystrophy Association, American Dental Association, the FDA, and NIH. P. Kaufmann reports no disclosures relevant to the manuscript. B. Darras has received publishing royalties from UpToDate and consulting fees from Isis Pharmaceuticals, Quest Diagnostics, Guidepoint Global Consultation, Easton Associates, Clearview Healthcare Partners, and Gerson Lehrman Group. He has received honoraria from the American Academy of Neurology and the Muscular Dystrophy Association. He has received research support from PTC Therapeutics, Isis Pharmaceuticals, NIH/NIAMS 2P01NS-40828-6A11, NIH/National Institute of Neurological Disorders and Stroke (NINDS) 1U10NS077269; NIH/NIAMS 1R01AR060850, SMA Foundation, Muscular Dystrophy Association, and Slaney Family Fund for SMA. He serves on the editorial boards of *Neurology* and *Pediatric Neurology*. W. Chung reports no disclosures relevant to the manuscript. D. Sproule receives research funding from PTC Therapeutics, the SMA Foundation, and NINDS-sponsored Neurological Sciences Academic Development Award (K12 NS01698). P. Kang received travel funding from Isis Pharmaceuticals and various nonprofit entities during the study period. He previously served as an occasional consultant for LEK consulting, Gerson Lehrman Group, and Leerink Swann, all commercial consulting firms. He has received honoraria for grant reviews from nonprofit entities and federal government agencies. He previously performed medico-legal consulting for Gross, Minsky & Mogul and currently does so for a federal government agency. He performs EMGs (15% effort). Dr. Kang receives honoraria for serving as an officer of the Massachusetts Medical Society, a nonprofit entity, and has received occasional honoraria from other nonprofit entities. He has received compensation from Oakstone Publishing and Springer, both commercial publishing firms. Dr. Kang receives research support from the NIH and the Muscular

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RESEARCH ARTICLE

Baseline results of the NeuroNEXT spinal muscular atrophy infant biomarker study

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Abstract

Objective: This study prospectively assessed putative promising biomarkers for use in assessing infants with spinal muscular atrophy (SMA). **Methods:** This prospective, multi-center natural history study targeted the enrollment of SMA infants and healthy control infants less than 6 months of age. Recruitment occurred at 14 centers within the NINDS National Network for Excellence in Neuroscience Clinical Trials (NeuroNEXT) Network. Infant motor function scales and putative electrophysiological, protein and molecular biomarkers were assessed at baseline and subsequent visits. **Results:** Enrollment began November, 2012 and ended September, 2014 with 26 SMA infants and 27 healthy infants enrolled. Baseline demographic characteristics of the SMA and control infant cohorts aligned well. Motor function as assessed by the Test for Infant

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Motor Performance Items (TIMPSI) and the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) revealed significant differences between the SMA and control infants at baseline. Ulnar compound muscle action potential amplitude (CMAP) in SMA infants (1.4 ± 2.2 mV) was significantly reduced compared to controls (5.5 ± 2.0 mV). Electrical impedance myography (EIM) high-frequency reactance slope (Ohms/MHz) was significantly higher in SMA infants than controls SMA infants had lower survival motor neuron (SMN) mRNA levels in blood than controls, and several serum protein analytes were altered between cohorts. **Interpretation**: By the time infants were recruited and presented for the baseline visit, SMA infants had reduced motor function compared to controls. Ulnar CMAP, EIM, blood SMN mRNA levels, and serum protein analytes were able to distinguish between cohorts at the enrollment visit.

Introduction

Spinal muscular atrophy (SMA) is the leading genetic cause of death in infants, exhibits a wide range of clinical severity and has an incidence of one in 11,000 live births.[1,2] SMA is caused by homozygous deletion or mutation in the *SMN1* (*survival motor neuron 1*) gene and retention of the nearly identical gene, *SMN2* (*survival motor neuron 2*), which results in reduced expression of full-length SMN protein.[3,4] In humans, *SMN2* is present in the same genomic region and differs from *SMN1* by a single-nucleotide substitution that results in the exclusion of exon 7 in approximately 90% of SMN transcripts.[5,6] The mRNA that results, SMNΔ7, produces a truncated protein that is nonfunctional and targeted for degradation.[7,8]

Clinically, SMA is characterized by skeletal muscle weakness and, in a substantial majority of severely affected individuals, respiratory insufficiency and premature death. Disease severity spans a wide range of phenotypes divided into five categories based upon maximal motor function: type 0, (neonates who present with severe hypotonia often with history of decreased fetal movements), type 1 (never sit independently), type 2 (sit but never stand independently), type 3 (ambulatory children), and type 4 (ambulatory adults).[9,10] *SMN2* copy number correlates inversely with clinical severity in humans and motor function and survival in murine models.[11–14] Thus, *SMN2* copy number is a prognostic biomarker that predicts future clinical outcome.

Clinical studies designed to increase the expression of the SMN protein are underway in infants with SMA (ClinicalTrials.gov: NCT02193074, NCT02292537, NCT02386553, NCT02122952, NCT02462759, and NCT02268552).[15,16] Natural history studies in the SMA type 1 population demonstrated shortened lifespan, with 68% mortality within the first 2 years of life.[9,10] With the advent of standardized care guidelines,[17] the mortality of SMA type 1 infants has been reduced at 2 years of age to 30%, with nearly half of these infants dependent upon noninvasive ventilation.[18] In a recent observational study, SMA infants who developed symptoms prior to 6 months of age demonstrated very poor motor function and significant motor loss electrophysiologically at the enrollment visit.[19] Thus, there is heightened need to identify and validate physiological and molecular biomarkers in the SMA type 1 population and to obtain longitudinal outcome measures for use in future SMA infant clinical trials.

We sought to determine the feasibility and reliability of testing specific putative physiological and molecular SMA biomarkers in infants with SMA and in age-matched healthy control infants. We performed a systematic, multi-center, longitudinal natural history study in SMA infants designed to mimic a hypothetical phase 3 interventional clinical trial. Our goals were: 1) to determine the natural history of motor function during the first 2 years of life in infants with SMA and in healthy infants, 2) to determine the natural history of putative electrophysiological and molecular biomarkers in infants with SMA and healthy infants 3) to determine the relationship between putative electrophysiological and molecular biomarkers to motor function in infants with SMA and healthy infants.

Subjects and Methods

This study was performed and supported by the National Network for Excellence in Neuroscience Clinical Trials (NeuroNEXT) Clinical Trial Network and originated from The Ohio State University Wexner Medical Center. The NeuroNEXT infrastructure consists of 25 clinical centers geographically distributed across the United States, a Central Coordinating Center at Massachusetts General Hospital and a central Data Coordinating Center at University of Iowa (Table S1). Fifteen sites (Table 1) began enrollment in November 2012. Guardians of all subjects

provided written, informed consent approved by the NeuroNEXT central institutional review board[20] at the enrolling sites.

Study design

This was a prospective, longitudinal natural history study of infants with genetically confirmed SMA and healthy control infants. Enrollment was restricted to infants who were 6 months of age or younger and were born between 36 and 42 weeks of gestation. The study was designed to mimic the inclusion and timing of future SMA clinical trials targeting treatment to SMA infants. Therefore, the diagnosis of SMA was made by study investigators or community neurologists and confirmed with clinical genetic testing prior to enrollment. Asymptomatic subjects who had been genetically tested prior to the enrollment were permitted. Subjects were excluded if they required noninvasive ventilatory support (i.e., BiPAP) for more than 12 hours/day, had a comorbid illness or were enrolled in an SMA therapeutic clinical trial. SMA infants taking any therapies thought to increase SMN expression, such as valproic acid, were excluded from the study. The absence of an *SMN1* gene deletion/mutation was confirmed for each healthy control infant.

The baseline study visit occurred prior to the age of 6 months and as young as possible, following either genetic confirmation of SMA (with or without clinical symptoms at time of enrollment) or identification as a suitable normal control subject. Thereafter, study visits were scheduled to occur according to age at 3 (if applicable), 6, 9, 12, 18, and 24 months. In this report, we present the baseline visit results. Twenty-seven healthy infants were enrolled within 12 months; 26 infants with SMA were enrolled concurrently over 22 months. Confirmation of the *SMN1* exon 7 deletion and *SMN2* copy number were performed as previously described.[21] In addition, DNA from SMA subjects was screened for the *SMN2* gene positive modifier mutation c.859G>C.[22]

The order of study procedures was strictly adhered to at all fifteen enrolling sites to minimize site-to-site and visit-to-visit variability. Subjects were asked to present to the visit in morning, fully rested. Funds were available for family travel and accommodations near the study site to reduce the confounder of travel time and time of day. After a medical history and a brief general examination, infant motor function testing was performed, followed by electrical impedance myography (EIM) testing, followed by ulnar compound muscle action potential (CMAP) testing, followed by a single peripheral blood draw.

Table 1. Baseline characteristics of SMA and healthy control infant cohorts.

	SMA (N = 26)	Control (N = 27)
Demographics	*N* (%)	*N* (%)
Females	15 (58)	14 (52)
White race	24 (92)	24 (89)
Hispanic	6 (23)	3 (11)
	Mean (SD)	Mean (SD)
Age at enrollment (months)	3.7 (1.7)	3.3 (2.0)
Baseline visit weight (lbs)	13.4 (2.2)	13.4 (3.3)
Gestational age (weeks)	38.8 (1.5)	39.0 (1.4)
Birth weight (lbs)	7.2 (1.2)	7.0 (1.4)
Birth length (inches)	20.1 (1.2)	20.0 (1.0)
SMN2 copy number	*N* (%)	*N* (%)
1	0	12 (44)
2	16 (64)	13 (48)
3	5 (19)	1 (4)
4	1 (4)	0
Unknown	4 (15)	1 (4)
SMN2 gene modifier c.859G>C	0	0

Motor function testing

Infant motor function was assessed by certified physical therapists who were required to pass reliability training and testing prior to enrollment. All subjects were evaluated using the Test of Infant Motor Performance Screening Items (TIMPSI), a 29-item, 99 point scale evaluation of infant motor function that has been shown to be valid and reliable in infants with SMA type 1.[23] After testing, all subjects were required to have a 20-minute rest period that could include nursing/feeding. Subjects who scored less than 41 on the TIMSPI were then evaluated using The Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders (CHOP-INTEND) which is a validated 16-item, 64-point scale shown to be reliable in SMA type 1 subjects.[19,24] Subjects scoring 41 or greater on the TIMPSI were evaluated using the Alberta Infant Motor Scale (AIMS), a 58-item observational scale developed to assess motor development in children from birth until independent walking.[25,26]

Compound muscle action potential (CMAP)

Ulnar CMAP measurements were obtained from the abductor digiti minimi (ADM) muscle by trained electromyographers using standardized electrode placement on the basis of anatomical landmarks. The low-frequency and high-frequency filter settings were set to 10 Hz and 10 kHz, respectively. Skin temperature was maintained at >33°C. Two adhesive strip electrodes (Carefusion Disposable Ring Electrode with Leads, order number 019-439300), trimmed to the width of each subject's ADM muscle, were used for recording. The G1 recording electrode was placed on the ADM muscle at 1/3 of the distance measured from the pisiform bone to the fifth metacarpophalangeal joint with

the length of the electrode-oriented orthogonal to the direction of the muscle fibers. The G2 reference electrode was placed on the ulnar aspect of the fifth metacarpophalangeal joint. An adhesive ground electrode (Carefusion Tab Electrodes 1.0 meter leads, order number 019-406600) was placed on the dorsum of the hand. The ulnar nerve was supramaximally stimulated either at the wrist or just proximal to the ulnar groove at the elbow using pediatric sized bipolar probe. Square-wave stimulations of 0.2 msec duration and gradually increasing intensity were delivered to reach 120% of the intensity required to elicit a maximal CMAP response. Maximum values for negative peak (NP) amplitude and NP area were recorded.

Electrical impedance myography (EIM)

Measurements were obtained following the motor function tests using a multi-frequency (1000 Hz–10 MHz) impedance system (Skulpt Inc. EIM1103, San Francisco, CA). As this study was the first time EIM had been performed in infants, a novel probe was designed specifically for use in this population. Muscle groups were tested in a specific order as follows: right biceps, right wrist extensors, right quadriceps, right tibialis anterior, left biceps, left wrist extensors, left quadriceps, and left tibialis anterior muscles. Measurements were performed three times on each muscle before moving on to the next and the two closest sets of data averaged. All data were transferred in a blinded fashion to a central database. Predetermined EIM metrics based on data obtained in older healthy and SMA-affected children[27] were derived from the full set of impedance data and transferred to the DCC for analysis.

Blood processing

A single peripheral blood draw was then obtained as the last study procedure by an experienced pediatric phlebotomist. Given the challenge and small blood volume of infants, a strict order of blood samples was adhered to: 2 cc blood into a PAXgene tube for SMN mRNA determination, 8 cc blood into a CPT tube for plasma, and PBMC isolation followed by a 2 cc into a purple top for DNA extraction. The CPT tube was processed at each site as previously described[28] and PBMCs resuspended in freezing medium consisting of 10% DMSO in FBS prior to shipment to the central processing laboratory (Kolb Lab).

SMN mRNA quantification

Total mRNA was isolated from the PAXgene tube as previously described.[28] mRNA was converted to cDNA using random hexamer primers and AMV-RT (7041Z, Affymetrix) according to the manufacture's direction. SMN mRNA analysis was performed using Droplet Digital PCR (ddPCR) (Bio-Rad Laboratories, Hercules, CA). The following primers were used for detection of full-length SMN expression: hSMN_FL_Ex7_FP: 5′ CAAAAAGAAGG AAGGTGCTCA, hSMN_FL_Ex8_RP: 5′ TCCAGATCT GTCTGATCGTTTC, hSMN_FL_Ex7/8 probe: 5′ FAM-TT AAGGAGAAATGCTGGCATAGAGCAGCAC-MGB. SMN expression was normalized to HPRT expression using the PrimePCR™ ddPCR™ Expression Probe Assay for intron-spanning human HPRT1 with HEX assay (dHsaCPE5192872, Bio-Rad). Mulitplex reactions were performed with 2–5 μL of cDNA as required to obtain a sufficient number of positive droplets. Template, primers (900 nM final), probes (250 nM final), and 2 × ddPCR Supermix in 20 μL final volume were converted into droplets with the QX200 droplet generator (Bio-Rad Laboratories) and PCR was run on a classic MJ thermal cycler under standard conditions: 95°C for 10 minutes followed by 40 cycles of 94°C for 30 seconds, 60°C for 1 minute, and a final step of 98°C for 10 minutes. After PCR, droplet counts were measured on the QX200 droplet digital reader (Bio-Rad Laboratories). Concentration of sample was determined by fitting droplet counts to the Poisson distribution using QuantaSoft software (Bio-Rad Laboratories). SMN mRNA expression per sample was normalized by dividing the SMN concentration by the HPRT concentration and plotted as Relative Fluorescent Units (RFU).

SMN protein levels

For the SMN protein measurements, peripheral blood was drawn into a cell preparation tube and peripheral blood mononuclear cells (PBMCs) were isolated as previously described.[29] PBMCs were cryopreserved at each study site and then shipped to the central laboratory (Kolb Lab) where they were stored at −80°C. Once all baseline samples were collected, SMN protein was measured at PharmOptima (Portage, MI) using the company's proprietary electrochemiluminescence immunoassay based on the Meso Scale Discovery technology. The assay is a quantitative sandwich immunoassay, where a mouse monoclonal antibody (2B1[30]) functions as the capture antibody and a rabbit polyclonal anti-SMN antibody (Protein Tech, Cat. No. 11708-1-AP) labeled with a SULFO-TAG™ is used for detection. SMN levels are determined from a standard curve using recombinant SMN protein (Enzo Life Sciences, Cat. No. ADI-NBP-201-050). The dynamic range of the assay is 10 pg/mL to 10,000 pg/mL. PBMC samples were received by PharmOptima, frozen and were maintained at −80°C until thawed for enumeration. Samples were thawed quickly in a 37°C water bath in batches of eight samples per thawing

and enumeration event in order to avoid prolonged incubation prior to cell lysis. Samples were diluted 10-fold into PBS prior to enumeration via direct hemocytometric counting. Finally, cells were lysed at a density of 1 X 10^7 cells/mL. Lysates were maintained at −80°C until the time of assay.

SMA-MAP quantification

Plasma samples were isolated for the CPT tubes, frozen immediately and stored at −80°C in cryovials. Frozen samples were sent to a central processing laboratory at Myriad and processed to quantify 25 plasma protein analytes that have been identified as putative serum SMA biomarkers.[31,32] All samples were stored at −80°C until tested. The samples were thawed at room temperature, vortexed, spun at 4000 RPM for 5 minutes for clarification and volume was removed for MAP analysis into a master microtiter plate. Using automated pipetting, an aliquot of each sample was introduced into one of the capture microsphere multiplexes of the Multi Analyte Profile. The mixture of sample and capture microspheres were thoroughly mixed and incubated at room temperature for 1 hour. Multiplexed cocktails of biotinylated, reporter antibodies for each multiplex were then added robotically and after thorough mixing, were incubated for an additional hour at room temperature. Multiplexes were developed using an excess of streptavidin-phycoerythrin solution that was thoroughly mixed into each multiplex and incubated for 1 hour at room temperature. The volume of each multiplexed reaction was reduced by vacuum filtration and the volume increased by dilution into matrix buffer for analysis. Analysis was performed in a Luminex 100 instrument and the resulting data stream was interpreted using proprietary data analysis software developed at Rules-Based Medicine. For each multiplex, both calibrators and controls were included on each microtiter plate. Eight-point calibrators were run in the first and last column of each plate and 3-level controls were included in duplicate. Testing results were determined first for the high-, medium-, and low controls for each multiplex to ensure proper assay performance. Unknown values for each of the analytes localized in a specific multiplex were determined using 4 and 5 parameter, weighted- and nonweighted-curve fitting algorithms included in the data analysis package.

Statistical analysis

Continuous variables were summarized by means, standard deviation, minimum, and maximum values. Categorical variables were summarized by percentages. Comparisons of continuous variables between the SMA and healthy control cohorts were performed using two sample t-tests. Comparisons of categorical variables between the two cohorts were performed using chi-square tests. All statistical tests were two-sided and used a significance level of 0.05. No adjustments for multiple comparisons were made.

Pearson's correlation coefficients between subject's age at enrollment and all continuous outcomes were estimated separately for each cohort. Similarly, the correlations between motor function tests (TIMPSI and CHOP-INTEND) and biomarkers (CMAP, EIM, SMN mRNA, and SMA-MAP) were estimated separately for each cohort. Additional analyses restricted to the subgroup of SMA subjects with two copies of the *SMN2* gene were also performed. All analyses were performed using SAS® version 9.3 or later.

Results

Baseline demographics

The first site was activated and enrollment began in November 2012. All fifteen sites had passed certification for MFTs and CMAP and were activated by February 2013. Enrollment of 27 healthy infants was completed in October 2013 and enrollment of 26 SMA infants was completed in September 2014. The baseline visit was defined as the enrollment visit. Every infant was less than 6 months of age at the initial visit. The SMA and healthy infant cohorts aligned well on baseline demographic characteristics (Table 1). The average age of enrollment for the SMA and healthy cohorts was 3.7 months (SD = 1.7) and 3.3 months (SD = 2.0), respectively; 57.7% of the SMA infants and 51.9% of the healthy infants were female. Birth weight and height were nearly identical in the two cohorts. In the SMA cohort, 15 infants were found to have two copies of *SMN2* gene, five had three copies and a single infant had four copies. *SMN2* copy number was not determined in five SMA infants because of a failure to obtain sufficient blood sample for DNA testing on the baseline or subsequent visits. No infants in the SMA cohort for whom DNA was tested had the *SMN2* c.859G>C mutation. In the healthy cohort, we confirmed that no infant had a homozygous deletion or mutation in the *SMN1* gene. There were four healthy control infants who were carriers with one copy of *SMN1* gene and all of these infants had siblings with the diagnosis of SMA. Three control infants had three copies of the *SMN1* gene.

The month of onset of symptoms was obtained from the parent or guardian during the baseline visit (Table 2). The majority of SMA infants (9) had symptom onset in the second month of life. There were six infants with

Table 2. Age of symptom onset for SMA subjects.

	< 1 month	1–2 months	2–3 months	4–5 months	Not recorded	Total
SMA	6	9	4	1	6	26
SMA, *SMN2* = 2	6	5	3	1	1	16

symptom onset prior to 1 month of age and all of these infants had two copies of *SMN2*. All but one SMA infant for whom this data were collected had symptom onset prior to the 3 months of age. This data was not recorded in six SMA infants. When asked if the infants had feeding or swallowing problems at the time of the baseline visit, ten (38.5%) of parents or guardians responded, yes.

Motor function

All motor function values are plotted against age at time of assessment in Figure 1. Motor function was measured using the TIMPSI for all infants. The average TIMPSI score for the SMA cohort, 34.9 (SD = 20.9, $n = 26$, range = 14–94), was significantly lower than in the healthy cohort, 66.1 (SD = 22.6, $n = 27$, range = 15–96, $P < 0.01$). SMA infants with two *SMN2* copies had an average TIMPSI score of 27.2 (SD = 8.0, $n = 16$, range = 15–49), and there was no correlation with age (Table S2). Moreover, at enrollment no SMA infant with two copies of *SMN2* had a TIMPSI greater than 51. In the healthy control cohort, TIMPSI score had a positive correlation with age ($r = 0.80$, $P < 0.0001$). There was no difference noted in control infants with one, two, or three copies of the *SMN1* gene. All healthy control infants older than 10 weeks of age had TIMPSI scores above 51.

The CHOP-INTEND was utilized to measure motor function in infants scoring less than 41 on the TIMPSI after the TIMSPI and a mandatory 20-minute rest period. As a result, a total of 23 SMA infants and 14 control infants were assessed using the CHOP-INTEND. All 16 SMA infants with two copies of *SMN2* were assessed using the CHOP-INTEND. The average CHOP-INTEND score for the SMA cohort, 21.4 (SD = 9.6, $n = 23$, range = 10–52) was significantly lower than the control cohort, 50.1 (SD = 10.2, $n = 14$, ranged 32–62, $P < 0.01$). The average CHOP-INTEND score for SMA infants with two copies of *SMN2* was 20.2 (SD = 7.4, $n = 16$, range = 10–33) and the maximum score in this subgroup was 33. There was no correlation between CHOP-INTEND scores and age in the SMA or control cohorts. There was excellent correlation between the CHOP-INTEND and TIMPSI scores for SMA ($r = 0.866$, $n = 22$, $P < 0.0001$) and control cohorts ($r = 0.839$, $n = 9$, $P = 0.005$).

The AIMS was assessed in infants scoring 41 or higher on the TIMPSI following the mandatory 20-minute rest period. Consequently, only three SMA infants and 13 control infants were assessed using the AIMS. No SMA infants with two copies of *SMN2* received the AIMS. The average AIMS score for the SMA cohort (8.7, SD = 3.5) was lower than the control cohort (13.8, SD = 4.5). There was a positive correlation between AIMS scores and age in the control cohort ($r = 0.650$, $n = 13$, $P = 0.02$).

Baseline putative physiologic biomarkers

Ulnar CMAP recordings were well tolerated. However, the CMAP for one SMA infant was not obtained. The peak amplitude (mV) for each subject is plotted against age at assessment in Figure 2. The average CMAP peak amplitude for the SMA cohort, 1.4 mV (SD = 2.2, $n = 25$) was significantly lower than the control cohort, 5.5 mV (SD = 2.0, $n = 27$, $P < 0.01$). The average CMAP peak amplitude for SMA infants with two copies of *SMN2* was 0.5 mV (SD = 1.0, $n = 15$). The CMAP values obtained in the control infants did not correlate with the motor function ability as measured by the TIMPSI ($r = 0.006$, $n = 27$, $P = 0.9773$) and the CHOP-INTEND ($r = 0.4105$, $n = 14$, $P = 0.2725$). The CMAP values obtained in the SMA infants had a positive correlation with motor function ability as measured by the TIMPSI ($r = 0.785$, $n = 25$, $P < 0.0001$) and the CHOP-INTEND ($r = 0.556$, $n = 21$, $P = 0.0088$). Interestingly, in the subgroup of SMA infants with two copies of *SMN2* there is no correlation with TIMPSI ($r = 0.276$, $n = 15$, $P = 0.320$) or CHOP-INTEND ($r = 0.283$, $n = 15$, $P = 306$). The results for the ulnar CMAP area were also analyzed and comparisons between groups and correlations were consistent with the results for ulnar CMAP amplitude.

Electrical impedance measurements were well tolerated. The test was not performed in two control infants at baseline. Predetermined EIM outcomes were analyzed based upon prior studies using EIM in older children with SMA.[27] Baseline EIM outcomes are presented in Table 3. EIM outcomes were analyzed using 1) the average value of all muscles tested, 2) the average value of the proximal muscles tested (right and left biceps and quadriceps), or 3) the average value of the distal muscles tested (right and left wrist extensors and tibialis anterior muscles). Of the outcomes measured, high-frequency reactance slope (units) distinguished between SMA and

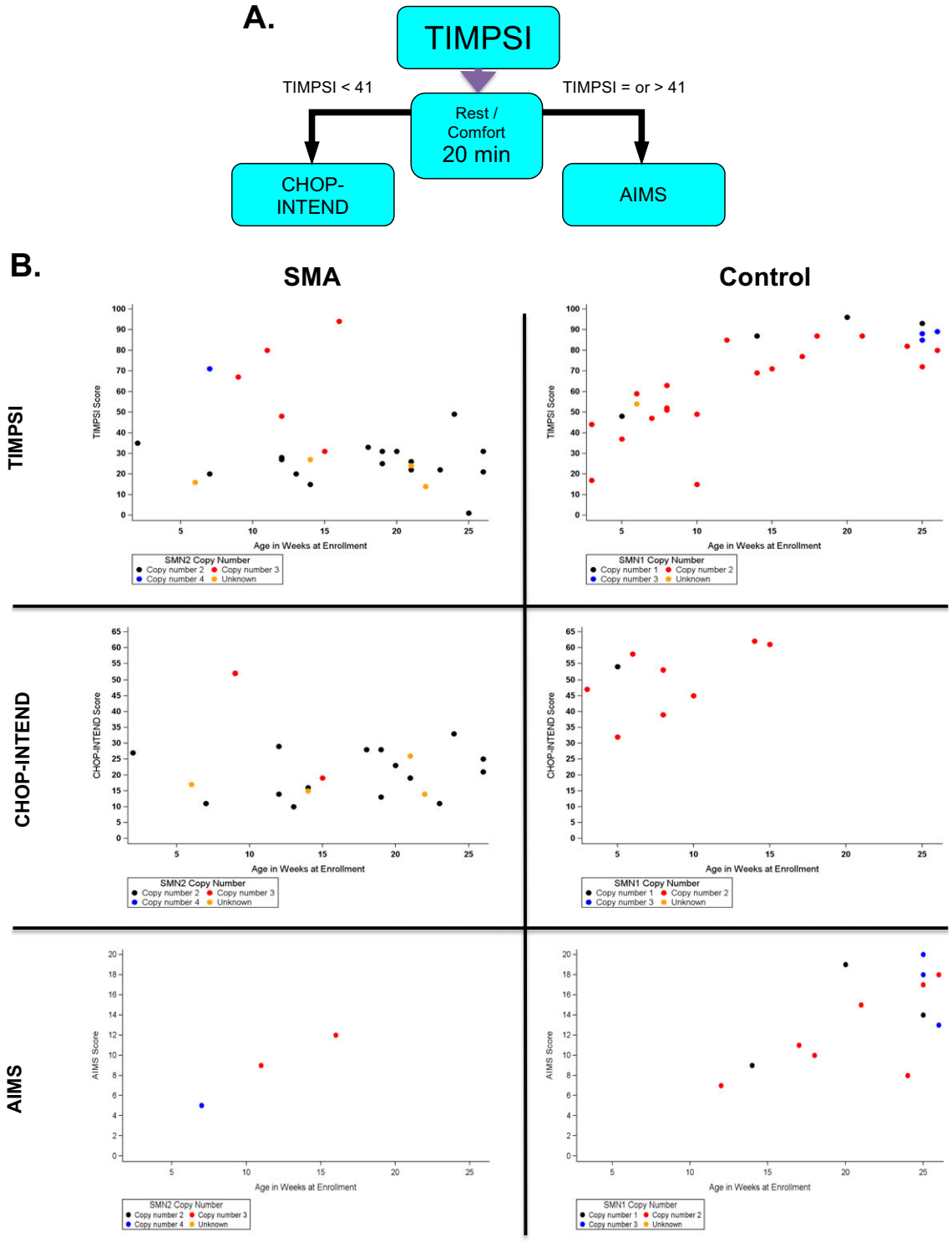

Figure 1. Motor function assessments in SMA and healthy infants in the first 6 months of life. (A) Motor function testing paradigm. All infants were tested using the TIMPSI. After the TIMPSI, a mandatory rest period of 20 minutes was followed by either the CHOP-INTEND or AIMS assessment. Infants who scored less than 41 on the TIMPSI were tested using the CHOP-INTEND, otherwise the infant was tested using the AIMS test. (B) Results of infant motor function tests for all infants as a function of the age at the time of enrollment visit. For the SMA cohort, the *SMN2* copy number for each infant is indicated by the color as indicated in the key by each graph. For the healthy cohort the *SMN1* copy number for each infant is indicated by the color as indicated in the key by each graph.

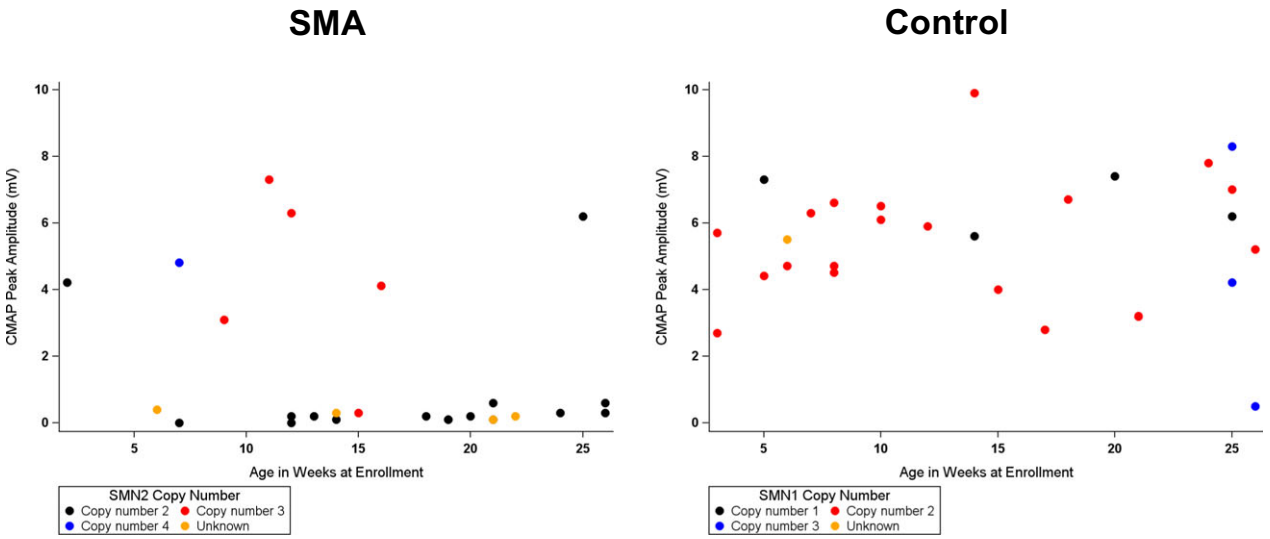

Figure 2. Ulnar compound muscle action potential is significantly reduced in SMA infants compared to healthy infants. Ulnar CMAP peak amplitude (mV) in SMA and healthy control infants as a function of the age at the time of enrollment visit. For the SMA cohort, the *SMN2* copy number for each infant is indicated by the color as indicated in the key by each graph. For the healthy cohort, the *SMN1* copy number for each infant is indicated by the color as indicated in the key by each graph.

Table 3. Baseline electrical impedance myography results in SMA and healthy control infants.

	SMA $N = 26$	SMA: 2 *SMN2* copy subgroup ($N = 16$)	Control $N = 25$	*P* value SMA vs Control	*P* value *SMN2* = 2 vs Control
All muscles grouped					
50k Phase (SD)	5.62 (2.54)	5.39 (1.67)	6.21 (1.64)	0.3317	0.1314
50k Resistance (SD)	104.8 (21.09)	108.0 (23.83)	99.11 (21.13)	0.3367	0.2190
50k Reactance (SD)	16.48 (27.83)	10.81 (4.91)	10.90 (3.72)	0.3204	0.9493
HF phase slope (SD)	13.76 (7.87)	15.53 (2.96)	13.33 (3.90)	0.8073	0.0616
HF reactance slope (SD)	**12.65 (4.39)**	**12.53 (4.50)**	**7.99 (3.82)**	**0.0002**	**0.0013**
LF reactance slope (SD)	−96.1 (2501)	**426.1 (170.4)**	**336.2 (113.2)**	0.3870	**0.0487**
Distal muscles grouped					
50k Phase (SD)	5.04 (2.35)	4.84 (1.69)	5.7 (1.89)	0.2724	0.1452
50k Resistance (SD)	100.9 (19.93)	103.9 (21.95)	98.84 (33.77)	0.7926	0.5961
50k Reactance (SD)	17.23 (40.54)	9.35 (4.17)	9.58 (3.59)	0.3457	0.8636
HF phase slope (SD)	15.16 (5.95)	**16.32 (2.80)**	**13.12 (6.11)**	0.2315	**0.0292**
HF reactance slope (SD)	**14.10 (4.75)**	**14.03 (5.16)**	**9.04 (4.22)**	**0.0002**	**0.0017**
LF reactance slope (SD)	181 (2963)	426.6 (253.4)	317.2 (171.9)	0.4006	0.1069
Proximal muscles grouped					
50k Phase (SD)	6.19 (2.96)	5.94 (1.78)	6.72 (1.62)	0.4309	0.1580
50k Resistance (SD)	109.0 (23.18)	112.0 (26.90)	99.33 (19.80)	0.1151	0.0895
50k Reactance (SD)	13.20 (7.86)	12.25 (5.81)	12.21 (4.01)	0.5725	0.9805
HF phase slope (SD)	12.35 (10.18)	14.75 (3.50)	13.54 (3.55)	0.5767	0.2941
HF reactance slope (SD)	**11.14 (4.86)**	**11.00 (4.79)**	**6.70 (4.37)**	**0.0016**	**0.0065**
LF reactance slope (SD)	78.95 (1591)	425.7 (162.4)	354.7 (131.5)	0.3869	0.1317

Bold rows highlight outcomes where *P* value is equal to or less than 0.05.

control cohorts regardless of how the muscles were grouped for analysis (Table 3).

Correlations of EIM outcomes from all muscles grouped with age, TIMPSI and CHOP-INTEND are tabulated in Table S2. In the control cohort, EIM outcomes 50k Phase, Resistance and Reactance and high-frequency reactance slope had a positive correlation with age (Table S2). Similarly, in the control cohort there were

many correlations between the TIMPSI motor function score and EIM outcomes. TIMPSI scores in the control infants had positive correlations with 50 kHz Phase ($r = 0.4968$, $n = 25$, $P = 0.0115$), Resistance ($r = 0.4769$, $n = 25$, $P = 0.0159$) and Reactance ($r = 0.6506$, $n = 25$, $P = 0.0004$). TIMPSI scores in the control infants had negative correlations with high-frequency reactance slope ($r = -0.4892$, $n = 25$, $P = 0.0131$). Interestingly, there was no correlation between EIM outcomes and CHOP-INTEND scores in control infants.

In the SMA cohort, there was a strong positive correlation between 50k Resistance and age ($r = 0.7649$, $n = 26$, $P < 0.0001$). This correlation also was seen in the subgroup of SMA infants with two copies of *SMN2* ($r = 0.7484$, $n = 16$, $P = 0.0009$). There were no correlations between TIMPSI or CHOP-INTEND and any of the EIM outcomes studied for the SMA cohorts (Table S2).

Baseline putative molecular biomarkers

Peripheral blood draws were tolerated although in some cases an insufficient amount of blood was drawn for all analyses. The SMN mRNA level, expressed as the ratio of SMN to HPRT transcripts, for each subject is plotted against age at assessment in Figure 3A. The average, baseline SMN/HPRT ratio in the SMA cohort, was 0.50 (SD = 0.14, $n = 19$) and was significantly lower than the SMN/HPRT ratio of control cohort, 1.27 (SD = 0.44, $n = 19$, $P < 0.0001$). The average SMN/HPRT ratio for SMA infants with two copies of *SMN2* was 0.47 (SD = 0.13, $n = 12$) and was also significantly lower than the control cohort ($P < 0.0001$). There was no correlation in either cohort between age and SMN mRNA level (Table S2). In the control cohort, there was no correlation between the TIMPSI score and SMN mRNA levels ($r = 0.244$, $n = 19$, $P = 0.315$). In the subgroup of control infants who were assessed using the CHOP-INTEND (these infants scored < 41 on the TIMPSI), there was a positive correlation between CHOP-INTEND score and SMN mRNA level ($r = 0.856$, $n = 7$, $P = 0.014$). In the SMA cohort, there were no correlations between the TIMPSI or CHOP-INTEND with SMN mRNA levels (Table S2)

The SMN protein levels were measured from PBMC samples. During the PBMC enumeration process involving direct microscopic examination, many samples were found to have significant numbers of platelets in the samples, in two samples platelets were found to be in a numerical excess of 10-fold to the PBMCs. Therefore, an additional low-speed (200 x g) centrifugation step was added resulting in a more purified PBMC sample. In three baseline PBMC samples, there were too few cells to count. The yield of the remaining samples ranged from 1 x 10^5 to 3 x 10^7 PBMCs. The SMN protein level for each subject is plotted against age of assessment in Figure 3B. The average, baseline SMN protein level in the SMA cohort (6601.7 pg/10^7 PBMCs, SD = 3592.8, $n = 18$) and was not significantly lower than the baseline SMN protein level of control cohort (8967.8 pg/10^7 PBMCs, SD = 5441.3, $n = 21$, $P = 0.1212$). In contrast, the average baseline SMN protein level for SMA infants with two copies of *SMN2* (5367.4 pg/10^7 PBMCs, SD = 3603.5, $n = 12$) was lower than the control cohort ($P = 0.0484$). There was no correlation in the control cohort between age and SMN protein level (Table S2). However, there was a negative correlation between age and SMN protein levels in the SMA cohort ($r = -0.632$, $n = 18$, $P = 0.0049$). In the control cohort, there was no correlation between the TIMPSI score and SMN protein level ($r = -0.101$, $n = 21$, $P = 0.664$) or between the CHOP-INTEND and SMN protein level ($r = -0.245$, $n = 8$, $P = 0.559$). In the SMA cohort, there were also no correlations between the TIMPSI or CHOP-INTEND with SMN protein levels (Table S2).

The concentration of 25 plasma protein analytes were determined from 18 SMA infants and 20 control infants at the baseline visit. The average baseline plasma analyte concentrations are tabulated in Table 4. When compared to the control cohort, the SMA cohort had lower concentrations of cadherin-13 ($P = 0.0277$), cartilage oligomeric matrix protein ($P = 0.0011$), Insulin-like growth factor binding protein 6 ($P = 0.0135$), peptidase D ($P = 0.0236$) and tetranectin ($P = 0.0493$). When compared to the control cohort, the SMA cohort had higher concentrations of myoglobin ($P = 0.0220$) and YKL-40 (0.0288). Comparisons between the control group and the SMA infants with two copies of *SMN2* improved the significance of the differences between groups for all analytes except for myoglobin (Table 4). In addition, significant differences were found between the control group and the subgroup of SMA infants with two copies of *SMN2* for complement component C1q receptor ($P = 0.0227$) and dipeptidyl peptidase IV ($P = 0.0260$).

There were nine analytes that had a negative correlation with age at enrollment in the control cohort and ten analytes that had a negative correlation with age at enrollment in the SMA cohort (Table S2). Only six analytes (AXL receptor tyrosine kinase, cartilage oligomeric matrix protein, complement component C1q receptor, Fibulin-1C, Tenascin-X, and Thrombospondin-4) showed this correlation in both the control and SMA cohorts. Interestingly, there were no analytes that demonstrated a positive correlation with age at enrollment in either cohort.

In the control infant cohort, there were negative correlations between the TIMPSI motor function score and the plasma concentrations of complement component C1q receptor ($r = -0.681$, $n = 20$, $P = 0.0010$), osteopontin

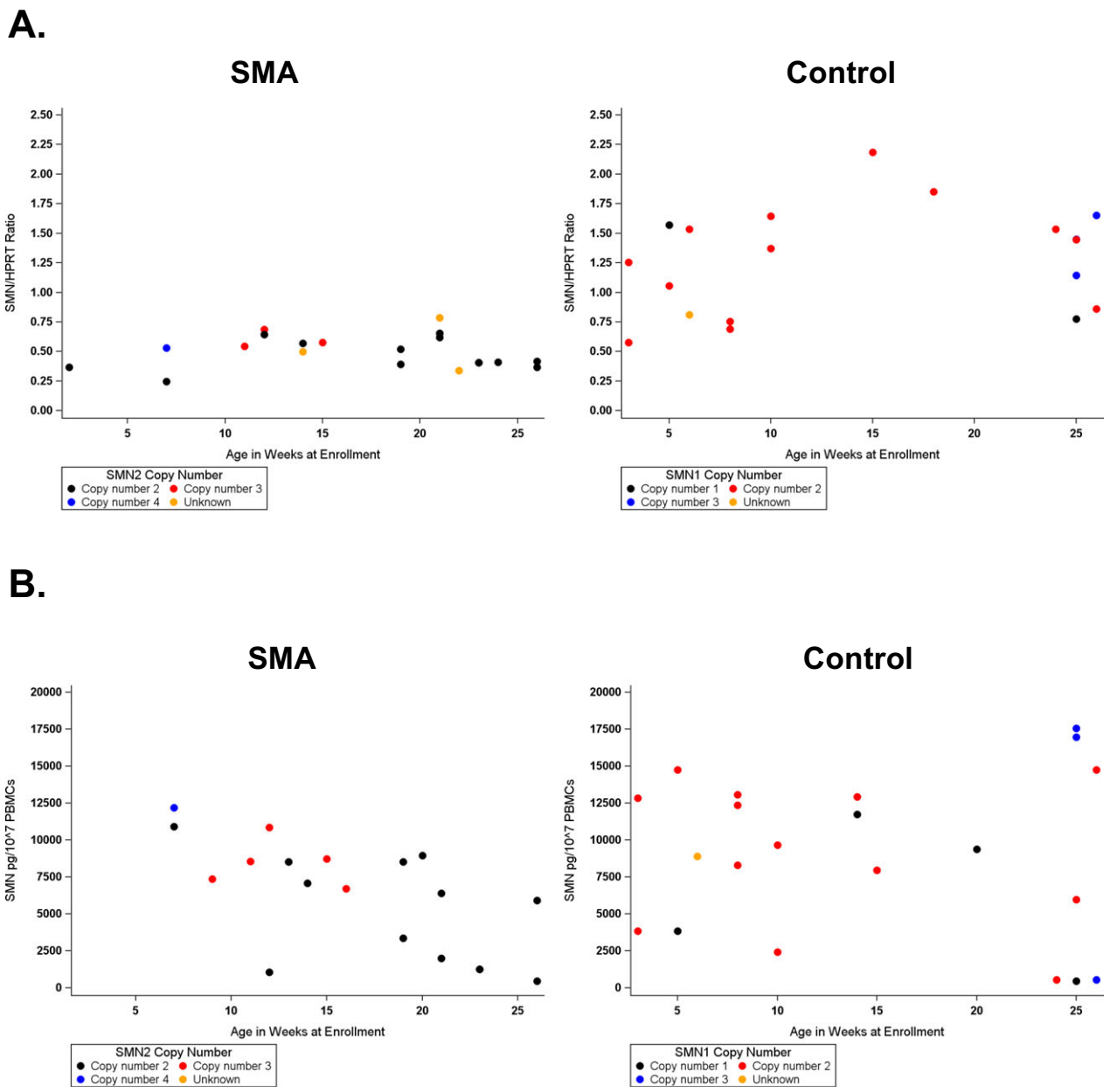

Figure 3. Peripheral blood SMN mRNA and protein levels in SMA and healthy control infants. (A) Full-length SMN mRNA levels from whole blood measured using ddPCR expressed as a ratio of SMN to HPRT. (B) SMN protein levels detected in PBMCs measured by SMN-ECL ELISA expressed as pg/10^7 cells. For the SMA cohort, the *SMN2* copy number for each infant is indicated by the color as indicated in the key by each graph. For the healthy cohort, the *SMN1* copy number for each infant is indicated by the color as indicated in the key by each graph.

($r = -0.528$, $n = 20$, $P = 0.0168$) and thrombospondin-4 ($r = -0.521$, $n = 20$, $P = 0.0187$). In the SMA cohort, there were positive correlations between the TIMPSI motor function score and the plasma concentrations of AXL Receptor Tyrosine Kinase ($r = 0.586$, $n = 18$, $P = 0.0107$), cartilage oligomeric matrix protein ($r = 0.834$, $n = 18$, $P < 0.0001$), dipeptidyl peptidase IV ($r = 0.603$, $n = 18$, $P = 0.0081$), endoglin ($r = 0.535$, $n = 18$, $P = 0.0223$), HER2 ($r = 0.544$, $n = 18$, $P = 0.0196$), Insulin-like growth factor-binding protein 6 (0.580, $n = 18$, 0.0117), PEPD ($r = 0.6037$, $n = 18$, $P = 0.0080$), thrombospondin-4 ($r = 0.615$, $n = 18$, $P = 0.0066$), and tetranectin ($r = 0.669$, $n = 18$, $P = 0.0024$). The only analyte that correlated with both the TIMPSI and the CHOP-INTEND score in the SMA cohort was cartilage oligomeric matrix protein (Table S2).

Table 4. SMA-MAP levels from blood samples in SMA and healthy control infants.

Analyte	SMA (N = 18)	SMA: 2 *SMN2* copy subgroup (N = 13)	Control (N = 20)	*P* value SMA vs Control	*P* value *SMN2* = 2 vs Control
Apolipoprotein B (Apo B) (μg/mL)	842.3 (310.3)	871.5 (313.2)	645.2 (369.0)	0.0850	0.0779
AXL receptor tyrosine kinase (AXL) (ng/mL)	20.17 (6.57)	18.62 (5.87)	23.37 (7.59)	0.1752	0.0650
C-Reactive protein (CRP) (μg/mL)	1.94 (6.28)	0.58 (0.66)	0.48 (1.06)	0.3423	0.7623
Cadherin-13 (T-cad) (ng/mL)	**6.83 (3.18)**	**6.67 (3.25)**	**9.72 (4.39)**	**0.0277**	**0.0399**
Cartilage oligomeric matrix protein (COMP) (ng/mL)	**388.4 (221.2)**	**298.1 (121.0)**	**617.5 (177.7)**	**0.0011**	**<0.0001**
Cathepsin D (ng/mL)	412.3 (243.5)	358.2 (233.1)	486.8 (494.8)	0.5549	0.3242
Complement component C1q receptor (C1qR1) (μg/mL)	14.62 (8.54)	**12.24 (6.78)**	**20.64 (13.17)**	0.1075	**0.0227**
Complement factor H-related protein 1 (CFHR1) (μg/mL)	1005 (675.4)	1000 (738.2)	942.9 (566.0)	0.7588	0.8031
Dipeptidyl peptidase IV (DPPIV) (ng/mL)	**232.4 (86.12)**	**215.1 (55.69)**	**282.1 (92.72)**	**0.0969**	**0.0260**
Endoglin (ng/mL)	3.31 (0.87)	3.08 (0.85)	3.32 (0.81)	0.9741	0.4143
Fetuin-A (μg/mL)	648.4 (187.2)	653.2 (163.7)	622.0 (141.5)	0.6241	0.5638
Fibulin-1C (Fib-1C) (μg/mL)	20.67 (4.95)	20.00 (5.58)	19.65 (5.62)	0.5596	0.8620
Human epidermal growth factor receptor 2 (HER-2) (ng/mL)	0.69 (0.23)	0.63 (0.18)	0.76 (0.29	0.4375	0.1571
Insulin-like growth factor binding protein 6 (IGFBP6) (ng/mL)	**116.3 (48.25)**	**106.2 (44.21)**	**153.9 (40.92)**	**0.0135**	**0.0034**
Leptin (ng/mL)	3.46 (2.59)	3.39 (2.72)	2.58 (1.65)	0.2177	0.3476
Monocyte chemotactic protein 1 (MCP-1) (pg/mL)	255.4 (79.99)	252.2 (78.17)	336.7 (160.2)	0.0541	0.0529
Myoglobin (ng/mL)	**32.71 (30.17)**	**30.56 (28.09)**	**14.46 (7.78)**	**0.0220**	**0.0645**
Osteopontin (ng/mL)	151.0 (63.92)	136.8 (67.08)	168.4 (46.75)	0.3426	0.1204
Peptidase D (PEPD) (μg/mL)	**9.39 (2.34)**	**8.84 (1.79)**	**11.15 (2.24)**	**0.0236**	**0.0038**
Placenta growth factor (PLGF) (pg/mL)	19.00 (2.85)	18.54 (1.85	20.10 (5.04)	0.4080	0.2183
Serum amyloid P-component (SAP) (μg/mL)	3.53 (1.69)	3.70 (1.84)	3.01 (1.72)	0.3564	0.2823
Tenascin-X (TN-X) (ng/mL)	184.8 (144.5)	151.5 (114.1)	351.0 (464.9)	0.1424	0.0798
Tetranectin (μg/mL)	**7.39 (1.62)**	**6.78 (1.23)**	**9.06 (3.20)**	**0.0493**	**0.0078**
Thrombospondin-4 (TSP4) (μg/mL)	22.51 (11.55)	18.86 (9.21)	28.66 (21.95)	0.2821	0.0876
YKL-40 (ng/mL)	**10.11 (3.96)**	**10.45 (3.90)**	**7.58 (2.85)**	**0.0288**	**0.0204**

Bold rows highlight analytes where *P* value is equal to or less than 0.05.

C-reactive protein plasma concentration correlated with the CHOP-INTEND (0.776, n = 15, 0.0007) but not the TIMPSI (r = 0.288, n = 18, P = 0.2457) in SMA infants.

Discussion

We were successful in our efforts to recruit SMA and healthy control infants into the study using 14 clinical sites geographically distributed across the US. Our ability to enroll in this challenging and vulnerable population illustrates the utility and power of the clinical trial infrastructure that the NeuroNEXT Network was designed to provide. Importantly, while some sites within the network had extensive experience in the SMA infant population, many sites did not. Thus, our data set may provide natural history data which are most relatable to large, multicenter SMA clinical trials involving sites with a heterogeneous experience level in infant SMA. Caution must be made when using this data as a "historical control" in future and current SMA infant clinical trials. The motivation of parents who enter their infant into an interventional trial compared to those who elect not to participate may bias the standard of care, the use of aggressive support and the timing of the initiation of hospice care.

By the time infants presented for the enrollment visit, SMA infants have reduced motor function compared to controls as reflected in both TIMPSI and CHOP-INTEND enrollment scores for the SMA cohort. This finding, while not surprising, is remarkably consistent with prior studies.[19,33] This consistency, obtained in a multicenter format similar to what would be expected in a large clinical trial context, is an important replication and validation of earlier single center studies (Finkel, Krosschell, and Swoboda, unpublished data). In addition, both the SMA cohort and control cohort data provide an informed baseline expectation for motor function in Type I infants and may eventually help to inform what should be considered a clinically important difference in the two motor function tests following an intervention.

Ulnar CMAP and EIM assessed using multiple sites bilaterally were both able to distinguish between cohorts at the enrollment visit. The CMAP results in the SMA infants closely match those seen in previous studies.[33,34] It

will be important to see how these already low values change as these infants age. The extent of the loss of CMAP response at the enrollment visit may not indicate that motor unit function is irreversibly lost at the baseline visit; however, it is clear that urgency is required to recruit infants into trials prior to significant CMAP loss, if feasible, to ensure the best possible outcomes. For the subgroup of SMA infants with two copies of *SMN2*, the CMAP values do not correlate with motor function, whereas when the more mildly affected infants with three or more copies of *SMN2* are included in the analysis, CMAP does correlate with motor function. This lack of correlation with motor function in the SMA infants with two copies of *SMN2* may be the result of a sampling error as the ulnar CMAP does not reflect the functional status of motor units involved in proximal muscle function. This study also demonstrates that, for healthy infants, CMAP responses appear stable from birth to 6 months of age, although a full analysis of the longitudinal responses in individual infants will provide more definitive evidence. An analysis of the normal development of CMAP responses for each infant at the end of the longitudinal study will provide important baseline data for future clinical trials.

Of all the predetermined EIM measures studied, only the high-frequency reactance slope distinguished SMA from healthy children; many of the standard measures that have shown differences in older children[27,35] did not do so in this group of infants. Moreover, EIM measures only correlated with motor function in the healthy children. Two factors may have impacted these results. First, there was no assessment of data quality. Unlike CMAP, with which the investigators were quite familiar, the impedance data were obtained virtually blindly; thus, poor-quality data (e.g. due to electrode contact problems) may have been included in this analysis. Second, following the design of the study, it has since become clear that very young individuals have different impedance spectral characteristics (including, e.g., peak reactance values far above the standard 50 kHz frequency) (Rutkove, unpublished observations). Thus, the predetermined metrics utilized in this study were likely not ideal for children of this age. Further analysis of the raw data will be necessary to identify optimized parameters for infants that can then be applied to the forthcoming longitudinal data analysis.

SMN mRNA levels were lower in SMA infants as expected, and there was no correlation between age and SMN levels in SMA or control cohorts. Surprisingly, there was a positive correlation detected in control infants between SMN mRNA levels and the scores on the CHOP-INTEND. It is worth noting, however, that only seven control infants had both the CHOP-INTEND and a blood draw for SMN mRNA levels. There was no correlation between SMN mRNA levels and the TIMPSI scores in 19 control infants. SMN protein levels were more variable than the SMN mRNA levels. There were no correlations between SMN mRNA levels and SMN protein levels as measured from PBMCs in either cohort ($r = -0.0184$, $n = 31$, $P = 0.9217$). We found variability in PBMC yield from patient samples and were not able to process some samples because of insufficient material. Since the start of this project, it is now clear that measurement of SMN protein levels from PBMC samples collected using the CPT tubes is not optimal and a whole blood methodology is now available.[36,37] The protocol was modified for subsequent longitudinal visits to include collection of whole blood so that future analysis of SMN protein in this study may be improved.

There were many serum protein analytes that distinguished between SMA and control cohorts. Most of these were in lower concentrations in the SMA infants compared to the control infants with the exception of myoglobin and YKL-40 that were found in higher concentrations in SMA infant serum compared to controls. While it is difficult to generalize the results of these disparate proteins, one general observation is that in the control cohort, if a protein analyte concentration correlated with age, then it was a negative correlation; the serum concentration of many of the analytes decreased with increasing age of enrollment. This overall trend was also seen in the SMA cohort with two exceptions (Apolipoprotein B and Serum Amyloid P-Component) suggesting that the natural history of most serum analytes studied here is to have reduced concentration with increasing age. Determination of the trends in individual infants with increasing age will help to clarify this possibility.

Future analysis of the longitudinal data sets from the SMA infant and healthy infant control cohorts described here will contribute to an understanding of the natural history of SMA infants and provide important control data for SMA infant interventional studies. It is clear from our initial data, that infants with SMA presenting prior to 6 months of age can be enrolled into studies readily. However, given the poor motor function and electrophysiological outcomes at enrollment, efforts should be made to enroll infants into interventional clinical trials as soon as possible after diagnosis, and ideally, prior to the onset of significant denervation.

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Author Contributions

SJK, AHMB, and JTK conceived the study. SJK, KK, WDA, SBR, KJS, STI, EK, AK, and JTK designed the study. KK, WDA, KJS, AS, BTD, RS, NK, DC, STI, JP, AC, CC, CM, WBB, KW, MT, PS, EF, and the NN101 SMA Biomarker Investigators (Table S1) acquired the clinical data. SRR, VLM, XW, PGZ, and TWP acquired the molecular data. MEC, MMM, AB, and the Neuro-NEXT Clinical Trial Network coordinated the data acquisition. SJK, CSC, and JWY analyzed the data. SJK, KK, WDA, SBR, and JTK wrote the manuscript.

Conflict of Interest

S.B.R. has equity in, and serves as a consultant and scientific advisor to, Skulpt, Inc. a company that designs impedance devices for clinical and research use; he is also a member of the company's Board of Directors. The company also has also licensed patented impedance technology of which S.B.R. is named as an inventor.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Enrolling NeuroNEXT sites. Enrolling NeuroNEXT clinical trial sites and NN101 site investigators and staff.

Table S2. Pearson correlation coefficients between baseline motor function test score and putative SMA biomarkers. Summary table of Pearson correlation coefficients. Shaded rows indicate a correlation with p value that is equal to or less than 0.05.

ONLINE FIRST

Observational Study of Spinal Muscular Atrophy Type 2 and 3

Functional Outcomes Over 1 Year

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Objective: To characterize the short-term course of spinal muscular atrophy (SMA) in a genetically and clinically well-defined cohort of patients with SMA.

Design: A comprehensive multicenter, longitudinal, observational study.

Setting: The Pediatric Neuromuscular Clinical Research Network for SMA, a consortium of clinical investigators at 3 clinical sites.

Participants: Sixty-five participants with SMA types 2 and 3, aged 20 months to 45 years, were prospectively evaluated.

Intervention: We collected demographic and medical history information and determined the *SMN2* copy number.

Main Outcome Measures: Clinical outcomes included measures of motor function (Gross Motor Function Measure and expanded Hammersmith Functional Motor Scale), pulmonary function (forced vital capacity), and muscle strength (myometry). Participants were evaluated every 2 months for the initial 6 months and every 3 months for the subsequent 6 months. We evaluated change over 12 months for all clinical outcomes and examined potential correlates of change over time including age, sex, SMA type, ambulatory status, *SMN2* copy number, medication use, and baseline function.

Results: There were no significant changes over 12 months in motor function, pulmonary function, and muscle strength measures. There was evidence of motor function gain in ambulatory patients, especially in those children younger than 5 years. Scoliosis surgery during the observation period led to a subsequent decline in motor function.

Conclusions: Our results confirm previous clinical reports suggesting that SMA types 2 and 3 represent chronic phenotypes that have relatively stable clinical courses. We did not detect any measurable clinical disease progression in SMA types 2 and 3 over 12 months, suggesting that clinical trials will have to be designed to measure improvement rather than stabilization of disease progression.

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SPINAL MUSCULAR ATROPHY (SMA) is the leading genetic cause of death in infancy, with an estimated incidence of 1 in 6000 to 1 in 10 000 live births.[1-4] Most patients have a homozygous *SMN1* deletion of exon 7, making diagnostic confirmation readily available.[5,6] *SMN2* is an inverted duplication that differs from *SMN1* by 5 nucleotides, the only critical difference being an 840C>T transition in exon 7 that alters splicing.[7] The resulting messenger RNA lacks exon 7 (Δ7*SMN2* messenger RNA) and produces a protein with reduced stability. However, the expressed SMN2 is partially able to rescue the phenotype.[8] The clinical severity is inversely related to *SMN2* copy number.[9,10] This observation has been replicated in transgenic mice by knocking out the *SMN* gene and introducing a human *SMN2* transgene.[11] The homozygous *SMN1* mutation affects motor neurons in the spinal cord and ultimately

leads to muscle atrophy and weakness. In all but the most severe infantile forms of SMA, there is histological and electrophysiological evidence of reinnervation that partially compensates for functional loss.[12,13]

Spinal muscular atrophy leads to predominantly proximal muscle atrophy and weakness often leading to secondary scoliosis, joint contractures, and restrictive lung disease.[12] In the more severe SMA phenotypes, noninvasive respiratory support is often needed to compensate for the presence of respiratory muscle weakness.[14,15] The continuous clinical spectrum of SMA has been divided into 3 types based on the age at onset and highest motor milestone achieved.[16,17] Patients with SMA type 1 (SMA 1) become symptomatic in infancy and never achieve the ability to sit. Even with proactive respiratory and nutritional management, they typically have a shortened life expectancy.[15,18] Patients with SMA type 2 (SMA 2) can sit but never walk independently. Patients with SMA type 3 (SMA 3) have a normal life expectancy and can walk but have varying degrees of disability.[12] Both patients with SMA 2 and 3 may lose motor milestones over time.[18,19]

The timing of motor neuron loss in SMA before or after birth is incompletely understood.[20,21] It is also unclear if SMA is a developmental or a neurodegenerative disease.[13,22,23] Similar to an observation in amyotrophic lateral sclerosis, a late-onset motor neuron disease, the motor unit loss in SMA appears to precede the clinical disease onset.[24,25] Electrophysiological studies suggest that there is active motor unit loss in the preclinical and early subacute phase of SMA, followed by a chronic phase with relative stability over time.[25] Unlike most adult neurodegenerative diseases, SMA appears to exhibit slow disease progression.[18,19,26] Young children may even gain motor milestones early in their course.[27] Yet decline in muscle strength and motor function eventually occurs in SMA 2 and 3.[18,28]

With advances in medical care, individuals with SMA 2 and 3 often have a normal life expectancy but remain severely disabled physically.[18,19,29] As a result of our increased understanding of the etiology and pathogenesis of SMA, several new treatments are now on the horizon of clinical investigation. Recent and carefully collected observational data can help in designing clinical trials. We now report the results of a prospective multicenter study to follow the clinical evolution of SMA.

METHODS

SETTING

The study was carried out by the Pediatric Neuromuscular Clinical Research Network for SMA, a consortium of clinical investigators at 3 clinical sites: Columbia University (Clinical Coordinating Center and Molecular Genetics Core), The Children's Hospital of Philadelphia and University of Pennsylvania, and Harvard University. The data management and statistical analyses were performed at the Muscle Study Group Coordination and Biostatistics Centers at the University of Rochester, Rochester, New York.

PARTICIPANTS

We included 65 patients with *SMN1*-associated SMA 2 or 3. Participants of any age were included, as long as they had been diagnosed with SMA before age 19 years. Exclusion criteria were unstable medical conditions that would preclude participation, significant respiratory compromise at baseline that would interfere with safe travel to the site of evaluation, and patient's location beyond a reasonable driving distance in the opinion of the site investigator. Participants were enrolled between May 25, 2005, and September 10, 2007. The diagnosis of SMA type for each subject was made by the principal investigator at each study site following generally accepted criteria of maximal motor function: patients with SMA 2 sat independently but never walked independently, and patients with SMA 3 achieved independent ambulation. No subject evolved from SMA 2 to SMA 3 during the course of the study. To avoid selection bias, all patients seen in the neuromuscular clinics who fulfilled the inclusion and exclusion criteria were offered enrollment. Additional recruitment efforts included a study Web site and interactions with family groups. The study was approved by the institutional review board at each site and all parents or participants provided signed informed consent or assent.

VISIT SCHEDULE

Participants were evaluated at baseline and at months 2, 4, 6, 9, and 12. The outcome measures summarized next were administered at each visit on a single day. For most participants, the order of outcome measure testing was the same across all visits, with the goal of testing motor function early on, followed by strength testing. Also, the same evaluators, in general, performed the evaluations for all participants at a given site over time. We established excellent interrater reliability between primary and backup evaluators.

OUTCOME MEASURES

Motor Function

We used 3 scales that have been used previously in SMA clinical research: the Hammersmith Functional Motor Scale (HFMS), the expanded Hammersmith Functional Motor Scale (HFMSE), and the Gross Motor Function Measure (GMFM). The HFMS is a 20-item scale that was specifically developed to measure function in patients with SMA 2.[30] Each item is scored on a 0 to 2 scale and the total score ranges from 0 to 40. The GMFM is another standardized instrument originally designed to measure change in gross motor function over time in children with cerebral palsy and later validated for SMA.[31] The 88 items of the GMFM are scored on a 4-point (0-3) ordinal scale. The scale is divided into 5 domains (lying and rolling; sitting; crawling and kneeling; standing; and walking, running, and jumping), and each domain score is expressed as a percentage of the maximum score for that domain. The total score is obtained by averaging the percentages across the 5 domains and ranges from 0 to 100.

The HFMSE is an expanded version of the HFMS that adds 13 items from the GMFM to capture aspects of ambulation, rescaled to the same 0 to 2 metric on which the HFMS items are scored. The HFMSE thus captures a wider range of functional abilities and has demonstrated reliability and validity in patients with SMA 2 and 3.[32] The HFMSE contains 33 items and has a total score that ranges from 0 to 66. After obtaining the GMFM and HFMS scores, we computed the sum of the HFMS and the 13 additional GMFM items to obtain the HFMSE score. In many participants, the HFMSE score is identical to the HFMS score because they could not perform any of the more challenging 13 tasks that distinguish the HFMS from the HFMSE.

Pulmonary Function

In children older than 5 years and in a few sufficiently cooperative children younger than 5 years, we measured forced expiratory vital capacity (FVC) as percentage of predicted for age and height or a surrogate for height (see later) using a spirometer (KoKo spirometer; nSpire Health Inc, Longmont, Colorado) with incentive visual reinforcement displayed on the computer screen.[33] We recorded 3 consecutive attempts for each participant, and the maximum result was taken as the measure of FVC. Previous studies in SMA have found that FVC is reliable.[34]

Muscle Strength

In cooperative children, we performed myometry using a handheld dynamometer to measure elbow flexion, knee extension, and knee flexion strength. Myometry was not included in our study protocol at the onset of the study, so this measure was introduced after the first visit in several participants.

ANTHROPOMETRICS

We measured standing height or, if not feasible, supine height by adding measured head, trunk, and leg segments. When height could not be measured (eg, because of severe scoliosis or contractures), we measured ulna length from the olecranon to the styloid process and calculated estimated height based on published data as a surrogate height measure.[35]

CONCURRENT MEDICATIONS AND MEDICAL EVENTS

Given that there is no known effective treatment for SMA, we did not exclude participants taking drugs or supplements intended as treatment for SMA. The use of concurrent medications was recorded at each visit. We also collected information on the timing of hospitalizations for spine surgery, respiratory infections, or other intercurrent events.

GENETIC TESTING

Confirmation of the *SMN1* exon 7/8 common deletion was carried out by polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism analysis of DNA using primers flanking *SMN1* and *SMN2* exon 7. Digestion of the PCR product with Dra I followed by agarose gel electrophoresis from subjects with SMA lacking *SMN1* exon 7 results in a loss of a fragment without a Dra I restriction site and a smaller fragment on gel electrophoresis that easily distinguishes the *SMN1* and *SMN2* genes. Using *SMN2*-specific primers and primers for the control gene *GAPDH*, we quantified *SMN2* copy number by real-time PCR using a light cycler to detect intensity of fluorescence in real time. Crossing points for each gene were determined and compared with *GAPDH* and then with the controls with a known *SMN2* copy number to determine the relative *SMN2*:*GAPDH* ratio. High specificity of the PCR product was ensured by performing a melting curve analysis to help distinguish between specific and nonspecific by-products, eg, primer dimers. Each sample was run in duplicate.

EVALUATOR TRAINING AND MEASUREMENT QUALITY

Dedicated evaluators and backup evaluators collected the outcome data at the clinical sites. Evaluators were trained at an initial meeting prior to study onset and had follow-up meetings and conference calls to maintain uniformity of evaluation procedures across sites. We developed a study manual for all procedures that was available to the evaluators in print and online. For pulmonary function measures, flow volume curves were uploaded into the central data management system and checked for technical limitations and poor patient effort by a single pulmonologist (A.C.) for all sites. When the curve was deemed of insufficient quality, the FVC result was not included in the data set because it was not thought to reflect the underlying physiologic event.

QUALITY CONTROL

The quality of study operations was enhanced by a comprehensive Web-based data entry and management system developed specifically for the Pediatric Neuromuscular Clinical Research Network SMA study. Data were entered at each site and managed centrally by the Muscle Study Group Coordination Center. The system identified out-of-range values and missing data at the time of entry. Further extensive data checking was accomplished after data entry. Regular queries were issued for missing or implausible data.

STATISTICAL ANALYSIS

Data were included for all participants who were enrolled in the observational cohort study at least 12 months prior to the cutoff date of June 1, 2009, and had at least 1 postbaseline evaluation. Only measurements up to 12 months postbaseline were included in the analyses. For each of the outcome measures, we performed formal analyses of the change over time using 2 different strategies: (1) a repeated-measures analysis of covariance model and (2) a mixed-effects linear regression model. The repeated-measures analysis of covariance model included SMA type as a covariate and time (categorical) as the independent variable of interest; this model describes the change in outcome from baseline to each separate visit, with month 12 being the visit of primary interest. The mixed-effects linear regression model included SMA type as a covariate and time (continuous) as the independent variable of interest; this model assumes a linear relationship between the outcome and time and allows subject-specific slopes and intercepts, with the average slope being of primary interest. Unstructured covariance patterns were assumed for both models. These models use maximum likelihood to estimate the parameters of interest using available data from all subjects, dealing with the problem of missing data in an appropriate way under the "missing at random" assumption. If a participant had scoliosis surgery during the 1-year follow-up period, observations obtained after the surgery were excluded from the analyses; the impact that such surgery had on the observed outcomes is summarized separately. The muscle strength outcomes were analyzed using only the mixed-effects linear regression model because of the irregularity in the timing of the visits for these outcomes, which were introduced partway into the study.

We examined potential baseline correlates of change over time by adding appropriate main effect and interaction terms to the mixed-effects linear regression model. The following baseline variables were considered (separately): age (3-12 and ≥13 years), sex, SMA type (2 or 3), ambulatory status (not walking or walking), *SMN2* copy number (2-3 or 4), HFMS score (<20 or ≥20), FVC (<70% or ≥70%), and use of agents to treat SMA (yes or no).

Intrarater reliability of the outcome measures was assessed using data from the baseline and month 2 visits. This was quantified by intraclass correlation coefficients, computed using 1-way random-effects analysis of variance models.

Table 1. Baseline Characteristics for Participants With SMA 2 and SMA 3

Variable	%		
	SMA 2 (n= 35)	SMA 3 (n=30)	Overall (n=65)
Age, y, mean (SD)	9.6 (7.6)	13.2 (10.3)	11.2 (9.1)
Age ≤12 y	77	57	68
Age <5 y	31	23	28
Male	40	50	45
Race			
White	69	80	74
Black	0	3	2
Mixed	23	10	17
Asian	6	3	5
Unknown	3	3	3
Center			
Columbia	46	50	48
Harvard	26	27	26
CHOP	29	23	26
Height, cm, mean (SD)	116.3 (23.5)	138.9 (29.3)	126.9 (28.5)
Weight, kg, mean (SD)	24.9 (13.1)	40.3 (23.9)	32.1 (20.3)
SMN2 copy number			
3	100	53	78
4	0	43	20
5	0	3	2
Walking	0	73	34
Sitting	77	100	88
BiPAP use	29	0	15
HFMS score, mean (SD)	9.0 (8.9)	33.5 (10.2)	20.1 (15.5)
HFMSE score, mean (SD)	10.9 (10.6)	45.9 (14.7)	26.5 (21.5)
GMFM score, mean (SD)	17.3 (13.3)	70.6 (25.2)	41.0 (33.0)
FVC, % of predicted, mean (SD)	46.1 (22.6)	96.9 (17.3)	70.5 (32.5)
Muscle strength, kg, mean (SD)			
Elbow flexion	2.5 (1.4)	11.4 (8.7)	6.8 (7.5)
Knee extension	0.8 (0.8)	3.8 (2.9)	2.3 (2.6)
Knee flexion	2.5 (1.4)	8.3 (6.1)	5.3 (5.2)

Abbreviations: BiPAP, bilevel positive airway pressure; CHOP, Children's Hospital of Philadelphia; FVC, forced vital capacity; GMFM, Gross Motor Function Measure; HFMSE, expanded Hammersmith Functional Motor Scale; HFMS, Hammersmith Functional Motor Scale; SMA 2, spinal muscular atrophy type 2; SMA 3, spinal muscular atrophy type 3.

RESULTS

RECRUITMENT AND RETENTION

We enrolled 71 participants with SMA 2 and 3 , but 3 of these (all younger than 2 years) were not evaluated with the functional assessments and 3 others did not have post-baseline evaluations (2 prematurely withdrew from follow-up and 1 had scoliosis surgery shortly after enrollment). Data from the remaining 65 participants (35 SMA 2 and 30 SMA 3) were included in the analyses. There were no deaths over the 12-month follow-up period. Of the 65 participants, 51 had a month 12 visit. Among the 14 participants without month 12 observations were 4 who missed the month 12 visit but remained in the study, 3 who had scoliosis surgery prior to the month 12 visit, and 7 who prematurely withdrew from follow-up. Reasons for premature withdrawal from follow-up included participation in a clinical trial at another institution (n=2), difficulties in coping with the burden of research visits in terms of time and travel (n=4), and family illness (n=1).

The mean (SD) age of the 65 participants was 11.2 (9.1) years, 45% were male, and 74% were white. Seventy-three percent of the participants with SMA 3 were walking at baseline. For participants with SMA 3 younger than 3 years, 68% were walking at baseline, compared with 82% for those at least 3 years old. Seventy-seven percent of participants with SMA 2 had the ability to sit at baseline, compared with 100% of participants with SMA 3. Twenty-nine percent of participants with SMA 2 (and no participants with SMA 3) were using bilevel positive airway pressure at baseline. Other baseline characteristics of participants are summarized by SMA type in **Table 1**.

TEST-RETEST RELIABILITY

Intraclass correlation coefficients for test-retest reliability, using data from the baseline and month 2 visits, were very high (>0.98) for the GMFM, HFMS, HFMSE, and FVC (liters).

MOTOR FUNCTION

As expected, participants with SMA 2 and SMA 3 differed substantially with respect to baseline motor function (Table 1). There was no appreciable mean change in motor function over the 12-month follow-up period as measured by the GMFM, HFMS, and HFMSE (**Table 2** and **Table 3**; **Figure 1** and **Figure 2**).

MUSCLE STRENGTH

Participants with SMA 2 and SMA 3 differed substantially with respect to baseline muscle strength, as measured by quantitative myometry (Table 1). No significant mean changes in elbow flexion, knee extension, or knee flexion strength were detected over 12 months (**Table 4**).

PULMONARY FUNCTION

Forced expiratory vital capacity was measured in 50 of the 65 participants (77%) and differed substantially between participants with SMA 2 and SMA 3 at baseline (Table 1). The average measured value of FVC (percentage of predicted) decreased slightly over time, by approximately 2% over 12 months (P=.23) (Table 3). The mean annual rate of change was –1.13% (95% confidence interval [CI], –4.18% to 1.91%; P=.46) (Table 4; **Figure 3**).

PREDICTORS OF PROGRESSION

Over the 12-month observation period, there were no significant associations between annual rates of change in motor function (GMFM and HFMSE) or pulmonary function (percentage of predicted FVC) and age, SMA type, baseline HFMS score, or baseline percentage of predicted FVC. The mean rate of change in FVC differed between female participants (–3.51% per year; 95% CI, –7.14% to 0.12%) and male participants (3.12% per year; 95% CI, –1.68% to 7.91%) (P=.03).

Table 2. Change Over Time for Participants With SMA 2 and SMA 3

Variable	Mean (SD) of the Change From Baseline to Each Visit				
	Month 2	Month 4	Month 6	Month 9	Month 12
HFMS	−0.83 (2.79) [n = 23]	−0.26 (1.51) [n = 23]	0.00 (2.41) [n = 29]	−0.30 (2.01) [n = 23]	−0.31 (2.24) [n = 26]
HFMSE	−0.52 (2.92) [n = 42]	0.37 (2.98) [n = 46]	0.27 (2.72) [n = 55]	0.00 (3.12) [n = 43]	0.15 (3.22) [n = 48]
GMFM	−0.43 (2.93) [n = 42]	1.33 (4.88) [n = 46]	0.46 (4.74) [n = 55]	1.03 (4.99) [n = 43]	0.78 (5.94) [n = 47]
FVC (% of predicted)	−1.73 (8.31) [n = 30]	−0.29 (7.82) [n = 34]	0.36 (9.81) [n = 39]	0.61 (8.74) [n = 36]	−2.49 (10.60) [n = 37]

Abbreviations: FVC, forced vital capacity; GMFM, Gross Motor Function Measure; HFMSE, expanded Hammersmith Functional Motor Scale; HFMS, Hammersmith Functional Motor Scale (participants with SMA 2 only); SMA 2, spinal muscular atrophy type 2; SMA 3, spinal muscular atrophy type 3.

Table 3. Analyses of Change Over Time for Participants With SMA 2 and SMA 3[a]

Variable	Mean Change (95% CI)	*P* Value
HFMS, mo		
6	−0.06 (−0.94 to 0.81)	.88
9	−0.39 (−1.14 to 0.37)	.30
12	−0.16 (−1.05 to 0.73)	.72
HFSME, mo		
6	0.27 (−0.43 to 0.97)	.44
9	−0.08 (−0.90 to 0.74)	.85
12	0.19 (−0.70 to 1.08)	.67
GMFM, mo		
6	0.42 (−0.80 to 1.64)	.49
9	0.39 (−1.12 to 1.90)	.61
12	0.79 (−0.99 to 2.57)	.38
FVC, % of predicted, mo		
6	0.46 (−2.34 to 3.26)	.74
9	0.57 (−2.07 to 3.20)	.67
12	−2.21 (−5.84 to 1.43)	.23

Abbreviations: CI, confidence interval; FVC, forced vital capacity; GMFM, Gross Motor Function Measure; HFMSE, expanded Hammersmith Functional Motor Scale; HFMS, Hammersmith Functional Motor Scale (participants with SMA 2 only); SMA 2, spinal muscular atrophy type 2; SMA 3, spinal muscular atrophy type 3.

[a] Mean changes, CIs, and *P* values were obtained from a repeated-measures analysis of covariance model that included SMA type as a covariate and time (categorical) as the independent variable of interest; see text for details.

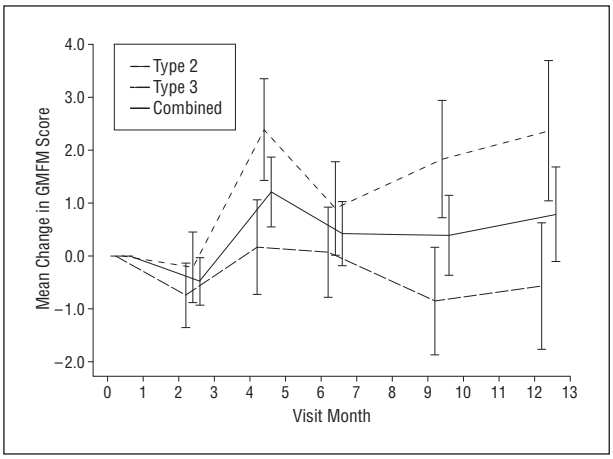

Figure 1. Mean change in Gross Motor Function Measure (GMFM) total score over time estimated using a repeated-measures analysis of covariance model. Error bars indicate 1 SE of the mean. Mean changes are plotted for spinal muscular atrophy (SMA) type 2 and SMA type 3 combined, SMA type 2 only, and SMA type 3 only.

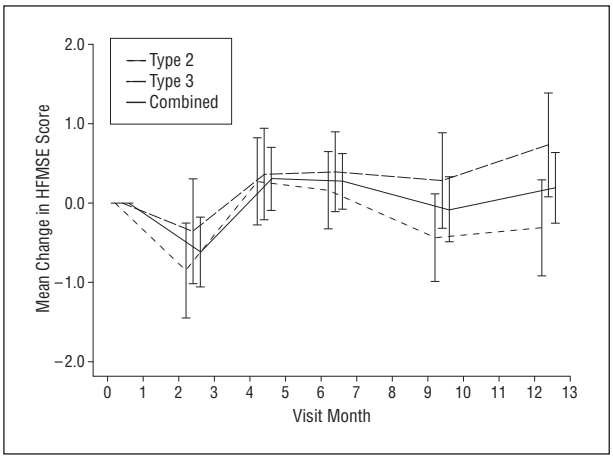

Figure 2. Mean change in expanded Hammersmith Functional Motor Scale (HFMSE) score over time estimated using a repeated-measures analysis of covariance model. Error bars indicate 1 SE of the mean. Mean changes are plotted for spinal muscular atrophy (SMA) type 2 and SMA type 3 combined, SMA type 2 only, and SMA type 3 only.

Ambulatory status at baseline was significantly associated with annual rate of change in motor function, with a significant increase in function over time in ambulatory participants compared with a slight decline in function over time in nonambulatory participants. For the GMFM score, the mean rate of change was 3.96 (95% CI, 1.20 to 6.72) in ambulatory participants and −0.70 (95% CI, −2.86 to 1.46) in nonambulatory participants ($P=.01$). For the HFMSE score, the mean rate of change was 1.62 (95% CI, 0.14 to 3.10) in ambulatory participants and −0.47 (95% CI, −1.66 to 0.72) in nonambulatory participants ($P=.03$). To explore these associations further, we reanalyzed the data and included an interaction term between ambulatory status and age (<5 years vs ≥5 years) in the mixed-effects linear regression model. We found that the difference in GMFM score slope between ambulatory and nonambulatory participants was 6.3 for those younger than 5 years compared with 4.2 for those at least 5 years of age. Similarly, for the HFMSE score slope, the difference between ambulatory and nonambulatory participants was greater in those younger than 5 years (3.8) than in those at least 5 years old (1.5).

All participants were homozygous for a deletion of *SMN1*. The distribution of *SMN2* copy number differed between participants with SMA 2 and SMA 3, with all participants with SMA 2 having 3 *SMN2* copies and participants with SMA 3 having between 3 and 5 *SMN2* copies;

Table 4. Analyses of Rate of Change Over Time for Participants With SMA 2 and SMA 3[a]

Variable	Mean Rate of Change, Slope (95% CI)	*P* Value
HFMS	0.06 (–0.82 to 0.93)	.90
HFMSE	0.25 (–0.65 to 1.15)	.58
GMFM	0.75 (–1.07 to 2.57)	.41
FVC, % of predicted	–1.13 (–4.18 to 1.91)	.46
Elbow flexion, kg	–0.58 (–2.62 to 1.47)	.57
Knee extension, kg	–0.07 (–0.98 to 0.84)	.87
Knee flexion, kg	–0.03 (–1.00 to 0.94)	.95

Abbreviations: CI, confidence interval; FVC, forced vital capacity; GMFM, Gross Motor Function Measure; HFMS, Hammersmith Functional Motor Scale (participants with SMA 2 only); HFMSE, expanded Hammersmith Functional Motor Scale; SMA 2, spinal muscular atrophy type 2; SMA 3, spinal muscular atrophy type 3.

[a] Mean rates of change, CIs, and *P* values were obtained from a mixed-effects linear regression model that included SMA type as a covariate and time (continuous) as the independent variable of interest; see text for details.

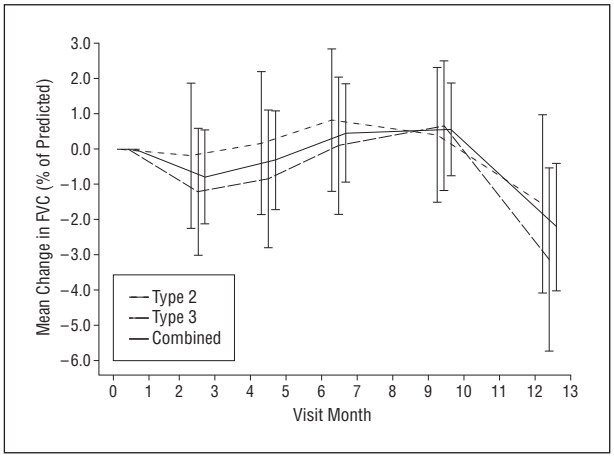

Figure 3. Mean change in forced expiratory vital capacity (FVC) (percentage of predicted) over time estimated using a repeated-measures analysis of covariance model. Error bars indicate 1 SE of the mean. Mean changes are plotted for spinal muscular atrophy (SMA) type 2 and SMA type 3 combined, SMA type 2 only, and SMA type 3 only.

most participants with SMA 3 carried 3 or 4 *SMN2* copies (Table 1). There was no significant association between any measure of the rate of disease progression and *SMN2* copy number.

Sixteen of the 65 participants (25%) used at least 1 medication that might be considered as a potential treatment for SMA at some time during the 12-month follow-up period. These medications included albuterol (6%), carnitine (11%), creatine (8%), hydroxyurea (3%), steroids (2%), and valproic acid (8%). There were no significant differences between those who did and did not take these medications with respect to the mean rate of change over time in motor function or pulmonary function.

Four participants had scoliosis surgery during the 12-month follow-up period. One 14-year-old participant with SMA 3 had surgery between months 6 and 12 and the HFMS score dropped from 21 to 8 after surgery. Only a slight decline in FVC was noted. A 6-year-old participant with SMA 2 had surgery between months 6 and 12 and the HFMS score dropped from 20 to 7 after surgery. This participant also had a decline in FVC from 0.98 L (67% of predicted normal) to 0.68 L (38% of predicted normal). A 5-year-old participant with SMA 2 had surgery shortly after enrollment, but the HFMS score at baseline remained very low throughout follow-up (ranging from 0-2); FVC was not measured in this participant. Finally, a 12-year-old participant with SMA 3 had surgery between months 9 and 12 and a preoperative HFMS score of 17. At the first postoperative study visit, the motor function testing could not be performed because of pain. The FVC changed only slightly in this participant.

COMMENT

We carried out a multicenter, prospective, observational cohort study in patients with SMA under conditions similar to those that would be used in a clinical trial with respect to having a fully developed manual of procedures, evaluator training, and data quality control. Our data suggest that SMA has a relatively stable course over a 12-month period in terms of motor function, pulmonary function, and muscle strength. There was no significant mean change in motor function when measured by 3 different instruments that have been validated for use in SMA: the HFMS, the HFMSE, and the GMFM. Our observational study, however, does not address the issue as to whether 1 scale may be more sensitive than another to the effects of an intervention. Pulmonary function appeared to decline slightly over 12 months, but the change was not statistically significant.

The motor function improvements observed in ambulatory participants may be due to a learning effect that may be more prominent in younger children. It may also in part be associated with the developmental gain of motor milestones in children who walk relatively late because of their underlying condition. The largest gain in motor function occurred in those who were walking and were younger than 5 years, although the number of participants younger than 5 years (n=18, with 5 ambulatory and 13 nonambulatory) was too small to permit definitive conclusions. In contrast, more participants with SMA 2 may have reached their highest level of motor function at the time of enrollment into our study. The slight gain in motor function was surprising, because loss in motor function for patients with SMA 3 had been described in previous studies.[19] A recent open-label study found improvement after drug treatment in younger patients with SMA.[36] While this may be due to biological factors, our results from this observational study suggest the potential for a learning effect or for developmental gains to confound the results of uncontrolled trials that evaluate motor function.

Our data suggest that female sex may be associated with a greater decline in pulmonary function, but not in motor function. This is unexpected because studies of the association between *SMN2* copy number and phenotype had suggested that female sex confers a mitigating effect on disease severity.[9] Additional studies are needed to either confirm or refute this finding.

Participants who had scoliosis surgery during the observation period had postsurgical declines in motor func-

tion. Because of the small number of participants who had surgery during the study period, this finding requires replication. The short-term loss in motor function may be due to reduced axial movements, which are invariably restricted as a result of the stabilizing nature of scoliosis surgery. It is unknown if this is a clinically meaningful functional impairment. From a clinical perspective, surgery is often needed to maintain long-term function and quality of life, and the gain in postural stability and the slowing of progressive worsening of the spine curvature are beneficial to patients in the long-term. From a research perspective, however, it may be important in future clinical trials to select participants who are unlikely to undergo scoliosis surgery during the observation period. For participants who do undergo surgery, exclusion of data collected postsurgery from the statistical analysis is an additional consideration.

A subset of our cohort (25%) used medications intended for the treatment of SMA. Thus, although none of these medications has been shown to have an impact on disease course, our data may not reflect a pure observation of the natural history of SMA. The decision to include participants using supplements and medications intended for the treatment of SMA was based on concerns about recruitment feasibility if the entry criteria had been restricted to entirely untreated patients, as well as the concern that participants may take medications without reporting this to the investigators.

Our results differ from those of previous studies in the United States and Europe that had found a slow decline in motor function in SMA 2 and SMA 3.[18,28,37] A French natural history study that used different measures of motor and pulmonary function found evidence of mild decline in both motor and pulmonary function at 2 and 4 years after baseline, but 1-year data were not reported.[26] The discrepant results may in part be due to differences in methods. Also, it is possible that we would have observed a significant decline in pulmonary function, for example, if we had observed participants for a longer period. The data that we are obtaining through ongoing long-term follow-up of cohort participants may allow a more direct comparison with the French study.

Our results showing relative stability of function over time suggest that future clinical trials will need to be designed to show treatment-associated improvement rather than a slowing or arrest of decline. This design is in contrast to prevailing trial designs for adult neurodegenerative disorders, which often aim to demonstrate treatment-associated slowing of disease progression. There is increasing evidence that key events in the pathogenesis of SMA occur in the distal compartments of the neuron and involve impaired axonal outgrowth and synaptic connections. Clinical trials aimed at detecting improvement would be most appropriate for potential therapies that could promote reinnervation by collateral sprouting or improve function and connectivity in surviving neurons with impaired function.

We have confirmed the feasibility and excellent reliability of the motor and pulmonary function measures used in this study. Importantly, we succeeded in enrolling a relatively large cohort of patients with SMA at only 3 sites in slightly more than 2 years. Given that there was no possibility for direct benefit for participants in this observational study, this success implies that people with SMA and their families are eager to embrace clinical research opportunities and contribute their time and effort.

Continued long-term follow-up of our cohort will determine if the functional stability observed over the initial 12 months continues over a longer observation period. It is also hoped that the planned analyses of additional outcomes including quality of life, electrophysiological outcomes, and muscle mass will enhance our understanding of the clinical longitudinal profile of SMA. The data from this cohort will be valuable for the design of future clinical trials in SMA.

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Natural History in Proximal Spinal Muscular Atrophy

Clinical Analysis of 445 Patients and Suggestions for a Modification of Existing Classifications

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Objectives: To describe the natural history in all types of proximal spinal muscular atrophy (SMA) and to propose a modified classification scheme that takes the long-term course of SMA into account.

Design: Patients with proximal SMA were studied prospectively and retrospectively in a genetic study that was based on clinical and family data.

Patients: Four hundred forty-five patients with SMA were ascertained since 1985 through various departments of neurology and neuropediatrics, institutes of human genetics, and the German muscular dystrophy association (Deutsche Gesellschaft für Muskelkranke, Freiburg, Germany).

Results: The study group was subdivided into patients with four types of SMA (ie, SMA types I, II, III, and IV) on the basis of achieved motor development and age at onset. Survival probabilities at 2, 4, 10, and 20 years of age were 32%, 18%, 8%, and 0%, respectively, in patients with SMA type I (those who were never able to sit) and 100%, 100%, 98%, and 77%, respectively, in patients with SMA type II (those who were able to sit but were unable to walk). Nineteen of 104 patients with SMA type II lost the ability to sit; this inability to sit was not of prognostic relevance. Patients with SMA type III (those who were able to walk [age at onset, younger than 30 years]) were subdivided into those with an age at onset before (SMA type IIIa) and after (SMA type IIIb) 3 years. The probabilities of being ambulatory at 10, 20, and 40 years after onset were 73%, 44%, and 34%, respectively, in patients with SMA type IIIa, and they were 97%, 89%, and 67%, respectively, in patients with SMA type IIIb.

Conclusions: The definition of long-term characteristics of SMA is helpful in providing medical care to families with members who have SMA and also in providing important information for future genotype-phenotype studies and therapeutic trials of patients with SMA. Our data indicate that the widely used classification schemes did not consider the broad spectrum of SMA so a practical modification was suggested.

(Arch Neurol. 1995;52:518-523)

PROXIMAL SPINAL muscular atrophy (SMA) is a genetically heterogeneous disease with paresis and muscular atrophy due to the loss of anterior horn cell function. The basic defect is still unknown.

The clinical picture is highly variable; it indicates a more continuous spectrum with ages at onset from before birth to adulthood rather than clearly separable subgroups. The variability can be observed not only in the age at onset but also as a feature of the individual course, with periods of only slow or even no remarkable progression in chronic forms. Consequently, the mainly used classifications with defined ages at onset and death, as well as achieved motor functions, are still a matter of controversial discussion.[1] Since "age at onset" is not a reliable criterion to predict a chronic rather than progressive disease in infancy, it is of little help in the prognostication of clinical courses.

The individual prognosis, especially in patients with an early onset of SMA, is often better than stated in most of the existing classification systems. Russman and colleagues[2] recently made the same conclusion in their study on the clinical course in 141 patients with proximal SMA.

Despite the fact that one gene has been mapped for different clinical forms of SMA to chromosome 5q, no markers exist to predict the course of SMA in a single patient. Furthermore, it is doubtful that

See Patients and Methods on next page

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PATIENTS AND METHODS

For the present study, patients (**Table 1**) were recruited from various departments of neurology and neuropediatrics, institutes of human genetics, and the German muscular dystrophy association (Deutsche Gesellschaft für Muskelkranke, Freiburg, Germany) since 1985. On receiving written informed consent, the participants answered a detailed questionnaire with respect to the clinical course of their disease and a questionnaire with regard to the family history. Medical histories were available for each affected person. The conditions of inclusion into the study were that all patients fulfilled the diagnostic criteria for proximal SMA, as recently defined by the International SMA Consortium.[6] The clinical questionnaire and medical reports provided detailed information about each patient, including age at onset, motor milestones, rate of progression, loss of functions, and respiratory and bulbar involvement.

Different variables with regard to progression and motor functions were defined as follows:

1. The age at onset was not necessarily that given by the patient or his or her family with regard to the question of age at onset. Onset was defined in this study as the age at which the first abnormalities were obvious, either from the medical reports or from the descriptions by patients or parents when the first signs of weakness were mentioned (eg, delayed motor development, loss of motor function).

2. According to the motor milestones that were used for classification, the ability to sit was achieved when a patient could maintain a sitting position without support steadily with a straight back. Infants are able to do so at 8 to 9 months of age (median age, 6½ months; 95th percentile, 9 months).[7,8] This age milestone has also been used by Pearn[9] to characterize motor functions in patients with SMA. The ability to walk was defined as unaided, regular walking. This motor milestone is achieved, at the latest, at the age of 18 months in the normal population.[10] There were different variables that indicated the beginning of walking difficulties: weakness in walking 100 m without support, obvious unstable gait, and/or frequent falling. There was not necessarily a time interval between the age at onset and the first walking difficulties, since the latter was often the first sign of weakness, especially in the group with an early onset of SMA type III. The age at which the patient was no longer ambulatory was defined as the inability to walk 100 m (even with walking aids); at this age, the patients required the use of a wheelchair for outdoor activities.

Families with affected persons in two generations, indicating an autosomal dominant inheritance, have been excluded, and most of them have been described before.[11]

According to the achieved motor milestones of the recently proposed classification of the International SMA Consortium, the study group was subdivided into four SMA types (Table 1).

Actuarial survival curves were calculated by the Kaplan-Meier method.[12] Because of the mode of ascertainment of patients in our study, there was a lack of severely affected patients who had an early death. Many parents whose children died in the first years of life were neither organized in the German muscular dystrophy association nor were they referred to our institute for DNA studies before 1990. This deficit of the severe end of SMA type I can be estimated on the basis of epidemiological data that have indicated nearly identical incidences of acute and chronic forms of SMA in northeastern England, Poland, and West Thuringia (Germany).[13-17] According to the definition of acute SMA that was used in the epidemiological studies, there was an estimated underrepresentation of approximately 90 patients within this group. Therefore, 90 deceased patients were added to the proportion of patients with SMA type I who died in the first 4 years of life. The survival curve of patients who never achieved the ability to sit (those with SMA type I) was calculated with and without this correction in our material (**Figure 1**).

Sex-specific differences in number were tested with the χ^2 test in every group. According to the age at onset, the Mann-Whitney U test was used. To detect whether there were differences in the intervals between the age at onset and the loss of the ability to sit (those patients with SMA type II) or to walk (those patients with SMA type III) between both sexes, Kaplan-Meier actuarial curves were compared with the log-rank or Mantel-Haenszel test.[12]

a prediction of the individual course in proximal SMA will be possible even after the identification of the gene—mainly because of two reasons: (1) Although the ages at onset in the majority of affected sibling pairs are similar and thus indicate a genetic determination, exceptions to this observation clearly exist.[3-5] (2) The ages at death are influenced mainly by exogenous factors, such as pulmonary infections, medical care, etc, and these factors differ even in siblings with a similar age at onset to a great extent.[5]

There is a need for statistical data to describe the natural history in patients with SMA as a basis for medical care and genetic counseling. In addition, the natural history of SMA has become of special importance in light of possible therapeutic trials (eg, with neurotrophic factors).

Based on detailed clinical data that were obtained from 445 patients with proximal SMA, we describe the natural history of patients who belonged to different subgroups, as defined by achieved motor functions (the ability to sit and walk independently). The definition of milestones alone is in accordance with those of corresponding types of the International SMA Consortium, which has classified SMA as follows: SMA type I (severe) with onset from birth to 6 months (patients who are never able to sit without support, with death occurring usually at younger than 2 years of age); SMA type II (intermediate) with onset before 18 months of age (patients who are able to sit but who are unable to stand or walk unaided, with death occurring usually at older than 2 years of age); and SMA type III (mild) with onset after 18 months of age (patients who are able to stand and walk, with death occurring in adulthood).[6]

RESULTS

The motor functions and ages at onset in patients with SMA types I through IV are summarized in Table 1. Sur-

Table 1. Definition of SMA Types I Through IV Based on 445 Patients in the Present Series*

SMA Type	Definition	n	No. of Patients		Age at Onset, y			
			Sex, M:F	Deceased	Mean	Median	SD	Range
I	Never sat alone	197	105:92	155	1.9 mo	1 mo	2.3 mo	0-10 mo
II	Sits alone, never walked	104	54:50	11	8.6 mo	8 mo	4.4 mo	0-18 mo
IIIa	Walks without support; age at onset, <3 y	73	41:32	3	17.9 mo	18 mo	7.2 mo	3-30 mo
IIIb	Walks without support; age at onset, 3-30 y	61	41:20	1	10.4 y; males, 11.4 y; females, 8.4 y†	10 y; males, 12 y; females, 6 y	5.6 y; males, 5.3y; females, 5.6 y	3-24 y; males, 3-24 y; females, 3-24 y
IV	Age at onset >30 y	10	2:8	2	44.8 y	46 y	7 y	33-54 y

*SMA indicates spinal muscular atrophy.
†P<.03.

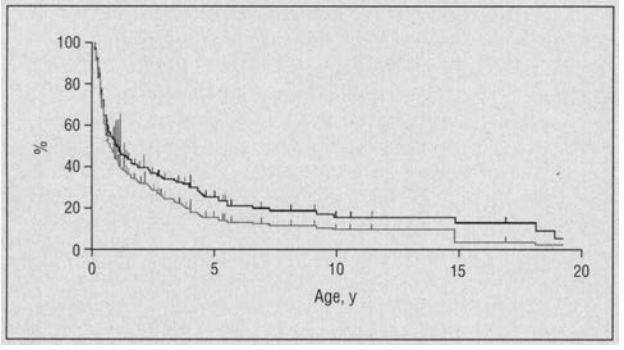

Figure 1. *Kaplan-Meier survival curves for patients with spinal muscular atrophy type I (those who were never able to sit). Solid curve indicates 197 patients in the study, including 155 deceased patients; dotted curve, corrected curve by including 90 additional patients with an age at death within the first 4 years of life (see text).*

Table 2. Survival Probability in SMA Types I and II and Probability of Still Being Able to Walk After Onset of SMA Types IIIa and IIIb*

SMA Type	Age, y				
	2	4	10	20	40
	Survival Probability at a Certain Age, %				
I†	32	18	8	0	0
II	100	100	98	77	...
	Probability of Being Ambulatory (Years After Onset), %				
IIIa	98	94.5	73	44	34
IIIb	100	100	97	89	67

*SMA indicates spinal muscular atrophy. Data were obtained from Kaplan-Meier actuarial curves and values are given as percentages.
†Corrected figures under inclusion of additional 90 deceased patients (see text).

vival probabilities of patients with SMA types I (corrected figures) and II are listed in **Table 2**.

As indicated in **Figure 2**, the loss of the achieved sitting function in 19 of 106 patients was not a prognostically relevant criterion, since nearly all patients were provided with sitting aids and spinal support. Most deceased patients were able to sit unaided throughout life.

Since only six of 144 patients who belonged to the groups with SMA types III and IV died prior to or during the study (**Figure 3**), survival probabilities could not be calculated at the time of the present study. The interval between the onset and the last available information for patients with SMA types III and IV was a mean± SD interval of 23.6±14.3 years, a median interval of 20 years 5 months, and an interval range from 2.8 to 65 years.

The probability of being able to walk after a certain duration of the disease (**Figure 4**) differed considerably between patients with SMA types IIIa (onset before the age of 3 years) and IIIb (onset after the age of 3 years). While 50% of the patients with SMA type IIIb were still ambulatory after a disease duration of more than 45 years, the corresponding value for patients with SMA type IIIa was 14 years. Further details are given in Table 2.

Because of the small number of patients with SMA type IV, no probabilities could be calculated. Three of 10 patients were confined to a wheelchair at 8 to 11 years following the first walking difficulties, while the remaining patients were still able to walk at 6 to 30 years after onset.

The interval between the inability to walk and the last available information in those patients with SMA types III and IV who stopped walking (n=57 [40%]) was a mean± SD interval of 12.1±7.9 years, a median interval of 10 years, and an interval range of 9 months to 34 years.

There was no significant sex difference with regard to the number of patients in each group, the ages at onset in patients with SMA types I, II, and IIIa, and the clinical severity in terms of survival probability (patients with SMA type I) and sitting and walking functions (patients with SMA types II and III, respectively). The only significant influence of gender was detected in the age at onset in patients with SMA type IIIb (Table 1). In this group, there was a predominance of male patients with a later onset of the disease (ie, female patients showed clinical symptoms on the average 3 years earlier than did male patients).

COMMENT

This study is largely based on retrospectively obtained data, especially with regard to patients with long-standing courses of the disease. This bears the risk that the data with regard to the different motor functions might not be reliable; thus, it may lead to an increasing inac-

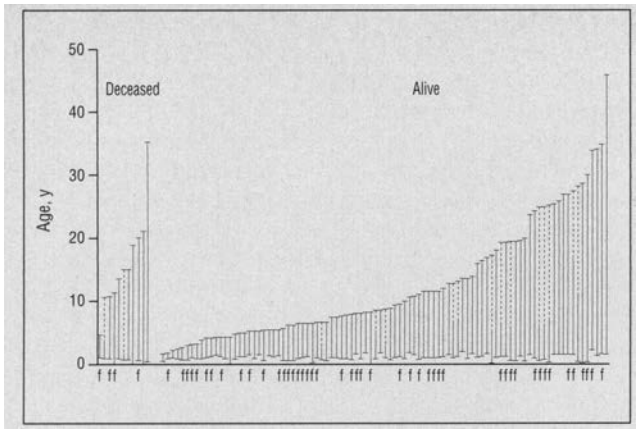

Figure 2. *Interval between age at onset and age at death (at left) or current age (at right) in 104 patients with spinal muscular atrophy type II (those who sat but never walked). Broken lines indicate the period during which patients were unable to sit unaided; f, female patients (the remaining are male patients).*

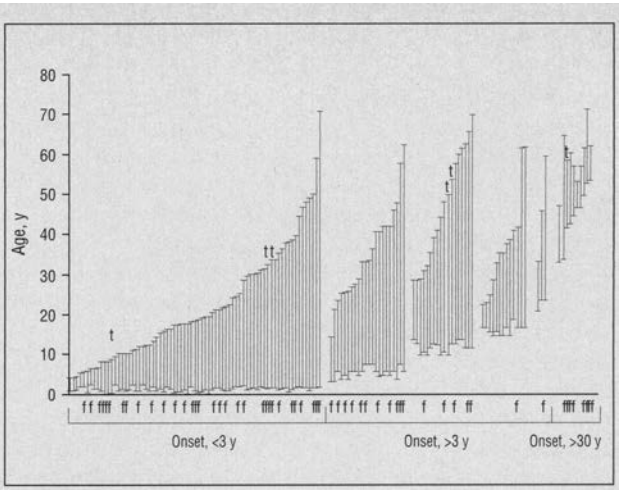

Figure 3. *Intervals between age at onset and current age in 144 patients with spinal muscular atrophy (SMA) types III and IV. t indicates deceased patients; f, female patients. The patients are subdivided into different age-at-onset groups (SMA type IIIa, age younger than 3 years; SMA type IIIb, age ranges from 3 to 10 years, 10 to 15 years, 15 to 20 years, and 20 to 30 years; and SMA type IV, age older than 30 years). Within these groups, they are arranged by increasing age at the last examination or age at death in the patients with SMA type III and by age at onset in patients with SMA type IV.*

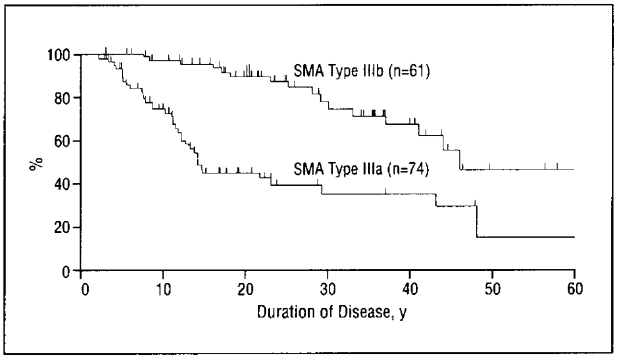

Figure 4. *Probability of patients being ambulatory after a certain disease duration (interval between age at onset and loss of ability to walk) in 134 patients with spinal muscular atrophy (SMA) type III. Separate Kaplan-Meier curves were calculated for SMA type IIIa (age at onset, younger than 3 years) and SMA type IIIb (age-at-onset range, 3 to 30 years).*

curacy that is correlated with the duration of the disease. There has to be a kind of compromise between the reliability of information and the quantity of data that give insights into the natural history of chronic SMA, since it is not possible to assess data from such material throughout many decades in a prospective study. Moreover, follow-up reports are not available from medical histories, because the patients are normally seen once in the neurological department for diagnostic evaluation. We think, however, that the bias of retrospective collection of data with respect to the major motor functions is small and does not effect the main conclusions.

Russman and colleagues[2] pointed out that in patients with SMA the prognosis is often better than generally defined in the existing classifications. In their study, 49 patients participated who had an onset of weakness before 6 months of age, whose life span was said to be only 2 to 4 years, and who were 4 months to 31 years of age.

In light of the current advances in linkage studies, the International SMA Consortium has defined strict diagnostic inclusion and exclusion criteria for DNA studies in patients with proximal SMA that are used in the application of a prenatal diagnosis, for example. As a basis for further studies (eg, the analysis of genotype-phenotype correlations), the International SMA Consortium has proposed a classification that is based on widely used systems.[18-21] Several attempts were made by other authors to subclassify their material.[22-27] Under the impression of a marked variability in clinical manifestation, some authors concluded that a sudivision is not useful[2,28,29] or that there are at least difficulties in drawing a clear-cut line between the different categories.[1,30]

The analysis of the whole clinical spectrum in SMA, including a large number of cases, clearly exhibits important deficits of the existing classifications as follows:

1. A certain percentage of patients are "unclassifiable"—most often because of "longer" survival. According to the classification of the International SMA Consortium and the defined ages at onset and death and achieved motor milestones, respectively, 24% (106/445) of our patients could not be classified at all. Most of them could not be classified because of survival beyond the age of 2 years in patients with SMA type I, with an age at onset within the first 6 months (60 of 178 patients), or because of achieved walking abilities in patients with an onset before the age of 18 months (46 of 240 patients).

2. A definite designation to either severe or intermediate SMA can sometimes only be made at a later stage of the disease (eg, after the age of 2 or 4 years). A "reclassification" is often necessary and leads to confusion among and distrust of physicians.

3. Life expectancy is often better than that proposed by age at death in the different types. The perspective of an early death is not justified for many patients, and it results in an unnecessary burden for the parents.

4. Life span only describes one aspect in the natural history of the disease. Others, like periods with pre-

served motor functions (eg, intervals between onset and the inability to walk), are other important characteristics in the description of the clinical course; these aspects are not mentioned in any of the used systems.

Although our results clearly underline the existence of a broad continuum of possible courses with regard to the age at onset and progression, a classification is necessary for practical reasons. We therefore propose a modified version (Tables 1 and 2) of the classification by the International SMA Consortium. This modified version is in accordance with the definition of SMA types I through IV based on the achieved motor functions, but it tries to avoid general age limitations for onset of the disease or life span. A similar approach was made by Tonali and colleagues.[26] The strictly defined ages should be replaced by relevant probabilities for survival and preserved motor functions in the different categories.

IN CONTRAST to the proposed classification of the International SMA Consortium, we have subdivided SMA type III (patients who learned to walk) into two subtypes that are defined by the age at onset before (SMA type IIIa) or after (SMA type IIIb) the age of 3 years—mainly because of the following two reasons:

1. Because of the wide range of ages at onset (from before 1 year to at least 20 years) in patients with SMA type III, including all patients who achieved the motor milestone of "standing alone," prediction of the prognosis in the whole group with SMA type III is not useful and justifies a subdivision of SMA type III. In our study, there appears to be a cluster of patients with an early onset of SMA type III that is characterized by a stable course with long periods of nonprogressive weakness, whereas the intrafamilial variability in patients with a later onset of SMA type III is much more pronounced. With one exception, there was no overlap between patients with an early and later onset of SMA type III in 13 affected sibships.[5]

2. There is evidence of genetic heterogeneity among patients with the ability to walk but who have onset before 3 years of age; this has been found in at least two systematic studies on the basis of segregation analyses.[31,32] In patients with SMA type IIIa, we found a mean± SD segregation ratio of 0.09±0.07 which differs significantly from the expected ratio in the case of recessive inheritance. The corresponding ratio that was found by Hausmanowa-Petrusewicz and colleagues[31] for a group of patients with an age at onset between 10 and 36 months was 0.039. These and other data indicate genetic heterogeneity, particularly in patients with SMA type IIIa, and lead to the assumption of a certain proportion of autosomal dominant new mutations within this group as the most plausible explanation,[32] with the consequence of modified risk figures to siblings and children of affected subjects.

The proposed classification system has been developed for practical reasons on the basis of the largest systematically analyzed series thus far, and it has been able to avoid the above-mentioned disadvantages. We have subdivided the group of patients with SMA type III into those with SMA subtypes IIIa and IIIb instead of creating new types, which allows for our classification system to be adapted easily to the types that have been defined by the International SMA Consortium, with only a minimum of necessary changes.

This modification does not represent a final version with regard to the survival and function probabilities, but it could be given a broader basis by including further systematic data from other groups.

The question of a possible influence of gender has been a matter of a controversial discussion.[33,34] There is a predominance of male patients in the whole group that is most pronounced in the mild forms of SMA types IIIa and IIIb. Only a small number of female patients experience the disease after puberty so that male patients have a statistically significant later onset of SMA type IIIb. Similar results were obtained by Hausmanowa-Petrusewicz[35] for patients with an onset between the ages of 3 and 17 years: 61 boys had onset of the disease at an average age of 8.37 years, whereas 34 girls had their first symptoms at the age of 4.25 years. It still needs to be clarified whether a "female-sparing factor," which might be associated with hormonal differences, is responsible for a remarkable decrease of the number of female patients in the group with a later onset.[34]

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Correction

Error in Caption on Cover. The caption on the cover of the February 1995 issue of the ARCHIVES is wrong. The first sentence was as follows: "Periventricular hyperintensives." This sentence should have been as follows: "Periventricular hyperintensities."

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A collaborative study on the natural history of childhood and juvenile onset proximal spinal muscular atrophy (type II and III SMA): 569 patients

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Abstract

We analyzed clinical data of 569 patients in two combined series with childhood and juvenile proximal SMA. This cohort included only patients who had achieved the ability to sit unaided (type II and III SMA). The survival rate among 240 type II patients (who sat but never walked) was 98.5% at 5 years and 68.5% at 25 years. SMA III ($n = 329$) (those who walked and had symptoms before age 30 years) was subdivided into those with an onset before and after age 3 years (type IIIa, $n = 195$; SMA IIIb, $n = 134$). In patients with SMA III, life expectancy is not significantly less than a normal population. The probabilities of being able to walk at 10 years after onset was 70.3%, and at 40 years, 22.0% in SMA IIIa. For SMA IIIb, 96.7% were walking 10 years after onset and 58.7% at 40 years. The subdivision of type III SMA was justified by the probability of being ambulatory depending on age at onset; the prognosis differed for those with onset before or after age 3 years. The data provide a reliable basis of the natural history of proximal SMA and support a classification system that is based primarily on age at onset and the achievement of motor milestones.

Keywords: Spinal muscular atrophy; Natural history; Survival probability; Prognosis

1. Introduction

Proximal spinal muscular atrophy of childhood and juvenile onset is a clinically heterogeneous disease. The incidence of more than 1:10000 makes it one of the most common autosomal recessive neuromuscular disorders. The vast majority (> 90%) of cases is recessively inherited with the responsible gene located on chromosome 5q and there is no evidence of genetic heterogeneity hitherto. The exact genetic mechanism and the underlying basic defect, however, are still unknown.

It is well known that proximal SMA is clinically heterogeneous with age of onset ranging from before birth to adult years, but there is still uncertainty about the natural history of the milder forms of childhood and juvenile onset SMA. Current classification systems do not provide sufficient data about the prognosis. Age at death is often used as the basis for classification criterion but is only a rough estimate for most cases of the severe form (type I SMA); even in SMA I, 32% of patients survive age 2 years (Zerres and Rudnik-Schöneborn, 1995). Age at death is even less appropriate for type III SMA where the life span is nearly normal. The classification of the International SMA consortium (International SMA Consortium, 1992) classifies the milder cases according to the ability to sit and walk. We have suggested a modification of current classification systems (Zerres and Rudnik-Schöneborn, 1995).

An earlier classification of the Warsaw group (Hausmanowa-Petrusewicz et al., 1968; Hausmanowa-Petrusewicz et al., 1984) could be adjusted and, in order to speak a common language in terms of SMA types, the Warsaw group agreed to adapt their classification to a generally accepted system primarily based on achieved milestones as suggested by the International SMA consortium. Increasing use of such a classification system based on achieved milestones makes it easier to compare different series and

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Table 1
Survival probabilities in patients with SMA type II (those who were able to sit but not to walk). Data were obtained from Kaplan–Meier actuarial curves

Study	*n*	Sex, M:F	Deceased	Survival probabilities at a certain age (%)				
				5	10	15	20	25 (years)
German	133	70:63	11	99.1	99.1	87.1	79.6	75.4
Polish	107	48:59	20	97.8	96.1	77.5	68.2	59.1
Combined	**240**	**118:122**	**31**	**98.5**	**97.8**	**82.8**	**75.1**	**68.5**

might avoid misinterpretation of data. Clinical classification has become an actual problem with the identification of molecular genetic alterations in the region of chromosome 5q (Hahnen et al., 1995).

We have now combined two series to form the largest study of patients with proximal SMA. This analysis was possible because we used a common database, included precisely defined features of the natural history of these patients.

2. Materials and methods

We included data from patients analyzed in Bonn and Warsaw (Table 1, Table 3) using a collaborative database for diagnostic aspects, clinical features, laboratory results, and family history.

German study: Starting in 1985, patients were recruited from departments of neurology and neuropediatrics, institutes of human genetics and the Deutsche Gesellschaft für Muskelkranke. On receiving written informed consent, the participants answered a detailed questionnaire with respect to the clinical course. Results of neurological examinations were documented for each affected person.

The clinical questionnaire and medical reports provided detailed information for each patient, including age at onset, motor milestones, rate of progression, and loss of functions (Zerres, 1989; Zerres and Rudnik-Schöneborn, 1995).

Polish study: Starting in 1960, patients with different types of infantile, childhood and juvenile onset SMA were recruited in the Department of Neurology, Warsaw Medical School and Muscle Clinic. All patients and their siblings were examined personally by the same group of physicians. Special clinical questionnaires were forwarded to the parents (Hausmanowa-Petrusewicz, 1978; Hausmanowa-Petrusewicz et al., 1992).

The inclusion and exclusion criteria of patients followed those of the International SMA consortium (International SMA Consortium, 1992). In both centers, we included only patients who met the diagnostic criteria for proximal SMA. Medical histories, including electromyographic, histopathologic and biochemical results, were recorded in computerized case report forms and combined with clinical information and pedigree data. Personal information was de-identified by the use of numerical codes to assure confidentiality.

Progression and motor functions were defined as follows: (1) The age at onset was not necessarily that given by the patient or family when asked 'at what age did the disease start?' Onset was defined in this study as the age at which the first abnormalities were obvious, either from medical reports or personal examination or from the de-

Table 2
Clinical features of prognostical relevance in type II SMA patients who were only able to sit versus those who achieved a standing position. 168 patients with complete information of the combined series are included

SMA II group (*n*)	Distal arm weakness [a] in the first 5 years (%)	Breathing difficulties in the first 5 years (%)	Pneumoniae in the first 5 years (%)
Sitting only (127)	33	12	28
Standing achieved (41)	12	0	24

[a] Weakness in the lower arm muscles leading to significant loss of hand mobility.

Table 3
Probability of being able to walk after onset of SMA types IIIa (those who achieved the ability to walk with an onset before the age of 3 years) and IIIb (onset between 3 and 30 years). Data were obtained from Kaplan–Meier survival curves

SMA type	Study	*n*	Sex, M:F	Probability of being ambulatory after a certain duration of disease (%)			
				10	20	30	40 (years)
IIIa	German	75	47:28	70.2	38.7	23.0	23.0
	Polish	120	57:63	70.7	25.8	20.6	20.6
	combined	**195**	**104:91**	**70.3**	**33.5**	**22.0**	**22.0**
IIIb	German	63	43:20	96.7	87.6	73.1	62.1
	Polish	71	46:25	94.1	81.3	66.9	53.6
	combined	**134**	**89:45**	**96.3**	**84.0**	**70.2**	***58.7***

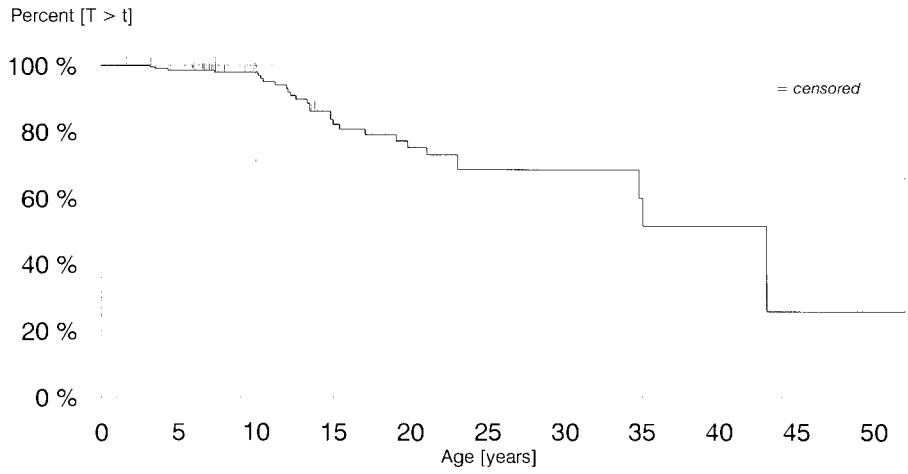

Fig. 1. Kaplan–Meier survival curve for 240 patients with spinal muscular atrophy type II (those who sat but never walked) of the combined study.

scriptions of parents about the first signs of weakness, such as age of motor milestones, loss of functions. (2) The ability to sit was achieved when a patient could maintain a sitting position without support, steadily and with a straight back. The ability to walk was defined as unaided walking. The age at which the patient was no longer ambulatory was defined as the inability to walk 100 m (even with walking aids); at this age, the patients began to use a wheelchair for outdoor activities.

We excluded patients with SMA I, who never sat unaided, and families with affected persons in two generations, indicating autosomal dominant inheritance. Actuarial survival curves were calculated by the Kaplan–Meier method (Matthews and Farewell, 1985).

3. Results

3.1. Type II SMA

Type II SMA is identified before age 18 months because the children do not pass the usual motor milestones. In many cases, the clinical course is marked by periods of apparent arrest. SMA II is defined by ability to sit alone. Other motor functions may vary; some children have early difficulty sitting or rolling over, whereas others learn to crawl or stand with support. None walked unaided; scoliosis and contractures generally developed early in the patients who were wheelchair dependent. Survival probabilities were calculated at different ages of 240 SMA II patients (Table 1, Fig. 1). Differences were seen for loss of function in the hands and pulmonary complications in type II patients who could or could not stand (Table 2). We found no difference in survival of the 'sitting' and the 'standing' groups of SMA II patients (data not shown).

3.2. Type III SMA

The mild form of childhood and juvenile onset SMA shows a wide range of clinical onset from the first year of life to the 3rd decade. Patients with type III SMA learn to walk without help, which distinguishes them from SMA II. Life span is not much reduced in SMA III; the course is characterized by slow progression and often with periods of apparent arrest. Dependent on the degree of weakness, spine deformities and contractures are frequent, mainly in patients who use a wheelchair. Among 329 type III SMA patients, only 10 patients died and the deaths were mainly

Table 4
Probability of being able to walk after onset of SMA III (those who achieved to walk with an onset before the age of 30 years). Data of combined study were obtained from Kaplan–Meier survival curves

Age at onset (months/years)	*n*	Probability of being ambulatory after onset of first symptoms (%)					
		5	10	15	20	25	30 (years)
0–17 mo	123	81.5	62.5	37.9	27.4	22.8	22.8
18–35 mo	72	97.2	83.7	48.9	41.3	30.3	26.0
3–4 y	26	100	89.7	72.4	72.4	72.4	60.3
4–6 y	24	100	100	89.2	89.2	70.1	58.4
6–10 y	25	100	94.7	94.7	74.2	66.8	66.8
10–15 y	34	100	96.6	96.6	96.6	91.7	87.7
15–30 y	25	100	100	94.1	85.6	85.6	57.0

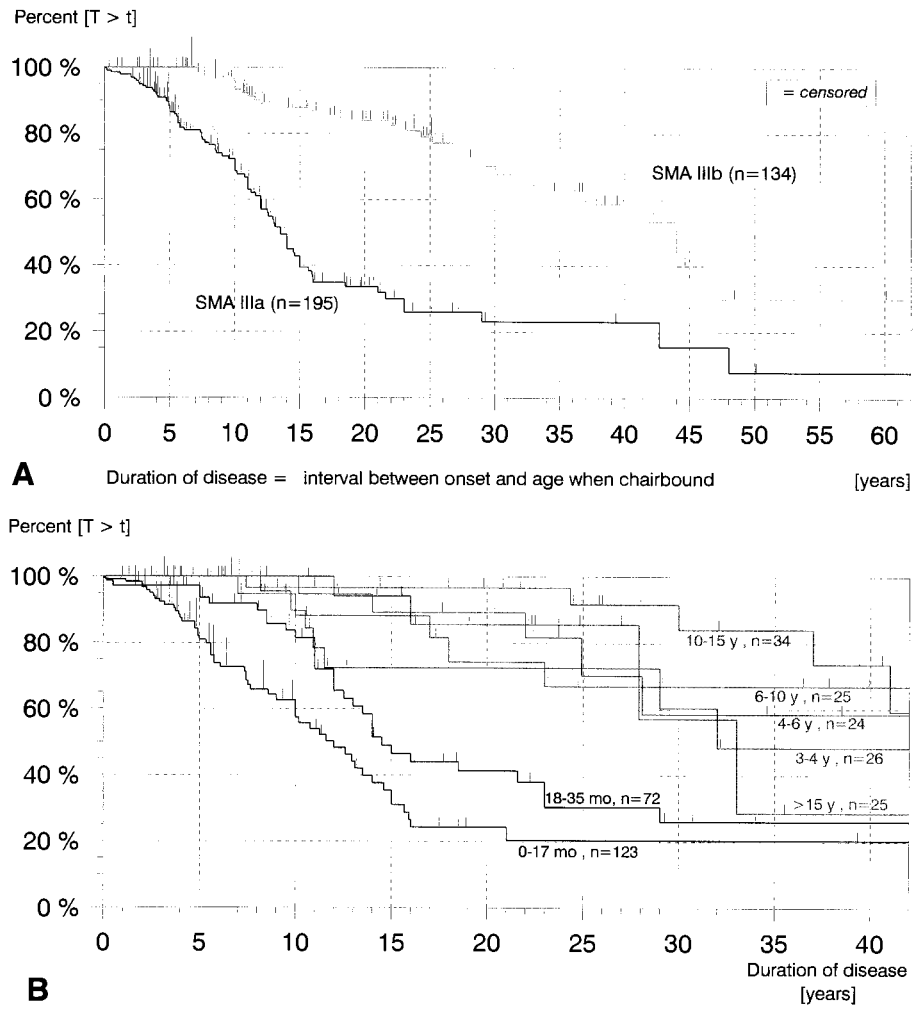

Fig. 2. (a) Probability of still being ambulatory after a certain disease duration (interval between age at onset and last information) in 195 SMA IIIa and 134 SMA IIIb patients (definition see text) of the combined study. Data obtained from Kaplan–Meier statistics. (b) Probability of SMA III patients still being ambulatory after a certain disease duration (interval between age of onset and last information) in 329 patients of the combined study. The slopes represent different age at onset groups (indicated at the right, mo = months, y = years), those corresponding to SMA IIIa are illustrated in black, those belonging to SMA IIIb in grey. Data obtained from Kaplan–Meier statistics.

on unrelated reasons. There was a statistically significant difference in the probability of being able to walk after onset of first symptoms between patients with an onset before 3 years (SMA IIIa) and after the 3rd birthday (SMA IIIb), details are shown in Tables 3 and 4, Fig. 2a, b. In general, the progression of weakness (i.e. the interval between onset and age when using a wheelchair) was correlated with the age at onset; the later the onset, the longer was the ambulant period (Table 4, Fig. 2b). Nevertheless, some patients with onset before age 3 showed little or no progression of weakness and therefore walked for many decades.

4. Discussion

The correlation between onset of SMA and severity has generally been accepted, especially for the most severe infantile SMA (type I) patients who never achieved the ability to sit. Among these, there is a striking difference in the survival curves for those with onset before and after age 2 months (Thomas and Dubowitz, 1994; Ignatius, 1994). Patients with an early onset generally have an earlier age at death and a narrower range of survival. However, in an individual patient, the prediction of life expectancy is not possible on the basis of the age at onset alone for two reasons. First, in contrast to the definition of 'age of death' in many classification systems, life span in many patients with early-onset SMA is often much better than expected (Russman et al., 1992; Zerres and Rudnik-Schöneborn, 1995). Second, in those with severe but stable SMA and a poor respiratory function, occasional events like pneumonia can be life-threatening. Among siblings, the age of death varies among deceased sib pairs with chronic childhood SMA despite a similar 'age at onset' and comparable motor functions (Rudnik-Schöneborn et al., 1994).

Type II SMA patients, who achieved the ability to sit, have a much better prognosis than type I patients who were never able to sit (Zerres and Rudnik-Schöneborn, 1995).

Survival probabilities for the German and Polish series separately are similar with a slightly better prognosis in the German study; the differences, however, were not statistically significant (Table 1). This difference may be due to slightly different observation periods, with an earlier start of collecting patients in the Polish study. Differences in life conditions and easier access to medical care (e.g. antibiotic therapy, ventilatory support) could explain the better prognosis in German patients. The loss of the ability to sit is not a negative prognostic sign for survival in SMA II patients, since life expectancy seems not to be shorted in those who lose the ability to sit than in those who can still sit (Zerres and Rudnik-Schöneborn, 1995). Patients with SMA II who can stand, however, have a better prognosis than those who do not. Those who stand generally do not have breathing impairment and rarely have distal upper limb weakness in the first 5 years of life. Severe pulmonary infections in the first years of life are similar in those who do or do not stand.

In type III SMA (ability to walk achieved) life expectancy is close to a normal population. Whether there is a slight difference in life expectancy between type IIIa and type IIIb cannot yet be answered, more information on the long term prognosis is needed. To determine the natural history of SMA III, we assume that loss of ambulation is important. We therefore calculated the probability of being ambulatory in relation to the duration of symptoms (Fig. 2b). Because of the great clinical heterogeneity in SMA type III, we divided patients into group IIIa (onset before age 3 years) and IIIb (onset between 3 and 30 years) (Fig. 2a). This approach proved be justified by the analysis of patients in different age-at-onset intervals. Among those with onset before and after three years of life, there was a striking difference in the probability of being ambulatory, for reasons still uncertain (Table 3). This applied to the overall group, but a prediction for a single patient is still not possible. There are type IIIa patients with a stable course who continue to walk for decades, as noted in earlier reports from both the Polish and German groups (Hausmanowa-Petrusewicz et al., 1992; Zerres and Rudnik-Schöneborn, 1995).

The combined material provides sufficient data for medical care and genetic counselling of SMA patients and their families. Molecular genetic results do presently not contribute much to explain the phenotypic heterogeneity of SMA. Although deletions in the responsible region on chromosome 5q can now be detected in most patients, no clear correlation has been established between the extent of deletions and the phenotype. Homozygous deletions of the most important candidate gene for infantile SMA, the survival motor neuron (SMN) gene, can be found in more than 90% of patients with autosomal recessive SMA (Lefebvre et al., 1995), whereby identical deletions are present in severe type I SMA and mild type III SMA. Molecular genetic studies in patients from the German series showed a smaller number of SMA III patients with SMN gene deletions (82%) as compared to SMA I (95%) and SMA II patients (94%) (Hahnen et al., 1995), providing evidence that further genetic factors are involved in the pathogenesis of SMA.

The data of this study, as well as those for type I SMA (Zerres and Rudnik-Schöneborn, 1995) show that a rigid classification system with defined age at onset and age of death is still limited, because life span describes only one aspect of the natural history. Others, like periods with preserved motor functions (e.g., intervals between onset and wheelchair), are other important characteristics in the clinical course. Strictly defined ages of onset and death, as used in many classifications should be replaced by the definition of achieved motor functions (ability to sit or walk) in addition to data about probability for survival and preserved motor functions (Tables 1–4). Follow-up information and data from other centers can easily be included into the classification used here.

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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

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Spinal muscular atrophy (SMA) is a common autosomal recessive disorder in humans, caused by homozygous absence of the survival motor neuron gene 1 (SMN1). SMN2, a copy gene, influences the severity of SMA and may be used in somatic gene therapy of patients with SMA in the future. We present a new, fast, and highly reliable quantitative test, based on real-time LightCycler PCR that amplifies either SMN1 or SMN2. The SMN1 copies were determined and validated in 329 carriers and controls. The specificity of the test is 100%, whereas the sensitivity is 96.2%. The quantitative analysis of SMN2 copies in 375 patients with type I, type II, or type III SMA showed a significant correlation between SMN2 copy number and type of SMA as well as duration of survival. Thus, 80% of patients with type I SMA carry one or two SMN2 copies, and 82% of patients with type II SMA carry three SMN2 copies, whereas 96% of patients with type III SMA carry three or four SMN2 copies. Among 113 patients with type I SMA, 9 with one SMN2 copy lived <11 mo, 88/94 with two SMN2 copies lived <21 mo, and 8/10 with three SMN2 copies lived 33–66 mo. On the basis of SMN2 copy number, we calculated the posterior probability that a child with homozygous absence of SMN1 will develop type I, type II, or type III SMA.

Introduction

With an incidence of 1/6,000 to 1/10,000 and a carrier frequency of 1/40 to 1/50, spinal muscular atrophy (SMA) is the second-most-frequent autosomal recessive disease in Europeans (Pearn 1980). It is a neuromuscular disorder caused by the degeneration of α-motor neurons of the spinal cord anterior horns, leading to progressive atrophy of proximal muscles, paralysis, respiratory failure, and infant death. Patients with SMA have been classified into three types, on the basis of age at onset and clinical severity (International SMA Consortium 1992; Zerres and Rudnik-Schöneborn 1995): type I (MIM 253300) is the most severe form, type II (MIM 253550) is the intermediate form, and type III (MIM 253400) is the mildest form.

The SMA-determining gene called the "survival motor neuron" gene (SMN) is present on 5q13 in two copies, SMN1 and SMN2, which differ by only five nucleotide exchanges within their 3′ ends (Lefebvre et al. 1995; Bürglen et al. 1996). Two of these base-pair exchanges, located in exons 7 and 8, allow SMN1 to be distinguished from SMN2 and currently are used for direct diagnosis of SMA (for review, see Scheffer et al. 2001). In a summary of 1,122 patients with type I, type II, or type III SMA, it has been shown that 94% revealed homozygous absence of SMN1 (for review, see Wirth 2000). Approximately half of the remaining patients were identified as compound heterozygotes (with deletions and intragenic SMN1 mutations), whereas the other half, without SMN1 mutations, were considered as distinct genetic entities (Wirth et al. 1999).

Each patient affected with SMA retains at least one SMN2 copy. An inverse correlation between SMN2 protein level and SMA severity has been reported in humans and transgenic SMA mice (Coovert et al. 1997; Lefebvre et al. 1997; Hsieh-Li et al. 2000; Monani et al. 2000). However, SMN2 fails to provide complete protection from SMA due to a single, translationally silent nucleotide difference in exon 7 that disrupts an exonic splicing enhancer and causes exon 7 skipping (Lorson et al. 1999; Monani et al. 1999). It has been shown that SMN2 full-length mRNA can be restored by the splicing factor Htra2-β1 and the nontoxic drug sodium butyrate (Hofmann et al. 2000; Chang et al. 2001). Therefore, prevention of exon 7 skipping by up-regulating the full-length SMN2 mRNA represents an exciting goal for SMA therapy.

Since homozygous absence of exon 7 of SMN1 is a

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Table 1

Reproducibility of Real-Time LightCycler Quantitative Analyses of SMN1 and SMN2, on the Basis of 10 Independent PCRs

Test and Subjects	SMN1/SMN2	No. of Ag1-CA/C212 Marker Alleles	No. of SMN Copies	Minimum/Maximum Average Values	Mean ± SD Values	Coefficient of Variation (%)
SMN1 quantitative test:						
Carrier	+/+	3/3	1	.989/1.141	1.072 ± .044	4.0
Carrier	+/+	3/3	1	.975/1.150	1.060 ± .056	5.2
Control	+/+	3/3	2	1.912/2.276	2.043 ± .105	5.1
Control	+/+	4/4	3	2.974/3.307	3.112 ± .119	3.8
SMN2 quantitative test:						
Type I SMA	del/+	1/1	1	.902/1.132	1.004 ± .083	8.2
Type I SMA	del/+	2/2	2	1.768/2.136	1.918 ± .104	5.4
Type I SMA	del/+	3/3	3	2.784/3.200	2.998 ± .136	4.5
Type III SMA	del/+	4/4	4	3.694/4.233	3.927 ± .172	4.3

common variant, SMA carrier testing based on direct quantitative SMN1 analysis is a major issue. So far, several different SMN quantitative PCR tests have been developed that include multiple steps and complicated procedures that are laborious and subject to errors (Velasco et al. 1996; McAndrew et al. 1997; Taylor et al. 1998; Wirth et al. 1999; Scheffer et al. 2000).

We therefore developed a fast and highly reliable quantitative test for either SMN1 or SMN2, on the basis of real-time PCR on a LightCycler instrument (Roche Diagnostics). This test allowed us for the first time to analyze a large number of patients affected with SMA for their SMN2 copy number and to correlate the SMN2 copy number with type of SMA and duration of survival. An important aim of the study was to examine whether the identification of SMN2 copy number in a patient with SMA could serve as a prognostic tool.

Subjects, Material, and Methods

Carriers and Noncarriers

One hundred twenty-four carriers (parents of multiplex families with SMA) and 65 unaffected sibs of patients with SMA (noncarriers), as determined by haplotype analyses, were used to validate the SMN1 copy number by real-time PCR. Furthermore, we used 140 DNA samples from control individuals (38 healthy spouses of SMA carriers and 102 unrelated unaffected individuals) to determine the rate of SMN1 duplication on one chromosome and the SMA carrier frequency.

Patient Samples

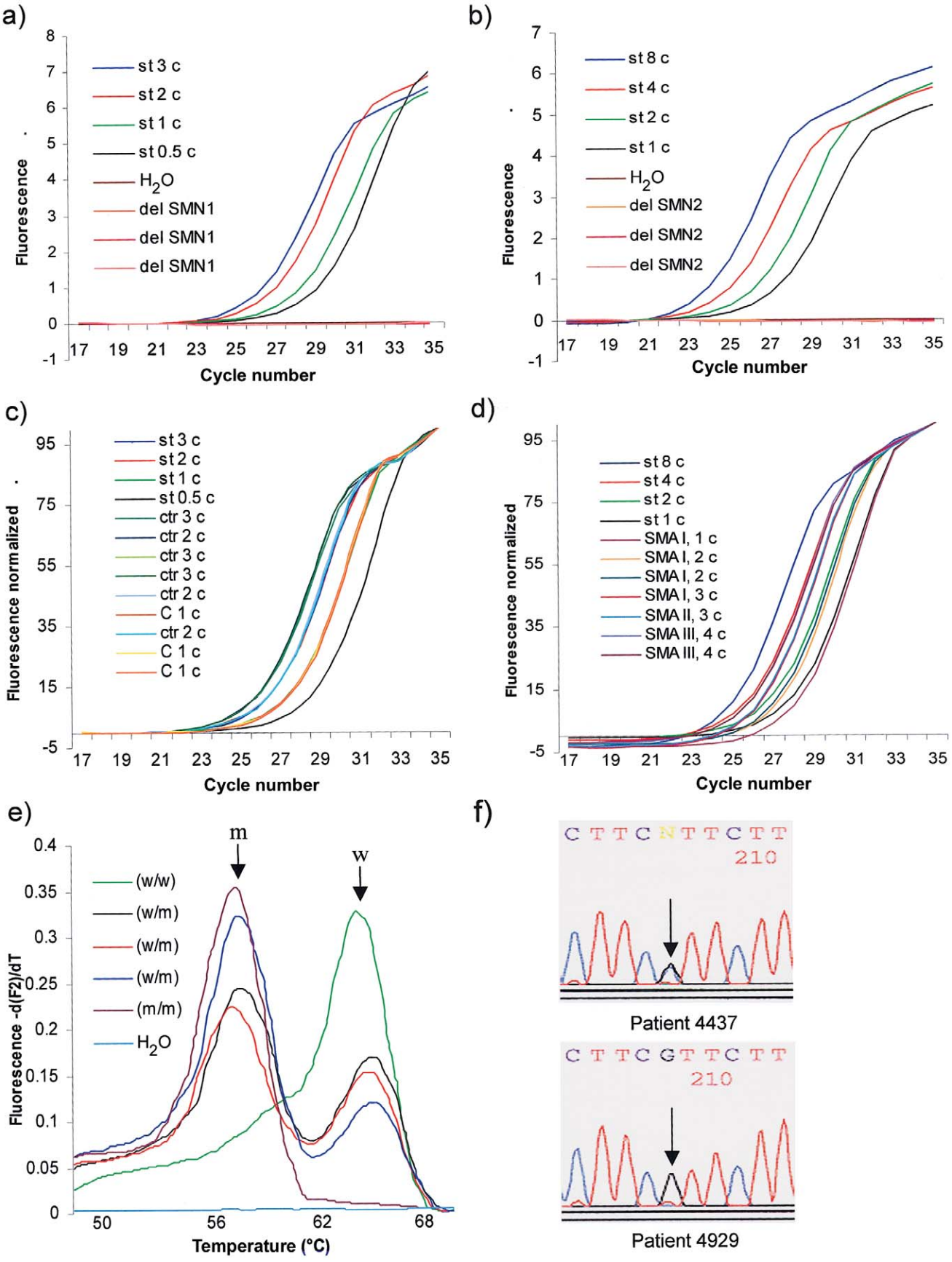
The quantification of the SMN2 copy number was performed on DNA samples from 375 patients with SMA who previously have been classified as follows: 188 have type I SMA, 110 have type II SMA, and 77 have type III SMA (Wirth et al. 1997, 1999). The age of survival was available from 113 patients with type I SMA. All patients fulfilled the diagnostic criteria defined by the International SMA Consortium (1992) and by Zerres and Rudnik-Schöneborn (1995) for proximal spinal muscular atrophy. Informed consent was obtained from all subjects.

DNA Isolation and Quantification and Haplotype Analysis

Genomic DNA was isolated from blood samples by the salting-out method (Miller et al. 1988). DNA was dissolved in TE^{-4} buffer (10 mM Tris/0.1 mM EDTA pH 8) and was stored for at least 5 d before measurement of DNA concentration. Photometric measuring of DNA was performed on a Gene Quant II (Pharmacia). Only samples that had a 260 nm:280 nm ratio in the range 1.75–1.85 were used. DNA was diluted first to 20 ng/μl and then to a final concentration of 5 ng/μl. The exact measurement of the DNA concentration (with maximum deviation of $\pm 3\%$) is the most important prerequisite for reliable determination of the gene copy number. The multicopy polymorphic markers Ag1-CA and C212 were used for haplotype analysis, as described elsewhere (Wirth et al. 1995).

Quantitative Real-Time PCR of SMN1 and SMN2 Copy Numbers

For determination of SMN1 and SMN2 gene dosages, we established a new quantification assay on the basis of real-time PCR. The quantification was performed with a LightCycler instrument (Roche Diagnostics) by use of the fluorescence-resonance energy-transfer technique. Online measurements of PCR products were based on the use of either SYBR Green I or specific hybridization probes labeled with 3′-fluorescein and 5′-LightCycler Red 640. The primers were designed to distinguish between SMN1 and SMN2, ending on or very close to the nucleotide differences between SMN1 and SMN2 (italicized letters) in exon 7 at position 6 and intron 7 at position +214. SMN1


a)
Fluorescence
Cycle number
st 3 c
st 2 c
st 1 c
st 0.5 c
H2O
del SMN1
del SMN1
del SMN1
b)
Fluorescence
Cycle number
st 8 c
st 4 c
st 2 c
st 1 c
H2O
del SMN2
del SMN2
del SMN2
c)
Fluorescence normalized
Cycle number
st 3 c
st 2 c
st 1 c
st 0.5 c
ctr 3 c
ctr 2 c
ctr 3 c
ctr 3 c
ctr 2 c
C 1 c
ctr 2 c
C 1 c
C 1 c
d)
Fluorescence normalized
Cycle number
st 8 c
st 4 c
st 2 c
st 1 c
SMA I, 1 c
SMA I, 2 c
SMA I, 2 c
SMA I, 3 c
SMA II, 3 c
SMA III, 4 c
SMA III, 4 c
e)
Fluorescence -d(F2)/dT
Temperature (°C)
m
w
(w/w)
(w/m)
(w/m)
(w/m)
(m/m)
H2O
f)
C T T C N T T C T T
210
Patient 4437
C T T C G T T C T T
210
Patient 4929

was amplified by use of the forward primer telSMNex7forw (5′-TTTATTTTCCTTACAGGGTTTC-3′) and the reverse primer telSMNint7rev (5′-GTGAAAGTATGTTTCTTCCACg*T*A-3′). SMN2 was amplified by using the forward primer cenSMNex7forw (5′-TTTATTTTCCTTACAGGGTTT*T*A-3′) and the reverse primer cenSMNint7rev (5′-GTGAAAGTATGTTTCTTCCACg*C*A-3′). The hybridization probe detects both SMN1 and SMN2 and consists of a primer pair with a 3′-fluorescein (SMN FL: 5′-AATGCTGGCAGACTTACTCCTTAATTTA FL-3′) and a 5′-LightCycler Red 640 (SMN LC: 5′-LCR640 AATGTGAGCACCTTCCTTCTTTTTG-3′). Lowercase letters indicate mutations introduced to obtain adequate gene-specific PCR.

The PCR was performed in a total volume of 10 μl, containing 7.5 ng (for SMN1) or 6 ng (for SMN2) of genomic DNA, 10 pmol of each primer, 1 μl of Faststart DNA Kit SYBR Green I (Roche Molecular Biochemicals), 4 mM $MgCl_2$ or Faststart Kit hybridization probes (Roche Molecular Biochemicals), 4 mM $MgCl_2$ and 2 pmol of each hybridization probe. The PCR conditions for SMN1 or SMN2 with SYBR Green I were: 95°C for 10 min followed by 35 cycles with 95°C for 15 s, 58°C for 5 s, 72°C for 25 s, and a fluorescent detection step at 76°C for 1 s. During the entire program, we used the maximum temperature transition rate of 20°C/s. This quantification program was followed by a melting program to detect the melting points for every PCR product for each sample. The analysis was performed with the second derivative maximum method of the LightCycler software.

The PCR conditions for SMN1 or SMN2 with hybridization probes were: 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 58°C for 10 s (in this step, the measurement takes place), and 72°C for 20 s. This PCR program is followed by a melting curve program consisting of 15 s of denaturation at 95°C, 30 s of annealing at 55°C, and a melting and continuous measuring step at 0.1°C/s up to 85°C. The analysis was performed with the second derivative maximum method of the LightCycler software.

During the log-linear phase, amplification can be described as $N = N_0(1 + E_{const})^n$ where N is the number of amplified molecules; N_0 is the initial number of molecules; E is the amplification efficiency; and n is the number of cycles. Since the amplification efficiency during the log-phase is constant, the initial concentration of the sample was calculated on the basis of the above formula, by use of human genomic DNA (external standard).

For SMN1, the DNA from a healthy individual with homozygous absence of SMN2 and two Ag1-CA and two C212 alleles was used as an external standard. This individual can be considered to possess two SMN1 copies. We used 1.5 μl of genomic DNA in concentrations of 1.25, 2.5, 5, and 7.5 ng/μl, respectively, corresponding to 0.5, 1, 2, and 3 fictive copies of SMN1.

For SMN2, the DNA of a patient with SMA who had homozygous absence of SMN1 and four Ag1-CA and C212 alleles was used as an external standard. This patient can be considered to possess four SMN2 copies. Genomic DNA (1.2 μl) was used in concentrations of 1.25, 2.5, 5, and 10 ng/μl, respectively, corresponding to one, two, four, and eight copies of SMN2. In addition, for a proper SMN2 standard, five further patients with SMA with three or four SMN2 copies were chosen and were compared to one another by defining each of the DNA samples as standards and the other DNAs as unknown. By means of this procedure, we were able to make sure that the initially postulated copy number was correct. All individuals used as standards and controls have also been tested by competitive multiplex quantitative PCR (Wirth et al. 1999). The standards were used to calculate the regression curve. In each experiment, the reproducibility of the calibration curve was analyzed by evaluation of the slope and the correlation coefficient of the curve. Sample concentrations were inferred on the basis of the regression curve in each experiment. All

Figure 1 Fluorescence versus cycle number, melting peaks, and sequence of new point mutation in SMN2. *a,* SYBR Green I fluorescence plot versus cycle number resulting from amplification of genomic DNA (external standard; st 0.5, st 1, st 2, st 3) of an unaffected individual carrying two SMN1 copies and homozygous absence of SMN2. In addition, four negative controls (three patients with SMA with homozygous absence of SMN1 and one water control) were tested. Slope = −4.112, error = 0.0279, and $r = -1.00$. *b,* SYBR Green I fluorescence plot versus cycle number resulting from amplification of genomic DNA (external standard; st 1, st 2, st 4, st 8) of a patient with SMA with four SMN2 copies and homozygous absence of SMN1. In addition, four negative controls (three normal individuals with homozygous absence of SMN2 and one water control) were analyzed. Slope = −4.057, error = 0.0469, and $r = -1.00$. *c,* Normalized SYBR Green I fluorescence plot of the external standard (st) for SMN1 and samples of carriers (C) and controls (ctr) with one, two, or three SMN1 copies (c). Each copy number was detected in three different DNA samples. Slope = −3.860, error = 0.0213, and $r = -1.00$. *d,* Normalized SYBR Green I fluorescence plot of the external standard (st) for SMN2 and of patients with type I, type II, and type III SMA with one, two, three, or four SMN2 copies. Slope = − 3,819, error = 0.0181, and $r = -1.00$. *e,* Melting-curve analysis of SMN2, with hybridization probe indicating the mutation 892G→C. The DNA of the patient with SMA with the homozygous 892G→C mutation in SMN2 shows a lower melting temperature (m/m, T_m = 58.0°C) than a patient with SMA with the wild-type SMN2 sequence (w/w, T_m = 64.5°C). Patients with SMA with heterozygous genotype (w/m) display two peaks. *f,* New intragenic SMN2 mutation 892G→C in patients with SMA, found by sequencing the DNA samples with melting peaks at 58.0°C. Reverse nucleotide sequences obtained from direct sequencing of genomic PCR products from patients 4437 (heterozygous) and 4929 (homozygous) for the mutation 892G→C; arrows indicate the positions of the mutation.

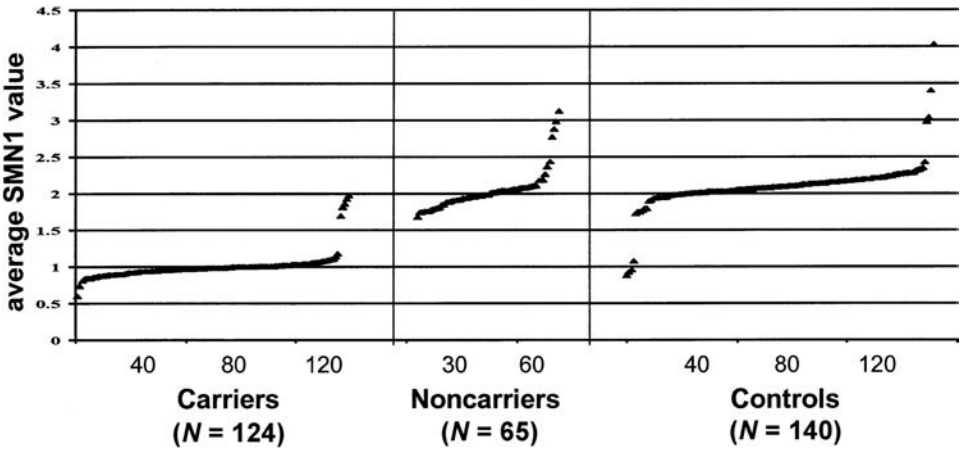


Figure 2 Plot of the average SMN1 values for carriers ($N = 124$), noncarriers (unaffected sibs of patients with SMA) ($N = 65$), and controls ($N = 140$).

samples were measured at least twice, by use of independent DNA dilutions of 5 ng/μl.

Sequencing of Genomic PCR Products

Mutations within the annealing region of the hybridization probe were detected by direct sequencing of genomic PCR products of SMN2 exon 7 of patients 4929 and 4437, amplified by the primers cenSMNex7forw and cenSMNex7rev. Sequencing was performed on an ABI Prism 377, according to the manufacturer's instructions.

Statistical Analysis

A special database in Access (Microsoft) was programmed for collection of all data for patients with SMA and their families (personal data, clinical features, and molecular genetic results, as well as data for direct calculation of mean ± SD LightCycler SMN1 and SMN2 values and coefficient of variation (CV). The SPSS program package was used to construct the Kaplan-Meier curves.

Prior probabilities for type I, type II, and type III SMA were based on cumulative frequencies found in cohorts of patients with SMA (Hausmanova-Petrusewicz et al. 1985). Odds ratios (ORs) were calculated using the SAS/STAT software, specifically the procedures FREQ and LOGISTIC in release 6.11 (SAS Institute 1996), according to the method of Hosmer and Lemesshow (2000). The ORs and their 95% Wald confidence limits were calculated for type I, type II, and type III SMA and for one to four copies of SMN2. The OR for one copy was used as a reference and was fixed to unity. ORs and prior probabilities were used in a Bayesian fashion to calculate posterior probabilities for type I, type II, and type III SMA, depending on the SMN2 copy number.

Results

Establishment of a Real-Time PCR for SMN1 or SMN2 on a LightCycler Instrument

On the basis of real-time LightCycler PCR, we developed a quantitative test that enables a fast and highly reliable differentiation between either one to four SMN1 copies or one to four SMN2 copies. To specifically detect either SMN1 or SMN2, primers were designed that end on or very close to the nucleotide differences between SMN1 and SMN2 in exon 7 at position 6 and intron 7 at position +214. To analyze the gene-specific amplification, DNA from individuals who carry SMN1 only or from patients with SMA who carry SMN2 only were used. The production of gene-specific amplicons (figs. 1*a* and 1*b*) allowed us to use both systems for online fluorescence resonance energy-transfer measurement: SYBR Green I and LightCycler hybridization probes labeled with 3′-fluorescein and 5′-LightCycler red 640. Since the data for both systems were almost identical and the SYBR Green I format is less expensive, we will present only data based on this PCR format.

The reproducibility of the method was determined in 10 independent PCR reactions on 10 different days. For SMN1, we used DNA from carriers with one copy or noncarriers with two or three copies, whereas, for SMN2, we used DNA from patients with type I, type II, and type III SMA with one to four SMN2 copies. The number of SMN1 or SMN2 copies in these individuals were also determined by the previously reported competitive multiplex quantitative PCR (Wirth et al. 1999), by haplotype analysis of the Ag1-CA and C212 marker alleles, and by quantitative analysis of the SMN genes in further relatives in three generations. The newly developed method allows

Table 2

LightCycler Mean Values ± SD and CVs of Both SMN1, Tested in 329 Carriers and Controls, and SMN2, Tested in 375 Patients with Type I, Type II, and Type III SMA

Test and No. of Copies	N[a]	Minimum/Maximum Average Values	Mean ± SD Values	CV (%)
SMN1:				
1	123	.599/1.176	.971 ± .077	7.9
2	198	1.683/2.436	2.053 ± .146	7.1
3	7	2.775/3.410	3.027 ± .200	6.6
4	1	4.035	4.035	...
SMN2:				
1	13	.860/1.390	1.146 ± .162	14.0
2	153	1.555/2.466	1.900 ± .174	9.2
3	166	2.518/3.411	2.866 ± .203	7.1
4	43	3.524/4.245	3.863 ± .183	5.0

[a] No. of individuals.

a clear differentiation between one to four SMN1 or SMN2 copies, without any region of overlap (table 1).

Validity of the Heterozygosity SMN1 Test

This test represents a fast molecular genetic tool for carrier screening both in families with SMA and in the general population, as well as for identification of compound heterozygous patients with SMA. A representative quantitative SMN1 PCR of the external standard and several unknown samples (carriers and controls) is shown in figure 1*c*. To validate the test, we analyzed 124 carriers (parents from multiplex families with SMA) and 65 unaffected sibs of patients with SMA (noncarriers, according to haplotype analysis). The mean value obtained for each person is plotted in figure 2. Maximum and minimum average values, mean values ± SD, and CVs are given in table 2. All but 5 of 124 carriers showed one SMN1 copy, as expected. The remaining five carriers presented two SMN1 copies. Among the unaffected sibs, 61 of 65 presented two SMN1 copies, whereas the remaining 4 carried three SMN1 copies. These data suggest that, in 9 of 254 normal chromosomes (124 from carriers and 130 from unaffected sibs), two SMN1 copies are present on one chromosome. To determine the frequency of two SMN1 copies per chromosome in a larger population, we analyzed 140 additional individuals from the general population (38 spouses of SMA carriers and 102 control individuals without muscle weakness; see fig. 2). Of 140 controls, 4 (2.85%) showed only one SMN1 copy and therefore were identified as SMA carriers. This corresponds to a heterozygosity frequency of 1 in 35. Additionally, we identified, in 3 of 140 controls, three SMN1 copies and, in 1 of 140 controls, four SMN1 copies. In summary, 14 (2.6%) of 530 normal chromosomes carry two SMN1 copies per chromosome (table 3).

Quantitative Analyses of SMN2 Copy Number in Patients with SMA

This fast and highly reliable test allowed us to analyze a large number of patients with SMA for their SMN2 copy number and to correlate these with the type of SMA and duration of survival. We analyzed the SMN2 copy number in 188 patients with type I SMA, 110 patients with type II SMA, and 77 patients with type III SMA with both SYBR Green I and the hybridization probe. In four patients, a discrepancy between the SMN2 copies determined by the two detection systems was observed. In addition, in four patients (three heterozygous and one homozygous), the PCR products detected with hybridization probes showed a different melting curve (fig. 1*e*). Direct sequencing of the SMN2 PCR products in two patients revealed a point mutation at nucleotide position 892, G→C, that causes an amino acid substitution from glycine to arginine at codon 287 (G287R) (fig. 1*f*). The nucleotide exchange is localized within the annealing region of the hybridization probe, thereby explaining the altered melting curves.

The quantitative analysis of the SMN2 copy number in 375 patients with SMA are given in tables 2 and 3. In 80% of patients with type I SMA, one or two SMN2 copies were found, 82% of patients with type II SMA carried three SMN2 copies, and 96% of patients with type III SMA carried three or four SMN2 copies. However, ~20% of patients with type I SMA showed three SMN2 copies, and none showed four SMN2 copies. Sim-

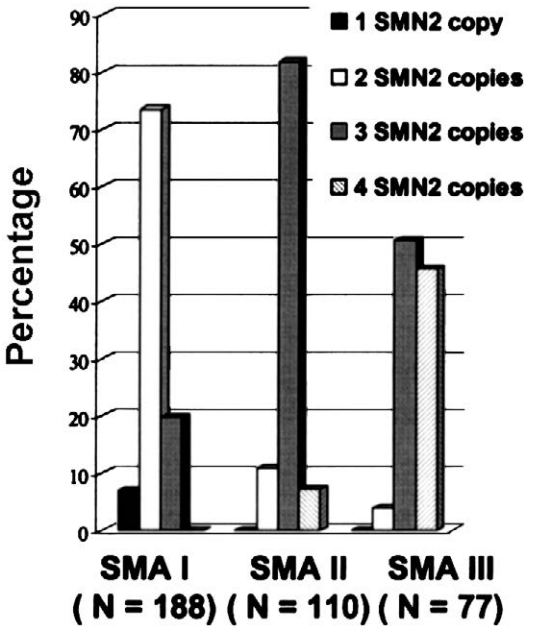


Figure 3 Diagram of the frequency of patients with type I, type II, and type III SMA versus SMN2 copy number.

Table 3

Distribution of Both SMN1 Copies in Carriers, Noncarriers (Unaffected Sibs of Patients with SMA), and Controls and SMN2 Copies in Patients with SMA

Test and Patient Status	Distribution, for SMN Copy Number				Total
	1	2	3	4	
SMN1:					
Carrier	119 (96.0%)	5 (4.0%)	0 (0.0%)	0 (0.0%)	124
Noncarrier	0 (0.0%)	61 (93.8%)	4 (6.2%)	0 (0.0%)	65
Controls	4 (2.9%)	132 (94.3%)	3 (2.1%)	1 (0.7%)	140
Total					329
SMN2:					
Type I SMA	13 (6.9%)	138 (73.4%)	37 (19.7%)	0 (0.0%)	188
Type II SMA	0 (0.0%)	12 (10.9%)	90 (81.8%)	8 (7.3%)	110
Type III SMA	0 (0.0%)	3 (3.9%)	39 (50.6%)	35 (45.5%)	77
Total					375

ilarly, among patients with type II SMA, ~11% showed two SMN2 copies, and ~7% showed four SMN2 copies, whereas, among patients with type III SMA, none showed one SMN2 copy, and ~4% revealed two SMN2 copies (fig. 3).

A strong correlation between SMN2 copy number and SMA phenotype was observed; however, this correlation is not absolute. This may be because of (1) true variation caused by modifying genes or other external factors, (2) intragenic mutations within SMN2, or (3) SMN2 genes partially deleted or duplicated as a result of deletions or gene conversions involving either the 5′ end or the 3′ end of the SMN genes. To verify the first hypothesis, we analyzed 13 families with SMA that had siblings carrying homozygous mutations of SMN1 but discordant phenotypes (Helmken et al. 2000*a*, 2000*b*). In each case, identical numbers of SMN2 copies were determined in haploidentical siblings. Although, in all these families, the unaffected or milder affected siblings were females, there is no overall significant difference in the distribution of SMN2 copies between sexes (table 4). For the second hypothesis, we already presented in this article an intragenic SMN2 mutation in exon 7 (G278R), revealing that intragenic mutations may indeed be a reason that can disturb the correlation between SMN2 copy number and SMA phenotype. To verify the third hypothesis, we analyzed, in our patients with SMA Ag1-CA and C212—two highly polymorphic markers localized at the 5′ end of the SMN genes (Wirth et al. 1995)—and correlated the total number of marker alleles in each individual with the SMN2 copy number (table 5). In 80% of patients with type I SMA, 77% of patients with type II SMA, and 64% of patients with type III SMA, identical numbers of Ag1-CA alleles and SMN2 copies were found, whereas C212 alleles were identical with SMN2 copies in 75% of patients with type I SMA, 62% of patients with type II SMA, and 63% of patients with type III SMA, respectively. As expected, Ag1-CA showed a slightly higher correlation, on the basis of its closer proximity to the 5′ end of the SMN gene, than C212. In some cases, false interpretation of the autoradiographies (duplicated alleles are sometimes not easy to recognize) may be the cause of the discordant data. However, these data also suggest that, at least in part, incomplete deletions or duplications of SMN2 copies may be responsible for the discrepancies seen in SMN2 copy number versus Ag1-CA/C212 alleles.

Furthermore, we checked for a correlation between number of SMN2 copies and age of survival in 113 patients with type I SMA (fig. 4*a*). In 9 of 113 patients with type I SMA, one SMN2 copy was found with a median age of survival of 7 mo; none survived longer than 11 mo. In 94 of 113 patients with type I SMA, two SMN2 copies were found, with a median age of survival of 8 mo. All but six patients with two SMN2 copies died before age 21 mo. In 10 of 113 patients, three SMN2 copies were determined, with a median age of survival of 37.5 mo; 8 of these 10 patients lived 33–66

Table 4

Distribution of SMN2 Copy Number in Females and Males of Patients with Type I, Type II, and Type III SMA

Sex and Disease	Distribution for SMN2 Copy Number				Total
	1	2	3	4	
Female:					
Type I SMA	7	66	13	0	86
Type II SMA	0	11[a]	38	1	50
Type III SMA	0	1	15	18	34
Male:					
Type I SMA	6	72	24	0	102
Type II SMA	0	1[a]	52	7	60
Type III SMA	0	2	24	17	43

[a] Significant difference between females and males: ORs (95% Wald confidence intervals) are, for females, 0.085 (0.037, 0.197) and, for males, 0.007 (0.001, 0.053).

Table 5

Correlation between SMN2 Copy Number and Ag1-CA and C212 Marker Alleles

Polymorphic Marker, Type of SMA, and SMN2 Copy Number	No. of Marker Alleles[a]					% of Patients[b]
	1	2	3	4	5	
Ag1-CA:						
Type I SMA:						
1	**1** / 3	10	2			
2	3	**114**	9	5		81
3	1	9	**27**			
Type II SMA:						
2		**7**	4	1		
3		11	**77**	1	1	77
4		1	6	**1**		
Type III SMA:						
2		**2**	1			
3		3	**28**	7		64
4		5	11	**19**		
C212:						
Type I SMA:						
1	**1** / 8	10	2			
2		**108**	11	4		75
3	1	8	**27**	1		
Type II SMA:						
2	1	**6**	4	1		
3		26	**60**	2		62
4		2	5	**1**		
Type III SMA:						
2		**2**	1			
3		6	**28**	4		63
4		4	13	**18**		

[a] Underlined, boldfaced numbers represent those values where identical SMN2 copy number versus Ag1-CA/C212 alleles were found.

[b] Percentage of patients with SMA with identical SMN2 copies and Ag1-CA/C212 alleles.

mo, and 6 of those 8 also revealed three Ag1-CA alleles, indicating that these patients with type I SMA owe their increased survival to the increased number of SMN2 copies. On the basis of Kaplan-Meier survival curves, we calculated the probability of survival for patients with type I SMA in accordance with their SMN2 copy number (fig. 4*b*).

Prediction of SMA Severity

Finally, we calculated the posterior probability of a child with homozygous absence of SMN1 developing type I, type II, or type III SMA, conditional on the number of SMN2 copies (table 6). A child with one SMN2 copy has a risk of >99%—and, with two SMN2 copies, a risk of 97%—of developing type I SMA. A child with three SMN2 copies has a risk of 82.8% of developing type II SMA, and a child with four SMN2 copies has a risk of 83.6% of developing type III SMA. These data demonstrate nicely that SMN2 copy number can be used as a prognostic tool.

Discussion

Here, we present the first quantitative test of SMN1 or SMN2 based on real-time PCR by use of the LightCycler Instrument. The possibility of direct measurement of the PCR product accumulated after each cycle, without any intermediate steps, ensures the high specificity of real-time PCR assays. We succeeded in developing the test so that gene-specific primers amplify only SMN1 but not SMN2 (or vice versa).

SMN1 dosage analysis is frequently requested in the context of genetic counseling, either as carrier testing or as a means of identifying patients with compound mutations (deletions and intragenic SMN1 mutation). Unfortunately, the presence of two SMN1 copies per chromosome or intragenic mutations in some of the SMA chromosomes slightly diminishes the sensitivity of the test (McAndrew et al. 1997; Wirth et al. 1999; Scheffer et al. 2000; present study). On the basis of all reported data, including this one, in 20/834 (2.4%) healthy chromosomes, two SMN1 copies were found. Consequently, 4.8% of normal individuals would be misinterpreted as noncarriers on the basis of the direct SMN1 test. The sensitivity of the test is 95.2%. In addition, 1.7% of SMA carriers reveal intragenic SMN1 mutations—given that 3.4% of patients with SMA show compound SMN1 mutations, deletion plus intragenic mutation (Wirth et al. 1999)—and would not be recognized by the SMN1 quantitative test. This reduces the sensitivity of the test to 93.5% for a person from the general population.

The quantification of SMN2 copies performed in this paper in 375 patients with type I, type II, and type III SMA demonstrates a significant correlation between SMN2 copy number and type of SMA, as well as duration of survival. Approximately 80% of patients with type I SMA showed only one or two SMN2 copies, suggesting that these patients mainly carry genuine deletions of SMN1, whereas SMN2 is present either on one chromosome (genotype 0:1 or 0:2) or on both chromosomes (genotype 1:1). Furthermore, for patients with type I SMA, we were able to show an inverse correlation between SMN2 copy number and duration of survival; patients with one SMN2 copy had a median survival of 7 mo, whereas those with two or three SMN2 copies survived 8 and 37.5 mo, respectively. Although, in the first months, the survival curves are relatively similar, the probability of survival for type I patients with one SMN2 copy is significantly lower than that for patients with two SMN2 copies (fig. 4*b*). The median age of survival for patients with one SMN2 copy might even

be biased, given the inclusion criteria for the quantitative analysis. We considered only those patients from whom DNA was isolated by the same method. Patients who died soon after birth had to be excluded sometimes, since only small amounts of DNA or DNA from muscle biopsies were available.

Our results of SMN2 copy number also fit well with our previously reported SMA model (Wirth et al. 1995), where we hypothesized that patients with type I SMA carry two "severe" SMA chromosomes and that patients with type II SMA carry one "severe" and one "mild" SMA chromosome, whereas patients with type III SMA carry two "mild" SMA chromosomes. In ~80% of patients with type I SMA, we identified one or two SMN2 copies, corresponding to deletions of SMN1 on both chromosomes. About 80% of patients with type II SMA carry three SMN2 copies, corresponding to a deletion of SMN1 on one chromosome and a gene conversion of SMN1 in SMN2 on the second chromosome, whereas ~45% of patients with type III SMA showed four SMN2 copies, corresponding to gene conversion on both chromosomes.

In previous work, Burghes (1997) reported a quantitative analysis of SMN2 copies in 21 patients with type I SMA, 38.1%, 42.9%, and 19% of whom had SMN2 copy numbers of one, two, and three, respectively, and in 58 patients with type II and type III SMA, 12%, 48.3%, 34.5%, and 5.2% of whom had SMN2 copy numbers of one, two, three, and four, respectively.

Table 6

Probabilities That an Unaffected Child Who Has Been Tested after Birth and Has Been Found to Carry a Homozygous Absence of SMN1 Will Develop Type I, II, or III SMA, on the Basis of Number of SMN2 Copies

Probability Type and No. of SMN2 Copies	Probability of Developing SMA		
	Type I	Type II	Type III
Prior probabilities	.51	.32	.17
Conditional probabilities (OR):[a]			
One copy	1.00	<.001	<.001
Two copies	1.00	.044	.0145
Three copies	1.00	18.4	4.19
Four copies	1.00	14.7	156.7
Joint probabilities:			
One copy	.51	<.00032	<.00017
Two copies	.51	.0141	.000255
Three copies	.51	5.89	.712
Four copies	.51	4.70	26.6
Posterior probabilities (%):			
One copy	>99.9	<.1	<.1
Two copies	97.26	2.7	.04
Three copies	7.2	82.8	10.0
Four copies	1.6	14.8	83.6

[a] The OR for one copy was used as a reference and was fixed to unity.

Since the number of patients with type I SMA is small and the numbers of patients with type II and type III SMA are not listed separately, it is difficult to compare the data. Similarly, Taylor et al. (1998) performed a

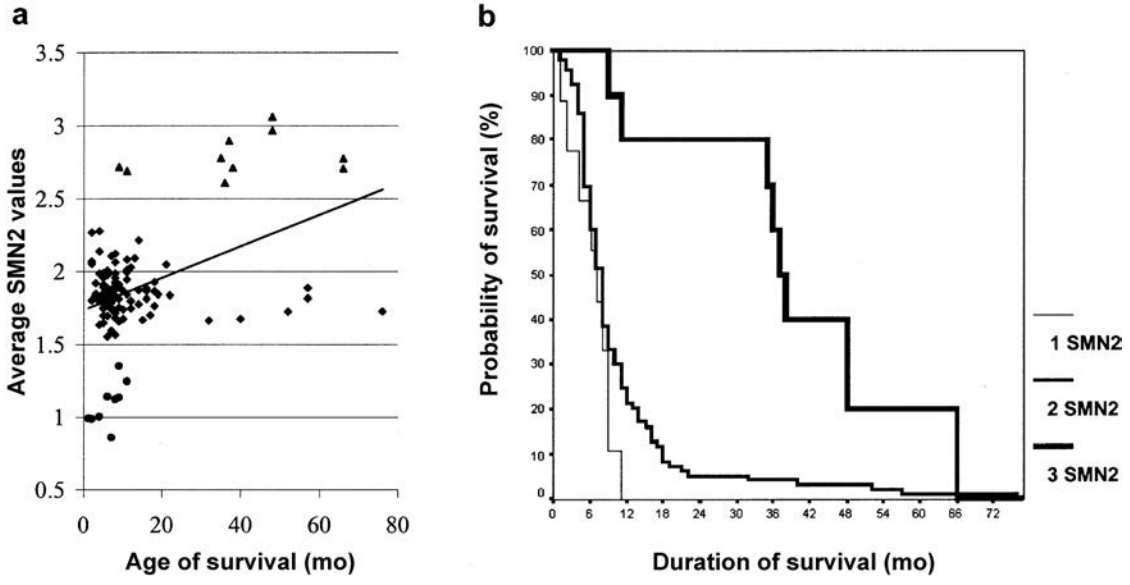


Figure 4 *a,* Age of survival of children with type I SMA, correlated with the average SMN2 value. Circles correspond to one SMN2 copy, squares to two SMN2 copies, and triangles to three SMN2 copies. The line represents a trendline for the age of survival versus the SMN2 copy number. *b,* Kaplan-Meier curves show the probabilities of survival for patients with type I SMA who carry one, two, or three SMN2 copies.

quantitative analysis of SMN2 in 26 patients with type I SMA and 23 patients with type II or type III SMA and showed a significant correlation between lower ratios for SMN2 versus MPZ (control gene) in type I and between higher ratios in type II and III. However, the authors stated that they were unable to assess exact copy number from those ratios.

The impressive progress achieved in the last few years in the understanding of the molecular basis of SMA and the recent identification of drugs that increase full-length SMN2 mRNA open the possibility of a therapy for patients with SMA in the near future. Quantification of the SMN2 copies in patients with SMA will be an essential prerequisite of therapy. Clinicians could start therapy and adapt drug treatment depending on the SMN2 copies before motor neurons are affected and children show first symptoms.

Our data show that, although various factors—such as modifying genes, external factors, intragenic SMN2 mutations, and incomplete SMN2 copies—can slightly influence the SMA phenotype, the risk calculation for a child with homozygous absence of SMN1 to develop a type I, II or III SMA can be reliably predicted. This study will open new diagnostic possibilities for routine laboratories and will be essential in clinical trials in patients with SMA.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for type I SMA [Werdnig-Hoffmann disease; MIM 253300], type II SMA [MIM 253550], and type III SMA [Kugelberg-Welander disease; MIM 253400])

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The changing natural history of spinal muscular atrophy type 1

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The changing natural history of spinal muscular atrophy type 1

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ABSTRACT

Background: Noninvasive ventilation has become increasingly available to spinal muscular atrophy (SMA) patients since the early 1990s. This is expected to have improved survival for SMA type 1 patients.

Objective: To assess whether there has been a change in survival in patients with SMA type 1 between 1980 and 2006.

Methods: We used deidentified, family-reported data from participants in the International Spinal Muscular Atrophy Patient Registry and obtained additional clinical information through a mail-in questionnaire. One hundred forty-three patients with SMA type 1 were included in the analysis. Survival of patients born in 1995–2006 (n = 78) was compared with that of patients born in 1980–1994 (n = 65), using the Kaplan–Meier method and Cox proportional hazards models with age at death as the outcome.

Results: Patients born in 1995 though 2006 had significantly increased survival compared with those born in 1980–1994 (log-rank test, $p < 0.001$). In a Cox model, patients born in 1995–2006 had a 70% reduction in the risk of death compared with those born in 1980–1994 (hazard ratio [HR] 0.3, 95% CI 0.2–0.5, $p < 0.001$) over a mean follow-up of 49.9 months (SD 61.1, median 22.0). However, when controlling for demographic and clinical care variables, year of birth was no longer significantly associated with age at death (HR 1.0, 95% CI 0.6–1.8, $p = 0.9$), whereas ventilation for more than 16 h/d, use of a mechanical insufflation–exsufflation device, and gastrostomy tube feeding showed a significant effect in reducing the risk of death.

Conclusion: Survival in spinal muscular atrophy type 1 patients has increased in recent years, in relation to the growing trend toward more proactive clinical care.

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GLOSSARY

BiPAP = bilevel positive airway pressure; **HR** = hazard ratio; **MI-E** = mechanical insufflation–exsufflation; **SMA** = spinal muscular atrophy; **YOB** = year of birth.

Spinal muscular atrophy (SMA) is one of the most common and devastating single gene disorders in childhood, with an incidence of approximately 1 in 6,700.[1] It is an autosomal recessive motor neuron disease caused by a homozygous deletion of the survival motor neuron gene on chromosome 5q13. Further classification of clinical subtypes is based on age at onset and highest motor milestone achieved. There is an association between age at onset and disease severity.[2] SMA type 1, or Werdnig–Hoffman disease, is the most severe subtype of SMA with age at onset before age 6 months. These children never acquire the ability to sit independently, and the majority were traditionally thought to die before their second birthday.[3,4]

In recent years, more proactive management, including pulmonary and nutritional interventions, has been incorporated into clinical practice.[5] Noninvasive pulmonary sup-

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port has been increasingly used since its introduction in the early 1990s, along with gastrostomy tube feeding that protects against aspiration and maintains adequate nutritional intake.[6] These advances in management may have had an impact on the natural history of SMA. There is, however, a paucity of studies providing survival data for SMA patients in the past 10 years, and many of the available studies do not include a North American population. Assessment of the possible impact of recent changes in care on the survival of SMA patients is important for clinical care, counseling, and clinical trials. The objective of this study was to assess whether there has been a change in survival in patients with SMA type 1 between 1980 and 2006. Specifically, we hypothesized that survival of recently born children with SMA type 1 has improved, and that this change is related to more proactive clinical care.

Table 1 Demographic and clinical characteristics of spinal muscular atrophy type 1 patients who remained alive and who died as of the censoring date

Variable	Alive (n = 63)	Deceased (n = 80)	Total (n = 143)
Male, %	47.6	52.5	50.3
Continent, %*			
North America	77.8	95.0	87.4
Other	22.2	5.0	12.6
Ethnicity, %			
White	84.1	82.5	83.2
African American	0.0	6.3	3.5
Hispanic	3.2	1.3	2.1
Other	12.7	10.0	11.2
Age at symptom onset, mean (SD), mo*	3.3 (1.7)	2.0 (1.5)	2.5 (1.7)
Age at diagnosis, mean (SD), mo*	6.1 (2.6)	3.4 (2.5)	4.6 (2.9)
Genetic testing, %*	92.1	53.8	70.9
Tracheostomy, %*	44.4	13.8	27.3
Ventilation, %*†	92.1	32.5	58.7
Ventilation > 16 h/d, %*†	52.4	18.8	33.6
MI-E device, %*	68.3	13.8	37.8
Gastrostomy tube feeding, %*	90.5	37.5	60.8
Amino acid diet, %*	56.5	10.0	30.3
YOB 1995–2006, %*	79.4	35.0	54.5

Total n: 141 for age at symptom onset, 142 for age at diagnosis, 141 for genetic testing, and 142 for amino acid diet.
* $p < 0.05$ for the comparison deceased vs alive.
† Includes noninvasive (e.g., bilevel positive airway pressure) and invasive ventilation.
MI-E = mechanical insufflation-exsufflation; YOB = year of birth.

METHODS **Patients and procedure.** Data were obtained from the International Spinal Muscular Atrophy Patient Registry, established in 1986, which is coordinated by the Department of Medical and Molecular Genetics at Indiana University and funded by the lay organization Families of SMA. Recruitment methods for the registry include Web sites (Indiana University and links to Web sites of lay organizations) and pamphlets. The registry prospectively collects data through voluntary participation of patients and their families who provide initial and annual follow-up information. The registry collects information on the patient's medical history, family history, and survival status. Information on socioeconomic status and details of genetic testing results have not been obtained. The registry questions are easily understood and are answered by a family member knowledgeable about the patient's medical condition or the patient himself/herself. All data entry is duplicated and cross checked, and data are queried for inconsistency or invalid entries. When a query cannot be resolved based on information provided in the questionnaire, staff from the Indiana Registry contact the family for clarification. To conform to the Health Insurance Portability and Privacy Act, all information provided by the Indiana Registry for this study was deidentified.

In addition to the demographic, clinical, and survival status information prospectively collected on all registry participants, a one-page retrospective questionnaire was developed to obtain more detailed clinical information, including relevant clinical care interventions, and to update the survival data obtained through the registry. Before sending the questionnaire to participants in this study, the questions were reviewed by SMA parents and improvements to clarity and language were made according to parent feedback. Questionnaires were mailed by registry staff to parents of all patients registered as having SMA type 1. The questionnaires were returned to the registry, where the information was deidentified before being sent for analysis. Information collected included SMA type (1, 2, or 3, based on highest motor milestone achieved), date of birth and death (month and year), age at symptom onset, age at diagnosis, genetic confirmation, tracheostomy, use of noninvasive [e.g., bilevel positive airway pressure (BiPAP)] or invasive ventilation (age started, number of hours used, age ventilation was used for more than 16 h/d), use of a mechanical insufflation–exsufflation (MI-E) device, gastrostomy tube feeding, and use of a "special amino acid diet." This diet has been advocated by parents who suggested this term to describe a diet based on elemental amino acids.[7]

Only patients with SMA type 1 were included in the analysis, defined as those with onset before age 6 months and who were never able to sit unsupported. The questionnaire was mailed to 391 registry patients who were reported as having SMA type 1. One hundred ninety-two questionnaires were returned, resulting in a 49.1% response rate. Of these 192 patients, 151 patients met the definition of SMA type 1; 9 patients had onset after age 6 months, and 32 patients did not meet the definition of SMA type 1 based on highest motor milestone achieved. One additional patient was excluded because no ventilatory support was used until age 21 years, making an SMA type 1 diagnosis clinically implausible. The remaining 150 patients included two pairs of twins, three

other pairs of siblings, and a set of three siblings. For each set of siblings, we retained the sibling first enrolled in the registry and excluded the other seven siblings from the analysis. Thus, the analysis included 143 SMA type 1 patients.

The protocol for this study was approved by the Columbia University Medical Center Institutional Review Board, the Indiana University Institutional Review Board, and the International Spinal Muscular Atrophy Patient Registry Oversight Committee.

Data analysis. Survival data were censored on September 1, 2006. Given that BiPAP was increasingly used after its introduction in the early 1990s, we dichotomized the sample by year of birth in patients born in 1980–1994 and those born in 1995–2006. This resulted in two groups of similar size (65 vs 78 patients). We compared demographic and clinical characteristics of SMA patients according to alive/deceased status as of the censoring date and according to year of birth using Student's t test for continuous variables and χ^2 test or Fisher exact test for categorical variables.

The Kaplan–Meier method was used to calculate survival probabilities and plot survival curves of patients born in 1980–1994 and those born in 1995–2006, and the log-rank test was used to compare survival in the two groups. These analyses were conducted using two outcomes: 1) age at death and 2) either age at death or age at ventilation for more than 16 h/d (includes noninvasive ventilation [e.g., BiPAP] and invasive ventilation). In each case, age as of the censoring date (September 1, 2006) was used for patients who did not experience the respective events, i.e., death and either death or ventilation for more than 16 h/d. In addition, Cox proportional hazards models were used to evaluate the association of year of birth (1995–2006 vs 1980–1994) with age at death. We first included only year of birth as a predictor variable in the model, and then repeated the analysis controlling for demographic and clinical care variables that were significantly different when comparing patients according to alive/deceased status and year of birth. A two-sided 0.05 level of significance was used in all analyses.

Table 2 Demographic and clinical characteristics of spinal muscular atrophy type 1 patients born in 1980–1994 and born in 1995–2006

Variable	YOB 1980–1994 (n = 65)	YOB 1995–2006 (n = 78)
Male, %	52.3	48.7
Continent, %*		
North America	96.9	79.5
Other	3.1	20.5
Ethnicity, %		
White	84.6	82.1
African American	3.1	3.8
Hispanic	1.5	2.6
Other	10.8	11.5
Age at symptom onset, mean (SD), mo*	2.2 (1.7)	2.8 (1.7)
Age at diagnosis, mean (SD), mo*	3.9 (2.9)	5.1 (2.7)
Genetic testing, %*	44.4	92.3
Tracheostomy, %	24.6	29.5
Ventilation, %*†	30.8	82.1
Ventilation > 16 h/d, %*†	21.5	43.6
MI-E device, %*	7.7	62.8
Gastrostomy tube feeding, %*	40.0	78.2
Amino acid diet, %*	7.7	49.4
Deceased, %*	80.0	35.9

Total n: 141 for age at symptom onset, 142 for age at diagnosis, 141 for genetic testing, and 142 for amino acid diet.
* $p < 0.05$ for the comparison year of birth (YOB) 1995–2006 vs YOB 1980–1994.
† Includes noninvasive (e.g., bilevel positive airway pressure) and invasive ventilation.
MI-E = mechanical insufflation-exsufflation.

RESULTS Patients whose parents did not respond to the mail-in questionnaire (n = 199) were not significantly different from patients whose parents responded to the questionnaire (n = 192) in sex, ethnicity, year of birth (1980–1994 vs 1995–2006), age at diagnosis, and proportion with genetic testing. Patients whose parents did not respond to the questionnaire were more commonly from continents other than North America (24.6% vs 16.1%, $p = 0.04$) than patients whose parents responded to the questionnaire.

The demographic and clinical characteristics of the 143 patients who met inclusion criteria are summarized in table 1. Of the 143 patients, 38 (26.6%) were deceased at the time of enrollment into the registry (47.7% among patients born in 1980–1994 and 9.0% among those born in 1995–2006). Eighty patients (55.9%) died before the censoring date, September 1, 2006 (mean age at death 20.2 months, SD 35.4, median 8.0, range 1.0–193.5). Patients who remained alive as of the censoring date had significantly higher age at onset and age at diagnosis, were significantly less often from North America, and were significantly more likely to have had respiratory or nutritional interventions than those who died (table 1). Similarly, when comparing patients according to year of birth, those born in 1995–2006 had significantly higher age at onset and age at diagnosis, were significantly less often from North America, and were significantly more likely to have had respiratory or nutritional interventions than those born in 1980–1994 (table 2). Among patients born in 1980–1994, 52 (80.0%) had died as of the censoring date (mean age at death 19.1 month, SD 38.7, median 7.3, range 1.0–193.5), and among those born in 1995–2006, 28 (35.9%) had died as of the censoring date (mean age at death 22.1 month, SD 28.6, median 10.0, range 2.5–112.0).

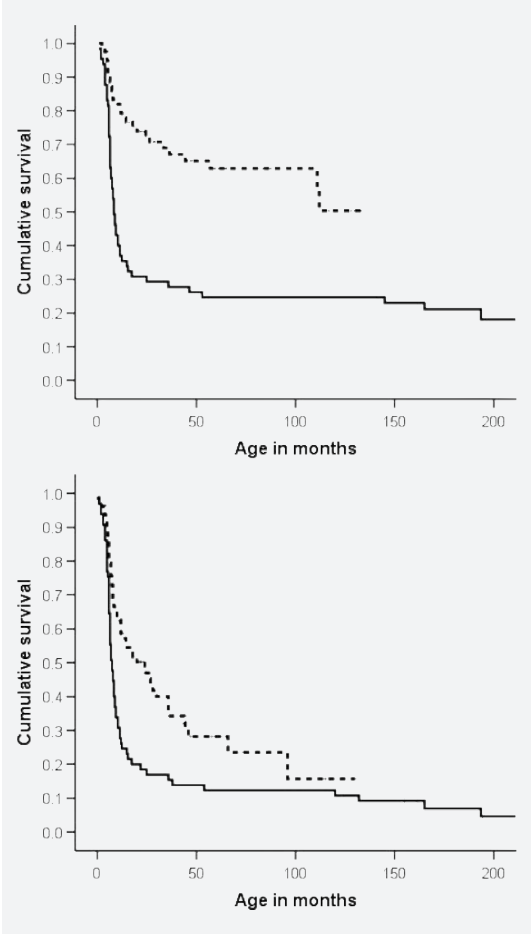
Survival plots are shown in the figure. When using death as the event, there is a significant difference in survival between patients born in 1980–1994 and those born in 1995–2006 (log-rank test statistic 22.9, $p < 0.001$). Median survival time was 8.5 months for patients born in 1980–1994 and indeterminate for those born in 1995–

Table 3 Survival probabilities (%)* at different ages (in months) of spinal muscular atrophy type 1 patients born in 1980–1994 and born in 1995–2006, for events death and death or ventilation† for more than 16 h/d

Event, by YOB	12 mo	24 mo	48 mo	120 mo	240 mo
Death					
YOB 1995–2006	79.3	73.9	65.1	50.3	—
YOB 1980–1994	36.9	30.8	26.2	24.6	18.1
Death or ventilation > 16 h/d					
YOB 1995–2006	58.6	47.0	28.2	15.7	—
YOB 1980–1994	26.2	18.5	13.8	10.8	4.6

* Kaplan–Meier method.
† Includes noninvasive (e.g., bilevel positive airway pressure) and invasive ventilation.
YOB = year of birth.

Figure Kaplan–Meier survival plots of spinal muscular atrophy type 1 patients born in 1995–2006 (dashed line) compared with those born in 1980–1994 (solid line), for events death (top panel) and death or ventilation for more than 16 h/d (bottom panel)



2006. The survival plot using death or ventilation for more than 16 h/d as the event shows a smaller difference between the two groups, but the difference remained significant (log-rank test statistic 11.2, $p = 0.001$). Median survival time was 7.5 months for patients born in 1980–1994 and 24.0 months for those born in 1995–2006. Survival probabilities for both events by year of birth are provided in table 3.

In a Cox proportional hazards model including only year of birth as a predictor variable, patients born in 1995–2006 had a 70% reduction in the risk of death compared with those born in 1980–1994 (hazard ratio [HR] 0.3, 95% CI 0.2–0.5, $p < 0.001$) over a mean follow-up of 49.9 months (SD 61.1, median 22.0). However, in a model controlling for demographic and clinical care variables, year of birth was no longer significantly associated with age at death (HR 1.0, 95% CI 0.6–1.8, $p = 0.9$). Whereas ventilation for more than 16 h/d, use of MI-E device, and gastrostomy tube feeding showed a significant effect in reducing the risk of death, the amino acid diet did not reach significance (table 4). When the analysis was repeated including only patients with genetic confirmation (n = 100), results were similar except for the amino acid diet (HR 0.6, 95% CI 0.2–1.6, $p = 0.3$).

DISCUSSION This study shows that survival of children with SMA type 1 born in 1995–2006 has increased compared with those born in 1980–1994. Although to a substantially smaller degree, this was also observed for survival time free of respiratory insufficiency (analysis using death or ventilation for more than 16 h/d as the event), suggesting that chronic ventilation accounts for part but not all of the recent improvement in survival. Further analyses indicated that ventilation for more than 16 h/d, use of MI-E device, and gastrostomy tube feeding had independent effects on survival and accounted for the observed effect of year of birth (1995–2006 vs 1980–1994) on survival.

Fifty percent of patients born in 1995–2006 have followed a special amino acid diet. This diet has become increasingly popular in recent years among parents of children with SMA, but its efficacy has not been studied. In our study, the amino acid diet had no significant effect on survival in the analysis including all patients, and even less so when the analysis was limited to those patients with genetic confirmation.

Previous studies providing survival data on SMA type 1 patients are summarized in table 5. Comparisons with our study are limited by methodologic differences. The studies before 1995 have reported a mean age at death of 8.8 to 10 months and outliers surviving up to 10 years.[8-10] In one study published in 1995, survival probabil-

Table 4 Lack of association of year of birth (1980–1994 vs 1995–2006) with age at death in spinal muscular atrophy type 1 patients, when controlling for demographic and clinical care variables*

Variable	HR (95% CI)	p Value
Age at symptom onset, mo	0.6 (0.5–0.7)	<0.001
Ventilation > 16 h/d†	0.3 (0.1–0.6)	0.002
MI-E device	0.2 (0.1–0.5)	0.001
Gastrostomy tube feeding	0.5 (0.3–1.0)	0.04
Amino acid diet	0.4 (0.2–1.1)	0.06
YOB, 1995–2006 vs 1980–1994	1.0 (0.6–1.8)	0.9

* All variables were included in the same Cox proportional hazards model; because age at symptom onset and age at diagnosis were correlated ($r = 0.7$, $p < 0.001$), only age at symptom onset was included in the model; continent (other vs North America) was included in an initial model but was excluded from the final presented model because its effect was not significant.
† Includes noninvasive (e.g., bilevel positive airway pressure) and invasive ventilation.
HR = hazard ratio; MI-E = mechanical insufflation-exsufflation; YOB = year of birth.

ities were 32% at 2 years, 18% at 4 years, 8% at 10 years, and 0% at 20 years.[11] The studies published after 2000 have reported an increased mean age at death ranging from 10.4 months to 4 years[12,13] and outliers surviving up to 24 years.[14] In a 2004 study, the survival probabilities were higher than those reported in 1995: 50% at 1 year, 40% at 2 years, 30% at 4 years, 30% at 10 years, and 30% at 20 years.[15] We found greater survival in our group of patients born in 1980–1994 as well as in the group born in 1995–2006 compared with the 1995 and 2004 studies, respectively (table 3). This suggests that the sample in our study may differ from the general SMA type 1 population.

Indeed, the possibility of selection bias is one limitation of our study: parents voluntarily signing up for a national registry and responding to questionnaires may be more likely to pursue proactive medical management. The same is also true, however, of parents signing up for clinical trials. A further limitation of the study is that the validity of the information obtained through the registry and the mail-in questionnaire has not been assessed against in-person interviews or medical records. Whereas the registry enrolled and followed SMA patients prospectively, the information obtained through the mail-in questionnaire was retrospective and relied on parents' recall of past medical events. Recall of specific events for patients born in 1980–1994 would tend to be less accurate than for those born in 1995–2006. Age at symptom onset and age at diagnosis were significantly lower in patients born in 1980–1994 than in those born in 1995–2006. Although we cannot exclude the possibility that patients born in 1980–1994 were more severely affected (as reflected by an earlier onset), the differences in age at symptom onset and age at diagnosis may also reflect recall bias due to the advent of genetic testing. When genetic testing was not available, the diagnosis was made clinically. For patients born in recent years, parents' recall may be driven by the age when genetic confirmation was obtained after the clinical diagnosis.

The changes in care over time suggest a shift in attitude among parents of SMA patients partici-

Table 5 Review of literature

Reference	Country	n	Survival data
Byers and Banker, 1961 (8)	United States	25	Age at death (n = 23): mean 10 mo, range 17 d–52 mo; survival (n = 2)*: mean 17 mo, range 10–24 mo
Ignatius, 1994 (9)	Finland	71	Age at death (n = 69): mean 8.8 mo, median 7 mo, range 2 mo–10 y
Thomas and Dubowitz, 1994 (10)	England	36	Age at death (n = 29): mean 9.6 mo, median 7 mo, range 1–24 mo
Zerres and Rudnik-Schoneborn, 1995 (11)	Germany	197	Survival probability: 32% at 2 y, 18% at 4 y, 8% at 10 y, 0% at 20 y
Borkowska et al., 2002† (14)	Poland and Germany	36	Age at death (n = 18): mean 11 y, range 5–24 y
Bach et al., 2002 (12)	United States	56	Age at death (n = 10): mean 10.4 mo, SD 3.4; survival (n = 46)*: mean 50.8 mo, SD 42.3
Ioos et al., 2004 (13)	France	68	Onset 0–3 months: age at death (n = 27): mean 18 mo, SD 29; onset 3–6 mo: age at death (n = 9): mean 4 y, SD 4
Chung et al., 2004 (15)	Hong Kong	22	Survival probability: 50% at 1 y, 40% at 2 y, 30% at 4 y, 30% at 10 y, 30% at 20 y

* Patients alive at the end of the study.
† Included only patients surviving >36 months.

pating in the International Registry, paralleling a shift in physician practice and attitude toward respiratory support in SMA type 1.[16] Major advances have been made in the past 10 years in our understanding of SMA, with identification of the SMN gene, development of animal models, and improved understanding of the SMN protein function. This research progress has translated into increased hope for therapeutic advances among families of children with SMA, who are more willing to pursue active medical management.

The results of this study are important for planning future clinical trials in SMA type 1. The recent change in survival time suggests that historic controls may not be suitable and that concurrent controls are likely needed for SMA type 1 trials. Comparing outcomes in current patients with data from historical controls may falsely suggest a treatment benefit. The association of ventilatory support with longer survival may indicate the need to use outcome measures other than time to death. Time to death or ventilation greater than 16 h/d may prove to be a better outcome measure in this patient population.

The results of this study may also impact clinical care. Physicians should be aware of the prolonged survival in SMA type 1 patients and offer families updated prognostic information when counseling. Now surviving into school age, more attention should be placed on assisting these children with adaptive measures to enable them to participate successfully in school and their environment.

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Natural History of Denervation in SMA: Relation to Age, *SMN2* Copy Number, and Function

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Denervation was assessed in 89 spinal muscular atrophy (SMA) 1, 2, and 3 subjects via motor unit number estimation (MUNE) and maximum compound motor action potential amplitude (CMAP) studies, and results correlated with *SMN2* copy, age, and function. MUNE and maximum CMAP values were distinct among SMA subtypes ($p < 0.05$). Changes in MUNE and maximum CMAP values over time were dependent on age, SMA type, and *SMN2* copy number. *SMN2* copy number less than 3 correlated with lower MUNE and maximum CMAP values ($p < 0.0001$) and worse functional outcomes. As *SMN2* copy number increases, so does functional status ($p < 0.0001$). Change in MUNE longitudinally over the time intervals examined in this study was not statistically significant for any SMA cohort. However, a decline in maximum CMAP over time was apparent in SMA2 subjects ($p = 0.049$). Age-dependent decline in MUNE and maximum CMAP was apparent in both SMA 1 ($p < 0.0001$) and SMA 2 ($p < 0.0001$) subjects, with age as an independent factor regardless of type. Maximum CMAP at the time of the initial assessment was most predictive of functional outcome ($p < 0.0001$). Prospective longitudinal studies in four prenatally diagnosed infants demonstrated significant progressive denervation in association with symptomatic onset or functional decline. These data highlight the potential value of such measures in increasing our understanding of pathophysiological factors involved in denervation in SMA.

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Spinal muscular atrophy (SMA, OMIM 253300) is a recessive motor neuron disease characterized by diffuse weakness and muscular atrophy. It is one of the most common neuromuscular conditions with an incidence of approximately 1 in 8,000 live births and a leading cause of hereditary infant mortality.[1–6] The disease most frequently manifests in infancy and early childhood. The clinical spectrum of onset and severity is broad, ranging from the infant with congenital arthrogryposis requiring ventilator support to the individual with onset of proximal leg weakness in adulthood. For ease of clinical classification, SMA patients are most commonly divided into types 1 to 3,[7] with further subclassification.[8] SMA 1 patients manifest weakness before 6 months and are defined by their inability to sit without support. Without substantial respiratory support, most of these children die before their second birthday. SMA 2 patients usually demonstrate weakness by 18 months. They are able to sit unsupported at some point in their clinical course, although they often later lose this ability. They suffer significant respiratory morbidity and early mortality. SMA 3 patients achieve the ability to ambulate independently. They generally have a milder course, with the potential for a normal life expectancy.[9]

Homozygous deletion of the survival motor neuron 1 (*SMN1*) gene is found in more than 94% of SMA cases regardless of type and plays a primary role in disease pathogenesis.[10,11] The *SMN1* gene resides in a duplicated inverted region on chromosome 5q. A near identical copy of the *SMN1* gene, *SMN2*, contains a single nucleotide change which alters splicing, drastically reducing the efficiency of exon 7 inclusion and resulting in truncated mRNA and protein products.[12] However, *SMN2* transcription results in approximately 10% full-length SMN protein. An increased number of *SMN2* gene copies, with the resulting increase in full-length mRNA levels and protein expression, is associ-

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ated with a less severe disease phenotype in humans and phenotypic rescue in an *SMN* knockout mouse model.[13–17] The SMN protein appears to play an important role in spliceosomal snRNP biogenesis, although the precise means by which a deficiency of the SMN protein affects neuronal viability remains unclear.[18] The identification of the unique mechanism by which the phenotype is modified in SMA has led to efforts to identify specific compounds which could shift splicing in the *SMN2* message toward a higher rate of exon 7 inclusion or upregulate SMN protein expression via an increase in SMN2 gene expression. Several compounds have been identified that upregulate *SMN* gene expression, some which are readily available for use in patients and may be of potential value in treatment.[19–22]

Design and implementation of effective therapeutic trials in SMA necessitates a better understanding of the pathophysiology underlying denervation, the impact of lowered SMN levels on neuronal health with regard to age and developmental status, and the potential reversibility of the direct and indirect effects of lowered SMN levels at various time points. Clinical classification in SMA implies greater lower motor neuron loss in types 1 and 2, and least in type 3. However, the magnitude and time course of such loss is not apparent from the usual clinical evaluation. Genetic testing has appropriately replaced electrophysiological testing for diagnostic purposes. However, a quantitative knowledge of the natural history of lower motor neuron loss is essential in guiding the design of clinical trials. In infants and young children, obtaining reliable and sensitive outcome measures of strength and functional status is a challenge. Assessments of strength may prove an insensitive measure for progression in a condition such as SMA in which clinical symptoms become apparent early but appear to stabilize over time. Preservation of strength in the setting of loss of function has been well documented.[23] Presumably, reinnervation of muscle fibers by the remaining motor neurons concurrent with progressive motor neuron loss allows for the relative maintenance of strength for prolonged periods of time despite disease progression. Functional scales may be better but are influenced by factors including developmental maturation, such that a child may continue to gain motor milestones or remain stable despite progressive denervation. For example, children with SMA 2 often lose the ability to roll, then regain it several months later as they learn strategies to overcome their strength limitations. In light of these factors, it is critical to determine ways in which to assess the health of remaining motor neurons, their capacity for axonal sprouting and reinnervation, the size of the remaining motor units, and ultimately the effect on muscle mass, strength, and function.

We prospectively evaluated the use of two electrophysiological measures in a distally innervated muscle group in SMA. We report natural history data for 89 SMA subjects with a wide range of age and clinical severity. In each subject, we obtained an estimate of the number of motor units innervating a distal muscle group (MUNE), maximum compound motor action potential amplitude (CMAP), general functional motor status, and *SMN2* copy number. These data support the applicability of assessment of MUNE and maximum CMAP amplitudes in children with SMA. Such data provide a measure of the overall health of motor neurons and their distally innervated muscle groups which may facilitate a better understanding of the natural history of SMA with regard to single motor unit size and stability. These data have been collected as part of a more comprehensive assessment protocol which includes repeated formal functional assessments, DEXA imaging of lean body mass and bone density, pulmonary function testing in children older than 5 years of age, analysis of nutritional status via 3-day dietary survey and growth parameters, and quantitative assays of SMN mRNA and protein levels from blood samples. Reliability assessment and correlation of these measures with MUNE, CMAP, and *SMN2* copy number data are ongoing but ultimately will provide additional information regarding the relevance of such measures to functional outcomes in SMA subjects.

Subjects and Methods

Informed consent (and informed assent in children older than 12 years of age) was obtained from each subject or their parents. This protocol was approved independently by the institutional review and general clinical research center review boards at the University of Utah School of Medicine and Primary Children's Medical Center.

Motor Unit Number Estimation and Maximum Ulnar Compound Motor Action Potential Amplitude

We used the multiple point stimulation (MPS) technique to estimate the number of motor units innervating hypothenar muscles, as previously described, using a proprietary software program on the Advantage A200 machine.[24,25] Although this machine is no longer available, similar software is available on the Comperio from Compumedics, from which we have obtained comparable results. The G1 electrode is moved for a minimum of three placements to ensure that maximum CMAP is selected. Individual single motor unit potentials (SMUPs) are identified by obtaining all-or-none responses to low-intensity stimulation. Ten consecutive observations of an individual SMUP are obtained to decrease the probability of multiplex units, and we attempt to identify at least 10 unique SMUPs. The MUNE value is the maximum CMAP negative peak area (NParea) divided by the mean SMUP NParea. Values are expressed for maximum CMAP and SMUP by both NParea and negative peak amplitude (NPamp).

Functional Assessment

Functional status was assessed at each visit to ascertain ability to sit with or without support, cruise, and walk independently and use of ventilatory support. An ordinal scale was applied to establish functional status at the time of data analysis, because frequently, SMA type as defined by maximal achievement of gross motor skills does not reflect current gross motor functional status. This ordinal scale allows documentation of critical outcomes including serious respiratory compromise or death during the course of the study. It is simplistic and follows the general delineations in function used for designating SMA type in patients. Scores were assigned as follows at the time of data analysis for this subject cohort: 0 = deceased or ventilator dependent more than 16 hours/day, 1 = unable to sit unsupported, 2 = sits independently when placed, 3 = cruises, walks with assistance or independently, and 4 = without evident muscle weakness.

Survival Motor Neuron Copy Number

Confirmation of homozygous *SMN1* deletion and assessment of *SMN* copy number was performed as previously described.[26]

Conscious Sedation

Intranasal or oral midazolam was used in children in whom anxiety or discomfort would interfere with reliable testing and to facilitate repeated evaluations. Dosing was 0.2 to 0.5mg/kg, maximum 5 mg per dose, repeated as needed up to 10mg. SMA 1 subjects typically have respiratory compromise which precludes such sedation. However, their low MUNE values enhance reliability and ease of testing.

Statistical Analysis

Maximum CMAP and MUNE values were normally distributed. Analysis of variance was used to determine differences between SMA types 1, 2, and 3 for CMAP and MUNE values. Because there are multiple comparisons the Ryan-Einot-Gabriel-Welsch multiple range test was used to maintain a statistical significance level of 0.05.

The intraclass correlation coefficient (ICC) was used to measure reliability of MUNE and CMAP measurements, based on a data pair (test and retest) for each patient. Test–retest pairs were performed on different days in 21 subjects, with retest occurring within 3 months of the first assessment. Assumptions were that the reviewers were fixed and that the sample was random.

Change Over Time Analysis

MUNE and CMAP values were repeated over time in individual subjects. Age of subject, time intervals between studies, and total number of measures varied for practical reasons. Data were analyzed using generalized estimating equations (GEE).[27] The validity of the GEE in evaluating sample sizes similar to this study has been established previously.[28] Given the normal distribution of MUNE and maximum CMAP values, the GEE with normal distribution, exchangeable correlation structure, and an identity link function was employed. The exchangeable correlation was used because the correlation from visit to visit was the same and not time dependent. Age, SMA type, and *SMN2* all were included in the model. To investigate the impact of each SMA type, we assigned a dummy variable to represent each type, and the reference point was type 3, because we expected the least change over time in that group. Age was included as a continuous variable and an age by SMA type interaction was assessed. To assess the robustness of the results a log transformation of age, MUNE and maximum CMAP were assessed. The results were the same as the nontransformed data. Outcome of the nontransformed analyses are reported for simplicity.

The Fisher-Freeman-Halton test was used to determine if functional status was independent of *SMN2* copy number. Evaluation of the linear relationship between the categories of functional status and number of *SMN2* copies was performed using the nonparametric linear by linear test.

Statistical analysis was not performed on prenatally identified cases, because the results were self-evident. In all instances, these subjects were ascertained in the context of an affected sibling.

Results

Motor Unit Number Estimation and Maximum Compound Motor Action Potential Amplitude Reliability

ICC to assess reliability for MUNE and CMAP, based on test–retest data pairs in 21 subjects obtained on different days within a 3-month time period confirmed excellent reliability ($p < 0.001$) for all measures. ICC for MUNE NPamp was 0.993, for MUNE negative peak area (NParea) 0.965, for CMAP NPamp 0.973, and for CMAP NParea 0.958.

Motor Unit Number Estimation and Compound Muscle Potential Amplitude Data

Cross-sectional data were available on 29 SMA 1, 44 SMA 2, and 14 SMA 3 subjects (one severely affected SMA 1 infant with congenital arthryogryposis was excluded, because CMAP was unelicitable). Serial longitudinal studies available in 19 SMA 1, 36 SMA 2, and 10 SMA 3 subjects, including three type 1 and one type 2 prospectively identified subjects. Excluding prenatally identified cases, mean MUNE and CMAP values differed substantially among the three types, with the lowest values observed in SMA 1 subjects, intermediate values in SMA 2 subjects, and the highest values in SMA 3 subjects ($p < 0.05$, Fig 1 and Table 1). However, there is overlap among types, and values varied widely with age and gross motor functional status. Cross-sectional data from 29 SMA 1, 44 SMA 2, and 14 SMA 3 subjects plotting age versus MUNE and CMAP values demonstrate an overall age-dependent decline in values, with SMA 2 subjects reaching values that overlap with the SMA 1 population (Figs 2 and 3). These data are consistent with observations from the change over time analysis in individual subjects (Fig 4).

Table 1. MUNE and CMAP Data from First Visit, Excluding Prenatally Identified Cases

Characteristic	SMA 1 (N = 26), Mean (SE)	SMA 2 (N = 43), Mean (SE)	SMA 3 (N = 14), Mean (SE)	*p*
Age (mo) (range)	18.9 (9.9) (1.08–263)	87.1 (20.8) (0.43–589)	146.6 (43.8) (28.4–604.8)	<0.05 (all distinct)
CMAP NPamp, mV (range)	0.34 (0.06) (0.03–1.20)	1.86 (0.24) (0.32–5.9)	6.24 (0.68) (2.7–10.4)	<0.05 (all distinct)
MUNE NParea, mVms (range)	6.3 (1.4) (1–40)	34.5 (6.9) (3–216)	102.1 (17.1) (17–231)	<0.05 (all distinct)

MUNE = motor unit number estimation; CMAP = compound motor action potential amplitude; SE = standard error; NPamp = negative peak amplitude; NParea = negative peak area.

Prenatally Diagnosed, Prospectively Identified Subjects

Longitudinal data were obtained in three SMA 1 and one SMA 2 subject identified in the context of an affected siblings. These subjects, identified as open diamonds in Figures 2 and 3, demonstrate severe and substantial postnatal progression of motor denervation as reflected by CMAP and MUNE values. This decline was temporally associated with a precipitous decrease in motor function in all three SMA 1 infants, with progression to generalized hypotonia and quadriparesis over a 1- to 2-week period, and death from respiratory failure by 6 months of age. This early and precipitous decline in SMA 1 subjects is in contrast with the normal MUNE and CMAP values in the SMA 2 subject observed as late as 9 months of age. She had a more subtle presentation between 9 and 12 months manifest as a failure to bear weight and a failure to show continued weight gain as expected. After a decline from 9 to 28 months, MUNE and CMAP values stabilized until her most recent assessment at 49 months, when MUNE values again decreased in the face of a stable CMAP value. She has remained stable with regard to gross motor functional abilities since formal testing was possible at 22 months of age.

Change Over Time Analysis and Effects of Age on Motor Unit Number Estimation and Compound Motor Action Potential Amplitude Data

We analyzed change in MUNE and CMAP values over time in subjects with SMA types 1, 2, and 3, excluding prenatally diagnosed subjects. SMA type and age were related to change in MUNE values over time for SMA 1, 2, and 3 subjects. Age and SMA type did not have a statistically significant interaction (Table 2).

Although change over time within individual SMA cohorts was not significant, age was a significant and independent factor regardless of SMA type ($p < 0.0001$), with a corresponding decrease in MUNE value of −0.074/month of additional age. However, the overall contribution of SMA type was greater than age (Table 3).

Analysis of CMAP data, in contrast, demonstrates a significant change over time which is clearly dependent on SMA type. SMA 1 and 3 subjects are essentially stable, whereas SMA 2 subjects demonstrate a modest decline of −0.007mV/month ($p = 0.0492$), even over the relatively short time frame of this study (Table 4).

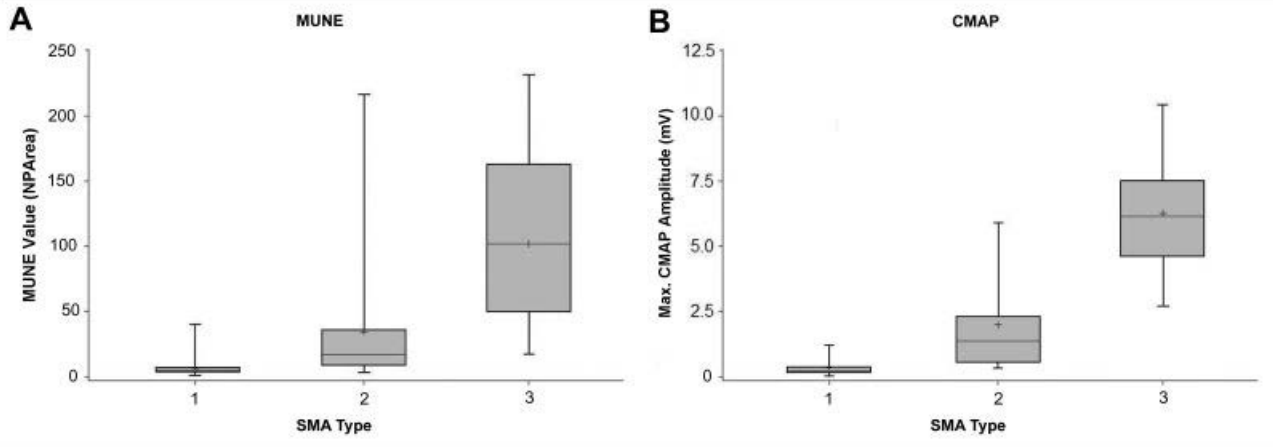

Fig 1. (A, B) MUNE and CMAP data, respectively, by SMA type in boxplot format. The shaded gray boxes represent the interquartile range (75th%–25th%) of the data. The whiskers demonstrate a measure of data variability, and the horizontal black lines within the gray boxes indicate median values, and plus signs indicated mean values, in each category. SMA = spinal muscular atrophy; MUNE = motor unit number estimation; CMAP = compound motor action potential amplitude.

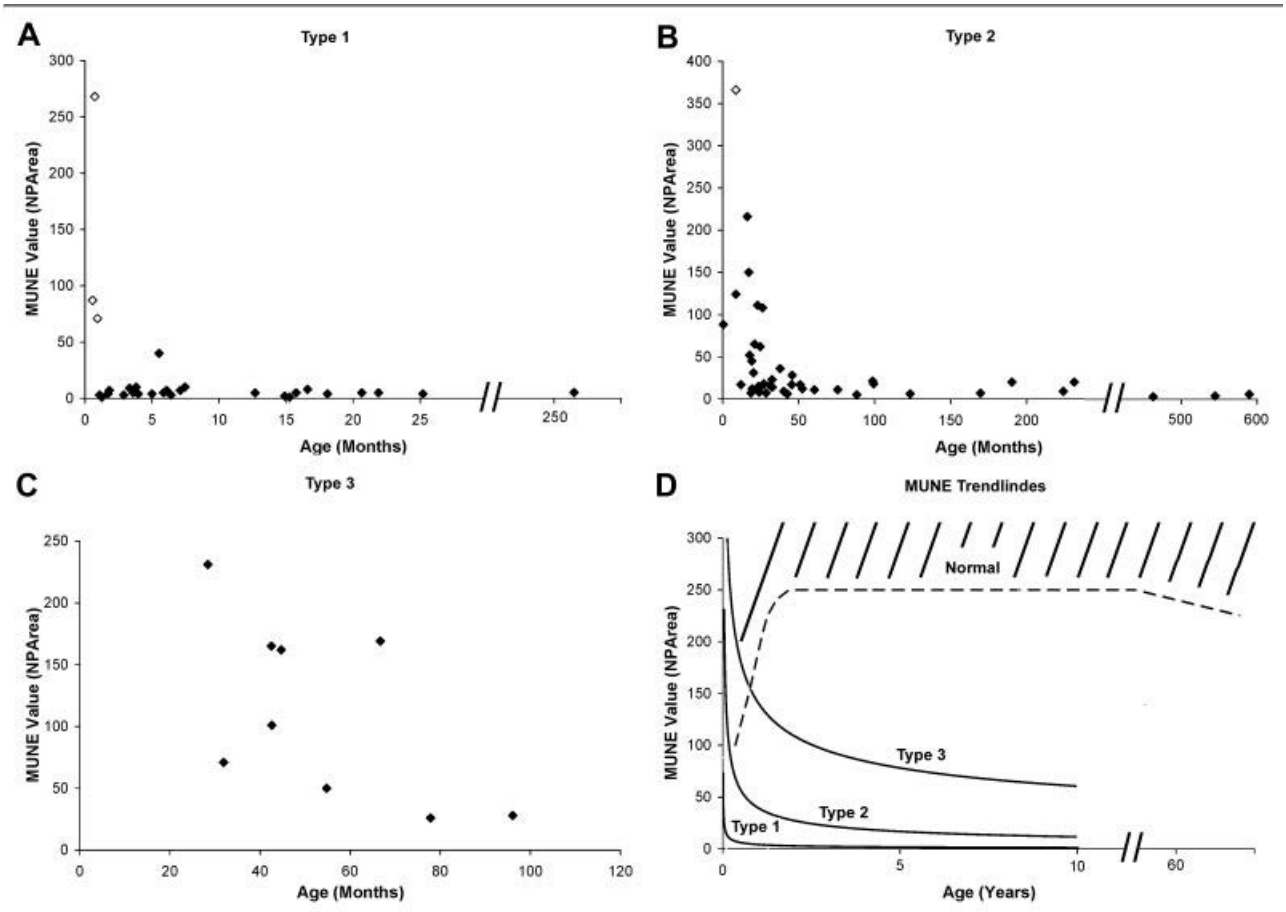

Fig 2. (A–C) Cross-sectional MUNE data obtained at first visit for spinal muscular atrophy type 1, 2, 3 subjects, respectively. Open diamonds indicate subjects identified prenatally via genetic testing because of a previously affected sibling. Solid diamonds indicate subjects with confirmation via genetic testing following symptom onset. (D) Power trendlines representing data for all three types. Estimated minimum normative values indicated by dashed line. MUNE = motor unit number estimation; NPArea = negative peak area.

Correlation of Survival Motor Neuron 2 Copy Number with Motor Unit Number Estimation and Compound Motor Action Potential Amplitude Data and Functional Status

A modifying effect of *SMN2* copy number on MUNE and CMAP values is clearly evident when contrasting subjects with two copies versus higher copy number (Table 5) are compared. Subjects with less than three *SMN2* copies performed significantly worse for both MUNE and CMAP values over time ($p < 0.0001$). However, there is substantial overlap in subjects with three and four copies. The linear by linear test indicated that as *SMN2* copy number increases so does functional status ($p < 0.0001$; Table 6). As pertinent examples, we have a 7.5-year-old SMA 3 subject with two *SMN2* copies. His clinical course has been severe relative to other SMA3 subjects. He was able to walk behind his chair with ankle-foot orthotics until 5 years of age but later lost this ability and underwent surgery for scoliosis at 7 years of age. He has the lowest CMAP and MUNE values to date for any of the SMA 3 subjects. Two SMA 1 subjects have three *SMN2* copies: a 23-year-old with a MUNE of 6, and a 22-month-old subject with a MUNE of 5. The 23-year-old subject has severe restrictive pulmonary disease, a tracheotomy with nocturnal ventilation requirement, and a gastrostomy-tube due to bulbar insufficiency. However, both subjects are clearly more robust than the SMA 1 subjects with two copies. Our weakest SMA 2 subject at just over 3 years of age has only two copies, with MUNE and CMAP values in the same range as our SMA 1 cohort. He was briefly able to sit unsupported before deterioration at around 6 months of age. At 38 months, he is quadriparetic with severe restrictive pulmonary disease. The highest functioning symptomatic subject in our study is a 50-year-old still ambulatory SMA 3 subject with five *SMN2* copies. She skied competitively into her teen years before the development of proximal leg weakness. Her son, also with five copies, is substantially weaker and lost independent ambulation at 23 years of age.

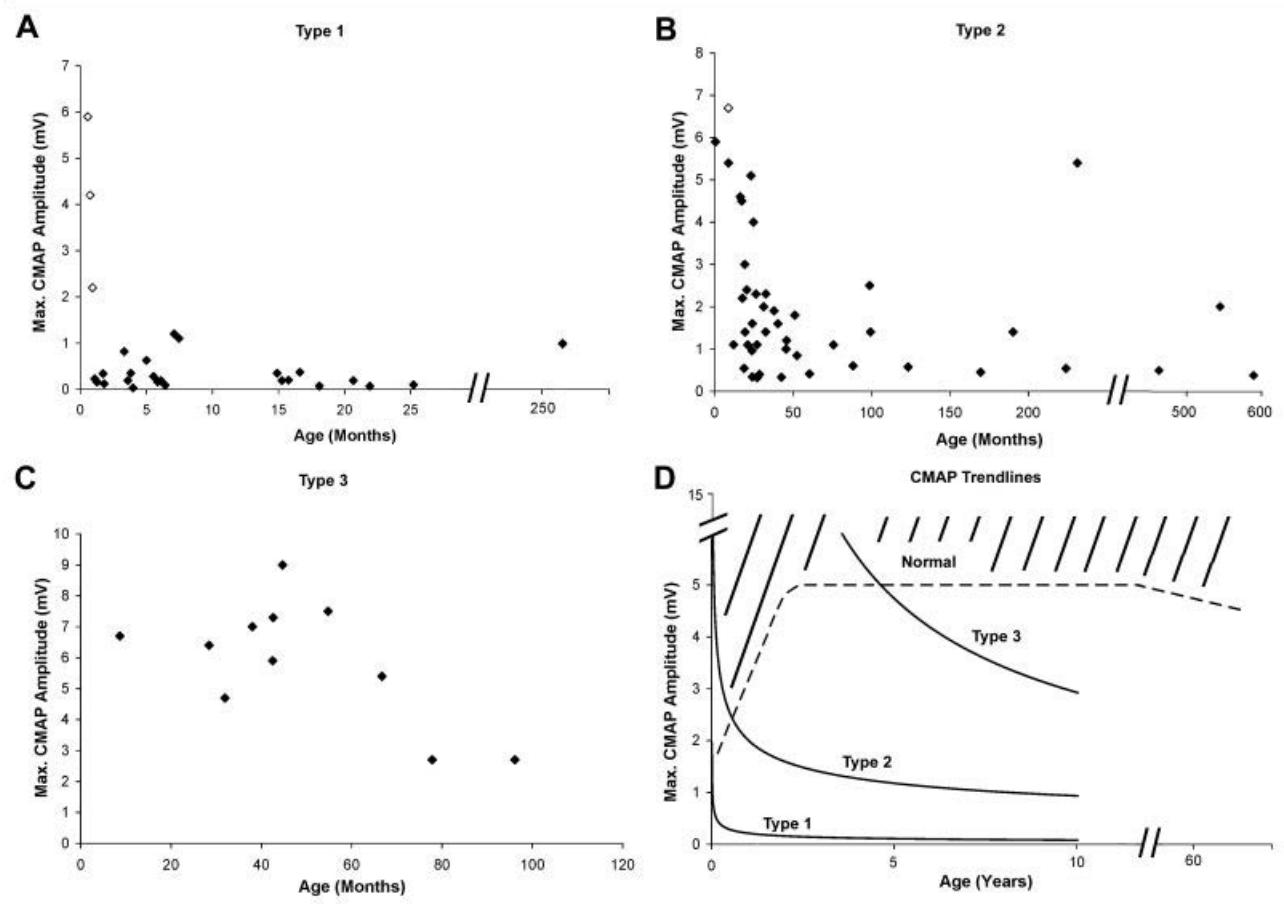

Fig 3. (A–C) Cross-sectional maximum CMAP data obtained at first visit for spinal muscular atrophy type 1, 2, and 3 subjects, respectively. Open diamonds indicate subjects identified presymptomatically via genetic testing because of a previously affected sibling. Solid diamonds indicate subjects with confirmation via genetic testing after symptom onset. (D) Power trendlines representing data for all three types. Estimated minimum normative values indicated by dashed line.[29] CMAP = compound motor action potential amplitude.

Role of Conscious Sedation in Data Collection

Conscious sedation was extremely well tolerated in the large majority of children with SMA type 2 or 3. Adverse events included occasional subjects with significant irritability after the procedure as sedation was waning. Transient oxygen desaturation less than 93% occurred in three subjects, with quick resolution after repositioning or encouragement to cough. Careful screening for current or recent illness and appropriate use of noninvasive preventive respiratory measures before and after the procedure helps ensure patient safety. Sedation is contraindicated in any child presenting with oxygen saturation less than 93% at baseline. Use of sedation has been critical in our hands in obtaining reliable MUNE data and in enhancing the acceptability of repeated testing for both parents and children, particularly in those subjects younger than 3 to 4 years of age.

Discussion

We demonstrate the feasibility and reliability of MUNE and CMAP measures in a large cohort of SMA subjects across a wide spectrum of age and disease severity. Our data support the value of these techniques in exploring issues related to disease pathophysiology and status of distal innervation over time in response to various therapeutic interventions.

We present to our knowledge the first prospective electrophysiological evaluation of prenatally identified SMA patients, demonstrating a substantial and unexpectedly precipitous decline in MUNE and CMAP values over time. In at least two of the SMA 1 infants, MUNE values, but not necessarily CMAP values, are already low at the time of initial testing. However, one SMA 1 infant and one SMA 2 child had normal values at the time of initial testing, supporting the concept that significant disease progression occurs in the postnatal period. If a therapeutic compound is identified that either rescues at-risk motor neurons or prevents ongoing motor neuron denervation, newborn screening may be indicated. Newborn screening for cystic fibrosis, despite a lack of a curative therapy, has led to significant improvements in quality of life in affected

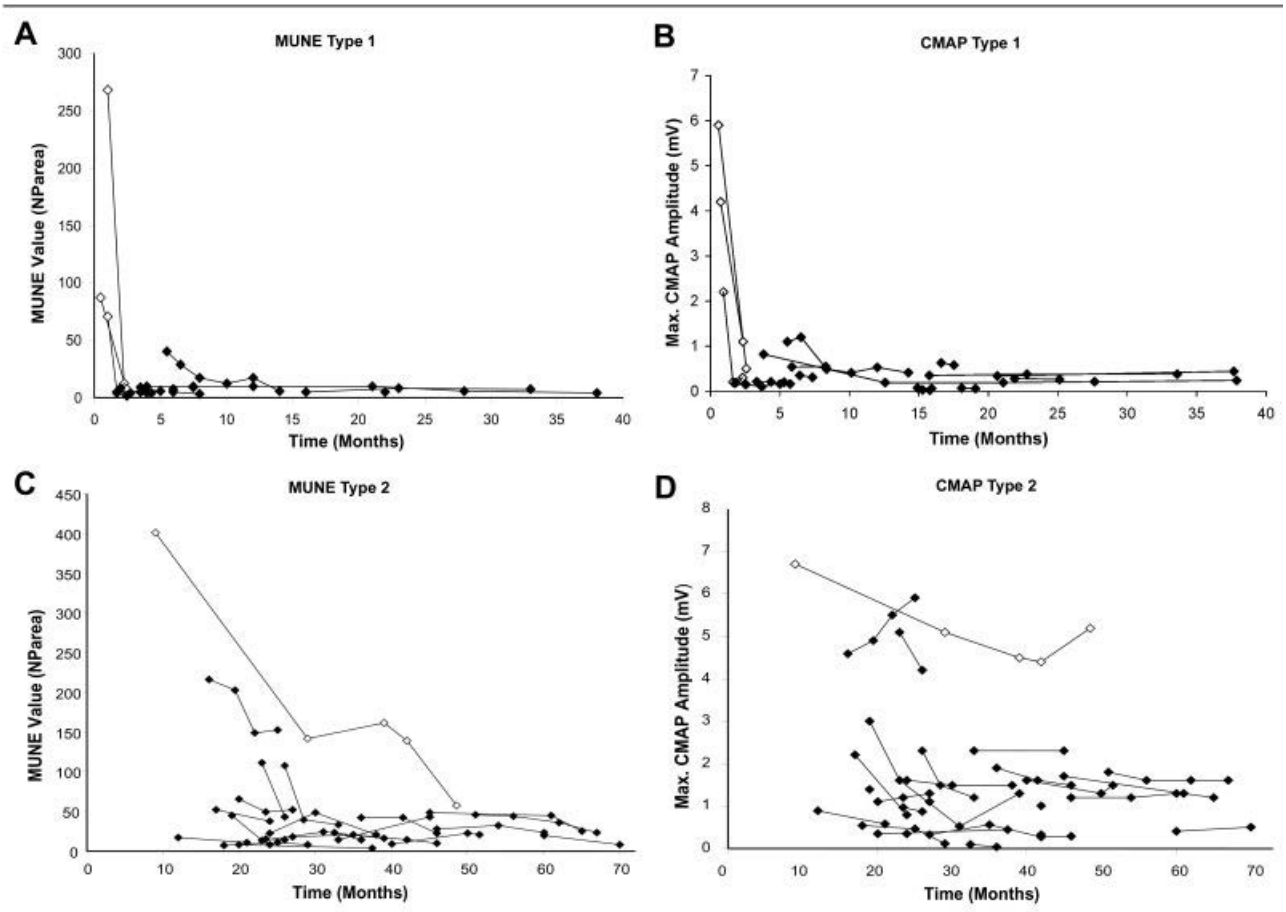

Fig 4. (A, C) Longitudinal MUNE and CMAP data, respectively, in type 1 subjects with more than one assessment. (B, D) Longitudinal MUNE and CMAP data, respectively, in type 2 subjects with more than one assessment. CMAP = compound motor action potential amplitude; MUNE = motor unit number estimation; NParea = negative peak area.

children because of early implementation of therapies such as nutritional support.

Analysis of individual trends within SMA types identifies two groups of subjects: those with relatively stable MUNE and CMAP values and those who demonstrate a significant decline in MUNE and CMAP values. This phenomenon appears to be largely age dependent, but of variable onset. It will be important for clinical trials to control for expected variability in response to a given therapeutic intervention, that is, a slowing of decline versus an increase in innervation or sprouting. The relative stability of MUNE over time across cohorts of SMA types given the relatively short time intervals is an important observation from a clinical trial design perspective, because most individual subjects of a specific type will be expected to remain stable over the time course of observation expected during a typical clinical trial. However, given that age is a significant factor in our model, age of enrollment is clearly a critical factor to consider.

MUNE and CMAP measures may provide sensitive indicators of health of motor neurons which are complementary to functional outcome measures, because some subjects with declining values are often quite stable in terms of overall functional status. MUNE and

Table 2. MUNE: Change with Time Analysis

Parameter	Estimate	Standard Error	*p*
Type 1	98.9	21.4	<0.0001
Type 2	67.6	22.4	0.0026
Age × type 1	−0.046	0.09	0.614
Age × type 2	−0.0085	0.02	0.667

MUNE = motor unit number estimation.

Table 3. MUNE vs Age Analysis

Parameter	Estimate	Standard Error	*p*
Type 1	100.4	15.4	<0.0001
Type 2	71.9	15.2	<0.0001
Age	−0.074	0.032	0.023
Intercept	−64.7	15.2	<0.0001

MUNE = motor unit number estimation.

Table 4. CMAP: Change with Time Analysis

Parameter	Estimate	Standard Error	*p*
Type 1	4.9	0.70	<0.0001
Type 2	3.3	0.74	<0.0001
Age × type 2 or 3 (type 1 absent)	0.0049	0.0031	0.1148
Age × type 1 or 3 (type 2 absent)	0.002	0.0008	0.0079
Age × type 2	−0.0068	0.0034	0.0492
Intercept	−3.03	0.75	<0.0001

CMAP = compound motor action potential amplitude.

CMAP measures may have independent value, because a potential therapy could demonstrate alternative mechanisms of improvement, including (1) distal axonal sprouting leading to increased territory and size of individual SMUPs without change in absolute MUNE value, or (2) reinnervation by axons from motor units whose axons had undergone distal degeneration but were "rescuable." Obtaining both measures may be useful in distinguishing agents that increase the reinnervation capacity of existing motor neurons (increasing size of individual SMUPs and CMAP amplitude) versus those that facilitate recovery of motor axons that have died back, allowing them to newly reinnervate muscle (increasing both MUNE and CMAP values). Such data may help to better predict the ultimate impact of a given therapy, because distal reinnervation can require considerable time. Baseline severity of denervation, duration of disease, and secondary complications such as contractures or end-stage muscle fibrosis will almost certainly be limiting variables with regard to therapeutic interventions.

Our data support a statistically significant correlation between maximum CMAP amplitude at the time of initial assessment and functional outcomes. These data may be valuable in providing relative prognostic information regarding an individual SMA subject's general health status, assessing where they lie on the curve relative to severity of denervation in a particular SMA cohort, and defining appropriate expectations regarding clinical improvement in response to therapeutic interventions.

SMN2 copy clearly plays a modifying role on MUNE and CMAP values and overall functional status, but other modifying factors are likely contributing in a complex fashion to disease phenotype. Despite the observation that *SMN2* dosage has a statistically significant modifying effect on function, predicting function or type in individual cases, particularly in the prenatal setting, remains a complex issue in light of the observed heterogeneity. However, our data support a clear distinction between those with more than two *SMN2* copies in overall MUNE and CMAP values, type, and functional status. An *SMN2* copy number higher than 2 may be much more relevant to long-term outcomes such as survival and maintenance of motor function over time rather than maximum motor function achieved, which is what is used currently for clinical classification into types. For instance, an SMA 1 child who has three *SMN2* copies may have a distinct survival advantage over one with two copies, although neither child will ever sit independently.

Based on the above observations and those of others, we propose stratification for therapeutic trials or post hoc adjustment of data analysis based on factors not limited to clinical subtype, including *SMN2* copy status less than 3, and recommend choosing patient cohorts with a narrower range of age and functional ability in any prospective clinical trial design. Although a quantitative knowledge of the natural history of lower motor neuron loss may be helpful in guiding the design of clinical trials, this does not imply that MUNE is necessarily the best outcome for clinical trials to assess efficacy. CMAP assessment may be sufficient for this purpose, because processes which increase either MUNE size or number will have a corresponding increase in CMAP amplitude. However, MUNE may be a valuable technique when asking specific questions about the mechanism by which a given therapy could lead to clinical improvement. Imperative in any clinical trial design is the use of the appropriate tool to answer the question of interest. For instance, a compound which has an impact on stabilizing the neuromuscular junction could have a significant effect on fatigue levels, resulting in a quality-of-life improvement. Use of

Table 5. Change in MUNE and CMAP over Time: Interaction with SMN2 Copy Number

MUNE				CMAP			
SMN2 Copy	Estimate	SE	*p*	SMN2 Copy	Estimate	SE	*p*
1 vs 2	−1.89	0.35	<0.0001	1 vs 2	−1.03	0.15	<0.0001
2 vs 3	−1.05	0.27	0.0001	2 vs 3	−0.78	0.11	<0.0001
3 vs 4	0.17	0.48	0.727	3 vs 4	−0.05	0.14	0.739
4 vs 5	−0.36	0.48	0.45	4 vs 5	−0.07	0.27	0.80

MUNE = motor unit number estimation; CMAP = compound motor action potential amplitude; SMN = survival motor neuron 1; SE = standard error.

Table 6. Evaluation of the Linear Relationship between the Categories of Functional Status and Number of SMN2 Copies Using the Nonparametric Linear by Linear Test

SMN2 Copy No.	Functional Status			
	0	1	2	3
1	1 (100%)	0	0	0
2	9 (41%)	12 (55%)	1 (4%)	0
3	0	7 (17%)	28 (68%)	6 (15%)
4	0	2 (14%)	11 (79%)	1 (7%)
5	0	0	1 (33%)	2 (67%)

Linear by linear test $p < 0.0001$.
0 = ventilator dependent > 16 hours/days or decreased; 1 = unable to sit unsupported; 2 = sits unsupported; 3 = cruises or walks.

MUNE and maximum CMAP values to evaluate such a compound would be irrational in this context but would be appropriate to address a question related to a potential improvement in sprouting and reinnervation capacity of remaining motor units in various SMA subpopulations. In light of new potential therapeutic avenues for motor neuron disease in general and SMA in particular, it is critical to identify additional approaches to assess the health of remaining motor neurons, their capacity for axonal sprouting and reinnervation, the size of the remaining motor units, and ultimately the impact on muscle mass, strength, and function.

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Newborn screening for spinal muscular atrophy: Anticipating an imminent need

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ABSTRACT

Spinal muscular atrophy (SMA) is the most common genetic cause of infant mortality. Children with type I SMA typically die by the age of 2 years. Recent progress in gene modification and other innovative therapies suggest that improved outcomes may soon be forthcoming. In animal models, therapeutic intervention initiated before the loss of motor neurons alters SMA phenotype and increases lifespan. Presently, supportive care including respiratory, nutritional, physiatry, and orthopedic management can ameliorate clinical symptoms and improve survival rates if SMA is diagnosed early in life. Newborn screening could help optimize these potential benefits. A recent report demonstrated that SMA detection can be multiplexed at minimal additional cost with the assay for severe combined immunodeficiency, already implemented by many newborn screening programs. The public health community should remain alert to the rapidly changing developments in early detection and treatment of SMA.

Introduction

Spinal muscular atrophy (SMA), the leading cause of heritable infant mortality,[1] is detectable by newborn blood spot screening (NBS).[2–7] SMA is presently an incurable condition characterized by degeneration of alpha motor neurons in the spinal cord. Over time, SMA results in progressive and symmetrical proximal weakness, followed by paralysis and ultimately, premature death. It is disheartening to examine SMA infants with advancing disease, their faces often bright and happy, and their full facial strength apparent because their eye and facial muscles are spared.

Abbreviations: AAV, adeno-associated virus; ASO, antisense oligonucleotides; DBS, dried blood spot(s); DcPS, human scavenger decapping enzyme; DHPLC, denaturing high-performance liquid chromatography; HRMA, high-resolution melting analysis; LNA, lock nucleic acid; MLPA, multiplex ligation probe amplification; NBS, newborn blood spot screening; NBSTRN, Newborn Screening Translational Research Network; RFLP, restriction fragment length polymorphism; scAAV, self-complimentary-associated adenovirus; SACHDNC, Secretary of Health and Human Services' Advisory Committee on Heritable Diseases in Newborns and Children; SCID, severe combined immunodeficiency; SMA, spinal muscular atrophy; SMN, survivor motor neuron; SNP, single nucleotide polymorphism(s); SSCP, single-strand conformation polymorphism; TREC, T-cell receptor excision circle(s)

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Among autosomal recessive disorders, SMA is the most common fatal disease and is second in birth prevalence only to cystic fibrosis.[1] The incidence of SMA is estimated at 1 in 6000–10,000, and the carrier frequency is 1/40–1/60.[8] SMA is a pan-ethnic disorder and overall represents the most widespread lethal genetic disease of children.[9]

The first article describing SMA was published in 1891 by Guido Werdnig, who meticulously detailed the clinical presentation, symptomatic progression, and pathological findings of a patient with infantile muscular atrophy relative to the gastrocnemius muscles and the spinal cord. He described the patient as a strong child, with lively movements, who succumbed to progressive weakness after a respiratory illness. Muscle pathology revealed a fatty tissue infiltrate, and the spinal cord showed atrophy of the anterior horn cells.[10] This report was followed by Johann Hoffmann's article in 1893 describing what he termed infantile progressive muscular atrophy.[11]

The molecular basis of SMA was recognized in 1990, when Gilliam et al.[12] showed that childhood-onset SMA is monogenic and linked to chromosome 5q, specifically 5q11.2–q13.3. At 5 years later, the site of the molecular lesion was identified as the *SMN1* gene encoding the survival motor neuron (SMN) protein.[13] This discovery was followed by an extraordinary scientific collaboration that untangled the complex genetics underlying SMA and revealed a unified theory of molecular pathogenesis. SMA became understood as a single-gene disorder with a closely related gene serving as a major phenotypic modifier. As investigators overcame the technical challenges that made SMA etiology so obscure, they focused on early identification, recognizing that the rapid onset and dire consequences of infantile SMA would require immediate intervention for any potential therapeutic actions to be effective. The development of methods that could detect SMA from newborn dried blood spots (DBS) was an integral part of the collaborative research.

These research efforts have led to more than a dozen potential therapies to treat SMA, including small molecules, antisense oligonucleotides, vector-based gene replacement, and stem cell transplantation. As this review goes to press, two such therapeutic treatments are in FDA Phase III clinical trials, which have been approved for evaluation in pre-symptomatic infants[14–17] (Table 1). Results from Phase I safety studies and Phase II efficacy trials in symptomatic children have been promising for both the therapies, suggesting stabilization and even modest improvement of symptoms.

This report reviews the current understanding of SMA pathogenesis, its clinical spectrum and pathognomonic features, the laboratory methods that have been used for detection and diagnosis, the current approaches to clinical management, and the promise of emerging therapeutic treatments. The current and future role of SMA-NBS is discussed, including the need for pilot studies that will allow the public health community to prepare for the availability of therapies with proven benefit in pre-symptomatic newborns.

Clinical presentation

Childhood SMA is commonly divided into three clinical groups—type I (OMIM 253300), type II (OMIM 253550), and type III (OMIM 253400)—based upon age of onset, maximum motor milestone attained, and age at death.[18–20] Adult-onset SMA, referred to as type IV (OMIM 271150), occurs at a much lower frequency.

SMA type I (Werdnig–Hoffmann disease) is the most severe phenotype, representing about 60–70% of all cases. Affected patients have severe and generalized weakness with hypotonia at birth or within the first few months of life, most definitely before the age of 6 months. All SMA type I patients are unable to sit unsupported. Because of the degree of weakness, most children die before 2 years of age, unless they receive substantial nutritional and respiratory support. The age of onset in SMA broadly correlates with age at death, indicating that the earlier onset of symptoms correlates with the severity of the condition.[21,22] The mortality rate is 75–95% by the second birthday.[18,22] These patients have normal cognition, and a somewhat higher average intelligence has been reported in comparison to other neuromuscular disorders such as Duchenne Muscular Dystrophy.[23,24]

SMA type II is characterized by the ability to sit unsupported; however, patients may lose this skill over time.

Table 1 – Therapies for spinal muscular atrophy in clinical trials (Accessed January 2015).

Therapy/phase	Title	URL link
Antisense oligonucleotides ISIS Pharmaceuticals Phase III	Randomized, double-blind, sham-procedure controlled study to assess the clinical efficacy and safety of ISIS 396443 administered intrathecally in patients with infantile-onset *spinal muscular atrophy*	https://clinicaltrials.gov/ct2/show/NCT02193074?term=sma&rank=26
Small molecule Trophos Phase III	Multicenter, randomized, adaptive, double-blind, placebo controlled study to assess safety and efficacy of *Olesoxime (TRO19622)* in 3–25 Year old *spinal muscular atrophy (SMA)* patients.	https://clinicaltrials.gov/ct2/show/NCT01302600?term=olesoxime+and+sma&rank=1
Gene therapy AveXis Inc Phase I	Gene transfer clinical trial for *spinal muscular atrophy* Type 1 delivering the survival motor neuron gene by self-complementary AAV9	https://clinicaltrials.gov/ct2/show/NCT02122952?term=sma&rank=39
Small molecule Roche Phase I	A multicenter, randomized, double blind, placebo controlled, multiple dose study to investigate the safety, tolerability, pharmacokinetics and pharmacodynamics of RO6885247 following 12 weeks of treatment in adult and pediatric patients with *spinal muscular atrophy* (MOONFISH).	https://clinicaltrials.gov/ct2/show/NCT02240355?term=sma&rank=51

Symptoms present before 18 months of age, and the affected children are never able to walk or stand. Life expectancy is beyond 4 years of age. SMA type III (Kugelberg–Welander disease) is the mildest childhood-onset phenotype, with symptoms presenting after 18 months of age. Type III patients are able to walk with assistance and potentially can have a normal life span.[18,20–22,25]

Further characterizations of the different types of SMA are based upon the copy numbers of the *SMN2* gene, a paralog of *SMN1* that produces SMN protein at much lower levels than *SMN1*. SMA type I patients typically have 2–3 copies of *SMN2*, SMA type II patients have 3–4 copies, and SMA type III patients have at least 4 copies of *SMN2*[19] (Table 2). Classification and determination of the various types of SMA are important for two reasons: first, to correlate clinical symptoms with genotype and second, to establish the natural history as a baseline for measuring the efficacy of therapeutic interventions.

Molecular genetics and pathogenesis

In humans, there are two nearly identical *SMN* genes present in 5q13: telomeric *SMN1* and centromeric *SMN2*. The two inverted sequences both code for the SMN protein and are more than 99% identical. Both genes have equivalent promoters and nine exons with a stop codon near the end of exon 7.[13,26,27] Homozygous absence of *SMN1* is found in about 95% of SMA patients, regardless of the type. The remaining 5% of the affected patients are compound heterozygotes for *SMN1* exon 7 deletions or small intragenic mutations.[5] The primary difference between *SMN1* and *SMN2* is a single nucleotide alteration in exon 7 (840C > T). This transition does not alter the amino acid sequence of the SMN protein produced by *SMN2*, but it affects the splicing process by disrupting the exonic splicing enhancer, resulting in exclusion of exon 7 in most mature *SMN2* mRNA transcripts[28,29] (Fig. 1). The lack of exon 7 disrupts the SMN protein domain that enables oligomerization, which prevents the protein's ability to interact with other proteins.[30] While the *SMN1* gene produces full-length transcripts and functional SMN protein, 90% of *SMN2* transcripts are translated into truncated SMN proteins (SMNΔ7).[26,28,31]

Interestingly, only the human genome contains *SMN2*, while all other animals have only one SMN protein-coding gene. In a transgenic mouse model, homozygous *Smn* knockout ($Smn^{-/-}$) results in lethality, indicating that SMN protein is necessary for cellular survival and function.[32] Inserting *SMN2* transgenically into a $Smn^{-/-}$ mouse results in a viable mouse pup that becomes SMA symptomatic soon after birth. The phenotype can vary depending on the copy number of *SMN2* inserted. Higher numbers reduce the severity of the condition, indicating that *SMN2* has a protective role in the disease phenotype.[33,34] These findings indicate that SMA results from inadequate production of SMN protein and that the *SMN2* gene is the phenotypic modifier.

Multiple studies have shown that *SMN2* copy number can alter the disease phenotype.[35–37] The small quantity of *SMN2* transcripts helps to explain the clinical observation that increased copy numbers of *SMN2* correlate with milder disease.[33–35] Interestingly, about 10–15% of the general population does not have any *SMN2*; however, this subpopulation has at least one intact *SMN1* gene and appears phenotypically normal. Conversely, three SMA carriers each with 5 copies of *SMN2* were unaffected, even though they had confirmed *SMN1* homozygous deletion. These carriers show that the expression of SMN protein from five *SMN2* copies is sufficient to compensate for the loss of *SMN1*.[38,39]

The *SMN2* copy number may not be a reliable indicator of disease severity if the *SMN2* genes have other mutations. In three adult patients with homozygous *SMN1* deletion and mild type III phenotype, a mutation of c.859G > C in exon 7 of the *SMN2* gene was found, resulting in increased number of full-length SMN protein production.[40] Conversely, a higher number of *SMN2* copies may not be beneficial if one or more contain mutations that reduce their ability to express a functional SMN protein.

SMN protein function

Full-length SMN protein is a 38-kDa polypeptide that is found in the cytoplasm and nucleus of all cells.[41,42] This protein is ubiquitously expressed at high levels during embryonic development. In most tissues, SMN protein expression diminishes in the postnatal period, but it remains highly expressed in the spinal motor neuron even in adulthood.

It has been well established that a deficiency of SMN protein leads to selective motor neuron loss, but the mechanism is unclear. SMN protein self-associates into oligomers, and it also associates with other proteins to form a larger macromolecule called the SMN complex. The SMN complex is known to interact with a variety of other proteins involved in pre-RNA splicing and assembly of the Sm-class spliceosomal small nuclear ribonucleoprotein (snRNP).[43–47] SMN protein also has an important role in axonal transport by forming a complex with beta-actin and profilin that regulates and stabilizes actin function.[48–51] Different hypotheses have been proposed to explain the importance of SMN protein in specific sets of neurons.[52,53]

Medical issues in SMA

Currently, there is no approved treatment that can effectively ameliorate the disease progress of SMA. However, there are

Table 2 – Types of SMA.

Type	Age at onset	Highest function	Age at death	SMN2 copies
I	Birth to 6 months	Never sit	<2 years	2
II	Birth to 18 months	Sit unsupported	>2 years	3–4
III	>18 months	Stand unsupported	Adult	3–4
IV	>21 years	Stand unsupported	Adult	4–8

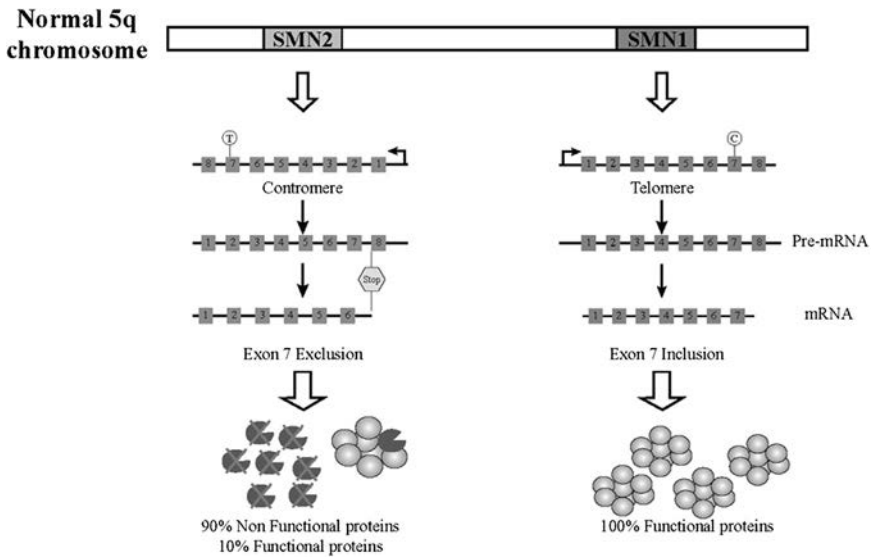

Fig. 1 – The human SMN1 and SMN2 genes are located on chromosome 5. Each gene encodes identical protein product; however, the substitution C to T in exon 7 of SMN2 gene alters exonic splicing enhancer, resulting in exclusion of exon 7 during splicing. Approximately 90% of the mature mRNA lacks exon 7 and consequently translate truncated SMN protein that is defective and degraded rapidly.[63] Schematic of the *SMN1* and *SMN2* genes and their proteins.

guidelines for clinical management based on evidence that the appropriate comprehensive pulmonary, orthopedic, rehabilitation, and nutritional care for patients with SMA reduces disease burden and improve natural history.[54] A team of multiple subspecialists is crucial in implementing these guidelines.[55] Early education and counseling for the family of patients is paramount in preventing SMA-associated complications.

The International Standard of Care Committee for Spinal Muscular Atrophy was formed in 2005 to establish necessary guidelines for the clinical care of patients with SMA. A consensus statement for the standard of care in spinal muscular atrophy in 2007 noted the need for multidisciplinary care for all affected patients. Further, it provided evidence-based recommendations for the common related medical problems in SMA patients, diagnostic strategy, assessment and monitoring requirements, and therapeutic intervention.[55,56]

Pulmonary complication is the major cause of mortality in SMA patients. Aspiration pneumonia results from bulbar dysfunction and from a weak cough and poor clearance. Hypoventilation can be prevented if anticipatory planning with a pulmonologist occurs as soon as the diagnosis is made. Family should be aware of the role of non-invasive ventilator support from weakened muscle strength as the disease progresses. General health maintenance to prevent pulmonary complications such as immunization against influenza, pneumococcus, and respiratory syncytial virus is highly recommended.[57] Those with recurrent pulmonary infection and poor secretion clearance could benefit from tracheostomy; however, this option needs to be weighed against the patient's quality of life.

Gastrointestinal complications such as gastro-esophageal reflux, delayed gastric emptying, and constipation can be present in those who are non-ambulatory. Gastrostomy tube placement is an option to promote adequate nutritional intake and prevent aspiration pneumonia; however, there is not a consensus in terms of the timing of its placement. Nutritional support is very important in SMA patients.

Routine rehabilitation assessment is important to maintain function and improve patient's quality of life. For those who are non-ambulatory, frequent stretching and bracing prevent contractures. Physical therapy can assist in safely attaining maximal mobility.

Scoliosis is largely unavoidable due to the nature of generalized muscle weakness in SMA, particularly in non-ambulatory type I and type II patients. When feasible, surgery is the treatment of choice, and with early anticipatory planning, appropriate timing of surgical intervention can be determined to prevent complications postoperatively.

Therapeutic progress and future promise

With greater understanding of the molecular genetics of SMA over the past 2 decades, therapeutic developments have focused on increasing the full-length SMN protein by enhancing *SMN2* gene expression, increasing the inclusion of exon 7 in *SMN2* transcripts, stabilizing the SMN protein, or replacing the *SMN1* gene. Figure 2 outlines the current progress of therapeutic strategies for the potential treatment of SMA.[58] Some of the more promising therapeutic modalities include antisense oligonucleotides, small-molecule drugs, gene therapy, and stem cell transplantation.

(1) Antisense oligonucleotides (ASO)

Increase in *SMN2* copy number correlates with a milder phenotype primarily because the higher number of *SMN2* (even with only 10% production efficiency by each copy) will result in more full-length SMN protein. The ASO therapeutic approach aims to alter the splicing process, thereby

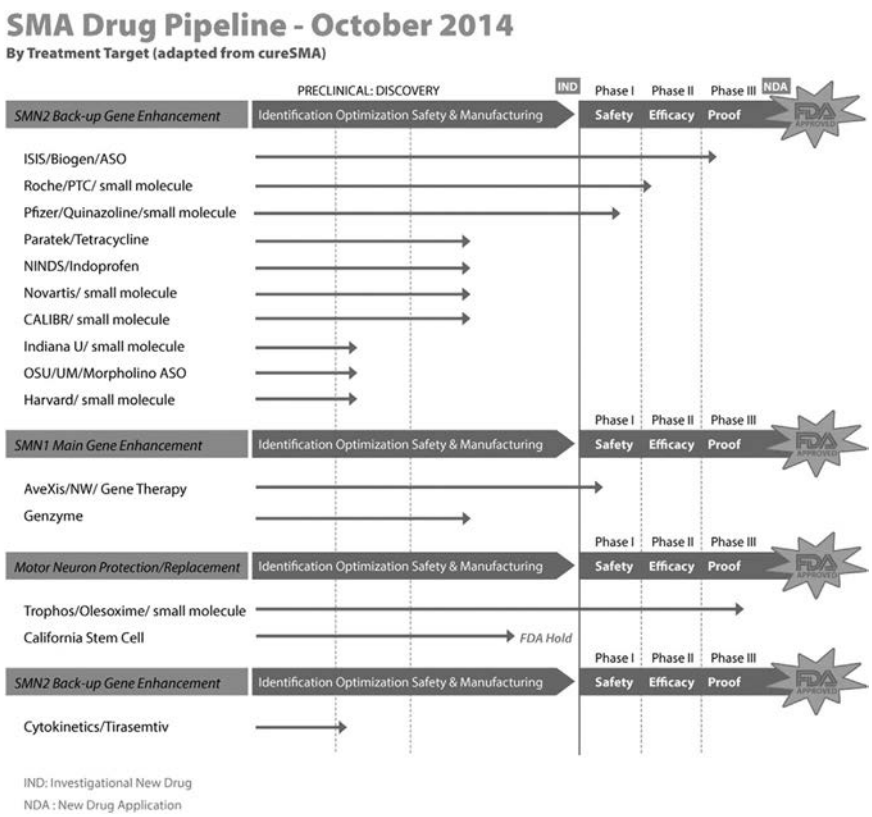

Fig. 2 – This chart outlines the current progress of therapeutic strategies for the potential treatments of SMA. With several therapies that have progressed from preclinical to clinical phase, an FDA-approved therapy is promising. IND, Investigational New Drug; NDA, New Drug Application. (Adapted with permission from SMA Drug Pipeline[58].)

increasing the inclusion of exon 7 in the *SMN2* transcripts, resulting in higher levels of functional SMN protein. ASO are therapeutic RNA molecules that block the cis-acting splice modifier or intron splice enhancer; either mechanism can enhance or disrupt the targeted splicing event. Specifically for SMA, hybridization of ASO-10-27 to the intronic splicing silencer N1 in *SMN2* intron 7 has been shown to increase the level of SMN protein *in vivo*.[59] As a proof of concept, interventricular administration of ASO-10-27 in the SMA mouse model has shown promising results. Treated mice showed an increase in the long-lasting *SMN2* exon 7 inclusion, as measured at both the mRNA and the protein levels in spinal cord motor neurons.[60] Moreover, treated mice had improved muscle structure, function, and ultimately, survival.[61]

In 2012, a Phase I human trial was launched with ASO-10-27 (renamed as ISIS-SMN_{RX}) in which 15 patients with SMA type II and 13 patients with SMA type III received a single intrathecal administration at varying dosages. The result established the drug to be safe and well tolerated in children with SMA. Additionally, treated children showed a dose-dependent improvement in the Hammersmith functional motor scale of muscle function. In a subsequent Phase II study, a total of 20 SMA infants received intrathecal administration of ISIS-SMN_{RX} at one of two different doses: four patients received a 6-mg dose and 16 patients received a 12-mg dose.[62] Results showed that the lumbar punctures were well tolerated and feasible. Also, there were no drug-related serious adverse events and no changes in safety profiles with repeated dosing. In this study, one accidental death and one permanent ventilation were recorded from the 6-mg cohort, whereas in the 12-mg cohort, three patients died due to respiratory infection. None of these events were considered drug related. Analysis of spinal cord tissue at autopsy showed that ISIS-SMN_{RX} was distributed throughout the central nervous system and was correlated with higher levels of full-length *SMN2* mRNA and proteins when compared to untreated infants.[63] Following these promising results, a Phase III clinical trial has begun to recruit patients for a randomized, double-blind, sham-procedure controlled study to assess the clinical efficacy and safety of ISIS-SMN_{RX}.[54]

(2) Small-molecule drugs

Certain low-molecular-weight drugs can promote increased levels of full-length SMN protein by activating the *SMN2* promoter, increasing its expression, and altering the splicing pattern of *SMN2* transcripts so as to favor the inclusion of exon 7.[64,65] Histone deacetylase inhibitor and quinazoline compounds increase *SMN2* mRNA levels and improve the disease phenotype in mouse models of SMA.[66–69] Other small-molecule drugs such as

aminoglycosides promote reading of the stop codon of SMNΔ7 transcripts, enabling the translation of the full-length functional SMN protein.[70] These molecules had shown promising results in mouse models and cell lines derived from SMA patients; however, when they were tested in clinical trials, SMA patients showed little benefit.[71–73]

An alternative class of small molecules including riluzole, olesoxime, and ceftriaxone function as neuroprotective compounds. Originally approved for use to treat patients with amyotrophic lateral sclerosis (ALS), riluzole blocks the sodium channels and inhibits glutamate, and it promotes the expression of neurotrophic factors that enhance motor neuron survival. A Phase I clinical trial showed this approach to be safe in SMA type I patients but without motor function improvement. A Phase II clinical trial of riluzole in children and young adults with SMA (ASIRI)[74] has been completed in France, but results have not been published. Olesoxime promotes neurite outgrowth and communication with the mitochondrial permeability transition pore.[75] A Phase II multicenter, randomized, and double-blinded study to assess safety and efficacy for SMA type II and type III has been completed, and the result of this trial was presented at the American Academy of Neurology in April 2014. Ceftriaxone prevents glutamate toxicity in a cell culture model of motor neuron degeneration, and it delayed motor neuron loss and improved muscle strength in an ALS mouse model. This compound is currently in clinical trials only for ALS. Another small-molecules member—quinazoline—increases full-length mRNA by inclusion of exon 7 in *SMN2* gene transcript, resulting in an increase in SMN protein levels. Its primary function is in the inhibition of human scavenger decapping enzyme (DcpS). The role of DcpS is best understood in the degradation of the 5′ cap during the 3′ to 5′ mRNA decay; it may have additional functions in nuclear–cytoplasm transportation and first intron pre-mRNA splicing.[76] Quinazoline is anticipated to progress to Phase I clinical trials (Table 1).

(3) Gene therapy

As a monogenic disease, SMA is a good target for vector-based gene replacement therapy to restore a normal form of the *SMN1* gene in SMA patients. The overall goal of gene therapy is to replace and permanently restore the deleted *SMN1* gene in the genome of the affected patients. As a result, more full-length SMN protein will be produced to promote normal function survival motor neurons, thereby improving phenotype and survivability.

In selecting a potential vector to deliver the *SMN1* gene, adeno-associated virus vectors (AAV) 8 and 9 appeared to be excellent contenders due to their ability to cross the blood–brain barrier through the vascular system in mouse models.[77–79] Additionally, self-complementary AAV (scAAV), a recombinant virus with a double-stranded DNA genome, results in early onset of gene expression. Several groups have shown this capability with the use of scAAV9, which appeared to have tropism toward motor neurons in the neonatal animals when injected intravascularly.[77,78] However, intracerebroventricular injection with SMN-scAAV8 has also been successfully used. Both modes of administration increased the median survival period; however, mice that received intravascular administration had a greater survival benefit of 250 days, compared to the 157 days in the intracerebroventricular injection group.[80–83]

With evidence that AAV9 could penetrate the blood–brain barrier, resulting in diffuse gene delivery via intravascular administration in animal models, this route of administration has been favored. But the potential side effects of this treatment modality are unknown, since a large vector load is given to increase the probability of penetration into the central nervous system. Alternatively, intrathecal administration of AAV9 has been accomplished in pigs and adult mice, with less vector load and improved gene expression.[84,85]

In 2014, FDA approved a Phase I clinical trial of a systemic AAV9-delivered human SMN gene to SMA type I infants up to 9 months old. The infantile-onset phenotype is considered the most appropriate candidates for immediate therapy.[86] This trial is currently recruiting patients.[15]

Even though the gene therapy approach offers great promise, its limitations are multiple. Similar to many neurodegenerative disorders, SMA is not diagnosed until a substantial amount of motor neuron loss and muscle atrophy has occurred. Like many other therapies, there is a critical therapeutic window in which treatment can increase SMN protein levels, leading to an improved or milder phenotype.[87–89] In the SMA mouse model, there is a crucial period in which a sufficient amount of SMN protein is required for motor neuron development.[8] In a study by Foust et al.,[90] administration of scAAV in SMNΔ7 mice on postnatal day 1 showed much more benefit as compared to postnatal day 5, and there was no benefit in postnatal day 10. The early death of SMA mice could be avoided with embryonic restoration of SMN protein expression, indicating that early administration of therapy can also prevent premature fatality.[91,92] Furthermore, the blood–brain barrier is more permeable during the perinatal period than later in infancy.[93]

(4) Stem cell therapy

Among the therapeutic approaches, stem cell transplantation also holds great potential for SMA patients. The primary goal for transplanted stem cells is to support endogenous motor neurons through the delivery of neuroprotective agents and to partially restore neuronal and non-neuronal cells.[94–96]

Neural stem cells obtained from the spinal cord administered intrathecally to SMA mice showed appropriate migration into the parenchyma and the

capability to generate a small proportion of motor neurons. These treated mice exhibited improved motor unit and neuromuscular function and showed a 38% increase in life expectancy. Furthermore, gene expression in these mice modified its SMA phenotype toward a wild-type pattern.[97]

Despite the positive results of neural stem cell transplantation in mice, its translational value in human is unclear due to limited availability of central nervous system tissues along with technical and ethical dilemmas.[98] These challenges led to the use of alternative stem cell therapy protocols, which includes the use of embryonic stem cells or induced pluripotent stem cells for transplantation, as their sources is vast and easily obtained. These cells have the ability to differentiate *in vitro* and *in vivo* into neural stem cells and motor neurons.[99–101] The findings of improved SMA phenotype in mice following the intrathecal transplantation of embryonic stem cell-derived neural stem cells included proper migration to target tissue in the spinal cord, neuroprotective function, and a 58% increase in lifespan.[102] A protocol to test neuronal stem cells in SMA patients is currently on hold by the FDA. In a similar neurodegenerative disorder such as amyotrophic lateral sclerosis (ALS), Phase I clinical trials of intraspinal stem cell transplantation have been completed and results have been published indicating that this method is feasible and well tolerated.[103] However, as of early 2015, no clinical trials of stem cell therapy in SMA patients are being conducted.

Need for early identification and treatment

The major obstacle in assessing the efficacy of any potential therapeutics for SMA is the recruitment of affected individuals in the early stages of the disease, prior to the loss of irreplaceable motor neuron units. For SMA type 1, such loss typically begins in the perinatal period, with severe denervation in the first 3 months of life and loss of more than 90% of motor units within 6 months of age.[104] This rate of neuronal loss means that the benefit of potential therapeutics can be assessed only by prompt treatment of affected newborns.

Newborn blood spot screening for spinal muscular atrophy

NBS began in the 1960s as a public health initiative to identify newborns with phenylketonuria and intervene with dietary restrictions before they suffered cognitive deficits. Since then, it has been expanded to detect over 30 congenital disorders for which newborns benefit from pre-symptomatic identification.[105] In the case of SMA, early diagnosis through SMA-NBS could assist overall medical care, ultimately reducing morbidity and mortality with the currently available tools, analogous to the benefits of newborn screening for cystic fibrosis.[56] Looking forward, the identification of therapeutic interventions that are effective pre-symptomatically would create a compelling need for SMA-NBS.

Molecular methodologies for the detection and diagnosis of SMA

No biochemical marker has been identified for SMA; however, several approaches to detecting *SMN1* deletion and intragenic mutations have been developed. Some methods are used for diagnostic purposes or carrier testing to detect the presence or absence of *SMN1* as well as quantification of *SMN1* and *SMN2* copy number. Other methods are used for screening purposes and identifying the majority of the affected individual by detecting *SMN1* deletions. All SMA cases identified through SMA-NBS methods must be confirmed with a reliable diagnostic test. A summary of current diagnostic and screening methods is listed in Table 3. These methodologies include single-strand conformation polymorphism (SSCP),[13] restriction fragment length polymorphism (RFLP),[106] denaturing high-performance liquid chromatography (DHPLC),[107–109] multiplex ligation probe amplification (MLPA),[110–112] and competitive PCR.[36,113–115] Only a few methods such as DHPLC and high-resolution melting analysis (HRMA) are useful for identifying intragenic mutations in *SMN1*. Digital PCR, a recently introduced methodology with a growing range of applications, is being used to identify SMA individuals and to quantify *SMN1* and *SMN2* copy number.[116–118]

Several methodologies have been utilized for screening applications with dried blood spot (DBS) specimens and for diagnostic applications; including liquid microbead suspension arrays,[5,6] high-resolution DNA melting analysis,[3,119,120] and real-time PCR.[7,37,121–125] Most screening assays will detect all individuals with the complete deletion of *SMN1* or mutations around exon 7; however, these assays will miss approximately 5% of affected individuals with other intragenic *SMN1* mutations.[4,126] Conversely, all of these methods would probably yield a screen-positive result in the rare phenotypically unaffected individuals with homozygous *SMN1* deletions but four or more copies of *SMN2*.

In order for an SMA detection method to be suitable for public health NBS programs, it must be cost-efficient, capable of high-throughput, and easy to implement in NBS laboratories. Screen-positive results should be confirmed as quickly as possible and reported routinely within 5 days of specimen receipt. Results should be comparable between laboratories.[127] Quality assurance must be established to ensure assay performance. A major component of quality assurance is the use of DBS reference materials from the Centers for Disease Control and Prevention (CDC).[128] Such materials for SMA screening have been developed at CDC and are currently undergoing multi-laboratory validation.

High carrier frequency for SMA (1/40–1/60) is also an issue to be considered in NBS. Identifying and reporting carriers from screening tests could potentially overwhelm follow-up activities; therefore, it may be disadvantageous to identify carriers through the initial screen.

The following sections describe three methods that have potential for use in traditional NBS laboratories.

Liquid microbead assays

Liquid microbead suspension arrays have been used for several disorders such as cystic fibrosis and hemoglobinopathies.[129,130] These methods involve PCR of the target

Table 3 – Molecular methods for SMA

Method	Application	Year[a]	Reference
Single-strand conformation polymorphism (SSCP)	*SMN1* deletion	1995	13
Restriction fragment length polymorphism (RFLP)	*SMN1* deletion	1995	106
Completive PCR	*SMN1* and *SMN2* gene quantitation	1997	36,113–115
Real-time PCR[b]	*SMN1* deletion	2002	2,7,37
Absolute quantitative PCR	*SMN1* and *SMN2* gene quantitation		37,121–124,138
Relative quantitative PCR			
Denaturing high-performance liquid chromatography (DHPLC)	*SMN1* deletion *SMN1* and *SMN2* gene quantitation Intragenic mutation detection	2002	108–110
High-resolution melting analysis[b]	*SMN1* deletion *SMN1* and *SMN2* gene quantitation Intragenic mutation detection	2009	3,119,120
Multiplex ligation probe amplification (MLPA)	*SMN1* and *SMN2* gene quantitation	2006	110–112,139
Liquid microbead assay[b]	*SMN1* deletion	2007	5,6
Digital PCR[b]	*SMN1* and *SMN2* gene quantitation	2011	116–118

[a] Year of earliest publication.
[b] Molecular methods have been validated on dried blood spots.

sequence with synthetic nucleobases, allele-specific target extension of the unique base in *SMN1* exon 7, microbeads that hybridize to tags on the extension region, and fluorescent signal detection. An advantage of using these assays is the large multiplex capability of 80–100 targets.

An initial study was done on 367 DBS samples using two different liquid bead assays: the MultiCode-PLx protocol and the Tag-it protocol.[6] Scatter plot distribution plots showed distinct clusters that separated the SMA-affected samples from the carrier and unaffected samples. This method was then adapted to the high-throughput protocol that was able to screen 400–500 samples daily and used to test DNA extracted from 40,103 anonymized DBS.[5] Results from this study showed 4 samples were detected with homozygous *SMN1* deletions and deletion was confirmed using competitive PCR. The estimated incidence of the general population affected by SMA was 1 in 10,026, consistent with previous literature. Carriers are not distinguished from unaffected individuals in these assays, and it is not useful for *SMN1* or *SMN2* copy number quantification. The reported cost of reagents and consumable for these methods were less than $10 for the MultiCode-Plx assay and less than $5 for the Tag-it assay. The time to run these assays from postextraction to final detection is about 3 h for the MultiCode-Plx assay and about 6 h for the Tag-it assay. The repeat rate for the high-throughput protocol was less than 0.02%.[4]

High-resolution DNA melting analysis (HRMA)

HRMA has been used to evaluate individuals for SMA in screening and diagnostic applications.[3,119,120] The difference in *SMN1* and *SMN2* copy number present in the specimens will create various unique HRMA profiles, distinguishing a heterozygous PCR product from a homozygous product. Therefore, the *SMN1* homozygous deletions can be readily identified since these samples will generate the same profile independent of *SMN2* copy number. Dobrowolski et al.[3] analyzed 1000 residual newborn blood spots and showed that unaffected individuals have distinct HRMA profiles from those of SMA individuals with *SMN1* homozygous deletion. This study also showed that *SMN2* copy number can be determined by mixing DNA from a homozygous *SMN1*-deleted patient with DNA that has a single copy of *SMN1* and *SMN2*, then matching the HRMA profiles with the corresponding profile of a carrier sample with a known *SMN2* copy number. Therefore, this method has the ability to identify *SMN1* and *SMN2* deletions and also to quantify copy number for both the genes. SMA carriers will be identified using the method, since they will generate different profiles dependent on the *SMN2* copy number. As reported, the assay takes under 3 h, and the cost of reagents and consumables is under $3 per sample. Preliminary results using HRMA were reported from a pilot study in nine hospitals in Utah, Colorado, and Illinois; 16,736 specimens were tested, but no affected individuals were identified.[131]

Real-time PCR

Several real-time quantitative PCR assays have been developed for SMA diagnosis, some of which have been applied to DBS specimens.[7,125] Some assays can only identify *SMN1* deletion (Fig. 3), while others can identify and quantify *SMN1* and *SMN2* copy numbers. The main challenge in developing a PCR assay for *SMN1* absence is the cross-reactivity with the nearly identical *SMN2* sequences; both genes are amplified, so the probe must be highly specific for *SMN1*.[26,132] A multiplex, real-time PCR designed to detect homozygous deletions of *SMN1* and a reference sequence in an RNaseP subunit gene was tested on DNA extracted from 153 DBS specimens.[7] Results showed 100% concordance with clinical status, identifying all of 57 affected individuals, while excluding 39 carriers and 56 unaffected individuals. Successful reduction in cross-reacting fluorescent signals due to probe binding to *SMN2* target was achieved by using an unlabeled *SMN2* probe as a blocker, in combination with a labeled *SMN1* probe with a minor grove binder group at the 3′ end.

Real-time PCR is also used to identify newborns with severe combined immunodeficiency (SCID) by measuring T-cell receptor excision circles (TREC). The TREC assay is currently used by many NBS laboratories in the United States and

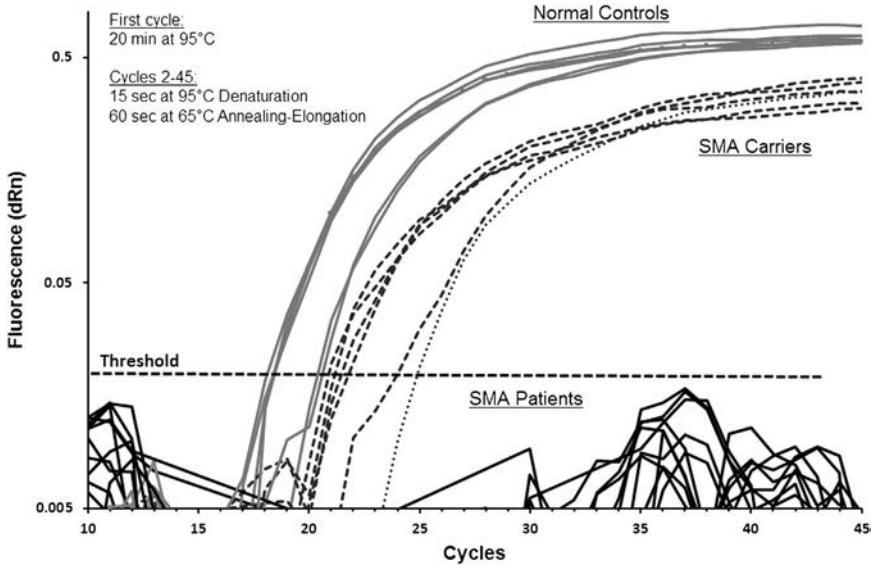

Fig. 3 – Real-time qPCR method for *SMN1* deletion detection. PCR amplification curves from normal controls, SMA parental carriers, and SMA patients with homozygous *SMN1* deletion. No amplification of *SMN1* above cycle threshold was observed in SMA patients. Real-time qPCR method for *SMN1* deletion detection. (Adapted with permission from Tayloret al.[2])

globally. A recent publication established that *SMN1* genotyping could be multiplexed with the TREC assay to simultaneously detect SMA and SCID.[2] The assay described does not require DNA extraction, and the real-time PCR reaction is carried out with the DBS punch in the PCR tube with the PCR reagents. A reference sequence in the *RPP30* gene is used as a control for amplification. The multiplex TREC–*SMN1*–*RPP30* assay has the advantage of using an existing NBS platform, which enhances its robustness, assures adequate throughput, and minimizes incremental costs. Detecting SMA by adding *SMN1*-specific PCR reagents to the TREC assay increases the cost by less than five cents per test; the cost of consumables and reagents to screen for both diseases is the less than $1 per specimen. The turnaround time from sample preparation to data analysis is less than 3 h. This assay reduces the cross-reactivity between *SMN1* and *SMN2* sequences by increasing the stringency of probe hybridization using a novel locked nucleic acid (LNA) technology. LNA oligonucleotides increase assay specificity through restriction of the ribose conformation in the oligonucleotide backbone, allowing shorter probe sequences and the use of higher annealing temperatures.[133] In the TREC–*SMN1*–*RPP30* assay, an LNA probe was used to discern the single nucleotide polymorphism (SNP) in intron 7 of *SMN1* from *SMN2*. Specimens from SMA patients with homozygous *SMN1* deletion produced no signal above the cycle threshold, making the analysis of results very quick and easy to interpret. Samples from carriers are essentially indistinguishable from unaffected individuals in this assay; all samples produce robust signals when at least one copy of the *SMN1* gene is present. The double-blinded study of this method with 26 residual DBS specimens showed 100% concordance with the clinical status.

Confirmatory and follow-up testing

After initial screening, a second-tier test may be used to quantify the number of *SMN1* and *SMN2* genes in the screen-positive sample to further characterize the SMA genotype and provide prognostic information. The second-tier assay may be based on lower-throughput platforms such as digital PCR,[118] since the number of the samples to be tested will be smaller. Second-tier testing will confirm screen-positive samples as well as identify newborns who are at the highest risk for infantile onset.

Legal, ethical, and social issues

Studies have been conducted to examine the legal, ethical, and social issues with population-based pilot studies for SMA. A study focused on the parental decision-making process in population-based pilots by engaging a focus group in public acceptability in an opt-out research pilot study.[134] Parental attitudes toward NBS for Duchenne Muscular Dystrophy and particularly SMA in a recent survey also showed overwhelming parental support for NBS, even without further development in treatments.[135] The Newborn Screening Translational Research Network (NBSTRN) Bioethics and Legal Issues Work Group has assessed questions related to parental consent for NBS pilot studies and concludes that a signed consent document is appropriate in some circumstances for pilot screening.[134] Furthermore, with known diagnosis, genetic counseling can be useful and educational; however, the approach and timing of the counseling will need to be refined.[86]

In 2008, SMA was submitted for formal consideration to the US Health and Human Services Secretary's Advisory Committee on Heritable Diseases in Newborns and Children by a collaborative group of physicians, researchers, the SMA community, and the Families of SMA. The consensus of this committee's internal Nomination and Prioritization Workgroup was that the addition of SMA to the uniform screening panel was premature at that time.[136] The Workgroup recommended the implementation of prospective pilot studies of the screening method by one or more traditional public health laboratories to validate the reproducibility of the preliminary findings in research settings. The Workgroup

also noted that the time required for such validation would allow for an assessment of potential therapies of drugs and other treatment benefits rather than just relying on the nutritional support and respiratory care options. Since then, clinical trials have made considerable progress, and the validation of screening methods in public health NBS laboratories is just beginning. As of this publication, an initial pilot has been completed by the Wadsworth Center of the New York State Department of Health[137] with further validation planned, and a population-based pilot study in Taiwan that began in November 2014 had detected two cases within 4 months (personal communication: Yin-Hsiu Chien, National Taiwan University Hospital, February 2015).

Summary and future directions

The molecular basis for SMA, the leading genetic cause of death in children, was uncovered some 20 years ago. Since then, scientific progress has spurred the development of innovative therapies that are just beginning clinical trials in pre-symptomatic patients. NBS pilot studies to establish technical reliability and operational feasibility in the context of public health programs will be essential to prepare for the prospect of effective pre-symptomatic treatment. Such pilot studies will provide the information needed for evidence-based review, both by NBS programs and their advisory committees, when considering the inclusion of SMA in routine NBS panels. As clinical trials progress and public health programs prepare, the vision of preventing the burden of death and disability caused by SMA will become a reality.

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Emerging therapies and challenges in Spinal Muscular Atrophy

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ABSTRACT

Spinal muscular atrophy (SMA) is a hereditary neurodegenerative disease with severity ranging from progressive infantile paralysis and premature death (Type I) to limited motor neuron loss and normal life expectancy (Type IV). Without disease–modifying therapies, the impact is profound for patients and their families. Improved understanding of the molecular basis of SMA, disease pathogenesis, natural history and recognition of the impact of standardized care on outcomes has yielded progress towards the development of novel therapeutic strategies and are summarised. Therapeutic strategies in the pipeline are appraised, ranging from *SMN1* gene replacement to modulation of *SMN2* encoded transcripts, to neuroprotection, to an expanding repertoire of peripheral targets, including muscle. With the advent of preliminary trial data, it can be reasonably anticipated that the SMA treatment landscape will transform significantly. Advancement in presymptomatic diagnosis and screening programmes will be critical, with pilot newborn screening studies underway to facilitate preclinical diagnosis. The development of disease–modifying therapies will necessitate monitoring programmes to determine the long–term impact, careful evaluation of combined treatments and further acceleration of improvements in supportive care. In advance of upcoming clinical trial results, we consider the challenges and controversies related to the implementation of novel therapies for all patients and set the scene as the field prepares to enter an era of novel therapies.

Introduction

Spinal muscular atrophy (SMA) is characterised by muscle weakness and severe physical disability due to motor neuron degeneration in the spinal cord and brainstem. It continues to represent the leading genetic cause of infant death due to respiratory insufficiency, with a pan–ethnic incidence of approximately one in 11,000 live births and a carrier frequency of one in 40–67.[1] The most common form of SMA is caused by mutations in the Survival Motor Neuron 1 (*SMN1*) gene, resulting in SMN protein deficiency.[2] The almost identical Survival Motor Neuron 2 (*SMN2*) gene produces a small amount of functional SMN protein and SMN2 copy number is recognised as a major modulator of the SMA phenotype.[3] There have been significant advances in the understanding of the underlying pathogenic process in SMA. Concomitantly there has been progress in defining disease progression and natural history, with a concerted effort in developing outcome measures and clinical trial readiness. Consequently novel genetic therapies, aimed at modulating SMN protein expression have resulted in significant clinical improvement in SMA patients for the first time, thereby providing much needed hope for the treatment of this devastating disease. As such, the present Review will focus on recent advances made towards developing novel therapeutic strategies and focus on and future challenges as the field enters into a new treatment era.

Progress in understanding the natural history of spinal muscular atrophy

SMA has a broad range of age of onset, severity, rate of progression, and variability between and within subtypes (table 1).[4, 5] In type I SMA, earlier age of onset is associated with worse prognosis and mortality;[6] the median age to death or ventilation (>16 hours per day) is 13.5 months and 10.5 months for patients with 2 copies of *SMN2*.[5] Patients with SMA type II have a better prognosis than those with type I disease, with 93% surviving to 25 years.[6] After age

15, a relative stability in function develops with subsequent gradual decline over time.[7] Age of onset is also a predictor of functional ability, with patients with classified as SMA IIIa having a 73% probability of walking 10 years after diagnosis, while SMA type IIIb patients have a 97% probability of walking 10 years after diagnosis.[8]

A better understanding of the natural history of SMA has been crucial to the development of relevant outcome measures and implementation of clinical trials. Understanding variability in the rate of clinical progression in SMA related to age, SMA type and ambulation status will assist in the development of appropriate motor function scales that are able to monitor subtle but clinically meaningful changes (see review 10). The clinical heterogeneity in motor function is a challenge and connected with the range of possibilities for change in short time frames. The pattern of age–related changes in motor function in SMA types II and III is non–linear, and there are different patterns of progression between ambulant and non–ambulant patients.[7, 9] In non–ambulant patients, variable improvement in motor function occurs up to 4–5 years of age, before functional ability (eg in upper limbs) declines between 5 and 15 years. After age 15, a relative stability in function develops with subsequent gradual decline over time.[7] Decline in motor and respiratory function within a 12 month period was often minor, although progression was variable between individuals[7, 9]. This slow rate of progression, particularly in milder phenotypes, poses a major challenge to clinical trials in SMA because most trials need to be completed within 1–2 years. Rather than SMA phenotype, ambulant status may be more relevant to the trajectory of disease progression and consequently in trial design and outcome measure development. For instance different outcome measures are required to monitor clinically important differences among ambulant and non–ambulant cohorts, yet it will also be important to better connect scales that measure different functional levels to be able to more accurately demonstrate improvements.[10]

In addition to motor function scales, neurophysiological studies have provided insights into clinical progression, timing of motor neuron loss and compensation in SMA, however these studies have predominantly focused on later stages of disease. Electrophysiological outcomes compound muscle action potential (CMAP) amplitude and motor unit number estimation (MUNE) correlate with age, SMA type, functional status and SMN2 copy number.[11] Gradual decline in motor and respiratory function may in part be related to physical growth in SMA types II and III, with the provision that CMAP amplitude remains stable.[9] In addition, the CMAP amplitude may also remain constant despite reduction in MUNE values, a finding explained by the presence of motor unit loss with compensatory collateral sprouting and supported by axonal excitability studies.[12] Transcranial magnetic stimulation techniques assessing central motor networks also suggest adaptive changes.[13] Neurophysiological studies are rare in presymptomatic infants but suggest motor function is initially relatively preserved.[11, 14] In SMA type I this loss of motor function is followed by early and precipitous reductions in CMAP and MUNE responses. The onset, time course and extent of motor neuron loss has not been established in SMA types II and III, yet is important in determining whether there is a specific therapeutic window. Preclinical studies of SMN restoring therapies, such as gene therapy and antisense oligonucleotides to correct SMN2 mRNA splicing, provide support for the utilisation of electrophysiological biomarkers for treatment stratification, determining response and defining therapeutic windows.[15]

Clinical Care

In parallel with preclinical advances, continued improvements in multidisciplinary care and technological advances have altered the natural history of SMA since the Consensus Statement for standards of care in SMA was published in 2007 (table 2 and appendix 1),[16]

however, even in areas of general consensus, marked variability in the implementation of the standards of care, particularly in the use of ventilation, nutritional support and scoliosis surgery have been observed.[17, 18] For patients participating in clinical trials, it is crucial to standardise the management of modifiable factors, particularly nutrition and respiratory support, as differences in care practices may impact outcome. However, at the most severe end of the SMA phenotypic spectrum, in the setting of uncertain therapeutic efficacy, this remains a challenge. Advances in drug development are likely to impact the standards of care for SMA, particularly as successful disease modification will inevitably alter natural history and necessitate new standards of supportive care and interventions.

Respiratory management

Respiratory complications are the major cause of morbidity and mortality in SMA. The onset of peripheral hypoventilation may be asymptomatic and initially occur during sleep, but with deterioration day–time respiratory dysfunction develops. Objective measures of respiratory function are not routinely performed in children younger than four to six years due to complexity of the required manoeuvres; potential alternative measures of respiratory function, eg, sniff nasal inspiratory pressure and forced oscillation techniques, have been proposed in this population as they are non-invasive and require less patient cooperation.[19] Attempts to identify night time hypoventilation using pulmonary testing in SMA patients have been largely unsuccessful,[20] highlighting the continued benefit of overnight polysomnography. Assessment frequency needs to be individualised, based on current functional status and rate of disease progression, and should be supplemented with other clinical observations, such as assessment for paradoxical breathing and chest wall growth, among others.

The major respiratory complications faced by SMA patients include impaired cough—resulting in reduced clearance of lower airway secretions—hypoventilation, chest wall and lung underdevelopment and recurrent infections that exacerbate muscle weakness. While contentious, proactive management with non-invasive ventilation, even before the onset of paradoxical respirations has led to improved survival, prevention and improvement in chest wall deformity, and improved quality of life.[21, 22] In addition, optimising airway clearance is important for acute and chronic management of SMA patients with secretion mobilisation techniques such as assisted coughing,[23-25] physiotherapy and postural drainage.[26] The decision to progress to invasive ventilation with tracheostomy remains an ethical dilemma and considerable variability exists between countries with no consensus in guidelines.[17, 18] The goal of interventions should always be to improve quality of life with the provision of support and assistance to parents in making difficult decisions consonant with their values and beliefs.

Nutritional Support

Malnutrition is prevalent in SMA; with bulbar dysfunction and deterioration of nutritional status preceding and exacerbating respiratory failure with disease progression.[27, 28] Appropriate nutritional management of SMA patients is critical for improving quality of life and optimising survival,[29] although no clear consensus exists on the timing of initiation of nutritional support.

Orthopaedic considerations

Scoliosis is a common complication of SMA, present in 60–95% of patients, secondary to progressive muscle weakness. In SMA types I and II, scoliosis occurs earlier and a more severe, progressive curvature is evident compared to SMA type III.[30] Progression of scoliosis

may exacerbate respiratory dysfunction, gastrointestinal reflux, and increase postural discomfort.[30] Management of scoliosis includes non–surgical options, such as physical therapy, bracing and seating modification, and depending on the strategy used, may slow but not necessarily prevent curve progression. Additionally, surgical approaches are utilised in progressive scoliosis, most frequently in type II and III SMA. Posterior spinal fusion is typically implemented after skeletal maturity during adolescence in SMA, with iliac fixation used to assist correction of pelvic obliquity. Innovative surgical techniques utilising growing rods (eg, vertical prosthetic titanium rib or magnetic rods) enable spinal growth while avoiding repeated invasive surgeries, however medium to longer term implications remain unclear. While surgery does not reverse the respiratory reserve lost due to scoliosis, it leads to improved life quality[31] and can slow deterioration of respiratory function.[32] Finally, with an intrathecally administered therapy showing promise in Phase 3 clinical trials, construction of bony windows may be considered to facilitate drug administration.

Genetic and environmental insights into pathogenesis

While mutations in *SMN1* characterise SMA, disease severity is also linked to a number of genetic modifiers. These modifiers are of relevance in enabling patient stratification in clinical trials, better prediction of an individual's prognosis, and establishing newborn screening. Patients have variable copy numbers of the *SMN2* gene, a related gene that differs from *SMN1* by only five nucleotides, altering splicing and leading to transcription of a non–functional SMN protein lacking exon 7 in the majority of transcripts (Figure 1). [33] SMN2 copy number is the main determinant of phenotype, although not solely sufficient to predict severity. [34] Sequence variations within the *SMN2* gene and upregulation of modifier proteins such as plastin 3 may also positively modify phenotype.[35-37] In addition, nutritional

deficiency, oxidative stress and hypoxia, partly due to gastrointestinal dysfunction, may cause widespread splicing alterations, including *SMN2* and accelerate SMA progression.[38-40]

How low levels of SMN cause SMA

Recent insights into the role of SMN within motor neurons have furthered our understanding of the implications of SMN deficiency. [41] The best characterised role of the SMN complex is in the assembly of Sm proteins (a distinctive family of RNA associated small proteins) onto small nuclear RNAs (snRNAs), forming small nuclear ribonucleoproteins (snRNPs) which are essential components of pre–mRNA splicing machinery in cells.[42] SMN deficiency and therefore reduced snRNP assembly capacity are proposed to cause aberrant splicing or transport of RNPs to the detriment of motor neurons.[43] A recent study showed transcriptional dysregulation in motor neurons isolated from very young presymptomatic SMA mice that preferentially affected a small subset of genes involved in synaptogenesis and maintenance of neuromuscular junctions (NMJs).[43] Furthermore, some of these dysregulated motor neuron relevant genes showed underlying splicing changes, strengthening a potential link between aberrant splicing and motor neuron vulnerability.[43]

A second view of SMA pathogenesis contends that SMN has axonal function independent of splicing that may be disrupted in SMA. Consequently, SMN deficiency may impair targeting and local translation of axonal mRNAs essential for motor neuron development and maintenance. [44, 45] Furthermore, SMN regulates several other fundamental cellular processes in the neuronal cytoplasm that are critical for maintaining axonal and synaptic health, including endocytic pathways, local translation, mitochondrial transport and targeting to axons and ubiquitin homeostasis.[46-51]

Animal models of SMA

Current understanding of SMA pathogenesis has been generated largely as a result of the availability of mouse models of SMA. These have been largely generated by targeting the endogenous mouse *Smn* gene, whilst using transgenic strategies to add variable copy numbers of the human *SMN2* transgene.[52] These animal models tend to phenocopy severe forms of the human disease, resulting in the majority of animal–based work focussing on early postnatal phenotypes. Recently, alternative strategies have been employed to generate mice modelling less severe forms of SMA, allowing investigation of disease pathogenesis and preclinical drug testing more relevant to Type II and III SMA.[53, 54] Additional animal models of SMA are also beginning to play an important role in SMA research, including *Drosophila* and zebrafish,[55, 56] and recent developments suggesting that large animal models (eg, pigs) may be forthcoming.[57]

While alpha motor neurons in the spinal cord remain the primary pathological target in SMA [58] there is now accumulating evidence suggesting that other cells, tissues and organs contribute to disease symptoms (Figure 2).[59, 60] For example, there is now experimental evidence suggesting a non–cell–autonomous contribution to motor neuron degeneration from astrocytes and Schwann cells.[61, 62] Likewise, low levels of SMN in skeletal muscle have been implicated in SMA pathogenesis with significant disruption of the molecular composition of skeletal muscle evident in pre-symptomatic severe SMA mice in the absence of detectable changes in lower motor neurons.[63] One potential unifying factor may be a deficiency in the development of vasculature in SMA; the resulting hypoxia would likely impact motor neurons as well as skeletal muscle and possibly contribute to the gastrointestinal defects (gastroesophageal reflux, constipation and delayed gastric emptying) commonly seen in SMA

patients. [64]Although the mechanisms mediating the effects of vascular depletion have not been fully elucidated, hypoxia has been identified as a modifier of *SMN2* splicing potentially explaining some of the splicing alterations observed in SMA.[38, 39, 65]

Disease–associated phenotypes have been reported across a range of other organs in SMA mice (in some cases supported by data from human patients). These include functional and structural cardiac defects,[66] abnormal development of the gastrointestinal tract, liver and spleen [64, 67, 68] and irregular bone remodelling and skeletal pathology.[69] These findings suggest that successful treatment of SMA may require systemic targeting of a range of affected tissues. However, how these findings will translate to humans is uncertain, for example heart defects are rare in humans.

Defining the therapeutic window in animal models

In severe SMA mice, induction of SMN expression at gestation or in the early postnatal period substantially improves survival, while later induction is less effective.[70, 71] Mice are resistant to SMN depletion after early postnatal stages, suggesting there is a period of sensitivity to low SMN levels and that high SMN levels are required during this early postnatal stage.[70, 72] Notably, in mouse models, the time period when SMN function is required coincides with the neonatal period of NMJ establishment, development and maturation, suggesting that the mechanistic underpinnings of the therapeutic window are based on the pathways driving normal NMJ maturation. These observations imply that early correction of SMN levels in SMA types II and III is likely to be necessary and sufficient to protect the neuromuscular system, and lifelong expression of SMN may not be required.

While most preclinical studies investigating SMN restoration in animals were limited to evaluating presymptomatic administration of gene therapy with viral vectors or antisense oligonucleotides to correct SMN2 splicing and enhance SMN expression, which in most cases robustly improves the health of SMA mice, some studies have tested the impact of pre– and post–symptomatic SMN restoration. Systemic delivery of these approaches largely rescued SMA mice's motor function, neuromuscular physiology and life span when delivered within the first three postnatal days (P0–3), but were less effective beyond P5 and gene therapy was not effective at P10, confirming the presence of a narrow therapeutic window.[73, 74] However, SMN restoration using intravenous injection of self–complementary adeno–associated virus (scAAV9)–SMN vectors given at symptom onset had a marked effect with amelioration of severe proximal weakness and electrophysiological indices, in a porcine model of SMA[57] which may represent a more relevant model for predicting efficacy in humans. Even so, pre-symptomatic delivery prevented the development of symptoms, suggesting that therapeutic windows are still critical in this model. While it is accepted that animal models cannot recapitulate human SMA precisely, the translation of concepts of motor neuron degeneration to humans suggests presymptomatic or early-symptomatic restoration of SMN (during NMJ maturation) will likely produce the best response to therapy. An unresolved issue remains as to whether commencing therapy in older patients will be effective. The time course and extent of motor neuron loss in type III or IV has never properly been mapped, largely due to a paucity of robust animal models of less severe forms of SMA. Encouragingly, recent results from models of milder SMA phenotypes suggest that some therapeutic efficacy may be possible even at late disease stages.[75] While the therapeutic window for SMA types III and IV has not been defined, the normal early motor development may suggest it is linked to age of presentation and broader than types I and II. Preliminary clinical trial data are emerging and indicating that with SMN repletion motor neurons may

not be irreversibly doomed. However, animal models recapitulating severe SMA show rapid postnatal motor neuron attrition and reduced efficacy with delayed treatments, such that the optimal success may ultimately arise from presymptomatic provision of therapy.[73, 76]

Therapeutic developments

The pipeline of therapies for SMA encompasses four different strategies, including *SMN1* gene replacement, modulation of *SMN2* encoded full–length protein levels, neuroprotection, and targeted improvements of muscle strength and function (Figure 3). Translational research continues to progress and clinical trials have recently reporting positive preliminary results related to safety and efficacy of the newest approaches (Table 3). This follows a number of negative clinical trials of repurposed drugs, including valproic acid and acetyl–L–carnitine, phenylbutyrate hydroxyurea, riluzole, and somatotropin,[77-79] despite promising preclinical data. Importantly, these negative studies have informed clinical trial design, validated the reliability and feasibility of specific outcome measures and highlighted the importance of patient stratification.

In a mouse model of severe SMA, postnatal intravenous gene therapy using a viral vector rescued motor function and neurophysiology and extended survival from two weeks to beyond 250 days.[70] A Phase 1/2a clinical trial for AVXS–101(a self-complementary AAV9 carrying the SMN gene under the control of a hybrid CMV enhancer/chicken-β-actin promoter) in SMA type I infants has completed enrolment and initial observations in safety, survival and motor function have been promising with all patients event free (death or continuous non–invasive ventilation greater than 16 hours per day) and stabilisation of pulmonary outcomes reported.[80] Modest improvements in motor function were observed in patients receiving a low dose of the study drug and greater improvements were shown in

patients receiving the proposed therapeutic dose; two patients achieved normal motor function 4.9 and 10.3 months following treatment as measured by the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND), a marked change from the natural history of SMA type I.[81]

The most advanced compound known to increase production of fully functional SMN protein is nusinersen (IONIS–SMN_{RX}), an antisense oligonucleotide administered intrathecally that modifies the splicing of *SMN2*. A Phase 1 open label study of nusinersen in 28 SMA II and III patients aged 2 to 14 years reported that the drug was well tolerated with no safety concerns identified.[82] Transient back pain and post lumbar puncture headache were of a similar frequency to previous reports in infants and children undergoing lumbar puncture. Favourable time and dose-dependent increases in muscle function were reported in patients nine to 14 months after dosing.[83] Observations from a phase 2 open label study of nusinersen in 20 infants with SMA type I show increases in motor function, ranging from stable independent sitting to walking (the latter patients having 3 copies of SMN2 not the standard type I SMA copy number of 2), with no evidence of a therapeutic plateau in motor skills yet. [84]Interim analyses of Phase 3 clinical trials evaluating nusinersen, including infants with SMA type I (ENDEAR) and children with SMA type II (CHERISH) have reached primary endpoints with improvement in motor milestones and favourable safety profiles. Regulatory filings have recently been submitted and an expanded access programme initiated for SMA type I. The effect of presymptomatic administration of nusinersen is currently being evaluated and will provide pivotal insights into therapeutic windows. Further advances include the development of peptide-mediated oligonucleotides to enable systemic therapy and overcome difficulties with CNS delivery via repeated intrathecal injections, with recent efficacy demonstrated in rodent models. [85]

Orally bioavailable small molecules are being developed for selective *SMN2* splicing correction and several (RG 7916 and LM1070) are entering early phase clinical trials. Administration of these compounds to mice with severe SMA increased SMN protein levels, motor function, and survival (from 18 to beyond 150 days).[76, 86] Further clinical trials are also needed to define the efficacy of salbutamol, a β agonist that promotes exon 7 inclusion in SMN2 transcripts, following encouraging early results from several pilot studies in SMA patients.[87] Increasing the expression of *SMN2* with small molecules such as quinazoline-derived compounds moderately increased SMN mRNA and protein levels, and survival in severe SMA mice.[88] However, plans to progress beyond phase 1 clinical trials have been terminated, reflecting challenges in translating disease-modifying benefits from mouse to human.

Olesoxime has entered clinical trials in SMA patients following demonstration of its neuroprotective properties motor neurons in cell culture and SMA mice. Phase 2 trials have been completed and while the primary endpoint was not statistically significant, a greater percentage of patients were stable or improved compared with placebo suggesting that olesoxime may slow decline in motor function over two years in already symptomatic patients with SMA types II and III.[89] However, further data is needed to determine whether this is a clinically meaningful effect.

Additional strategies focused on improving neuromuscular function and physical performances in SMA patients are also being assessed in clinical trials. Among these is CK-2127107, that slows calcium release from fast skeletal muscle troponin and sensitizes the sarcomere to calcium thus increasing contractile response to nerve signalling; studies have

demonstrated its efficacy in mouse models of motor neuron disease and it appears safe in healthy human volunteers.[90] Additionally, exercise induced neuroprotection has recently been demonstrated in SMA–like mice with greater motor neuron survival, maintenance of neuromuscular junctions and improved neuromuscular excitability properties, accompanied by positive metabolic and behavioural changes.[91] The benefits and risks of different types of exercise are being evaluated in SMA patients and initial studies have demonstrated resistance training is feasible, safe and well tolerated and aerobic training increases oxidative capacity.[92, 93] Further outcomes will be important in planning patient therapy and rehabilitation.

A number of potential SMN independent therapeutic targets have been identified in preclinical studies. These include the compounds Fasudil and Y–27632 that regulate actin cytoskeleton integrity though ROCK inhibition,[94, 95] the antioxidant flavonoid quercetin that suppresses beta–catenin signalling,[96] BAY 55–9837 that indirectly stabilises *SMN* mRNA[97] and compounds that activate the mTOR pathway.[98] In addition RNA sequencing of motor neurons may identify novel downstream targets of splicing alterations. Stabilisation of endogenous SMN protein provides a further therapeutic strategy, with STL-182 showing promising pre-clinical efficacy.[99]

Conclusions and future directions

There have been tremendous advances in therapeutic development in SMA, with treatment options rapidly evolving and preliminary results of clinical trials in patients producing new hope. In parallel, there has been substantial progress in understanding clinical disease progression, and natural history to accelerate the implementation of clinical trial. Rodent models suggest that requirements for normal SMN levels are paramount during development of the motor unit, with SMN restoring therapies most effective early. The translation of these

concepts to humans is needed to determine whether therapy in later stages of disease is beneficial. Critical for timely access to novel disease modifying treatments is the rapid recognition of clinical manifestations and diagnosis, with presymptomatic diagnosis to guard against disease onset and progression the ultimate aim. Population based newborn screening pilot programmes are determining the feasibility and reliability of presymptomatic diagnosis and effective molecular methods have been validated on dried blood spots, including real time PCR and high–resolution melting analysis.[100-103]

Improvements in multidisciplinary clinical care together with advances in technology have changed the natural history for patients with SMA. With new therapeutics emerging it is likely that profound shifts in management approaches will transpire in severe SMA. These will also necessitate additional validation of non–motor standardised and reliable outcome measures, particularly respiratory assessments, as these are functionally meaningful and contribute substantially to morbidity. Secondary complications such as scoliosis and contractures may further limit the value of existing motor outcome measures. Further, individual motor function scales are relevant to specific levels of SMA severity and it will be important to better connect scales that measure different functional levels to be able to more accurately demonstrate improvements. While motor function scales are a major focus and most relevant to SMA, strength testing, electrophysiological assessments, metabolomic and proteomic outcomes measures are also being integrated into natural history studies and clinical trials in SMA.[104]

With the tantalising prospect of novel therapies moving closer to clinical reality, more questions arise, compelling the formation of collaborative and linked future monitoring programmes to determine the impact of these therapies. Longer term monitoring programmes

should also include assessments of cognition, growth, autonomic function, adverse events and enable a comparison and evaluation of combined treatments. These will serve to understand how novel therapies may affect phenotype over the longer term and the duration of effect– will they reduce progression, stabilise or improve function? How will they affect requirements for permanent ventilation or age of death? What is the potential variability of responsiveness of different motor neuron subpopulations due to drug distribution or inherent differences in reversibility? Further, the possibility that reinnervation may stress remaining motor neurons, resulting in a post–polio like condition with late deterioration, must be considered. It is likely that combined therapies increasing SMN levels while also enhancing and preserving neuromuscular function and preventing additional systemic pathology will provide the best approach. In the setting of a first in class approved therapy, continued progress in developing second generation and combination therapies will require innovative approaches in trial design. In addition new challenges are arising with emerging therapies, including difficulties with access to treatment associated with the complexities, costs and expertise required with intrathecal administration. Further efforts to ascertain optimal routes of drug delivery and distribution and defining the therapeutic window will be essential.

As the field looks towards a new treatment era, it is necessary to focus on timely access to novel, disease–modifying therapy and endeavouring to develop therapies for patients with SMA of all ages and severities.

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The authors contributed equally to the literature search and the writing and formatting of the Review, and to critically reviewing the manuscript.

Potential Conflicts of Interest

Dr Farrar has received an honoraria from Biogen. Drs Carey, Park, Turner, and Professors Vucic, Swoboda and Kiernan have no conflicts of interest. Professor Gillingwater is named on a patent application filed by the University of Edinburgh covering the use of beta–catenin inhibitors for the treatment of SMA (DD/P206869GB, filed 10/7/2013).

Table 1: Classification and subtypes of Spinal Muscular Atrophy.

Type	Age of onset	Maximal motor milestone	Motor ability and additional features	Prognosis^
SMA 0	Before birth	None	Severe hypotonia; Unable to sit or roll*	Respiratory insufficiency at birth; Death within weeks
SMA I	2 weeks (Ia) 3 months (Ib) 6 months (Ic)	None	Severe hypotonia; Unable to sit or roll #	Death/ventilation by 2 years
SMA II	6–18 months	Sitting	Proximal weakness; unable to walk independently	Survival into adulthood
SMA III	< 3 years (IIIa) > 3 years (IIIb) >12 years (IIIc)	Walking	May lose ability to walk	Normal lifespan
SMA IV	>30 years or 10 – 30 years	Normal	Mild motor Impairment	Normal lifespan

- *Need for respiratory support at birth; contractures at birth, reduced fetal movements
- # Ia joint contractures present at birth; Ic may achieve head control
- ^ Prognosis varies with phenotype and supportive care interventions

Table 2 Current management of Spinal Muscular Atrophy

	Assessments	Interventions
Respiratory	Cough effectiveness; respiratory muscle function tests; overnight oximetry; forced vital capacity (>6 years). Overnight polysomnography if disordered breathing suspected. Acute respiratory infections	Referral to respiratory specialist Routine immunisations Annual influenza vaccination Airway clearance techniques and cough assistance – chest physiotherapy, postural drainage, mechanical or manual cough assistance Non–invasive ventilation (nocturnal and/or daytime if indicated)* Antibiotics intensified airway clearance, increased ventilation support*
Gastrointestinal and Nutritional	Feeding and swallowing assessment Assess caloric intake Assess for signs of reflux or aspiration Assess for constipation	Nutritional supplementation, modifying food consistency, optimizing oral intake, positioning and seating alterations. Nasogastric, nasojejunal or percutaneous gastronomy – as soon as reduced oral intake is recognised Nissen fundoplication (if indicated) Hydration, regular oral aperients
Orthopaedic and rehabilitation	Posture, Mobility, Function Contractures Scoliosis Hip subluxation/dislocation	Equipment to assist with mobility, self–care and function Physiotherapy, standing frames, orthoses Spinal surgery†
Psychological	Assess for depression/anxiety	Counselling, pharmacotherapy

The management of SMA incorporates a multidisciplinary and supportive approach, including neurologists (adult and paediatric), respiratory physicians, geneticists, gastroenterologists, palliative care physicians, rehabilitation specialists, orthopaedic surgeons and allied health

*The appropriate level of interventional support to prolong life, particularly in SMA type1, is controversial and the consensus statement [71] recognises the importance of discussions with the family to explore and define potential quality of life and palliative care issues. The philosophy and introduction of proactive respiratory support in patients with SMA type 1 varies considerably and practice varies internationally.

†There is no consensus on management of scoliosis or hip subluxation/dislocation in non–ambulant patients.

Table 3: New therapeutic approaches in spinal muscular atrophy: Current clinical trials

Approach	Study Description	Preliminary Results
SMN1 GENE REPLACEMENT		
AVXS–101* [†]	Phase 1/2a gene transfer of SMN1 in SMA type I infants Two cohorts treated with a single dose of AVXS-101 delivered intravenously	Safety, survival and motor function have been promising with all patients event free (death or continuous non–invasive ventilation greater than 16 hours per day) and stabilisation of pulmonary outcomes [80]
MODULATION OF SMN2 FULL LENGTH PROTEIN		
Nusinersen (IONIS–SMN$_{RX}$)*	10 Phase 1 to Phase 3 studies Nusinersen administered via intrathecal injection in SMA type I infants (0–6 months) and later–onset type II / III participants (age range 2–14 years)	Favourable safety, tolerability and encouraging clinical efficacy [83, 84]. Intrathecal administration tolerated, no drug related adverse events;[82]. Phase 3 ENDEAR study in SMA type I and Phase 3 CHERISH study in childen aged 2-12 years with SMA type II - Primary endpoint met in each study at interim analysis with statistically significant improvement in motor milestones.
RG7916 *(RO7034067)*	Phase 2 in adult and paediatric patients with Type II and Type III SMA with oral delivery.	
LMI070	Phase 1/2 study in infants with type I SMA (1–7 months) of oral LMI070	
NEUROPROTECTION – PROMOTE SURVIVAL OF MOTOR NEURONS		
Olesoxime (TRO19622)	Phase 2 studies in 3–25 year olds with type II or non ambulant type III SMA	A greater percentage of patients were stable or improved compared with placebo, however the primary endpoint was not met (p=0.07) .[89]
Exercise	Pilot study of a physio–therapeutic approach tailored to type II and III SMA patients aged 5–10 years.	
Exercise	Muscle strengthening program using hand weights and resistance bands in combination with a home based cycle ergometry in type III patients aged 8–50 years.	
ENHANCING NERVE OR MUSCLE FUNCTION		
CK–2127107	A Phase 2 oral compound in SMA type II–IV (aged 12 years+).	
Pyridostigmine	Phase 2 study in SMA type III (aged 6 years+).	
4 aminopyridine	Phase 2/3 study assessing changes in walking ability and endurance in 18–50 year olds with SMA type III.	

*AVXS–101 and Nusinersen have been granted US FDA and ERA orphan drug status and FDA fast track approval

†AVXS–101 has been granted FDA Breakthrough Therapy Designation. Following a FDA Type B meeting on 30 Sep 2016 a single arm pivotal trial has been announced.

Figure 1. Genetics of Spinal Muscular Atrophy

In humans, the SMN protein is encoded by the *SMN1* and *SMN2* genes. The C to T substitution in exon 7 of *SMN2* is translationally silent but alters splicing such that the majority of SMN2 transcripts lack exon 7 and the truncated protein is unstable. Normally, *SMN1* produces abundant SMN protein. In SMA, homozygous mutation of *SMN1* results in only small amount of functional SMN protein contributed by the varying copy numbers of SMN2.

Figure 2. Pathophysiological findings in SMA

Multiple functional abnormalities in motor networks have been identified in SMA mice and humans including defects in astrocytes, Schwann cells motor neurons and skeletal muscle. Disease–associated phenotypes have also been reported across a range of other organs in SMA mice (in some cases supported by data from human patients). including cardiac structural and functional abnormalities, gastrointestinal tract dysfunction and irregular bone remodelling. One potential unifying factor may be a deficiency in the development of vasculature in SMA with the resulting hypoxia likely impacting a range of cell types.

Figure 3. Therapeutic targets for SMA being investigated in clinical trials.

SMN1 gene replacement therapy utilises a self–complementary adeno–associated viral vector (**AAV9–SMN**) that crosses the blood brain barrier following intravenous administration. Compounds that increase the production of fully functional SMN protein by modifying the splicing of SMN2 include the orally available small molecules **RG7916** and **LMI070** and the intrathecally administered antisense oligonucleotide **nusinersen** which acts by displacing heterogenous nuclear ribonucleoprotein (hnRP) proteins from the intronic splicing silencer site on the SMN2 pre–mRNA. The neuroprotective effects of **olesoxime**, through altered

mitochondrial permeability, and **exercise**, through greater motor neuron survival, maintenance of neuromuscular junctions and improved neuromuscular excitability properties are being investigated. Additional strategies focused on improving neuromuscular function and physical performance include **CK–2127107**, a fast skeletal troponin activator that sensitises the sarcomere to calcium and increases the contractile response to nerve signalling, and **4–aminopyridine** and **pyridostimine** that may facilitate neurotransmitter release and increase its synaptic duration.

Appendix 1: Additional evidence base for management of spinal muscular atrophy subsequent to 2007 standards of care

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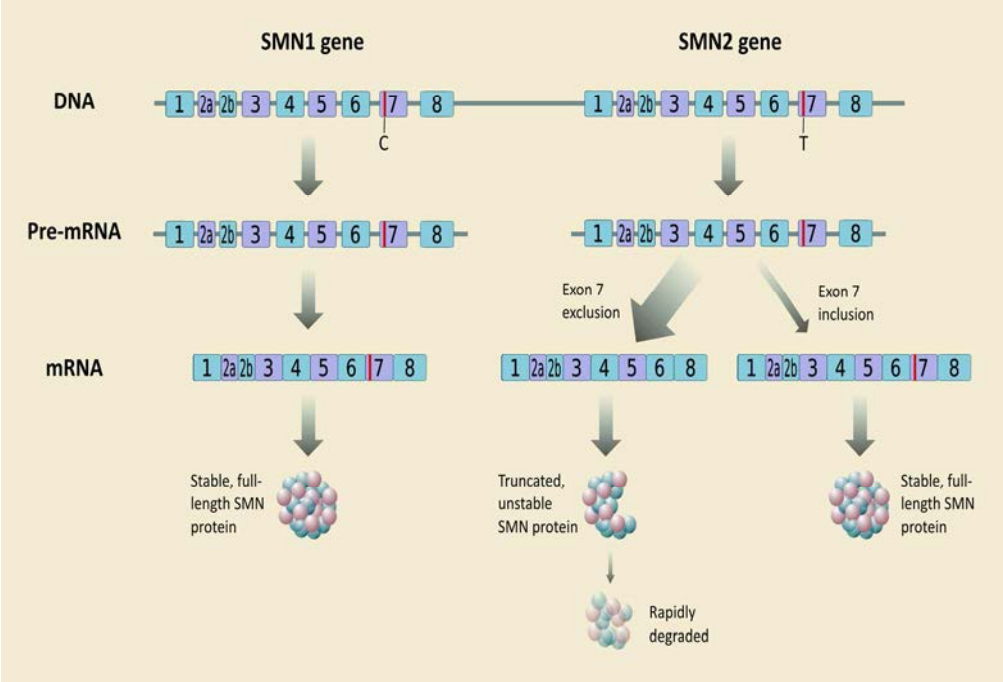
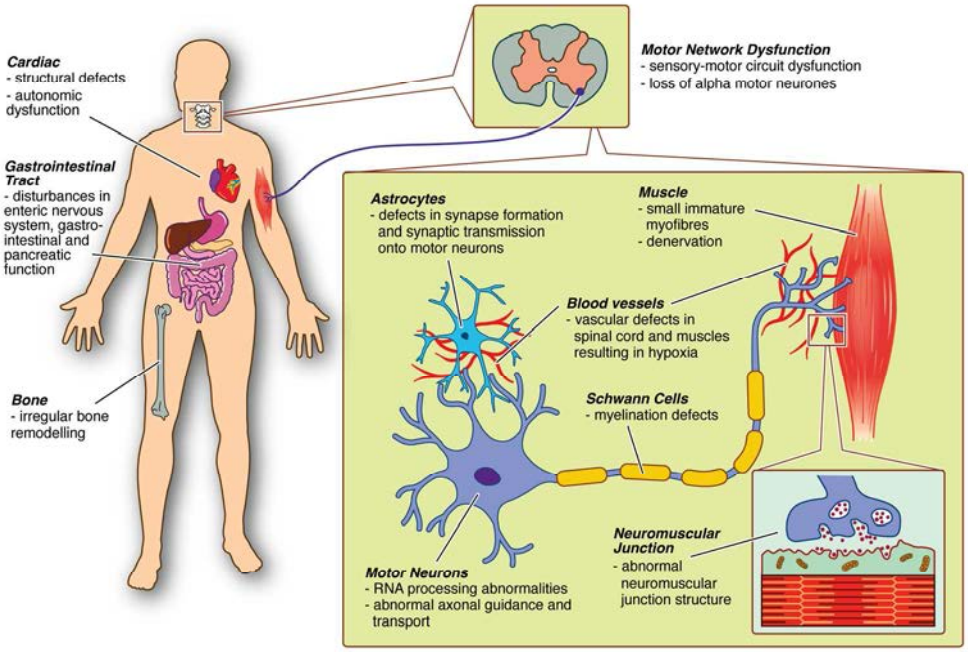
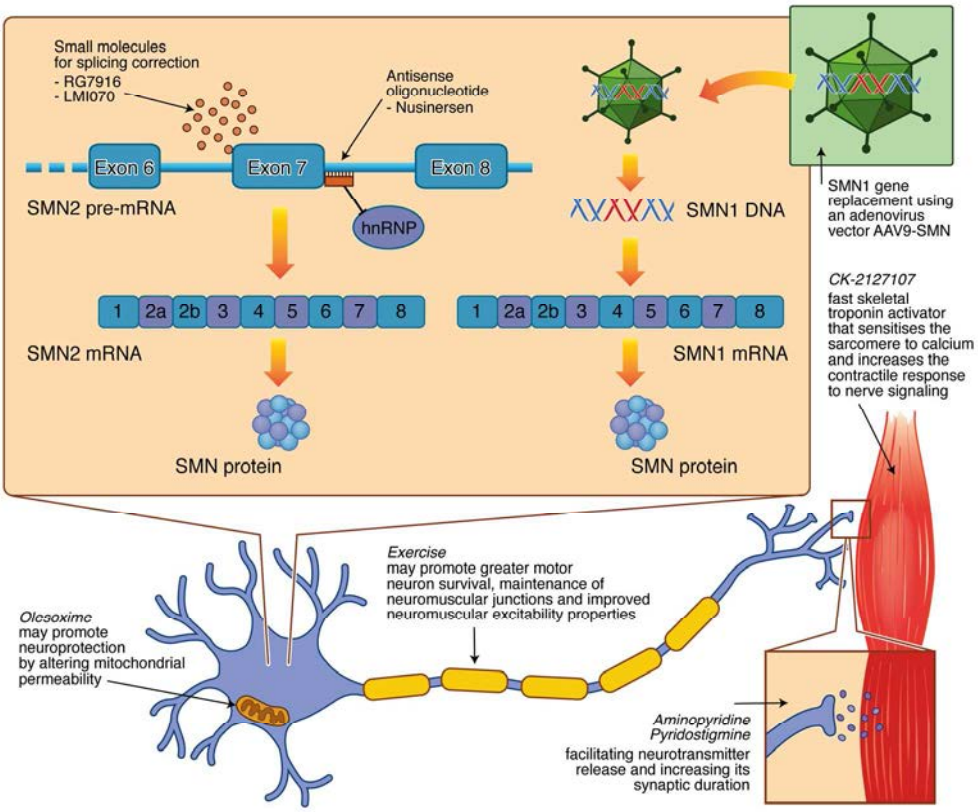


Genetics of Spinal Muscular Atrophy
In humans, the SMN protein is encoded by the SMN1 and SMN2 genes. The C to T substitution in exon 7 of SMN2 is translationally silent but alters splicing such that the majority of SMN2 transcripts lack exon 7 and the truncated protein is unstable. Normally, SMN1 produces abundant SMN protein. In SMA, homozygous mutation of SMN1 results in only small amount of functional SMN protein contributed by the varying copy numbers of SMN2.
Figure 1
708x482mm (72 x 72 DPI)



Pathophysiological findings in SMA
Multiple functional abnormalities in motor networks have been identified in SMA mice and humans including defects in astrocytes, Schwann cells motor neurons and skeletal muscle. Disease–associated phenotypes have also been reported across a range of other organs in SMA mice (in some cases supported by data from human patients). including cardiac structural and functional abnormalities, gastrointestinal tract dysfunction and irregular bone remodelling. One potential unifying factor may be a deficiency in the development of vasculature in SMA with the resulting hypoxia likely impacting a range of cell types.

Figure 2
175x120mm (300 x 300 DPI)



Therapeutic targets for SMA being investigated in clinical trials.
SMN1 gene replacement therapy utilises a self–complementary adeno–associated viral vector (AAV9–SMN) that crosses the blood brain barrier following intravenous administration. Compounds that increase the production of fully functional SMN protein by modifying the splicing of SMN2 include the orally available small molecules RG7916 and LMI070 and the intrathecally administered antisense oligonucleotide nusinersen which acts by displacing heterogenous nuclear ribonucleoprotein (hnRP) proteins from the intronic splicing silencer site on the SMN2 pre–mRNA. The neuroprotective effects of olesoxime, through altered mitochondrial permeability, and exercise, through greater motor neuron survival, maintenance of neuromuscular junctions and improved neuromuscular excitability properties are being investigated. Additional strategies focused on improving neuromuscular function and physical performance include CK–2127107, a fast skeletal troponin activator that sensitises the sarcomere to calcium and increases the contractile response to nerve signalling, and 4–aminopyridine and pyridostimine that may facilitate neurotransmitter release and increase its synaptic duration.

165x138mm (300 x 300 DPI)

Appendix 1: Additional evidence base for management of spinal muscular atrophy subsequent to 2007 standards of care [1]

	Assessment/Intervention	Outcome and clinical implications
Respiratory		
Anderson et al[2]	Sniff nasal inspiratory pressure to measure respiratory function	Sniff nasal inspiratory pressure and forced oscillation technique assessments were feasible and reliable and possible measures of respiratory function in SMA, including young children.
Gauld et al[3]	Forced oscillation technique.	
Stehling et al[4]	Mechanical insufflation/exsufflation	Regular use of mechanical insufflation/exsufflation may improve vital capacity in SMA patients
Han et al [5]	Home mechanical ventilation in SMA I	Reduction in respiratory morbidity
Chatwin et al [6]	Non–invasive ventilation in 13 SMA I patients	A symptom and goal directed approach assists in making informed decisions about discharge home in patients with ventilator dependency and palliation of respiratory symptoms
Benson et al[7]	Tracheostomy in SMA I	Variation in physician recommendations for intervention between countries
Gastrointestinal and Nutritional		
Chen et al[8]	Feeding and swallowing in SMA II/III	Feeding and swallowing difficulties in up to 30%, associated with being underweight and aspiration pneumonia
Messina et al[9]	Feeding and nutrition in SMA II	Difficulties with jaw opening, chewing and swallowing occur in up to 30% of SMA II patients
Mannaa et al [10]	Survival in SMA I	There was a positive trend in survival over a 16 year period. Advances in pulmonary care and aggressive nutritional support may be a factor.
Durkin et al[11]	Early laparoscopic fundoplication and gastrostomy in 12 infants with SMA I	Improved nutritional status and trend toward less long–term aspiration–related events
Orthopaedic		
Chandran et al[12]	Early treatment with titanium growing rods	Preliminary observational case report in 11 children with SMA that growing rods were effective in the treatment of scoliosis
Heydar et al[13]	Magnetic Controlled Growing Rods	Safe and effective technique in correction scoliosis in 18 patients with early onset scoliosis
Dede et al[14]	Self–growing Instrumentation With Gliding Connectors	Growth with growing rods achieved in 3 children with neuromuscular early onset scoliosis – case report
Abol Oyoun et al[15]	Vertical expandable prosthetic titanium rib implants in neuromuscular scoliosis	Preliminary report of use in 2 children with SMA, further follow up needed

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Topical Review

Delay in Diagnosis of Spinal Muscular Atrophy: A Systematic Literature Review

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ABSTRACT

BACKGROUND: Spinal muscular atrophy is a rare genetic disease with devastating neurodegenerative consequences. Timing of diagnosis is crucial for spinal muscular atrophy because early diagnosis may lead to early supportive care and reduction in patient and caregiver stress. The purpose of this study was to examine the published literature for diagnostic delay in spinal muscular atrophy. **METHODS:** A systematic literature search was conducted in the PubMed and Web of Science databases for studies published between 2000 and 2014 that listed any type of spinal muscular atrophy and without molecular, mouse, or pathology in the keywords. Mean and/or median age of onset and diagnosis and delay in diagnosis was extracted or calculated. All estimates were weighted by the number of patients and descriptive statistics are reported. **RESULTS:** A total of 21 studies were included in the final analysis. The weighted mean (standard deviation) ages of onset were 2.5 (0.6), 8.3 (1.6), and 39.0 (32.6) months for spinal muscular atrophy types I, II, and III, respectively, and the weighted mean (standard deviation) ages of confirmed spinal muscular atrophy genetic diagnosis were 6.3 (2.2), 20.7 (2.6), and 50.3 (12.9) months, respectively, for types I, II, and III. For studies reporting both age of onset and diagnosis, the weighted diagnostic delay was 3.6, 14.3, and 43.6 months for types I, II, and III, respectively. **CONCLUSIONS:** Diagnostic delay is common in spinal muscular atrophy. The length of delay varied by severity (type) of spinal muscular atrophy. Further studies evaluating this delay and tools such as newborn screening are warranted to end the diagnostic delay in spinal muscular atrophy.

Keywords: spinal muscular atrophy, diagnosis delay, systematic review, diagnostic odyssey, SMA type I, SMA type II, SMA type III
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Introduction

Spinal muscular atrophy (SMA) is a neuromuscular disease that affects approximately 1 in 6000 to 1 in 11,000 live births in the United States with a high carrier frequency of 1 in 40 to 60.[1-4] SMA is an autosomal recessive disorder caused by mutations in the survival motor neuron (*SMN*) 1 gene and is characterized by degeneration of the motor neurons in the spinal cord, which results in progressive muscular atrophy and weakness.[1,3] It has been shown that another gene, *SMN2*, codes for a protein similar to that encoded by the *SMN1* gene and multiple copies of *SMN2* can somewhat compensate for the loss of the *SMN1* gene and alleviate the severity of clinical symptoms observed.[5,6]

SMA is classified into several types based on the age of onset of the disease and the degree of motor function achieved by the affected individual.[1,3] Approximately 50% of patients diagnosed with SMA have type I, which has an early onset; these infants usually do not survive beyond the first 2 years without intervention.[1] Type I patients have the most severe form of SMA with extensive muscle weakness, are never able to sit without support, and have increasing difficulty over time with swallowing and feeding, and respiratory difficulties.[1,3] In type II SMA, the onset of symptoms occurs slightly later than in type I and, although type II children are generally able to sit without support and some may stand, they are never able to walk independently. Type II SMA patients can present with varying severity of bone

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TABLE 1.
Characteristics and Findings for Included Studies: Studies Reporting Means

Author, Year	Country	Type of Study	SMA Type	No. of Patients	Sex	Mean (SD) Age at First Symptoms, Months	Mean (SD) Age at Confirmed Diagnosis, Months	Genetic Information and *SMN2* Copies
Reported age of onset only								
Rudnik-Schöneborn et al., 2001[12,*]	Poland, Germany	Retrospective chart review with questionnaire survey	II	175	NR	Normal sitters, n = 128: 6.6 (2.3) Delay sitters, n = 47: 9.6 (2.7)	NR	57 type II and 186 type III patients genetically confirmed
			III	266		NR		
Harada et al., 2002[5]	Japan	Clinical research	I	11	8 M, 3 F	3.3 (1.7)	NR	1 copy = 1 type I patient; 2 copies = 7 type I patients; 3 copies = 3 type I and 14 type II patients and 1 type III patient
			II	14	7 M, 7 F	9.2 (2.9)		
			III	1[†]	1 F	36		
Cuscó et al., 2003[13]	Spain	Clinical research	I	1	1 F	1	NR	1 copy = 1 type I and 1 type II patient; 2 copies = 2 type III patients; 3 copies = 2 type II and 2 type III patients 5 patients without *SMN1* deletion excluded
			II	3	2 M, 1 F	8.7 (5.5)		
			III	4	1 M, 3 F	46 (30.1)		
Armand et al., 2005[14]	France	Clinical research	II	2	1 M, 1 F	12.5 (0.7)	NR	
Arkblad et al., 2009[15]	Sweden	Clinical research	I	15[‡]	6 M, 13 F	1.9 (1.0)	NR	2 copies = all type I patients; 3 copies = 10 type II and 4 type III patients; 4 copies = 1 type II and 10 type III patients
			II	11	8 M, 3 F	8.7 (2.6)		
			III	14[‡]	8 M, 7 F	55.0 (65.5)		
Rudnik-Schöneborn et al., 2009[16]	Germany	Retrospective chart review with questionnaire survey	I	66	35 M, 31 F	1.4 (NR)	NR	1 copy = 4 patients; 2 copies = 57 patients; 3 copies = 5 patients
Sproule et al., 2012[17]	US	Retrospective chart review	I	28	14 M, 14 F	2.6 (1.4)	NR	
			II	16	12 M, 4 F	7.0 (3.6)		
Duman et al., 2013[18]	Turkey	Retrospective chart review	I	15	11 M, 4 F	3.7 (2.8)	NR	
Farrar et al., 2013[8]	Australia	Clinical research	I	20	11 M, 19 F	2.1 (2.2)	NR	Type II and III patients average 2.7 copies (SD 0.6; range 1-3).
			II	31	16 M, 15 F	11.7 (4.1)		
			IIIa	14	6 M, 8 F	18.0 (5.4)		
			IIIb	5	1 M, 4 F	137.0 (26.0)		
Reported age of confirmed diagnosis only								
Kinali et al., 2004[19]	UK	Clinical research	II	4	2 M, 2 F	NR	19.5 (4.0)	
			III	8	5 M, 3 F		49.8 (16.7)	
van Bruggen et al., 2011[20]	The Netherlands	Clinical research	II	11[§]	4 M, 7 F	NR	13.1 (6.0)	
Fujak et al., 2013[21]	Germany	Clinical research	II	99	65 M, 61 F	NR	19.2 (10.8)	47 patients were not genetically confirmed
			III	27			37.2 (18.0)	
Finkel et al., 2014[22]	US	Clinical research	I	7	NR	NR	2.8 (2.0)	
Reported both age of onset and age of confirmed diagnosis								
Oskoui et al., 2007[23]	North America and other countries	Registry review	I	143	72 M, 71 F	2.5 (1.7)	4.6 (2.9)	42 patients were not genetically confirmed

TABLE 1. (*continued*)

Author, Year	Country	Type of Study	SMA Type	No. of Patients	Sex	Mean (SD) Age at First Symptoms, Months	Mean (SD) Age at Confirmed Diagnosis, Months	Genetic Information and *SMN2* Copies
Park et al., 2010[24]	Korea	Retrospective chart review	I	14	8 M, 6 F	3.4 (1.6)	4.3 (1.4)	1 patient was not genetically confirmed
Ge et al., 2012[25]	China	Retrospective chart and registry review with questionnaire survey	I II III	107 105 25	61 M, 46 F 62 M, 43 F 17 M, 8 F	3.1 (2.7) 8.7 (3.8) 21.1 (11.7)	9.0 (12.8) 23.0 (15.0) 64.7 (49.3)	

Abbreviations:
F = Female
M = Male
NR = Not reported
SD = Standard deviation
SMN = Survival motor neuron
* Included in both Tables 1 and 2 because the study reported mean age of onset for type II patients and median age of onset for part of type III patients.
† Excluded one adult-onset patient.
‡ Number of patients based on participants' reported age of onset and/or diagnosis.
§ Excluded one prenatally diagnosed patient.

weakness (such as scoliosis), weakness in swallowing or chewing, and respiratory problems.[1] The survival rate of type II patients is higher than type I.[7,8] Patients with type III SMA have less severe symptoms and are able to walk and reach the major motor milestones, but often lose the ability to walk over time as the disease progresses.[1,9] These patients then need wheelchairs and may develop scoliosis, obesity, and other problems related to lack of mobility.[1,9] Because symptoms for type III SMA appear later in childhood, type III is generally diagnosed later than type I or type II SMA.[1,3] The type IV SMA category includes those patients with mild disease and who are diagnosed in early adulthood (age ≥18 years).[1]

Although awareness of SMA is increasing, diagnostic delay is common as SMA symptoms can vary widely in onset and severity and can resemble other diseases.[10] This also may be due to the potential lack of expertise in this area for many health care professionals who may often rule out other diagnoses before considering SMA. Data on the frequency and extent of the diagnostic delay in SMA are limited. The objective of this systematic review was to evaluate the diagnostic delay in SMA and to identify potential factors for this delay based on the published literature.

Materials and Methods

A systematic review of the literature was conducted using the PubMed and Web of Science databases. Articles in English that were published between January 1, 2000, and August 21, 2014, were identified using the search terms: ("spinal muscular atrophy" OR "Werdnig-Hoffmann") AND ("type 1" OR "type I" OR infantile) NOT (pathology OR molecular OR mouse OR mice) for SMA type I; and "spinal muscular atrophy" AND ("type 2" OR "type II" OR "type 3" OR "type III") NOT (pathology* OR molecule* OR mouse OR mice) for SMA types II and III. This time frame was chosen because the genetic test for *SMN1* became available in the late 1990s.[11] Included in the analysis were articles that reported age of first symptom onset and/or age of confirmed SMA genetic diagnosis. Studies with a prenatal SMA diagnosis, no confirmed genetic case, or adult onset patients (≥18 years of age) and publications of case reports or case series were excluded.

Age of symptom onset, age of diagnosis as confirmed by genetic testing, and diagnostic delay were evaluated across studies. Age of onset was defined as the age of the patient when the first symptom was observed as reported by parents or caregivers using questionnaires or collected from medical charts in chart review studies. The age of diagnosis was defined as the age of the patient when SMA diagnosis was confirmed with genetic testing. Diagnostic delay was defined as the time between the age of onset and age of confirmed diagnosis and was calculated directly if both age of onset and diagnosis were reported in the same study. The mean and standard deviation (SD) of age of onset, confirmed diagnosis, and diagnostic delay for studies that reported mean ages were weighted by the number of patients in each study. The weight for each study was the proportion of patients in that study among all patients with the available age information. For example, the weight for Harada et al.[5] applied in the weighted mean age of onset calculation for type I was 11 (number of patients in Harada et al.[5]; Table 1) divided by the total number of patients. The weighted mean age was then calculated as the sum of the product of ages reported in the study and the associated weights for the study. For studies that only reported median ages, the range of the medians was extracted. If both median and mean were reported, the mean was used in the analysis. As a sensitivity analysis, studies that reported median ages were analyzed and reported separately. An analysis of potential overlap in study populations in publications using the same dataset also was conducted. Age of onset and diagnosis also were examined by type of SMA, region (North America, Europe, and Asia Pacific), and year of publication.

Results

The initial search and screening by title resulted in 355 publications (Fig 1). After reviewing the abstract, 204 publications were excluded because they did not meet the search criteria. The remaining 151 publications were reviewed in detail and an additional 130 were excluded because they did not meet the inclusion criteria. After

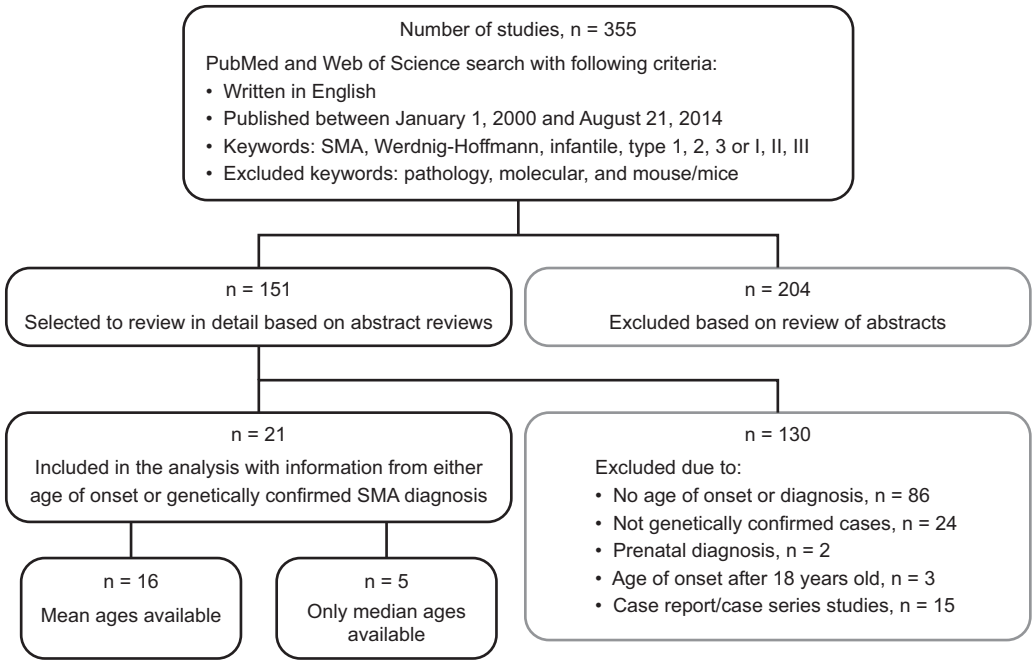

FIGURE 1.
Selection of studies for review. SMA, spinal muscular atrophy.

excluding case reports, case series, and publications with only adult onset patients, a total of 21 publications were included in the final analysis (Tables 1 and 2, Fig 1). Of these, 11 articles reported only age of onset, five reported only age of confirmed diagnosis, and five reported both age of onset and confirmed diagnosis (Tables 1 and 2). Although some publications included mean age information on more than one type of SMA, 11 articles studied SMA type I, 11 studied SMA type II, and 7 studied SMA type III (Table 3). Evaluation of potential overlap in patients from publications reporting data from the same dataset revealed no double counting of patients in the studies. Clinical research studies were the most common type of study (12; 57.1%), followed by retrospective chart review and registry review (six; 28.6%), and retrospective chart review with questionnaire survey (three; 14.3%). Six studies were conducted in North America, eight in Europe, five in Asia, one in Turkey, and one in Australia (Tables 1 and 2).

The weighted mean ± SD age of onset was 2.5 ± 0.6 months (range 1.0-11.0 months; number of patients, n = 420) for SMA type I, 8.3 ± 1.6 months (range 2.0-18.0 months; n = 357) for SMA type II, and 39.0 ± 32.6 months (range 5.0-192.0 months; n = 63) for SMA type III (Table 4, Fig 2). Weighted mean ± SD age of confirmed diagnosis was 6.3 ± 2.2 months (range 0.6-9.0 months; n = 271), 20.7 ± 2.6 months (range 1.2-72.0 months; n = 219), and 50.3 ± 12.9 months (range 3.0-82.8 months; n = 63) for SMA types I, II, and III, respectively (Table 4, Fig 2).

For the subset of studies that reported both age of onset and age of diagnosis, SMA type III patients had the longest delay (43.6 months; n = 25), followed by type II (14.3 months; n = 105), and type I had the shortest delay in diagnosis (3.6 months; n = 264; Table 4). For SMA type I, the difference between the weighted mean age of confirmed diagnosis (6.3 months) and the weighted mean age of onset (2.5 months) was 3.8 months, which was similar to the diagnostic delay measured in the subset of studies that included both age of confirmed diagnosis and mean age of onset (3.6 months; Table 4). Similarly, for SMA type II, the difference of 12.4 months was comparable to the delay in diagnosis observed in the subset of studies that included both age of confirmed diagnosis and mean age of onset (14.3 months). However, in SMA type III the difference between mean age and age of onset was 11.3 months versus 43.6 months from studies that evaluated both of these outcomes. In the studies that reported only medians (n = 6), the range of reported median age of onset was 1.2-3.0 months for SMA type I, 7.5-15.0 months for type II, and 8.0-24.0 months for type III (Table 5). The median age of diagnosis was 2.3-6.0 months for SMA type I, 11.5-13.2 months for type II, and 42.0 months for type III (Table 5).

A subgroup analysis by region (data not shown) indicated that patients in North America appeared to have been diagnosed earlier than those in Europe or the Asia Pacific region. The weighted mean age of onset was greatest for SMA type III in North America compared with those in Europe and the Asia Pacific region. An analysis of delay in diagnosis by year of publication did not show any clear trends (data not shown). A further analysis of age of onset by *SMN2* copy number also was inconclusive due to the small number of studies (n = 3) reporting *SMN2* copy numbers and mean age of diagnosis (data not shown).

Discussion

This is the first extensive systematic literature review to study the diagnostic delay in SMA. Our review included a wide range of studies from several regions and our results

TABLE 2.
Characteristics and Findings for Included Studies: Studies Reporting Medians

Author, Year	Country	Type of Study	SMA Type	No. of Patients	Sex	Median (range) Age at First Symptoms, Months	Median (range) Age at Confirmed Diagnosis, Months	Genetic Test Information and *SMN2* Copies
Reported age of onset only								
Rudnik-Schöneborn et al., 2001[12,*]	Poland, Germany	Retrospective chart review with questionnaire survey	II III	175 266	NR	NA 8 for delayed walking (walked after 18 month of age, n = 27)	NR	57 type II and 186 type III patients genetically confirmed
Arai et al., 2005[26]	Japan	Clinical research	I II III	7 2 1	5 M, 2 F 1 M, 1 F 1 F	2 (1-7) 11.3 (7-16) 15	NR	1 type I and 1 type II patient not genetically confirmed
Yuan et al., 2014[27]	China	Retrospective chart review	I II III	52 46 34	29 M, 23 F 26 M, 20 F 16 M, 18 F	1.2 (0-6) 12 (0-18) 23.5 (18-156)	NR	96/132 patients had genetic test; among these: 34/35 of type I, 30/32 of type II, and 23/29 of type III patients had *SMN1* deletion
Reported age of confirmed diagnosis only								
Lemoine et al., 2012[28]	US	Retrospective chart review	I	49	29 M, 20 F	NR	Proactive respiratory care group, median: 4.5 (IQR 1.8-6.5) Supportive respiratory care group, median: 2.3 (IQR 1.3-4.8)	2 copies = 48 patients
Reported both age of onset and age of confirmed diagnosis								
Kaufmann et al., 2012[9]	US	Clinical research	II III	41 38	16 M, 25 F 21 M, 17 F	9.6 (IQR 6-12) 24 (IQR 12-36)	13.2 (IQR 10.8-16.8) 42 (IQR 30-68.4)	3 copies = 41 type II and 20 type III patients; 4 copies = 17 type III patients; 5 copies = 1 type III patient
Finkel et al., 2014[7]	US	Clinical research	I II	34 45	19 M, 15 F 18 M, 27 F	3 (IQR 2-4) IIa: 11.0 (IQR 7-12) IIb: 8.5 (IQR 6-12)	6 (IQR 4-7) IIa: 11.5 (IQR 9-14) IIb: 13.0 (IQR 10-18)	2 copies = 23 type I and 2 type II patients; 3 copies = 9 type I and 43 type II patients

Abbreviations:
F = Female
IQR = Interquartile range
M = Male
NA = Not applicable
NR = Not reported
SD = Standard deviation
SMN = Survival motor neuron
* Included in both Tables 1 and 2 because the study reported mean age of onset for type II patients and median age of onset for part of type III patients.

show that there is an apparent delay between when a patient with SMA shows symptoms to when the diagnosis is confirmed. From the results of this review, the only factor directly related to the length of delay in diagnosis was the type of SMA. The shortest delay in diagnosis was observed for SMA type I patients and the longest delay was for type III patients, indicating that severity of disease has an impact on time to diagnosis. Although we analyzed delay of diagnosis by geographic region and year of study publication, we did not find a clear correlation between delay in diagnosis and these factors, likely due to the small sample size.

It has been noted that the delays in diagnosis of SMA resulted from patient visits to multiple health care professionals to rule out the possibility of other illnesses before genetic testing for SMA was performed and a confirmed diagnosis was obtained.[10] This “diagnostic odyssey” from the time first symptoms are noticed to a confirmed genetic diagnosis of SMA puts patients and caregivers through physical and mental stress.[10] Although it is not clear what kind of functional loss occurs during the delay, a later diagnosis may result in a missed opportunity for optimal early intervention for SMA. Early diagnosis and care of SMA also can lead to lower patient and caregiver burden; therefore, tools for improving the appropriate and early detection of SMA, such as newborn screening, may be warranted.[29-31] Regular newborn screening is currently not standard practice in the United States, although the SMA-determining gene was identified in 1995 and the test is available.[11,31,32] The idea of newborn screening has a high level of support among parents of children who have SMA and among expecting parents.[33,34]

A long delay to diagnosis has been noted in other pediatric diseases as well. For example, a median delay of

TABLE 3.
Studies Evaluated by SMA Type and Type of Study

	Type I	Type II	Type III
SMA type*			
Total no. of studies included in the analysis	11	11	7
No. of studies only reporting age of onset	7	7	4
No. of studies only reporting age of confirmed diagnosis	1	3	2
No. of studies reporting both age of onset and age of confirmed diagnosis	3	1	1
Type of study*			
Retrospective chart review and registry review	4	1	0
Retrospective chart review with questionnaire survey	2	2	1
Clinical research	5	8	6

Abbreviation:
SMA = Spinal muscular atrophy
* Numbers are not mutually exclusive because one study could have reported on more than one type of SMA. Only studies with mean ages available are included (Table 1).

1.4 months to 12.6 years from symptom onset to diagnosis has been noted in patients with Pompe disease,[35] whereas a delay of 6 months to more than 4 years has been documented in patients with Duchenne muscular dystrophy.[36] It is important to identify ways to reduce these delays to diagnosis for all pediatric diseases to provide earlier intervention for disease management or appropriate treatment. As shown in other childhood diseases,[35] earlier treatment has been associated with better outcomes and suggests that in a neurodegenerative condition such as SMA, an earlier diagnosis, particularly for type I and II patients, will be immensely helpful to increase the chance of survival using optimal care and supportive interventions.

A strength of this study is the application of stringent criteria by including only those publications that based diagnosis on confirmed genetic testing and excluding any case studies or case series. These criteria allowed us to obtain robust estimates (i.e., less affected by special cases in case report studies) for patients that were properly diagnosed with current technology, such as genetic testing. Moreover, the weighted mean age of onset and weighted mean age of diagnosis appeared to match between studies that reported only the age of onset or age of confirmed diagnosis and those that reported both age of onset and confirmed diagnosis, which indicates the validity of the findings. However, this study has a few limitations. The number of publications evaluated overall is small, and of these only a few focused on SMA type III; therefore, it is difficult to draw any conclusions for type III. Also, the age of onset may be affected by parental recall bias and could be more common in SMA type III patients because of the longer diagnostic delay. Therefore, more studies on age of onset and diagnosis of SMA, particularly type III, are needed to provide a reliable estimate of the diagnostic delay in these patients.

TABLE 4.
Weighted Mean Age of Onset, Confirmed Diagnosis, and Diagnostic Delay* in SMA

	Type I	Type II	Type III
Age of onset, months			
No. of patients for weighted mean	420	357	63
No. of studies for weighted mean	10	8	5
Mean (SD)	2.5 (0.6)	8.3 (1.6)	39.0 (32.6)
Range	1.0-11.0	2.0-18.0	5.0-192.0
Age of confirmed diagnosis, months			
No. of patients for weighted mean	271	219	60
No. of studies for weighted mean	4	4	3
Mean (SD)	6.3 (2.2)	20.7 (2.6)	50.3 (12.9)
Range	0.6-9.0	1.2-72.0	3.0-82.8
Delay in diagnosis, months			
No. of patients for weighted mean	264	105	25
No. of studies for weighted mean	3	1	1
Mean (SD)	3.6 (1.9)	14.3 (0.0)	43.6 (0.0)
Range	1.0-5.9	14.3†	43.6†

Abbreviations:
SD = Standard deviation
SMA = Spinal muscular atrophy
* Case reports and studies reporting only median age were excluded; data weighted by total number of patients evaluated in studies that met the search criteria.
† Reported in only one study.

Conclusions

This systematic review clearly indicates that there is a delay in diagnosis of SMA and that the length of delay varies by the severity (type) of SMA. Based on the publications examined for this review, it is difficult to conclude if other factors are related to the delay. Newborn screening, which has resulted in improved outcomes for pediatric patients

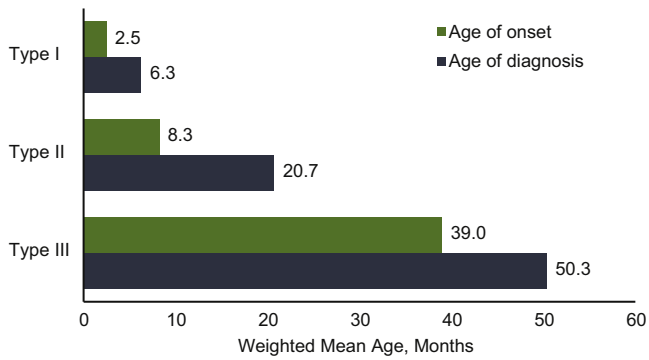

FIGURE 2.
Age of onset and diagnoses by type of SMA. SMA, spinal muscular atrophy. (The color version of this figure is available in the online edition.)

TABLE 5.
Range of Median Age of Onset, Diagnosis, and Diagnostic Delay in SMA

	Type I	Type II	Type III
Age of onset, months			
No. of studies	3	5	3
No. of patients	93	134	99
Range of reported median,	1.2-3.0	7.5-15.0	8.0-24.0
Age of diagnosis, months			
No. of studies	2	2	1
No. of patients	83	86	38
Range of reported median	2.3-6.0	11.5-13.2	42.0*

Abbreviations:
SD = Standard deviation
SMA = Spinal muscular atrophy
* Reported in only one study.

with other diseases such as cystic fibrosis,[30] may provide an opportunity for earlier diagnosis of SMA and could be the most effective solution to end this SMA diagnostic odyssey. Future studies are necessary to confirm the observations in this report, to examine the extent of functional decline during the prediagnosis period, and to evaluate the potential negative impact the process of obtaining an appropriate diagnosis has on patients and families of patients with SMA.

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Conflict of interest: C-W.L. was an intern at Biogen when this study was conducted. S.K. and W-S.Y. are full-time employees of Biogen.

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Results from a phase 1 study of nusinersen (ISIS-SMN$_{Rx}$) in children with spinal muscular atrophy

OPEN

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ABSTRACT

Objective: To examine safety, tolerability, pharmacokinetics, and preliminary clinical efficacy of intrathecal nusinersen (previously ISIS-SMN$_{Rx}$), an antisense oligonucleotide designed to alter splicing of *SMN2* mRNA, in patients with childhood spinal muscular atrophy (SMA).

Methods: Nusinersen was delivered by intrathecal injection to medically stable patients with type 2 and type 3 SMA aged 2–14 years in an open-label phase 1 study and its long-term extension. Four ascending single-dose levels (1, 3, 6, and 9 mg) were examined in cohorts of 6–10 participants. Participants were monitored for safety and tolerability, and CSF and plasma pharmacokinetics were measured. Exploratory efficacy endpoints included the Hammersmith Functional Motor Scale Expanded (HFMSE) and Pediatric Quality of Life Inventory.

Results: A total of 28 participants enrolled in the study (n = 6 in first 3 dose cohorts; n = 10 in the 9-mg cohort). Intrathecal nusinersen was well-tolerated with no safety/tolerability concerns identified. Plasma and CSF drug levels were dose-dependent, consistent with preclinical data. Extended pharmacokinetics indicated a prolonged CSF drug half-life of 4–6 months after initial clearance. A significant increase in HFMSE scores was observed at the 9-mg dose at 3 months postdose (3.1 points; $p = 0.016$), which was further increased 9–14 months postdose (5.8 points; $p = 0.008$) during the extension study.

Conclusions: Results from this study support continued development of nusinersen for treatment of SMA.

Classification of evidence: This study provides Class IV evidence that in children with SMA, intrathecal nusinersen is not associated with safety or tolerability concerns. *Neurology*® 2016;86:890–897

GLOSSARY

AE = adverse event; **AUC** = area under the concentration time curve; **DSMB** = data and safety monitoring board; **HFMSE** = Hammersmith Functional Motor Scale Expanded; **LP** = lumbar puncture; **PedsQL** = Pediatric Quality of Life Inventory; **SMA** = spinal muscular atrophy; **SMN** = survival of motor neuron.

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized primarily by motor neuron degeneration, resulting in muscular atrophy and weakness involving limbs, and more variably, bulbar and respiratory muscles.[1,2] The natural history of SMA includes several major phenotypes that are characterized by age at onset and achieved motor abilities.[3] SMA is caused by deletions or loss-of-function mutations in the survival of motor neuron (*SMN1*) gene located on chromosome 5q13.[4] Humans also have a paralogous *SMN2* gene that has an identical coding sequence to *SMN1* but differs by 11 nucleotides.[5] One of the nucleotide changes is a C to T transition within exon 7, resulting in 80%–90% of the transcripts, excluding exon 7, producing a truncated protein that is rapidly degraded.[5] As a result, the limited amount of full-length protein produced is insufficient to compensate for loss of the *SMN1* gene, causing the SMA phenotype.[5]

Editorial, page 884

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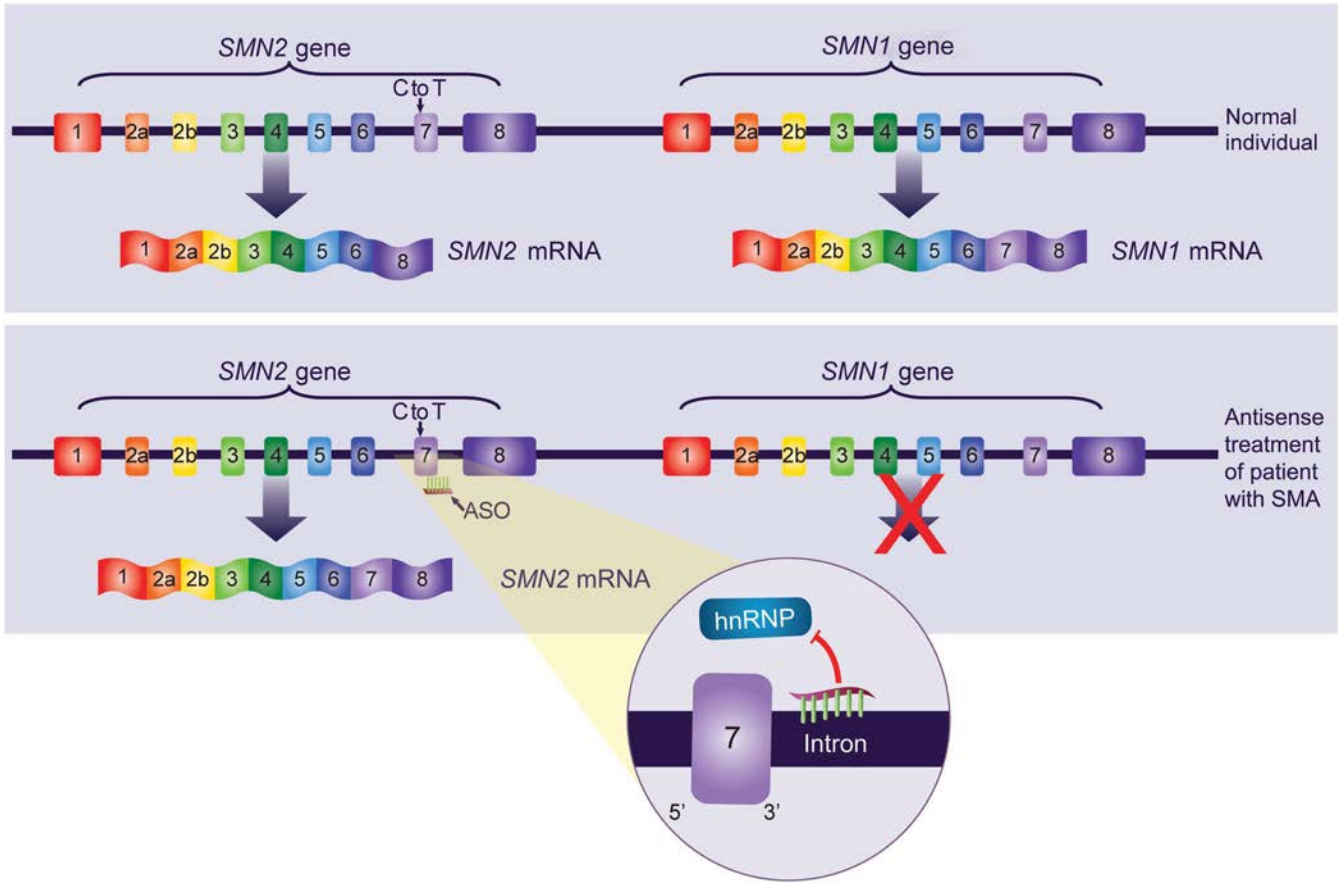
Nusinersen (previously ISIS-SMN_{Rx} and also known as ISIS 396443) is an antisense oligonucleotide designed to bind to the *SMN2* pre-mRNA and promote inclusion of exon 7 (figure 1).[6,7] In mouse models of SMA, nusinersen enhanced exon 7 inclusion, increased SMN protein production, and improved function.[6–9] Nusinersen is delivered by intrathecal injection as antisense oligonucleotide drugs do not cross an intact blood–brain barrier when delivered systemically.[10] This first-in-human, open-label, single-ascending dose study was designed to assess safety, tolerability, pharmacokinetics, and clinical effects of intrathecal nusinersen in patients with childhood SMA.

METHODS Standard protocol approvals, registrations, and participant consents. Written informed parental consent and assent was obtained for all participants as required. Studies were initiated after institutional review board approvals of the participating study centers and carried out in accordance with Good Clinical Practice guidelines. A data and safety monitoring board (DSMB) monitored the studies. Studies were registered with ClinicalTrials.gov (NCT01494701; NCT01780246).

Study design. In this open-label, escalating-dose, phase 1 study, participants received a single intrathecal injection of 5 mL nusinersen following standard lumbar puncture (LP) techniques (over 1–3-minutes). Four dose levels (1, 3, 6, 9 mg) were evaluated in sequential groups: dose escalation was dependent on DSMB review of safety data through study day 8 for the preceding dose group. Participants were eligible to re-enroll in an open-label extension study at 9–14 months after their initial nusinersen dose to receive additional study drug; data included in this report include baseline evaluations for the follow-up study only.

Participants. Male and female patients aged 2–14 years with symptomatic SMA and documented *SMN1* homozygous gene deletion were eligible to participate. Participants were enrolled at 4 study centers: Columbia University, University of Utah, Boston Children's Hospital, and University of Texas Southwestern Medical School. Inclusion/exclusion criteria were designed to enroll patients who were medically stable and in whom safety concerns could be clearly assessed. Briefly, participants needed to be able to complete all study procedures, meet age-appropriate

Figure 1 Mechanism of action of nusinersen



Nusinersen is a 2′-*O*-(2-methoxyethyl) modified ASO drug designed to target an hnRNP-A1/A2–dependent splicing silencer, ISS-N1, in intron 7 of the *SMN* pre-mRNA. Nusinersen displaces hnRNP proteins from the ISS-N1 site on the *SMN2* pre-mRNA, facilitating accurate splicing of *SMN2* transcripts (e.g., increasing the synthesis of transcripts containing exon 7) and resulting in increased production of full-length SMN protein. ASO = antisense oligonucleotide; hnRNP = heterogenous nuclear ribonucleoprotein; ISS = intronic splicing silencer; mRNA = messenger RNA; SMA = spinal muscular atrophy; SMN = survival of motor neuron.

institutional guidelines for LP procedures, and have a life expectancy of >2 years per investigator judgement. Participants were excluded for respiratory insufficiency, hospitalization for surgery or pulmonary event within the past 2 months, active infection at screening, history of brain or spinal cord disease or bacterial meningitis, presence of implanted CSF drainage shunt, clinically significant laboratory abnormalities, any ongoing medical condition that would interfere with the conduct and assessments of the study, or treatment with another investigational drug ≤1 month of screening.

Study procedures. LP was performed under anesthesia/sedation per institutional guidelines. Before intrathecal injection of study drug, 5–6 mL of CSF was collected for analysis. Following injection, participants were observed for 24 hours. Follow-up visits were performed on days 8 and 29 for all participants and on day 85 for participants in the 6- and 9-mg dose groups. A second LP to collect CSF for safety and pharmacokinetics was performed on day 8 in the 1-, 3-, and 6-mg dose groups and on days 8 or 29 in the 9-mg dose group (n = 5 at each time point). Participants were assessed 9–14 months postdose at enrollment into the long-term extension study, using assessments identical to those employed in the single-dose study.

Safety assessments. Safety assessments included collection of adverse events (AEs), physical/neurologic examinations, vital signs, clinical laboratory tests (serum chemistry, hematology, urinalysis; analyzed centrally at PPD, Wilmington, NC), and ECGs. CSF safety laboratory tests (cell counts, protein, glucose) were assessed at 7 or 28 days and 9–14 months postdose (analyzed in local laboratories), and CSF cytokines (interleukin-6, tumor necrosis factor–α, monocyte chemotactic protein 1; Aushon BioSystems, Billerica, MA) at 7 or 28 days postdose. Plasma samples for participants in the 6- and 9-mg cohorts were collected on days 1, 8 or 29, and 85 and 9–14 months postdose for the analysis of anti–nusinersen antibodies using an immunogenicity assay validated to be specific for the detection of anti–nusinersen antibodies (Charles River Laboratories, Wilmington, MA).

Pharmacokinetic and SMN protein assessments. Plasma pharmacokinetic specimens were collected on day 1 predose, at 1, 2, 4, 6, 8, 12, and 20 hours postdose, and on day 8 in the 6- and 9-mg cohorts. CSF pharmacokinetic specimens were collected on day 1 predose, days 8 and 29, and 9–14 months. Nusinersen concentrations were determined using a variation of the hybridization ELISA method[11] or an electrochemiluminescence method; both methods were validated (PPD). SMN protein concentration was determined using an Erenna Immunoassay System validated for human CSF (Singulex, Inc., Alameda, CA). CSF elimination half-life was estimated for each individual using concentrations determined at 7 or 29 days and 9–14 months by using the following equation:

$$t_{1/2} = \frac{\ln(2)}{\ln\left(C_t/C_0\right)/(t_0 - t)},$$

where $t_{1/2}$ is half-life in days; C_t and C_0 are CSF concentrations at 9–14 months and 7 or 29 days, respectively; and $(t_0 - t)$ is time in days between measurements.

Clinical outcome measures. Exploratory clinical assessments included Hammersmith Functional Motor Scale Expanded (HFMSE)[12,13] performed at baseline, day 29, day 85 (6- and 9-mg cohorts), and at 9–14 months; and Pediatric Quality of Life Inventory (PedsQL) Measurement 4.0 Generic Core Scales and 3.0 Neuromuscular Module[14,15] at baseline, day 29, and day 85 (6- and 9-mg cohorts). For HFMSE, physical therapist evaluators were trained annually with a standardized procedure manual. Intraclass correlation coefficients for interrater reliability among all evaluators were very high (>0.951; computed using 1-way random effects analysis of variance models of 3 videotaped assessments).

Sample size and statistical analysis. Sample size was selected based upon prior experience with phase 1 single-dose studies of antisense oligonucleotide. There was no statistically based rationale for the number of participants chosen. All participants who received the single dose were included in safety, tolerability, pharmacokinetic, and efficacy analyses. Exploratory efficacy endpoints were assessed using Wilcoxon signed-rank test.

Classification of evidence. This study provides Class IV evidence that in children with SMA, a single dose of nusinersen up to 9 mg is not associated with safety or tolerability concerns on a follow-up of 9–14 months. A single dose of 9 mg nusinersen resulted in an increase in HFMSE scores in children with SMA; however, the study was open-label and results would need to be confirmed in a controlled trial.

RESULTS

Participants. Six participants were enrolled in the 1-, 3-, and 6-mg dose groups, and 10 participants in the 9-mg group. All participants completed treatment and follow-up evaluations. Demographics/background disease characteristics are presented in table 1. Twenty-four of 28 participants in the phase 1 study were enrolled in the long-term

Table 1 Baseline characteristics of study participants

	Total nusinersen (N = 28)	1 mg nusinersen (n = 6)	3 mg nusinersen (n = 6)	6 mg nusinersen (n = 6)	9 mg nusinersen (n = 10)
Age (min-max), y	6.1 (2-14)	7.7 (3-14)	5.3 (4-7)	6.0 (2-12)	5.8 (2-10)
Male, %	39	83	17	17	40
Caucasian, %	82	83	100	83	70
Type 2/3, n	15/13	2/4	3/3	3/3	7/3
Ambulatory, %	36	50	33	33	30
SMN2 copy no. 3/4/5, n	25/2/1	4/2/0	6/0/0	6/0/0	9/0/1

Abbreviation: SMN = survival of motor neuron.

extension study 9–14 months after initial dose and thus, their demographics were similar to those in the single-dose study.

Safety and tolerability. There were no serious AEs during the course of the study and no participant experienced an AE leading to discontinuation. Overall, 89% of participants reported AEs (table 2); most AEs were mild in severity, with only 5 participants reporting an event that was moderate in severity and none that was severe. The most frequently observed AEs by prevalence were headache (39.3% of participants), post-LP headache (21.4%), and back pain (17.9%). There were 2 AEs considered potentially related to the study drug: a mild paresthesia in the 1-mg group and a report of palpitations in the 3-mg group, both of which resolved spontaneously without sequelae. The incidence of post-LP headache was 10.9% (6 events reported for 55 LPs performed). No clinically significant changes in vital signs, neurologic or physical examinations, clinical laboratory tests, or ECGs were reported. There were no clinically significant changes in CSF safety laboratory tests or CSF cytokines. All samples were negative for the presence of anti–nusinersen antibodies, indicating that an immunogenic response to nusinersen was not observed at 9–14 months after a single intrathecal dose of nusinersen.

Pharmacokinetic profile. Nusinersen concentrations were measurable over 24 hours postdose in plasma and 7 days postdose in CSF (figure 2), as well as 29 days postdose in CSF in the 9-mg group (2.33 ± 0.928 ng/mL). Nusinersen concentrations 7 days after dosing increased dose dependently but less than dose proportionally (3.7-fold) over the evaluated dose range. A substantial portion of intrathecally administered nusinersen rapidly transferred from the site of administration (CSF) into systemic circulation, with dose-dependent mean peak plasma levels observed within a few hours after dosing. After peaking, nusinersen plasma concentrations declined slowly ≤20 hours after intrathecal dosing, followed by a slower period of decline over the next 7 days. By 7 days postdose, concentrations in the 6-mg cohort had decreased to below the limit of quantification (1 ng/mL), and to <1% of the 20-hour postdose concentration in the 9-mg cohort. Dose-normalized total plasma exposure measures decreased ~3-fold over a ~3-fold increase in total body weight. A similar relationship between dose-normalized total plasma exposure and age was observed. No differences were observed between males and females for untransformed total plasma exposure (area under the concentration time curve from 0 to 20 hours [$AUC_{0–20h}$]) measures stratified by cohort or for dose-normalized total plasma exposure ($AUC_{0–20h}$) measures. At the 9- to 14-month evaluation, CSF nusinersen concentrations were still measurable. Apparent terminal half-life in CSF was estimated to be 132–166 days (mean ± SD days: 132 ± 42, 1-mg group; 135 ± 14.8, 3-mg group; 163 ± 26.5, 6-mg group; 177 ± 41.3, 9-mg group).

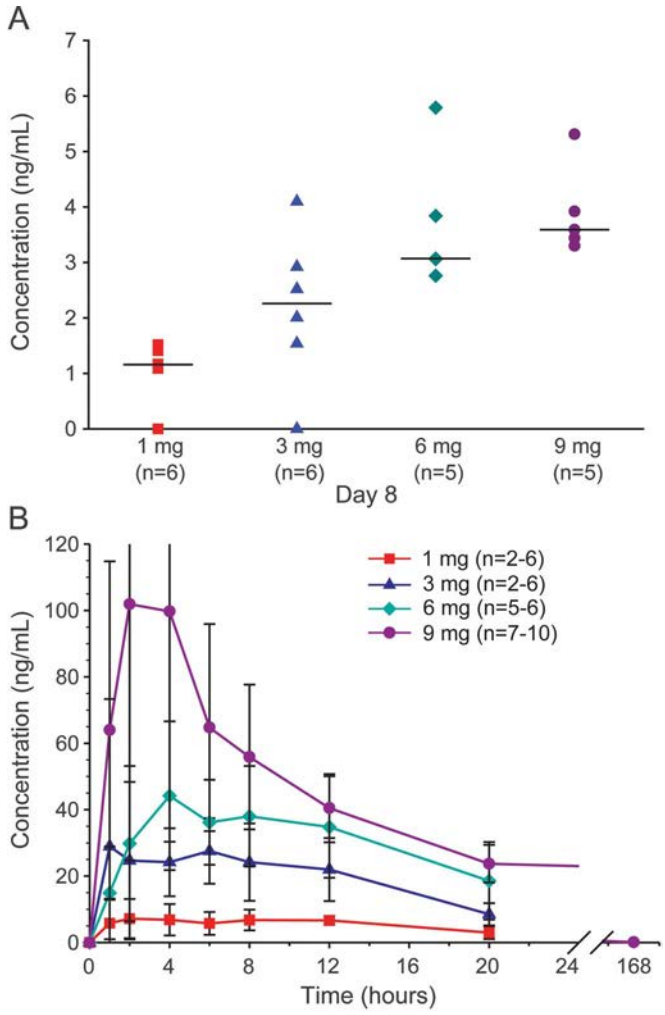
Analysis of CSF samples for SMN protein levels indicated no change in the 1- or 3-mg groups, but that SMN protein levels more than doubled at 9–14 months postdose compared with baseline in the 6- and 9-mg groups (6-mg group: baseline, 0.27 ± 0.03 pg/mL; 9–14 months, 0.56 ± 0.12 pg/mL; not statistically significant; 118% mean increase; 9-mg group: baseline, 0.31 ± 0.18 pg/mL;

Table 2 Adverse event summary by preferred term, % (number of events)[a]

Adverse event term	Total nusinersen (N = 28)	1 mg nusinersen (n = 6)	3 mg nusinersen (n = 6)	6 mg nusinersen (n = 6)	9 mg nusinersen (n = 10)
Any serious adverse event	0	0	0	0	0
Any adverse event	89 (72)	100 (25)	67 (15)	100 (11)	90 (21)
Headache	39 (12)	67 (4)	0	50 (3)	40 (5)
Post-LP syndrome	21 (7)	17 (2)	33 (2)	33 (2)	10 (1)
Back pain	18 (7)	33 (2)	33 (4)	0	10 (1)
Pyrexia	14 (4)	17 (1)	0	17 (1)	20 (2)
Constipation	11 (3)	33 (2)	17 (1)	0	0
Upper respiratory tract infection	11 (5)	0	17 (1)	17 (1)	10 (3)
Nausea	11 (3)	17 (1)	33 (2)	0	0
Vomiting	11 (3)	17 (1)	33 (2)	0	0

Abbreviation: LP = lumbar puncture.
[a] Adverse events listed are those that occurred with a frequency >10% in nusinersen-treated participants (i.e., occurring in >2 participants).

Figure 2 CSF and plasma concentrations of nusinersen



Measured nusinersen concentrations for each dose group are shown. (A) CSF at 7 days postdose. (B) Plasma over 24 hours (all groups) or 7 days (6- and 9-mg groups) postdose (mean ± SEM). As anticipated, plasma levels were below the limit of detection of the assay at day 8 postdose.

9–14 months, 0.59 ± 0.22 pg/mL; $p = 0.06$; 161% mean increase).

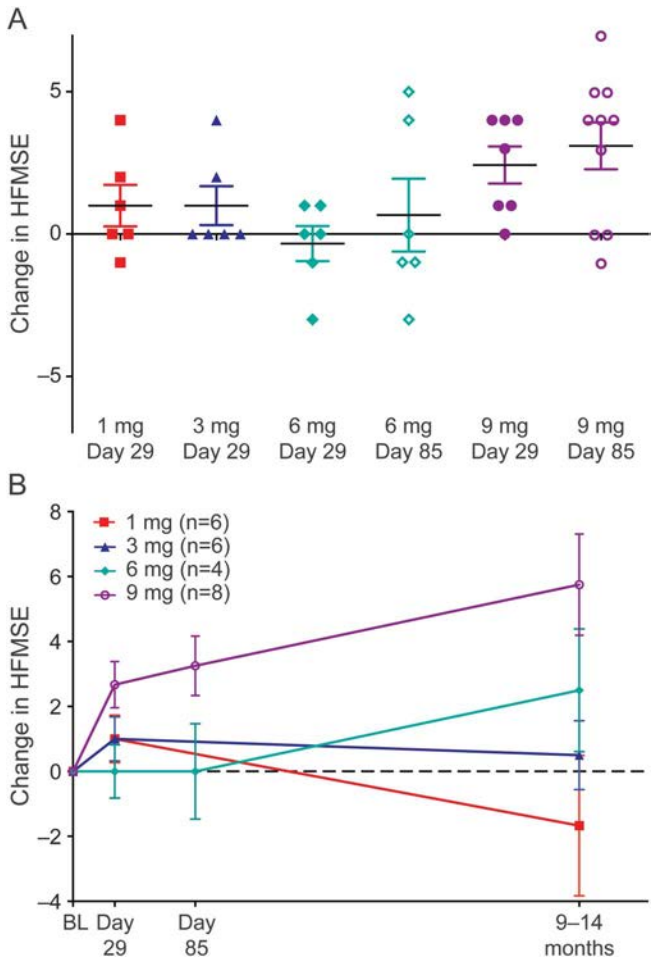
Clinical outcomes. There were no significant changes in HFMSE assessments (figure 3) from baseline at day 29, day 85, or 9–14 months postdose in the 1-, 3-, or 6-mg groups (mean change: 1-mg group, +1.0 point at day 29 and –1.7 point at 9–14 months; 3-mg group, +1.0 point at day 29 and +0.5 point at 9–14 months; 6-mg group, +0.7 point at day 85 and +2.5 at 9–14 months). In contrast, the 9-mg group demonstrated improved HFMSE scores from baseline at day 85 (mean increase of 3.1 points or 17.6% increase; $p = 0.016$), with 7/10 (70%) participants exhibiting an increase of 3–7 points. Observed improvements in HFMSE scores of ≥3 points were equally distributed by severity; 5/7 type 2 participants and 2/3 type 3 participants showed this level of improvement. Similarly, HFMSE score improvement was distributed broadly across the age range, with 3 children aged <5 years and 4 children aged ≥5 years showing improvement. At long-term follow-up, mean change in HFMSE score from baseline to 9–14 months was 5.8 points (32.8% increase; $p = 0.008$; n = 8). Six of 8 participants had an increase of ≥3 points. No participants in the 9-mg cohort declined in HFMSE score, and range of improvement was 1–14 points.

A slight improvement for the PedsQL Generic Core Scales was observed compared with baseline in the 9-mg group at day 85 (mean percent change: 9.8% [patient] and 8.4% [parent], not statistically significant). Similarly, a slight improvement in the PedsQL Neuromuscular Model was observed compared with baseline in the 9-mg group at day 85, with the change being greater for the patient compared with the parent report (mean percent change: 17.7% [patient] and 4.6% [parent], not statistically significant). No meaningful changes were observed in the other dose groups.

DISCUSSION Nusinersen was well-tolerated when given as a single intrathecal bolus injection to children with SMA and no safety concerns were identified. No serious AEs or discontinuations due to AEs were reported. The most commonly reported AEs were headache, post-LP headache, and backache; however, none of these events was considered to be related to nusinersen. No clinically significant abnormalities were noted on neurologic examinations or laboratory safety assessments, including CSF safety assessments at 7 or 28 days and 9–14 months postdose. These results are consistent with previous but limited clinical studies on direct CNS delivery of antisense oligonucleotide drugs, which appeared to be similarly well-tolerated.[16,17] In this study, the LP injection procedure itself was well-tolerated and was shown to be feasible in children with SMA. Incidence rate of post-LP headache overall was 10.9%, which is consistent with the expected post-LP headache rate of ~10% in children from published literature,[18–20] further taking into account that patients in the study underwent 2 LPs (except for 1 patient who had only 1 LP) and that patients with SMA often have scoliosis or spinal deformities, making LPs more challenging. Eighteen of the children with SMA underwent 3 repeated LPs, with no increase in AEs or tolerability issues reported, further supporting the feasibility of this approach. The presence of hardware from scoliosis surgery in some patients with SMA, however, may limit the feasibility of intrathecal injections in the broader population of patients with SMA.

CSF and plasma drug concentrations of nusinersen were measurable and dose-dependent. No readily

Figure 3 Changes in Hammersmith Functional Motor Scale Expanded (HFMSE) scores by treatment assignment



(A) Individual change in HFMSE score from baseline for all participants. (B) Mean change in HFMSE score from baseline in participants followed through 9–14 months postdose. Bars represent mean ± SEM for each dose group. BL = baseline.

apparent correlations were observed between age or total body weight and CSF concentrations, consistent with literature reports indicating that CSF volume is similar in all children aged >2 years,[21] and suggesting that fixed doses are appropriate in this pediatric population, with no dose scaling necessary. CSF and plasma drug levels were reasonably consistent with predicted values (within 2- to 3-fold) based on preclinical (monkey to human) CSF volume scaling. Dose-dependent mean peak plasma levels were observed within a few hours after dosing and then typically declined slowly ≤20 hours after intrathecal dosing. The slow decline may be because of CSF turnover and transfer of any remaining drug not taken up into CNS tissues from CSF into systemic circulation, coupled with the expected rapid and extensive distribution of systemically available drug from the plasma to systemic tissues. A consistent finding of a long drug half-life in CNS tissue for nusinersen has been observed in mice (145–191 days) and monkeys (139 ± 54 days)[22] and was confirmed in humans by the observed CSF half-life of between 4–6 months in children with SMA. This observation is critical in that it provides support for infrequent administration of nusinersen for maintenance of drug levels (i.e., once every 4–6 months) following drug loading of the target tissue.

Although preliminary and requiring confirmation by a larger controlled study, improvement in patients' motor function as evidenced by increases in HFMSE score was observed following a single intrathecal dose of 9 mg nusinersen, with a 3.1-point mean increase from baseline at 3 months and a 5.8-point mean increase at 9–14 months. This improvement in motor function is not expected to occur as part of the disease process, as the predicted natural history trajectory of the HFMSE in type 2 and type 3 patients with SMA indicates stability over 1 year[23] and a small decline over 2 years (−1 point).[24] Though trials in patients with SMA evaluating other investigational therapies have ultimately been unsuccessful and several previous open-label trials have shown strong placebo effects, variations on the HFMSE have been previously used as the primary outcome measures in SMA clinical studies.[25,26] It has been projected, including as an assumption in previous trials, that a change of 3 points over a 6-month period would constitute a clinically meaningful change in this endpoint,[26] and thus, the 5.8-point increase observed at 9–14 months in the 9-mg group in this study clearly falls within the meaningful range. Consistent with the intended biological mechanism of nusinersen, an increase in CSF SMN protein concentrations correlated well with the improvement in motor function. In addition, the study findings reaffirm the sensitivity of the HFMSE to detect change in functional motor outcome and inform the length of time that is required to observe improvement in motor function after treatment.

Nusinersen dose levels for this first-in-human study were selected based on preclinical pharmacokinetic observations from monkey studies and consideration of the target tissue concentration anticipated for drug pharmacology from SMA transgenic mouse models.[22] As nonhuman primates do not express the *SMN2* gene, it was not possible to measure pharmacology in that animal species. Based upon pharmacology and pharmacokinetic results in *SMN2* transgenic mice, the target tissue concentration to produce 50%–90% *SMN2* exon 7 inclusion was estimated to be between 1 and 10 μg/g in spinal cord tissues.[10] The lowest dose selected for this clinical study (1 mg) was predicted to achieve the low end of this range, while the highest dose (9 mg) was predicted to achieve spinal cord tissue concentrations at the upper end of this range. However, no dose-limiting safety issues were identified in this study,

suggesting that doses higher than the 9 mg tested in this study should be considered.

The favorable risk-benefit profile from this first-in-human clinical study of nusinersen in children with SMA provides encouragement for further development of nusinersen for the treatment of SMA, for which no therapeutic options currently exist. Ongoing multiple-dose studies, which include incorporation of higher doses, are currently being conducted in both infants and children with SMA.

AUTHOR CONTRIBUTIONS

C.A.C., K.J.S., B.T.D., S.T.I., D.C.D., D.A.N., and K.M.B. designed the trial. C.A.C., K.J.S., B.T.D., S.T.I., J.M., and D.C.D. conducted the trial. D.A.N. and K.M.B. performed the analysis of the data. C.A.C., D.A.N., F.B., and K.M.B. interpreted the data and wrote the manuscript. All authors critically revised and approved the final manuscript.

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Results from a phase 1 study of nusinersen (ISIS-SMN$_{Rx}$) in children with spinal muscular atrophy

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Intrathecal Injections in Children With Spinal Muscular Atrophy: Nusinersen Clinical Trial Experience

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Abstract

Nusinersen (ISIS-SMN_{Rx} or ISIS 396443) is an antisense oligonucleotide drug administered intrathecally to treat spinal muscular atrophy. We summarize lumbar puncture experience in children with spinal muscular atrophy during a phase 1 open-label study of nusinersen and its extension. During the studies, 73 lumbar punctures were performed in 28 patients 2 to 14 years of age with type 2/3 spinal muscular atrophy. No complications occurred in 50 (68%) lumbar punctures; in 23 (32%) procedures, adverse events were attributed to lumbar puncture. Most common adverse events were headache (n = 9), back pain (n = 9), and post–lumbar puncture syndrome (n = 8). In a subgroup analysis, adverse events were more frequent in older children, children with type 3 spinal muscular atrophy, and with a 21- or 22-gauge needle compared to a 24-gauge needle or smaller. Lumbar punctures were successfully performed in children with spinal muscular atrophy; lumbar puncture–related adverse event frequency was similar to that previously reported in children.

Keywords

lumbar puncture, spinal muscular atrophy, antisense oligonucleotide, drug delivery

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Advances in the identification of the genetic basis of neurologic diseases have enabled the emerging development of therapies to treat these diseases based on the known genetic mechanisms and deficiencies. However, drug delivery to the central nervous system remains a key challenge. Intrathecal injection via lumbar puncture provides a direct route of delivery that has traditionally been focused on malignancy-directed chemotherapeutics and pain management,[1] but is increasingly being used in clinical trials assessing neurologic therapies.[2,3]

Nusinersen (ISIS-SMN_{Rx} or ISIS 396443) is an antisense oligonucleotide currently in development for spinal muscular atrophy, an autosomal recessive motor neuron disease associated with progressive muscular atrophy and weakness involving limbs and, more variably, bulbar and respiratory muscles.[4,5] Spinal muscular atrophy affects approximately 1:10 000 births,[6] and is classified into clinical subtypes (types 0-4) differentiated by age of onset and highest motor function.[7] Nusinersen is designed to alter splicing of *SMN2* messenger RNA and increase the amount of functional SMN protein produced, thus compensating for the genetic defect in the *SMN1* gene.[8,9] Nusinersen, currently under evaluation in phase 3 clinical trials in infants and children with spinal muscular atrophy, is administered via lumbar puncture and intrathecal injection directly into the cerebrospinal fluid, from where it distributes to the spinal cord and the brain.

Although lumbar puncture is routinely performed for diagnostic and therapeutic purposes in children and infants,[10] it is infrequently performed in patients with spinal muscular atrophy. Lumbar puncture is generally a safe and straightforward procedure, but side effects, such as headache, back pain, and transient or persistent cerebrospinal fluid leakage (post–lumbar

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puncture syndrome), have been documented.[11-13] In addition, in children with spinal muscular atrophy who often experience complications such as scoliosis, lumbar punctures might be technically more challenging to perform. The objective of this analysis was to summarize our clinical trial experience with lumbar punctures for intrathecal nusinersen drug delivery in a phase 1 study in children with spinal muscular atrophy[14] and to develop recommendations for procedures in a pediatric population with a severe neuromuscular disease.

Methods

Standard Protocol Approvals, Registrations, and Participant Consents

These trials were conducted in compliance with the Declaration of Helsinki, the International Conference on Harmonisation Good Clinical Practice guidelines, the European Union Clinical Trials Directive, and local regulatory requirements. Approval for the study protocols and all amendments were obtained from Columbia University Medical Center Institutional Review Board (Approval #AAAI6758 and #AAAK5458). Written informed consent and assent (if applicable) were obtained before any evaluations were conducted for eligibility. The trials are registered on ClinicalTrials.gov (NCT01494701 and NCT01780246).

Phase 1 and Extension Study Designs

The phase 1 study was a first-in-human, open-label, escalating-dose study to assess the safety, tolerability, and pharmacokinetics of a single intrathecal dose (1, 3, 6, or 9 mg) of nusinersen in children with spinal muscular atrophy. Each dose cohort had 6 to 10 participants (N = 28). Upon completion, all participants had the opportunity to enroll in a subsequent extension study and receive additional dosing with nusinersen. The methods and results of the phase 1 study and its extension are detailed elsewhere.[14] Briefly, medically stable spinal muscular atrophy participants 2 to 14 years of age were enrolled in 4 sites in the United States (Boston Children's Hospital, Boston, MA; Columbia University Medical Center, New York, NY; UT Southwestern Medical Center–Children's Medical Center Dallas, Dallas, TX; and University of Utah School of Medicine, Salt Lake City, UT). Eligible participants had to be able to complete all study procedures, meet age-appropriate institutional guidelines for lumbar puncture procedures, and have a life expectancy of >2 years. Participants were excluded for serious respiratory insufficiency, hospitalization for surgery or pulmonary event within the past 2 months, active infection at screening, history of brain or spinal cord disease or bacterial meningitis, presence of implanted cerebrospinal fluid drainage shunt, clinically significant laboratory abnormalities, any ongoing medical condition that would interfere with the conduct and assessments of the study, or treatment with another investigational drug within 1 month of screening. Patients with scoliosis were allowed to participate if, in the opinion of the investigator, a lumbar puncture could be performed safely.

Lumbar Puncture Procedures

A total of 3 lumbar punctures were scheduled during the 2 trials for drug delivery and/or follow-up collection of cerebrospinal fluid for safety and pharmacokinetic analyses. Drug was administered via intrathecal injection of a 5-mL bolus over 1 to 3 minutes. The protocol recommended a 22- to 25-gauge spinal anesthesia needle (21-gauge needle allowed if participant's weight or condition dictated) and that the lumbar punctures were performed at the L3-L4 disc space or 1 level above or 1 to 2 levels below, as needed. In all cases, 5 to 6 mL of cerebrospinal fluid was collected before drug injection. Participants were encouraged to lie flat for an hour after the procedure. Anesthesia and/or sedation and fluoroscopy or ultrasonography were permitted to facilitate the procedure and varied by institution at the discretion of the investigators at each site.

Participants underwent the first lumbar puncture on day 1 for cerebrospinal fluid collection and nusinersen dosing, the second lumbar puncture on day 8 or day 29 for cerebrospinal fluid collection, and the third lumbar puncture during the extension study for cerebrospinal fluid collection and redosing with nusinersen 9 to 14 months after the initial lumbar puncture.

Table 1. Baseline Demographics of Participants (N = 28).

Characteristic	
Female, n (%)	17 (61)
Mean (range) age, y	6.1 (2.0-14.0)
Mean (SD) weight, kg	23.6 (14.7)
SMA, n (%)	
type 2	15 (54)
type 3	13 (46)
Ambulatory, yes, n (%)	10 (36)
Scoliosis, yes, n (%)	13 (46)
Spinal rods, n (%)	1 (4)

Abbreviation: SMA, spinal muscular atrophy.

Safety and Tolerability

In the phase 1 single-dose study, participants were initially monitored for safety and tolerability for 29 days (1-mg and 3-mg cohorts) or 85 days (6-mg and 9-mg cohorts) post dosing. Participants enrolled in the extension study were monitored over 169 days post dosing. Safety reporting included adverse events related to lumbar puncture, and a subgroup analysis was performed to compare reported lumbar puncture–related adverse events by needle size, participant age, and spinal muscular atrophy type.

Results

A total of 28 children were enrolled and received dosing in the phase 1 study; 15 children had spinal muscular atrophy type 2 and 13 had spinal muscular atrophy type 3 (Table 1). At baseline, participant mean (range) age was 6.1 (2.0-14.0) years, 17 were female, 10 were ambulatory, and 13 had scoliosis. One child had vertical expandable prosthetic titanium rods inserted and the pedicle screws were inserted into T2, T3, and T4 with fixation of the growing rods to the pelvis, leaving the lumbar spine area spared. The first and second lumbar punctures were performed on 28 and 27 children in the parent study, respectively, and the third on 18 children who re-enrolled in the extension study. Of the 10 children not re-enrolling, 6 re-enrolled in a multiple-dose study of nusinersen and 4 decided not to enroll. Of these, 1 child did not re-enroll for reasons related to the lumbar puncture procedure. This was a

Table 2. Comparison of Lumbar Puncture Procedural Differences Between the Study Sites.[a]

Lumbar Puncture Procedural Details	Columbia University (n = 28)	University of Utah (n = 25)	Boston Children's Hospital (n = 10)	University of Texas (n = 10)
Needle size(s) used	• 21, 22, 25, or 27 gauge	• 21, 22, or 25 gauge	• 24 or 22 gauge	• 22 or 25 gauge
Needle type(s)	• Whitacre 3.0-inch spinal needle	• Whitacre spinal needles	• Sprotte 35-mm 24-gauge spinal needle • Quincke 25-mm 22-gauge spinal needle	• Quincke 1.5- to 3-in. spinal needle
Placement	• L2-L3, L3-L4, L4-L5	• L2-L3, L3-L4, L4-L5	• L3-L4, L4-L5, L5-S1	• L3-L4 or L4-L5
Fluoroscopy, yes/no	• Yes[b]	• No[c]	• No[c]	• Yes[d]
Ultrasound, yes/no	• No	• No	• Yes	• No
Anesthesia and/or sedation, yes/no	• Yes	• Yes	• Yes	• Yes
If yes, type(s)	• Inhalational anesthesia: sevoflurane ± nitrous oxide • IV sedation: remifentanil, midazolam, propofol, fentanyl • Other: 1% lidocaine	• Inhalational anesthesia: sevoflurane ± nitrous oxide • IV sedation: midazolam ± ketamine	• Inhalational anesthesia: sevoflurane and/or nitrous oxide • IV sedation: propofol	• Inhalational anesthesia: sevoflurane and nitrous oxide • IV sedation: propofol • Topical: EMLA cream
Specialty of performing clinician	• Neuroradiologist (lumbar puncture) • Neurologist (drug administration) • Pediatric anesthesiologist (anesthesia/sedation)	• Neurologist (lumbar puncture/drug administration) • Anesthesiologist, nurse practitioner, or sedation nurses (anesthesia/sedation)	• Pediatric anesthesiologist (entire procedure)	• Pediatric anesthesiologist (entire procedure)
Participant position	• Prone position on the angiography table	• Left lateral decubitus position or supporting sitting	• Lateral decubitus position	• Left lateral position
Place of procedure	• Interventional diagnostic suite (part of the department of radiology)	• Rapid treatment unit or outpatient clinic suite within the hospital setting	• Operating room	• Operating room
Monitoring	• ECG, pulse oximetry, non-invasive blood pressure, end-tidal CO_2, temperature	• ECG, oxygen saturation, and expired CO_2[e] • Oxygen saturation and heart rate monitoring[f]	• ECG, pulse oximetry, oscillometric blood pressure, and end-tidal CO_2 • Ventilation was spontaneous or assisted	• ECG, pulse oximetry, blood pressure, temperature, and end-tidal CO_2

Abbreviations: ECG, electrocardiogram; IV, intravenous.
[a]n's indicate the number of lumbar punctures performed at each site (total 73).
[b]Fluoroscopy was used routinely per institutional practice.
[c]Only in some participants.
[d]In participants with severe scoliosis only.
[e]For children receiving lumbar puncture.
[f]For children receiving IV sedation.

12-year-old girl with spinal muscular atrophy type 2 who had severe scoliosis at baseline and in whom the second fluoroscopically guided lumbar puncture procedure was not performed successfully at day 8 in the phase 1 study.

Participants were enrolled and treated in 4 sites; the institutional lumbar puncture procedures used in each site are reported in Table 2. Both cutting-tip and atraumatic (pencil-point) spinal needles were used and the general practice was to

Table 3. Summary of Procedural Details per Lumbar Puncture.[a]

Lumbar Puncture Procedural Details	First Lumbar Puncture (n = 28)	Second Lumbar Puncture (n = 27)	Third Lumbar Puncture (n = 18)	Total No. of Lumbar Punctures (N = 73)
Gauge of needle				
21	2 (7)	1 (4)	0	3 (4)
22	13 (46)	14 (52)	8 (44)	35 (48)
24	4 (14)	2 (7)	1 (6)	7 (10)
25	9 (32)	9 (33)	9 (50)	27 (37)
27	0	1 (4)	0	1 (1)
Needle insertion site				
L2-L3	4 (14)	8 (30)	5 (28)	17 (23)
L3-L4	11 (39)	13 (48)	8 (44)	32 (44)
L4-L5	12 (43)	5 (19)	4 (22)	21 (29)
L5-S1	1 (4)	1 (4)	1 (6)	3 (4)
Use of fluoroscopy, yes	14 (50)	12 (44)	6 (33)	32 (44)
Treatment for post–lumbar puncture syndrome, yes[b]	5 (18)	1 (4)	1 (6)	7 (10)

[a]Values are n (%).
[b]Participants were treated for post–lumbar puncture headache with acetaminophen, ibuprofen, and/or caffeine citrate.

Table 4. Summary of Reported Lumbar Puncture–Related Adverse Events.

	First Lumbar Puncture (n = 28)		Second Lumbar Puncture (n = 27)		Third Lumbar Puncture (n = 18)		All Lumbar Punctures (N = 73)	
Adverse Event	Patients, n (%)	Events, n	Patients, n (%)	Events, n	Patients, n (%)	Events, n	Total, n (%)[a]	Total Events, n
Patients reporting ≥1 adverse event	9 (32)	20	8 (30)	10	6 (33)	8	23 (32)	38
Headache	4 (14)	4	4 (15)	4	1 (6)	1	9 (12)	9
Back pain	2 (7)[b]	3	3 (11)	3	3 (17)	3	8 (11)	9
Post–lumbar puncture syndrome	5 (18)[b]	6	1 (4)	1	1 (6)	1	7 (10)	8
Nausea	2 (7)	2	0	0	0	0	2 (3)	2
Puncture site pain	1 (4)	1	1 (4)	1	0	0	2 (3)	2
Paresthesia	1 (4)	1	0	0	0	0	1 (1)	1
Pain in extremity	0	0	1 (4)	1	0	0	1 (1)	1
Procedural nausea	0	0	0	0	1 (6)	1	1 (1)	1
Procedural pain	0	0	0	0	1 (6)	1	1 (1)	1
Vomiting	1 (4)	1	0	0	0	0	1 (1)	1
Puncture site reaction	1 (4)	1	0	0	0	0	1 (1)	1
Dehydration	1 (4)	1	0	0	0	0	1 (1)	1
Hypotension	0	0	0	0	1 (6)	1	1 (1)	1

[a]Total number of adverse events in all 73 lumbar punctures performed.
[b]Participants reporting >1 adverse event after each lumbar puncture were counted only once for the lumbar puncture.

perform the procedure in the lateral decubitus or prone position by pediatric anesthesiologists, neurologists, or neuroradiologists in either inpatient or outpatient settings. All sites performed the procedure under intravenous (midazolam, ketamine, fentanyl, remifentanil, and/or propofol) or inhaled anesthesia/sedation (sevoflurane and/or nitrous oxide). Some sites also reported the use of topical anesthesia (EMLA cream) or locally injected lidocaine (Table 2).

A total of 74 lumbar punctures were attempted and 73 procedures were performed. The majority of lumbar punctures were carried out using either a 22- (48%) or 25-gauge (37%) needle inserted in the L3-L4 (44%) or L4-L5 (29%) space. Nearly half (44%) of the lumbar punctures were guided using fluoroscopy (Table 3).

Of the 73 lumbar punctures performed, the majority (n = 50; 68%) had no complications; lumbar puncture–related adverse events were reported in 23 (32%; Table 4). The most common adverse events were headache (9 events), back pain (9 events), and post–lumbar puncture syndrome (8 events; post–dural puncture headache with or without vomiting), and all events resolved without long-term complications. The timing of the adverse events varied; 67% (n = 6) of the headaches occurred 12 to 72 hours post lumbar puncture, whereas back pain was reported 0 to 48 hours post procedure (Figure 1). Fifty percent

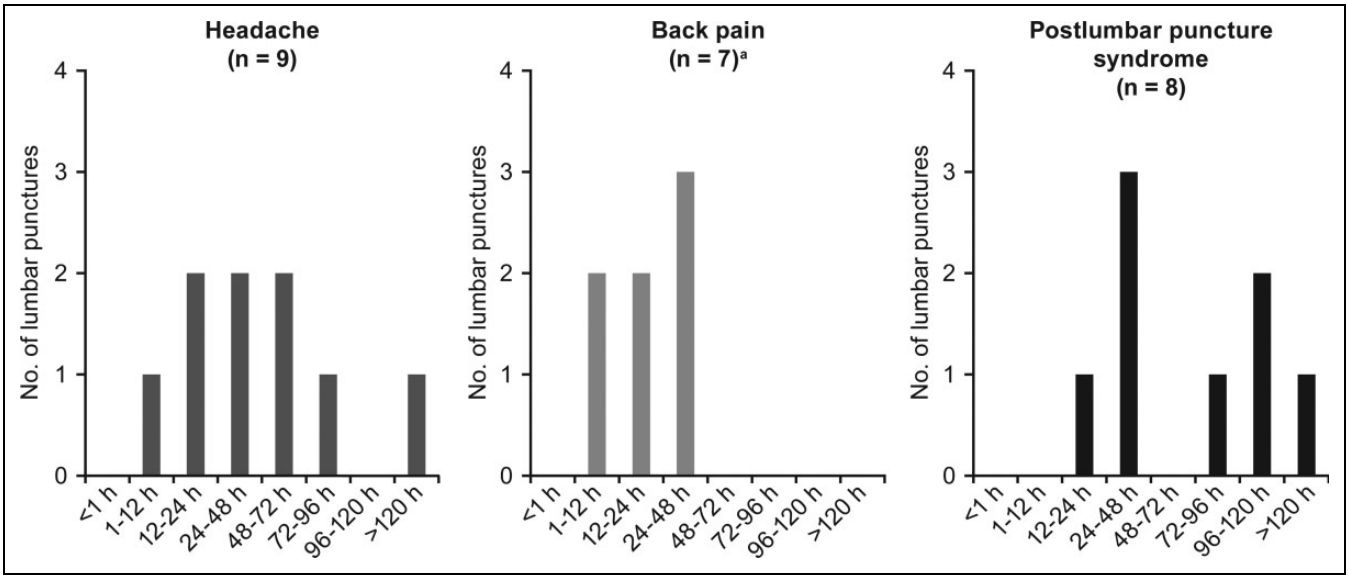

Figure 1. Time of onset of headache, back pain, and post–lumbar puncture syndrome. Most common lumbar puncture–associated adverse events (N = 73) and their time of onset are shown. [a]Time of onset for 2 cases of back pain were not reported.

(n = 4) of the post–lumbar puncture syndrome events occurred between 12 and 48 hours, with the remaining occurring after 72 hours (Figure 1). All 8 incidences of post–lumbar puncture syndrome were managed with acetaminophen, ibuprofen, and/or caffeine citrate for headache. All 8 cases of post–lumbar puncture syndrome resolved with conservative therapy, and none of the 7 participants needed an epidural blood patch. In 1 case, a blood patch was performed prophylactically during the second lumbar puncture procedure in a patient who had experienced a post–dural puncture headache following the first procedure. No post–dural puncture headache resulted after the second procedure. Resolution of the post–lumbar puncture syndrome occurred between 4 hours and 5 days, with the majority of the events (50% of cases) lasting 1 to 2 days.

A subgroup analysis compared lumbar puncture complications with needle size, participant age, and spinal muscular atrophy type and demonstrated that headache, back pain, and post–lumbar puncture syndrome were observed more frequently when a 21- or 22-gauge needle was used, in older children (8-14 years of age), and in children with spinal muscular atrophy type 3 (Table 5).

Discussion

Lumbar puncture–related adverse events are well documented in children and infants,[11-13] with the most common complications being back pain and headache with an incidence of 11% to 40% and 12% to 33%, respectively.[11,12,15] Although the incidence of post–lumbar puncture syndrome in children is not well reported, 4% to 11% of the reported headaches are classified as post–dural puncture headaches.[11,12,15] In addition to headache, post–lumbar puncture syndrome can also include transient effects of backache, dizziness, nausea with or without vomiting, numbness, and lower extremity weakness.[16,17] In rare cases, more severe symptoms have been reported, such as intracranial hypotension, epidural hematoma, and cauda equina syndrome.[18,19] In this phase 1 study and its extension, 73 lumbar punctures were performed for cerebrospinal fluid collection and/or intrathecal drug administration in children with spinal muscular atrophy. Approximately one-third of the procedures were associated with adverse events, most commonly with headache, back pain, and post–lumbar puncture syndrome, and the majority occurred within 72 hours after the procedure. A subgroup analysis demonstrated that the highest incidence of adverse events was reported in older children, children with spinal muscular atrophy type 3, and when a larger 21- or 22-gauge needle was used.

Using a 24-gauge needle or smaller atraumatic needle inserted with the bevel parallel to the dura fibers had been suggested to considerably reduce damage to the dura and consequently decrease the risk for cerebrospinal fluid leak after lumbar puncture,[20,21] including in children.[11,22] Needle type (atraumatic or traumatic) may have a greater impact on the reported incidence of post–dural puncture headache than needle size. Turnbull et al[21] found the incidence of post–dural puncture headache varied from 0.6% to 4% (22 gauge) and 0% to 14.5% (25 gauge) with an atraumatic (Whitacre) needle versus 36% (22 gauge) and 3% to 25% (25-gauge) with a traumatic cutting-tip (Quincke) needle, respectively. Although using an atraumatic needle may reduce the incidence of post–lumbar puncture syndrome, some studies have identified increased failure rate[23,24] and paresthesia[23] when using an atraumatic needle versus a cutting-tip spinal needle. However, differences in success rate were not found in other studies.[15,22,25]

Nearly half of the lumbar punctures were successfully carried out in children with spinal muscular atrophy using a 24-gauge spinal needle or smaller and, consequently, were

Table 5. Summary of Reported Lumbar Puncture–Related Adverse Events by Subgroup.[a]

Adverse Event	Gauge of Needle: 21/22 (n = 38)	Gauge of Needle: 24/25/27 (n = 35)	Patient Age: 2-7 y (n = 47)	Patient Age: 8-14 y (n = 26)	Patients With SMA type 2 (n = 39)	Patients With SMA type 3 (n = 34)	Total No. of Procedures (N = 73)
Headache	8 (21)	1 (3)	3 (6)	6 (23)	4 (10)	5 (15)	9 (12)
Back pain	5 (13)	3 (9)	4 (9)	4 (15)	2 (5)	6 (18)	8 (11)
Post–lumbar puncture syndrome	5 (13)	2 (6)	2 (4)	5 (19)	2 (5)	5 (15)	7 (10)
Nausea	2 (5)	0	1 (2)	1 (4)	1 (3)	1 (3)	2 (3)
Puncture site pain	2 (5)	0	1 (2)	1 (4)	2 (5)	0	2 (3)
Paresthesia	1 (3)	0	0	1 (4)	0	1 (3)	1 (1)
Pain in extremity	1 (3)	0	1 (2)	0	1 (3)	0	1 (1)
Procedural nausea	0	1 (3)	1 (2)	0	1 (3)	0	1 (1)
Procedural pain	1 (3)	0	0	1 (4)	0	1 (3)	1 (1)
Vomiting	1 (3)	0	1 (2)	0	1 (3)	0	1 (1)
Puncture site reaction	1 (3)	0	1 (2)	0	1 (3)	0	1 (1)
Dehydration	1 (3)	0	1 (2)	0	1 (3)	0	1 (1)
Hypotension	1 (3)	0	1 (2)	0	1 (3)	0	1 (1)

Abbreviation: SMA, spinal muscular atrophy.
[a]Values are n (%).

associated with reduced incidence of headache and post–lumbar puncture syndrome compared with procedures performed using a 21- or 22-gauge needle. After initial experiences, all investigational sites switched to using a smaller needle size, except when use of a larger needle size was required (eg, scoliosis). The higher incidence of adverse events, particularly headaches, observed among older children and/or in children with spinal muscular atrophy type 3 were likely related to the use of larger bore/gauge spinal needles, cutting-tip needles (eg, Quincke type), multiple attempts, and/or due to technical difficulties resulting from increased body weight and the presence of scoliosis or excessive lumbar lordosis.

In most cases, post–dural puncture headache can be successfully treated with conservative therapy, consisting of bed rest in prone or lateral position, hydration, oral or intravenous caffeine, anti-nausea or antiemetic therapy, and/or analgesics.[20,21,26,27] However, in children with post–dural puncture headaches persisting >48 hours or that worsen despite the use of conservative therapy, a therapeutic epidural blood patch may be indicated.[27,28] Although there is conflicting evidence, some studies also support the use of a prophylactic blood patch to prevent post–dural puncture headache; however, none of these studies included children.[29] In this study, all post–dural puncture headaches resolved with conservative treatment of bed rest, adequate hydration, and administration of caffeine and other analgesics, and therapeutic epidural blood patches proved unnecessary.

Other considerations that may facilitate lumbar puncture success in children include patient positioning and the use of spinal ultrasound. Few studies in children and infants have compared the feasibility of lumbar puncture in upright versus lateral recumbent position. Both the seated and lateral decubitus position with hip flexion increased the interspinous space compared with hip neutral positions and may increase lumbar puncture success rate.[30-33] The use of spinal ultrasound may facilitate the lumbar puncture procedure,[34,35] and is favored over fluoroscopy in children, particularly if repeated procedures are planned, to minimize radiation exposure and cost.[36] In this study, lumbar punctures were performed in the lateral decubitus or prone position, and nearly half of the lumbar punctures were successfully done without ultrasound and/or fluoroscopy. However, in patients with spinal muscular atrophy with scoliosis, spinal rods, or other hardware, the use of imaging may be warranted to facilitate the procedure and increase the success of intrathecal medication delivery. Lumbar punctures were successfully performed in the participants with scoliosis (13 in the first procedure, 12 in the second procedure, and 11 in the third procedure) and in the one child who had spinal rods. The second fluoroscopy-guided procedure was unsuccessful in only 1 patient because of scoliosis. This suggests that in some cases scoliosis might hinder lumbar puncture success in children with spinal muscular atrophy, but the frequency needs to be confirmed in larger studies.

General anesthesia and/or sedation are routinely performed in children to reduce procedure-related anxiety, pain, and distress, and to increase the overall success rate of lumbar puncture.[37-39] However, increasing concerns about the impact of repeated anesthesia exposure on the developing nervous system in infants and young children must be carefully considered when repeated procedures are planned.[40] All 73 lumbar punctures performed in this study were performed using either intravenous (midazolam, ketamine, fentanyl, remifentanil, and/or propofol) or inhaled anesthesia/sedation (sevoflurane, nitrous oxide), with no associated complications reported. Children with spinal muscular atrophy who have moderate to severe muscle weakness and/or severe scoliosis are at higher risk for hypoventilation and respiratory compromise. Thus, if deep sedation or general anesthesia is used, assisted ventilation may

be required by either non-invasive or invasive means. The use of local topical or subcutaneous anesthesia may help decrease the requirements for sedation. Based on our experience in this study, we recommend the most minimal use of sedative medications or anesthesia to permit safe and effective completion of the procedure.

The limitations of this study include a small sample size and the limited number of lumbar puncture procedures performed. Further experiences with lumbar puncture in children and infants with spinal muscular atrophy are needed to add to this knowledge.

Conclusions

In summary, repeated lumbar punctures were successfully performed in children with spinal muscular atrophy in the initial nusinersen clinical studies. The frequency of lumbar puncture–related adverse events in children with spinal muscular atrophy was similar to that previously reported in children and infants, and were mainly limited to headache, back pain, and post–lumbar puncture syndrome. A 24-gauge needle or smaller was successfully used in children with spinal muscular atrophy to perform lumbar puncture with lower incidence of complications, suggesting that using a 24-gauge or smaller needle would likely reduce the chance of adverse events. Bed rest, adequate hydration, oral caffeine, and/or analgesics were sufficient to resolve post–lumbar puncture headache/syndrome without the need of a therapeutic blood patch in all patients. Provisions for ultrasound guidance may be warranted in future research protocols given the potential benefit of reduced radiation exposure compared to fluoroscopy, especially in cases of serial procedures for repeated drug delivery. Overall, we conclude that intrathecal delivery of medication is feasible, safe, and well tolerated. Our experience may prove useful for guiding the development of best practice strategies for safe and effective intrathecal delivery of nusinersen and/or other promising emerging therapies for spinal muscular atrophy.

Author Note

Clinical trial registration: ClinicalTrials.gov identifiers: NCT01494701 (An Open-label Safety, Tolerability, and Dose-Range Finding Study of ISIS SMN_{Rx} in Patients With Spinal Muscular Atrophy; URL: https://clinicaltrials.gov/ct2/show/NCT01494701) and NCT01780246 (An Open-label Safety and Tolerability Study of ISIS SMN_{Rx} in Patients With Spinal Muscular Atrophy Who Previously Participated in ISIS 396443-CS1; URL: https://clinicaltrials.gov/ct2/show/NCT01780246)

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Author Contributions

MH, KJS, NS, AK, and AF-G coordinated and supervised data collection and performed study procedures (eg, lumbar punctures or anesthesia) at the 4 clinical sites, critically reviewed and revised the manuscript, and approved the final manuscript as submitted. SX carried out the initial data analyses, reviewed and revised the manuscript, and approved the final manuscript as submitted. KB conceptualized and designed the study, participated in the data analysis, reviewed and revised the manuscript, and approved the final manuscript as submitted.

Declaration of Conflicting Interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: KJS has received funding for clinical trial contracts from Ionis Pharmaceuticals, Inc. She was working at the Department of Neurology, University of Utah, at the time of the study. SX is a full-time employee of Ionis Pharmaceuticals, Inc. KB was a full-time employee of Ionis Pharmaceuticals, Inc. at the time of the study and manuscript preparation.

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Ethical Approval

Approval for the study protocols and all amendments were obtained from Columbia University Medical Center Institutional Review Board (IRB) (Approval nos. AAAI6758 and AAAK5458). Written informed consent and assent (if applicable) were obtained before any evaluations were conducted for eligibility.

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Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study

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Summary

Background Nusinersen is a 2′-*O*-methoxyethyl phosphorothioate-modified antisense drug being developed to treat spinal muscular atrophy. Nusinersen is specifically designed to alter splicing of *SMN2* pre-mRNA and thus increase the amount of functional survival motor neuron (SMN) protein that is deficient in patients with spinal muscular atrophy.

Methods This open-label, phase 2, escalating dose clinical study assessed the safety and tolerability, pharmacokinetics, and clinical efficacy of multiple intrathecal doses of nusinersen (6 mg and 12 mg dose equivalents) in patients with infantile-onset spinal muscular atrophy. Eligible participants were of either gender aged between 3 weeks and 7 months old with onset of spinal muscular atrophy symptoms between 3 weeks and 6 months, who had *SMN1* homozygous gene deletion or mutation. Safety assessments included adverse events, physical and neurological examinations, vital signs, clinical laboratory tests, cerebrospinal fluid laboratory tests, and electrocardiographs. Clinical efficacy assessments included event free survival, and change from baseline of two assessments of motor function: the motor milestones portion of the Hammersmith Infant Neurological Exam—Part 2 (HINE-2) and the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) motor function test, and compound motor action potentials. Autopsy tissue was analysed for target engagement, drug concentrations, and pharmacological activity. HINE-2, CHOP-INTEND, and compound motor action potential were compared between baseline and last visit using the Wilcoxon signed-rank test. Age at death or permanent ventilation was compared with natural history using the log-rank test. The study is registered at ClinicalTrials.gov, number NCT01839656.

Findings 20 participants were enrolled between May 3, 2013, and July 9, 2014, and assessed through to an interim analysis done on Jan 26, 2016. All participants experienced adverse events, with 77 serious adverse events reported in 16 participants, all considered by study investigators not related or unlikely related to the study drug. In the 12 mg dose group, incremental achievements of motor milestones (p<0·0001), improvements in CHOP-INTEND motor function scores (p=0·0013), and increased compound muscle action potential amplitude of the ulnar nerve (p=0·0103) and peroneal nerve (p<0·0001), compared with baseline, were observed. Median age at death or permanent ventilation was not reached and the Kaplan-Meier survival curve diverged from a published natural history case series (p=0·0014). Analysis of autopsy tissue from patients exposed to nusinersen showed drug uptake into motor neurons throughout the spinal cord and neurons and other cell types in the brainstem and other brain regions, exposure at therapeutic concentrations, and increased *SMN2* mRNA exon 7 inclusion and SMN protein concentrations in the spinal cord.

Interpretation Administration of multiple intrathecal doses of nusinersen showed acceptable safety and tolerability, pharmacology consistent with its intended mechanism of action, and encouraging clinical efficacy. Results informed the design of an ongoing, sham-controlled, phase 3 clinical study of nusinersen in infantile-onset spinal muscular atrophy.

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Introduction

Classic proximal 5q spinal muscular atrophy (OMIM 253300), a progressive motor neuron disorder, is the most common genetic cause of childhood mortality,[1,2] having an incidence of about one in 11 000 livebirths.[3] About 60% of patients with spinal muscular atrophy are born with the severe form, infantile-onset type I, developing profound limb and trunk weakness before 6 months of age, and failing to rollover or achieve independent sitting. There is a high disease burden with substantial morbidity and mortality[4,5] from dysphagia, failure to thrive, hypoventilation, poor airway clearance due to weak cough, and lower respiratory tract infections.

Deletions or mutations in the *Survival Motor Neuron 1* (*SMN1*) gene cause spinal muscular atrophy.[6] Absence of the *SMN1* gene results in reliance on a nearly identical gene, *SMN2*, which differs from *SMN1* by 11 nucleotides. *SMN2* has a c.840C→T substitution at an exon splice enhancer site that regulates exon 7 inclusion,[7] so that only 10–25% of *SMN2* transcripts contain exon 7 and generate full-length functional SMN protein.[8] Although the role of SMN protein in motor neurons is incompletely understood and the concentration required for optimum functioning unknown, the phenotype of spinal muscular atrophy (type I, II, III, or IV)[9] is largely related to the number of *SMN2* gene copies present.[10]

Research in context

Evidence before this study

Spinal muscular atrophy is caused by deletions or mutations in the *Survival Motor Neuron 1* (*SMN1*) gene and is the most common genetic cause of childhood mortality. Infantile-onset spinal muscular atrophy presents clinically as a severe, progressive motor neuron disease, resulting in generalised weakness and impaired feeding and breathing. Survival is dependent upon a small amount of normal SMN protein translated by the backup *SMN2* gene, which, due to a splice site variant, usually excludes exon 7. Less than a quarter of these infants survive beyond 2 years of age without dependence upon ventilation support. There are no approved drug treatments for spinal muscular atrophy. Antisense oligonucleotides provide a targeted strategy for spinal muscular atrophy treatment by specifically binding to repressive sites within *SMN2* exon 7 or the flanking introns, thus promoting exon 7 inclusion and increased production of functional SMN protein. Nusinersen (also known as ISIS 396443 and ISIS-SMN$_{Rx}$) is a uniformly modified 2′-*O*-methoxyethyl phosphorothioate antisense oligonucleotide being developed for the treatment of spinal muscular atrophy. We searched PubMed using the keywords "nusinersen", "ISIS 396443", "ISIS-SMNRx ", and "ASO 10-27" with no date restrictions. Of the six publications identified, two reported the results of the phase 1 study described below, three described preclinical results using nusinersen, and one was a review article on SMA therapeutics. A phase 1, short-term, single-dose clinical study of intrathecally delivered nusinersen was previously done in children with spinal muscular atrophy.

Added value of this study

We report interim results of an ongoing open-label, phase 2, multiple-dose study of intrathecal nusinersen in patients with severe infantile-onset spinal muscular atrophy. We provide evidence that nusinersen has acceptable safety and tolerability when delivered by multiple intrathecal injections and shows promising clinical efficacy as evidenced by improvements in motor function, achievement of motor milestones, and permanent ventilation-free survival as compared with published natural history. Additionally, autopsies collected during the study indicate proof of target engagement and mechanism, as nusinersen altered *SMN2* splicing, with an increase in full-length transcript that includes exon 7, and an increase in SMN protein in spinal cord motor neurons as compared with untreated infants with spinal muscular atrophy as control.

Implications of all the available evidence

Our study shows favourable safety and tolerability, pharmacokinetics, proof-of-concept pharmacodynamics, and a promising clinical response of intrathecal nusinersen. Results informed the design of an ongoing large phase 3, randomised, sham-controlled study of nusinersen in infantile-onset spinal muscular atrophy. More broadly, the mechanistic effects of nusinersen at the mRNA and protein level in participants of this study provides proof of principle for the use of antisense therapeutics in the treatment of neurological disorders. Finally, results from this study suggest treatments that increased SMN protein might provide clinical benefit to patients with spinal muscular atrophy.

Antisense oligonucleotides provide a targeted strategy for treatment of spinal muscular atrophy by specifically binding to repressive sites within *SMN2* exon 7 or the flanking introns, thus promoting exon 7 inclusion, increased production of functional SMN protein, and rescuing the motor neuron pathology and improving survival in mouse models of spinal muscular atrophy.[11–14] Nusinersen (ISIS 396443 or ISIS-SMN$_{Rx}$) is a uniformly modified 2′-*O*-methoxyethyl phosphorothioate antisense oligonucleotide being developed for the treatment of spinal muscular atrophy (appendix).[11–14] A phase 1 study of nusinersen in children with type II and type III spinal muscular atrophy was previously done.[15] We present here the first study of nusinersen treatment for the more severe infantile-onset spinal muscular atrophy, reporting results from an ongoing phase 2 study.

See Online for appendix

Methods

Study design and participants

This open-label, escalating dose phase 2 study was designed to assess the safety and tolerability, pharmacokinetics, and clinical efficacy of nusinersen in infants with spinal muscular atrophy. Eligible participants were of either gender between 3 weeks and 7 months old with onset of spinal muscular atrophy symptoms between 3 weeks and 6 months, who had *SMN1* homozygous gene deletion or mutation, and who met additional eligibility criteria (appendix). The first four participants received loading doses of 6 mg equivalent nusinersen on days 1, 15, and 85, and then 12 mg equivalent doses on day 253 and every 4 months thereafter (6–12 mg group). The next 16 participants received 12 mg equivalent doses on the same schedule (12 mg group). Follow-up visits occurred on days 16, 29, 86, 92, 169, 254, 337, and 442, and then every 4 months. Participants also were monitored every 2–3 weeks by telephone for safety and ventilation status. *SMN2* copy number and *SMN2* gene sequencing were done on samples collected at day 85 (Athena Diagnostics, Marlborough, MA, USA).

The study was initiated after institutional review board approvals at the participating centres, in accordance with Good Clinical Practice guidelines. Written informed parental consent was obtained for all participants. An independent data safety monitoring board monitored the study.

Procedures

Nusinersen was diluted to a concentration of 1·2 mg/mL (6 mg dose equivalent) or 2·4 mg/mL (12 mg dose

equivalent) with artificial cerebrospinal fluid (CSF). The dose delivered was adjusted based on age such that each participant received a scaled dose equivalent (4–5 mL) based on their projected CSF volume.[16] The dose regime used was designed, based on the spinal cord tissue half-life in preclinical[17] and clinical[15] studies, to provide a loading period of dosing over the first 3 months to achieve a target spinal cord drug concentration, and then, after 6 months, chronic dosing once every 4 months to sustain the tissue concentration. Intrathecal dosing was done via lumbar puncture with topical anaesthesia using standard techniques for infants. Sedation was not used in infants younger than 2 years, but some older children required sedation due to movements during the procedure. Injections were delivered over 1–3 min using a 22-gauge spinal anaesthesia needle. Before each injection, about 5 mL of CSF was collected for analyses.

Outcomes

Safety assessments included adverse events, physical and neurological examinations, vital signs, clinical laboratory tests (serum chemistry, haematology, and urinalysis), CSF laboratory tests (cell counts, protein, and glucose), and electrocardiographs.

Clinical efficacy assessments included change from baselines of two assessments of motor function: the motor milestones portion of the Hammersmith Infant Neurological Exam—Part 2 (HINE-2),[18] a general categorical measure of infant developmental motor milestones done by a paediatric neurologist, and the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) motor function test,[19] a validated 16 item scale (0–64 points) designed specifically to capture motor function in infants with spinal muscular atrophy. Physical therapist evaluators were trained annually in the CHOP-INTEND using standardised procedure manual and videotaped assessments to establish and maintain strong inter-rater reliability. Compound muscle action potentials (CMAPs) were recorded for stimulation of the ulnar nerve[5,20,21] (recording from abductor digiti minimus) and peroneal nerve (recording from tibialis anterior), and change from baseline values were analysed. Survival, tracheostomy placement, and daily ventilation use were collected to assess the endpoint of age at death or permanent ventilation (defined as tracheostomy or ≥16 h ventilation per day continuously for at least 2 weeks in the absence of an acute reversible illness)[5,22] and was compared with a published natural history case series (appendix).[5]

Nusinersen concentrations were determined using an electrochemiluminescence method (PPD Bioanalytical, Wilmington, NC, USA), in plasma and predose CSF samples. Spinal cord and brain tissues were collected for pharmacokinetic and pharmacodynamic analyses from three infants who died during the study. Control thoracic spinal cord tissue for pharmacodynamics from four untreated infants with spinal muscular atrophy (aged 8–16 months; all with two *SMN2* gene copies) and three infants without the disease (aged 4–14 months) was provided by Charlotte Sumner (Johns Hopkins University, Baltimore, MD, USA). Nusinersen concentrations in tissues were determined using a variation of an enzyme-linked immunosorbent assay method.[23] Reverse transcription polymerase chain reaction (RT-PCR) analyses of tissues were done using a variation of published methods (appendix).[17,24]

Statistical analysis

The sample size was selected to examine the safety and tolerability of nusinersen. All participants were included in the safety and pharmacokinetic analyses. The per-protocol efficacy analysis included all participants who completed the loading doses and day 92 assessment. An additional participant with three *SMN2* gene copies was excluded from the CHOP-INTEND analysis due to a ceiling effect, having a baseline CHOP-INTEND score of 64, the maximum achievable. Excluded participants were in the 12 mg dose group (efficacy evaluable, n=14). HINE-2, CHOP-INTEND, and CMAP were compared between baseline and last visit following treatment using Wilcoxon signed-rank test. Age at death or permanent ventilation was estimated using a Kaplan-Meier survival curve and compared with natural history using the log-rank test. SMN protein staining intensity in neurons of treated infants with spinal muscular

	6–12 mg group (n=4)	12 mg group (n=16)	Total (n=20)
Gender			
Male	3 (75%)	9 (56%)	12 (60%)
Female	1 (25%)	7 (44%)	8 (40%)
Race			
White	3 (75%)	13 (81%)	16 (80%)
Black	0	1 (6%)	1 (5%)
Asian	0	1 (6%)	1 (5%)
Multiple Race	1 (25%)	0	1 (5%)
Other	0	1 (6%)	1 (5%)
Weight (kg)	7·1 (5·2–8·9)	6·7 (5·1–9·3)	6·8 (5·1–9·3)
SMN2 copy number (2/3/unknown)	4/0/0	13/2/1	17/2/1
Age at symptom onset (days)	47 (28–70)	63 (21–154)	60 (21–154)
Age at diagnosis (days)	74 (42–105)	80 (0–154)	78 (0–154)
Symptom onset to enrolment (days)	97 (39–151)	77 (15–130)	81 (15–151)
Age at enrolment (days)	145 (67–207)	140 (36–210)	141 (36–210)
CHOP-INTEND score	27 (22–34)	30 (17–64)	30 (17–64)
HINE-2 score	2 (1–3)	2 (1–12)	2 (1–12)
On Bi-PAP at entry	0	0	0
Gastrostomy tube at entry	1	1	2
Ulnar CMAP amplitude (mV)	0·37 (0·20–0·60)	0·53 (0–3·20)	0·50 (0–3·20)
Peroneal CMAP amplitude (mV)	0·67 (0·30–1·40)	0·52 (0–2·70)	0·55 (0–2·70)

Data are n (%), mean (range), or n, unless otherwise stated. Bi-PAP=bi-level positive airway pressure. CHOP-INTEND=Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders. CMAP=compound muscle action potential. HINE-2=Hammersmith Infant Neurological Exam—Part 2. *SMN2*=Survival Motor Neuron 2.

Table 1: **Demographics and baseline clinical characteristics of participants**

	6–12 mg group (n=4)		12 mg group (n=16)		Total (n=20)	
	Events (n)	Patients (n, %)	Events (n)	Patients (n, %)	Events (n)	Patients (n, %)
Any serious adverse event	8	3 (75%)	69	13 (81%)	77	16 (80%)
Any adverse event	110	4 (100%)	460	16 (100%)	570	20 (100%)
Pyrexia	5	3 (75%)	32	11 (69%)	37	14 (70%)
Respiratory infection	10	3 (75%)	28	11 (69%)	38	14 (70%)
Constipation	1	1 (25%)	9	8 (50%)	10	9 (45%)
Vomiting	2	2 (50%)	11	6 (38%)	13	8 (40%)
Joint contracture	15	3 (75%)	9	5 (31%)	24	8 (40%)
Pneumonia	2	1 (25%)	15	6 (38%)	17	7 (35%)
Scoliosis	2	2 (50%)	6	5 (31%)	8	7 (35%)
Respiratory distress	2	1 (25%)	10	6 (38%)	12	7 (35%)
Nasal congestion	3	1 (25%)	8	6 (38%)	11	7 (35%)
Respiratory failure	0	..	7	6 (38%)	7	6 (30%)
Rhinovirus infection	0	..	7	6 (38%)	7	6 (30%)
Nasopharyngitis	0	..	13	6 (38%)	13	6 (30%)
Gastro-oesophageal reflux	0	..	6	6 (38%)	6	6 (30%)
Otitis media	1	1 (25%)	7	5 (31%)	8	6 (30%)
Cough	2	2 (50%)	8	4 (25%)	10	6 (30%)
Diarrhoea	1	1 (25%)	13	4 (25%)	14	5 (25%)
Actelactasis	0	..	9	5 (31%)	9	5 (25%)
Increased secretions	0	..	7	5 (31%)	7	5 (25%)
Rash	4	1 (25%)	6	4 (25%)	10	5 (25%)

Treatment-emergent adverse events listed are those that occurred until Jan 26, 2016, with a frequency of more than 20% in nusinersen-treated participants (ie, occurring in more than four participants).

Table 2: **Adverse event summary**

	Age at death (days)	Gender	*SMN2* copy number	Nusinersen dosing	Days from last dose to autopsy
1	385	Male	2	6 mg on days 1, 15, 85; 12 mg on day 253	12
2	157	Male	2	12 mg on days 1, 15, 85	36
3	342	Female	2	12 mg on days 1, 15, 85	78

Table 3: **Demographics and drug exposure for the three autopsy participants treated with nusinersen**

atrophy was compared with untreated infants with spinal muscular atrophy using the two-factor nested ANOVA with subject effect nested within treatment effect. SAS version 9.4 was used for all statistical analyses. The study is registered at ClinicalTrials.gov, number NCT01839656.

Role of the funding source

The study was funded by Ionis Pharmaceuticals, Inc and Biogen. Employees of Ionis Pharmaceuticals, Inc (MY, FR, GH, ES, DAN, SX, CFB, and KMB) contributed to the study design, analysis and interpretation of data, and writing of the report. The corresponding author (RSF) had full access to all the data from the study and had final responsibility for the content of the report and decision to submit for publication.

Results

20 infants with spinal muscular atrophy were enrolled between May 3, 2013, and July 9, 2014. Demographic and baseline characteristics are summarised in table 1. *SMN2* copy number and gene sequencing was done on 19 infants: 17 of 19 patients had two copies of the *SMN2* gene and two patients three copies (both in the 12 mg dose group). No sequence variants in the *SMN2* gene were reported. One infant died before the day 85 assessment when samples for *SMN2* gene copy analysis were collected. Two screen failures occurred (one, a hypoxic-bradycardic event requiring intubation during screening; one cardiac abnormality) and one additional participant terminated from the study before dosing due to respiratory failure, secondary to an acute infection. We report interim results until Jan 26, 2016, which was about 18 months since the last participant was enrolled. At this time, the 6–12 mg group had been followed for 9–32 months and received four to nine doses of nusinersen, whereas the 12 mg group had been followed for 2–27 months and received two to eight doses.

Standard intrathecal injections of nusinersen resulted in no safety concerns. All participants experienced adverse events (570 events in total) during the study (table 2), with most being mild (359 events [63%]) or moderate (153 events [27%]) in severity. There were 77 serious adverse events reported in 16 participants, all considered by study investigators not related or unlikely related to the study drug, with the most common being respiratory distress or failure or respiratory infections, which are commonplace in infants with spinal muscular atrophy. No changes in neurological examination findings, laboratory assessments, vital signs, electrocardiogram parameters, or CSF safety parameters that were considered clinically significant and related to nusinersen were reported, with the exception of one mild event of transient, asymptomatic neutropenia and one mild event of vomiting, which were both considered possibly related to nusinersen by the investigators.

Incremental improvements in developmental motor milestones on the HINE-2 were observed for 16 of 19 participants (one of four participants in the 6–12 mg group; 15 of 15 participants in the 12 mg group) at the last visit compared with baseline (figure 1A, appendix). Change in HINE-2 score from baseline to last visit was significant for both cohorts combined ($p=0·0002$) and for participants in the 12 mg dose group ($p<0·0001$). Improvements of two or more levels per motor milestone category on at least one category were observed in 13 participants and were most often observed for grasping (13 participants), ability to kick (nine participants), and sitting (eight participants), but were also evident for head control (six participants), rolling (six participants), standing (five participants), crawling (two participants), and walking (two participants; figure 1B, appendix).

Motor function, assessed using the CHOP-INTEND, showed a mean increase of 11·5 points from baseline to

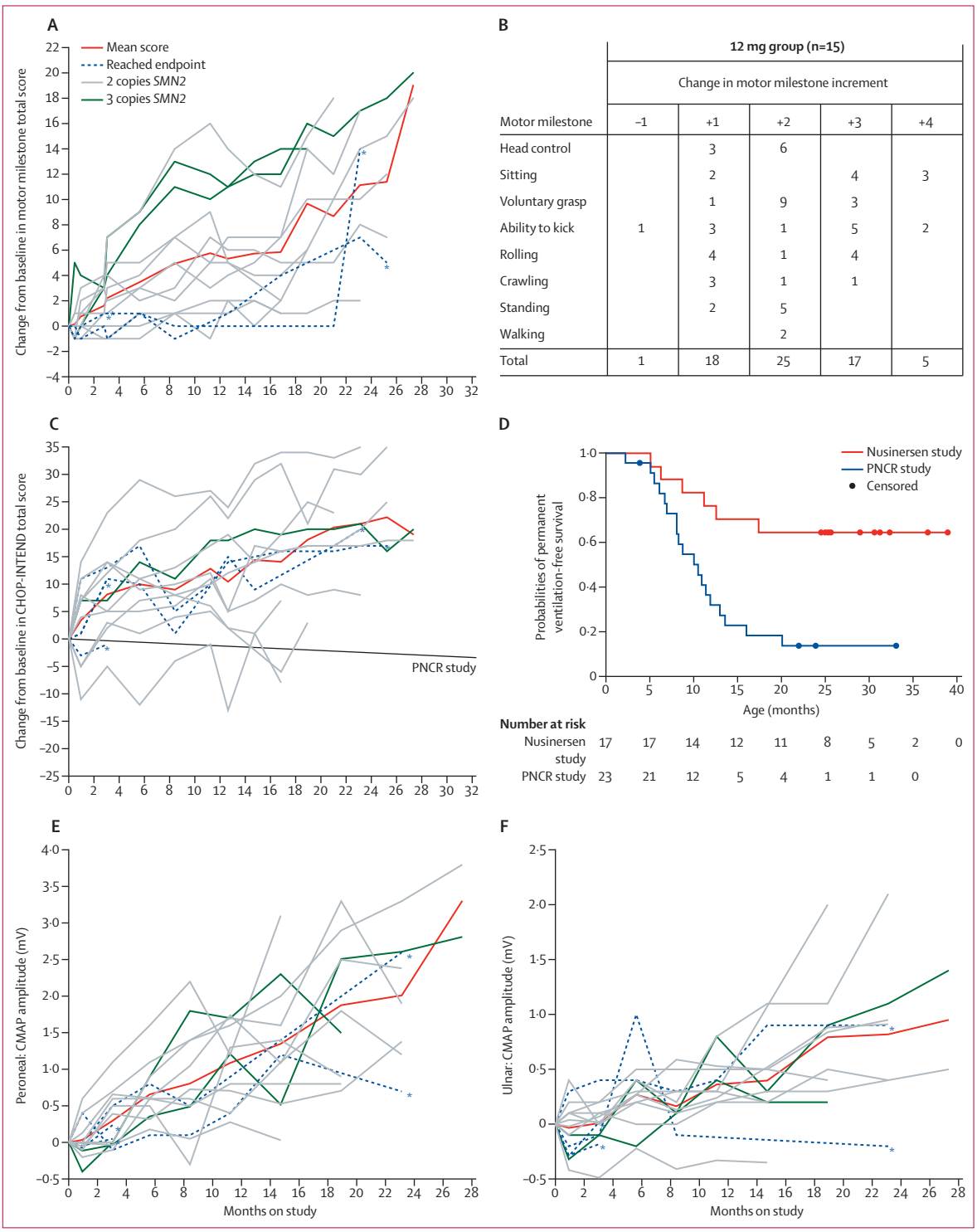
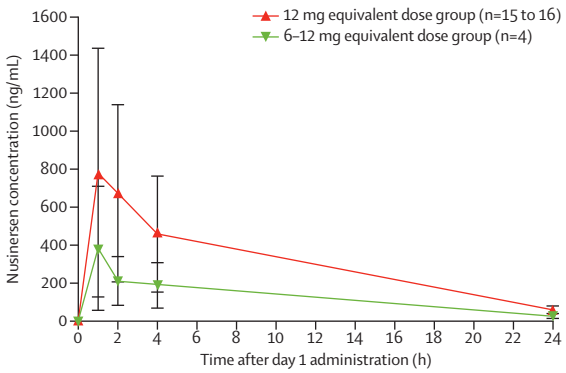


Figure 1: **Clinical effects in infants with spinal muscular atrophy**

(A) Change from baseline in the motor milestones as assessed by the Hammersmith Infant Neurological Exam –Part 2 (HINE-2) for participants in the 12 mg dose group. Red line=mean score. Dashed blue line=participants who reached the endpoint of death or permanent ventilation. Green line=participants with three copies of the *SMN2* gene. (B) Changes in individual milestone categories as assessed by the HINE-2 for the 12 mg dose group. (C) Changes in Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) infant motor function test for individual participants in the 12 mg dose group. Solid black line=Pediatric Neuromuscular Clinical Research (PNCR) natural history comparison for infantile spinal muscular atrophy.[5] Red line=mean score. Dashed blue lines=participants that reached endpoint of death or permanent ventilation. Green line=participants with three copies of *SMN2*. (D) Kaplan-Meier curves for participants with infantile-onset spinal muscular atrophy and two *SMN2* gene copies: nusinersen-treated versus untreated infants with spinal muscular atrophy from the PNCR natural history study (log-rank test, p=0·0014). (E and F) Change from baseline in peroneal (E) and ulnar (F) nerve compound muscle action potential (CMAP) negative peak amplitude for individual participants in the 12 mg dose group. Red line=mean score. Dashed blue lines=participants that reached endpoint of death or permanent ventilation. Green line=participants with three copies of *SMN2*.

A



B

Tissue	Observed nusinersen concentration (µg/g)		
	Infant 1	Infant 2	Infant 3
Lumbar cord	26·6	31·8	19·46
Thoracic cord	23·7	14·2	15·92
Cervical cord	15·5	23·3	11·93
Brainstem	13·8	8·13	3·68
Motor cortex	8·74	10·54	5·80

C

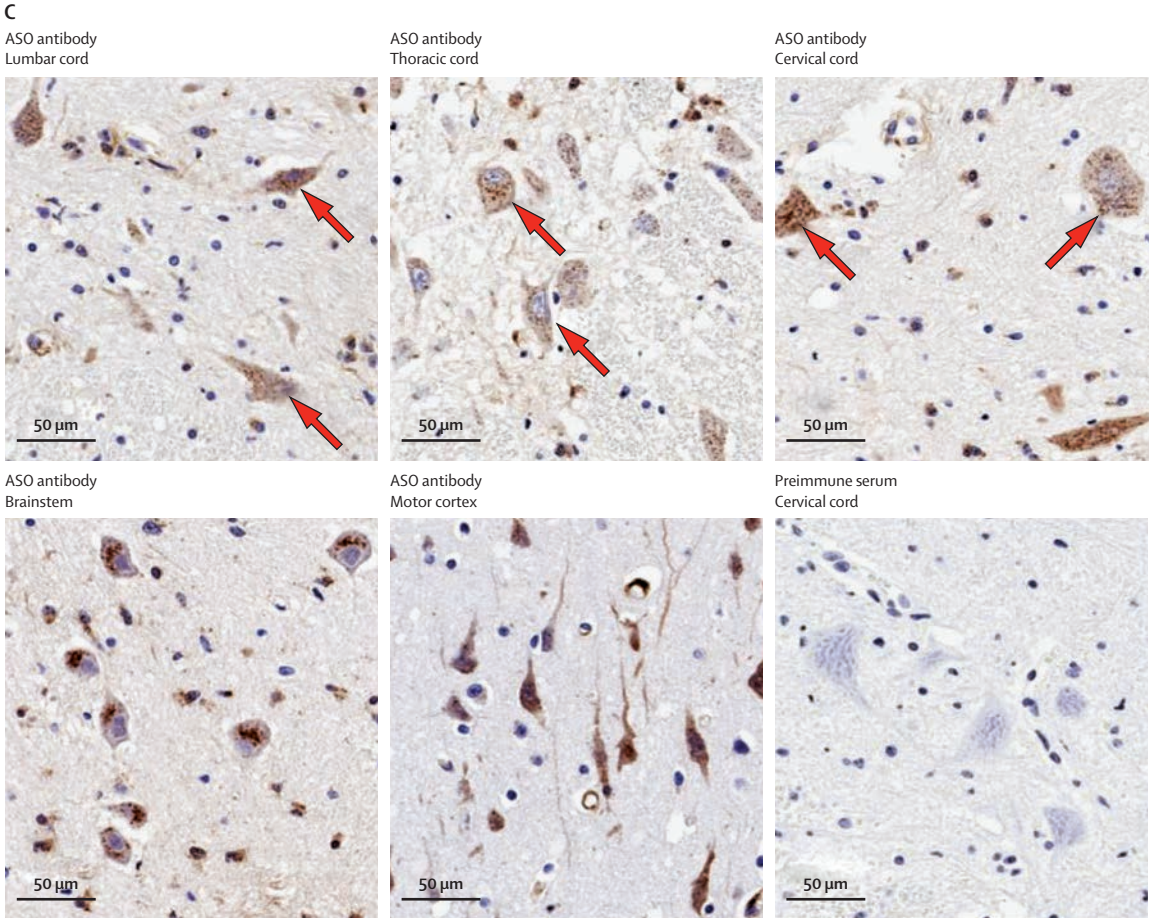


Figure 2: **Drug pharmacokinetics in infant plasma, cerebrospinal fluid, and spinal cord**
(A) Plasma concentrations of nusinersen in infants with spinal muscular atrophy from day 1 for the first 24 h after the first 6 mg or 12 mg equivalent intrathecal dose of nusinersen, measured with an electrochemiluminescence assay. (B) Concentrations of nusinersen determined in different autopsy tissues taken from the three infants with spinal muscular atrophy who died during the course of the study (table 3). The tissues were analysed for nusinersen concentration using a hybridisation enzyme-linked immunosorbent assay method.[23] (C) Distribution of nusinersen in spinal cord and brain tissues obtained from infant 2 treated with nusinersen. Tissue sections were stained with an antibody raised to phosphorothioate oligonucleotides and visualised using a peroxidase-conjugated anti-rabbit antibody as previously described.[26] Slides were counterstained with haematoxylin (blue). The brown staining in large neurons (red arrows) and, to a lesser extent, in smaller neurons and non-neuronal cells in the area, indicates the presence of nusinersen within these cells. ASO=antisense oligonucleotide.

last visit overall (p=0·0080; n=18), with 14 of 18 infants having an improvement (figure 1C, appendix). In the 12 mg group, 12 of 14 participants had an increase from baseline to last visit (mean increase 15·2 points; p=0·0013), compared with a natural history case series of infants with type I spinal muscular atrophy, in which a

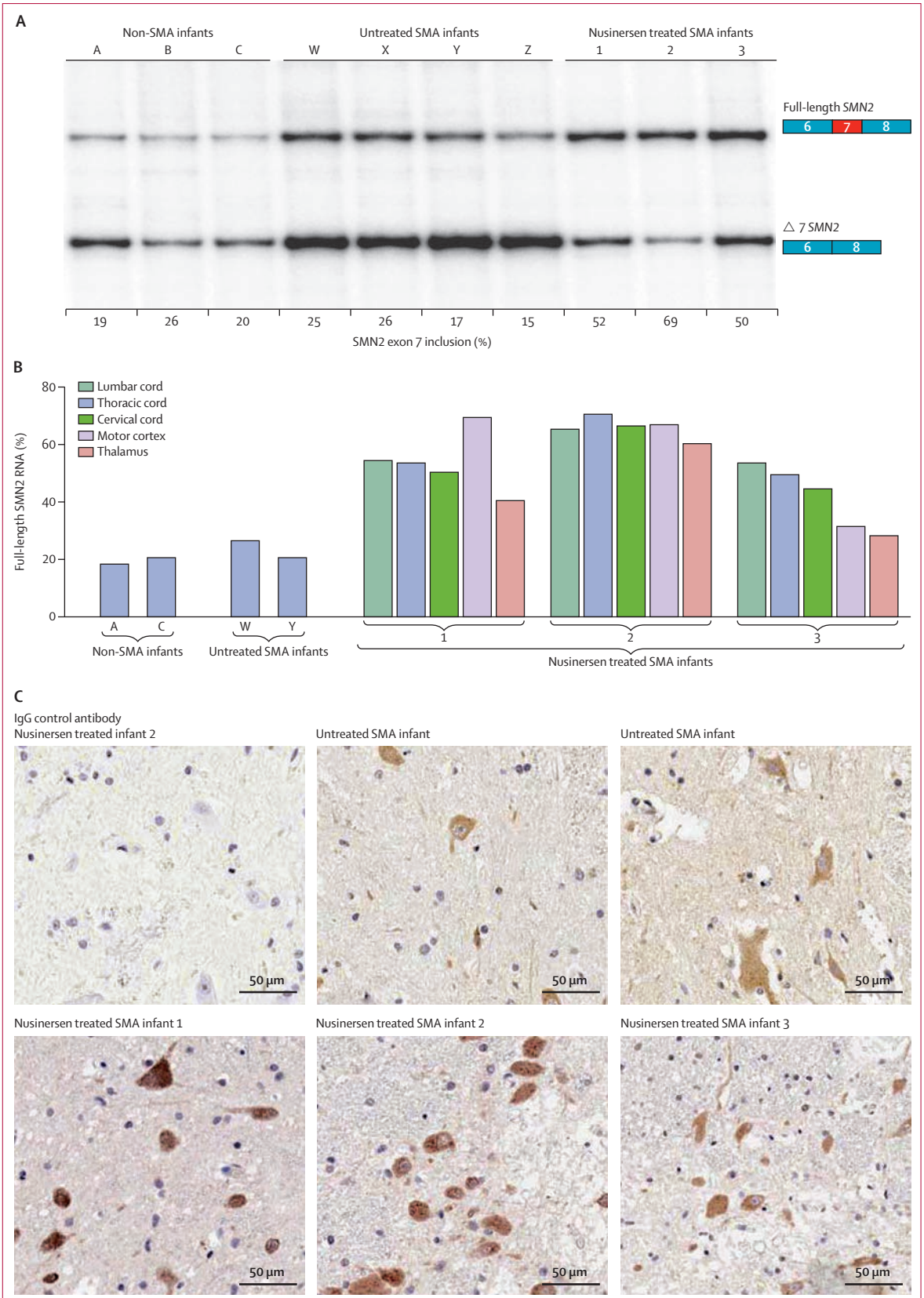


***Figure 3*: Drug pharmacodynamics in infant spinal cord samples**
(A) Amount of full-length and truncated (missing exon 7, Δ7) *SMN2* transcripts in thoracic spinal cord tissues determined by radioactive reverse transcription polymerase chain reaction (RT-PCR) assay. Tissues from three infants without spinal muscular atrophy (non-SMA) and four infants with spinal muscular atrophy (SMA) also were analysed to determine the percent of full-length *SMN2* transcripts in untreated infants. The numerical values under each lane represent the percentage of full-length *SMN2* transcript in the sample. (B) *SMN2* splicing analysis of spinal cord and brain regions by radioactive RT-PCR. RNA was isolated from brain and spinal cord tissue and analysed for full-length *SMN2* transcript by radioactive RT-PCR as described in the Methods. (C) Thoracic spinal cord tissue from two participants with infantile-onset spinal muscular atrophy and three nusinersen-exposed participants with spinal muscular atrophy stained for presence of SMN protein and detected using immunohistochemistry as described in the Methods. The staining was done in batch for these samples to allow comparison. SMN protein (brown) was readily observed in large neurons, which have location, morphology, and size consistent with motor neurons. IgG=immunoglobulin G.

mean decline of 1·27 points per year (95% CI 0·21–2·33) was observed (figure 1C).[5] A score greater than 40, a value rarely observed for symptomatic infants with type I spinal muscular atrophy with two *SMN*2 gene copies,[5,25] was observed in none of the 13 infants with two *SMN*2 gene copies at baseline and increased to seven of 13 participants at last visit in the 12 mg group.

As of the date of analysis, a median age of death or permanent ventilation had not been reached as most participants were surviving without permanent ventilation. A conservative analysis in which the median age at the endpoint or the data cutoff date (censoring) was calculated resulted in a censored median of 24·7 months in the 6–12 mg group and 25·2 months in the 12 mg group (which started enrolment about 5 months later). Of the seven participants who met the endpoint, one participant in the 6–12 mg group progressed to more than 16 h per day on bi-level positive airway pressure (Bi-PAP) at 8·7 months of age and one participant died from accidental asphyxia at 12·6 months. In the 12 mg group, one participant died from spinal muscular atrophy disease progression at 6·9 months, two participants died from progression of disease secondary to a recent pulmonary infection at 5·1 months and 11·2 months, and two participants had a tracheostomy at 6·3 months and 17·4 months. A log-rank test was done comparing infants with spinal muscular atrophy with two copies of *SMN2* from this study (n=17) to infants with spinal muscular atrophy with two copies of *SMN2* from the Pediatric Neuromuscular Clinical Research (PNCR) natural history case series (n=23; appendix).[5] Given all the caveats associated with comparison to a natural history case series, the log-rank test indicates a differentiation in age at death or permanent ventilation (p=0·0014; figure 1D).

Electrophysiological assessment using CMAP showed that in the 12 mg group, all participants exhibited an increase in peroneal CMAP and 12 of 15 patients in ulnar CMAP at last visit compared with baseline (figure 1E, F). Statistically significant increases in CMAP amplitude were observed for both nerves, with a mean increase of 742% (p<0·0001) or 1·56 mV in the peroneal CMAP amplitude and a mean increase of 377% (p=0·0103) or 0·62 mV in the ulnar CMAP amplitude.

CSF and plasma pharmacokinetics indicated that nusinersen cleared from the CSF into systemic circulation, consistent with normal CSF turnover, with dose dependent mean peak plasma concentrations observed about 1 h after dosing and declining over 24 h (figure 2A). CSF drug concentrations were still quantifiable 15–168 days after dosing, indicating prolonged exposure of the CSF and CNS tissues to nusinersen (appendix). Pharmacokinetic and pharmacodynamic analyses were also done on spinal cord and brain tissues collected for three infants who died during the study (table 3, figure 2B, C). Drug concentrations were greater than 10 µg/g (figure 2B), a concentration predicted to produce pharmacological effects,[17] in all areas of the spinal cord. Immunohistochemical staining for nusinersen confirmed localisation in neurons, vascular endothelial cells, and glial cells throughout the CNS, with neurons staining more intensely than other cell types (figure 2C, appendix). Nusinersen was also identified in peripheral tissues such as liver and kidney, consistent with clearance from the CSF into the systemic circulation (appendix).

Analysis of thoracic spinal cord tissues from untreated infants with spinal muscular atrophy or infants with no disease showed that 15–26% of the *SMN2* transcripts contained exon 7 (figure 3A, B). By contrast, 50–69% of the *SMN2* transcripts contained exon 7 in thoracic cord tissues from infants with spinal muscular atrophy exposed to nusinersen (figure 3A, B). This corresponded to a 2·6 times increase in full-length SMN2 transcripts compared with untreated infants with spinal muscular atrophy (appendix). Similar levels were also observed in multiple brain regions of infants with spinal muscular atrophy exposed to nusinersen (figure 3B, appendix). Infants exposed to nusinersen had an apparent increase in SMN protein staining in thoracic cord motor neurons compared with untreated infants with spinal muscular atrophy (figure 3C). Image analysis of thoracic spinal cord anterior horn indicated a 63·7% increase in SMN protein staining intensity in neurons of treated infants with spinal muscular atrophy compared with untreated infants with spinal muscular atrophy (mean optical density nusinersen-treated 0·1981 [SD 0·049], n=188 neurons analysed; untreated 0·1210 [0·023], n=122 neurons analysed; p<0·0001; appendix).

Discussion

In this study, multiple intrathecal doses of the antisense drug nusinersen were well tolerated in infants with spinal muscular atrophy, with no safety concerns identified for up to nine doses given over 32 months of treatment. Adverse events were generally consistent with those observed in fragile infants with type I spinal muscular atrophy. Similarly, nusinersen was well tolerated in older children aged 2–15 years with type II or type III spinal muscular atrophy.[15] Additionally, the intrathecal injections were successful and well tolerated in infants with spinal muscular atrophy, similar to the experience with intrathecal injections in children with spinal muscular atrophy.[27] This study is the first to give an antisense drug intrathecally to infants and thus adds to the growing body of clinical safety and tolerability data with this novel treatment modality.

Examination of postmortem tissue indicated that intrathecal nusinersen is distributed broadly throughout the spinal cord and brain, including target motor neurons, and that drug concentrations are above those predicted to produce the intended pharmacology.[12,13] Pharmacology consistent with the intended mechanism of action of nusinersen was observed in infants with spinal muscular atrophy treated with nusinersen, via an increase in the amount of full-length *SMN2* mRNA

transcript and a qualitative increase in SMN protein compared with untreated infants with spinal muscular atrophy. These data provide confirmation that the drug reaches target tissues and generates the desired molecular response of promoting inclusion of exon 7 in the transcript and are the first direct evidence for antisense pharmacology in the human CNS.

A promising clinical response to nusinersen in most, but not all, infants with spinal muscular atrophy was observed in all three categories examined: achievement of motor milestones and motor function, survival or permanent ventilation independence, and neuromuscular electrophysiology. Participants in the 12 mg dose group showed incremental achievement of motor milestones and improved motor function scores, as compared with an expected decline based on natural history,[5] suggestive of a favourable drug effect. Some of these infants developed the ability to sit independently and roll over independently and improved in head control, kicking, hand function, and standing and walking, changes that are beyond the motor repertoire expected for infants with type I spinal muscular atrophy according to natural history reports.[5,20,21] Although the improvements observed have not been reported to occur in type I spinal muscular atrophy infants, it should be noted that treatment with nusinersen has not restored normal age-appropriate function. Additionally, the peroneal and ulnar nerve CMAP amplitude increased over time, indicating an increase in electrically excitable muscle. These observations are unlike the natural history data, in which the ulnar CMAP is of uniformly low amplitude in symptomatic infants at the time of diagnosis and does not change over time.[5,20,21] The improved functional performance of infants treated with nusinersen and the increases in CMAP might represent improved function of remaining motor neurons with neuronal sprouting and re-innervation, or improved neuromuscular transmission at the neuromuscular junction.

Survival, including the surrogate of avoiding the need for permanent ventilation, was divergent as compared with natural history cohorts.[5,28] It is important in this context to also consider the standard of care because it has been shown that this will have an effect in infants with spinal muscular atrophy.[29,30] In this phase 2 study, the standard of care guidelines from 2007[9] were used as a benchmark during the course of the study, but parents were allowed to pursue a palliative care approach. This same approach of allowing both palliative and proactive care (gastrostomy tube and noninvasive ventilation support) was used in the PNCR (US) and German natural history case series.[5,28] In the PNCR case series, recently diagnosed patients with two *SMN2* gene copies had a median age at reaching a similar endpoint of 10·5 months, whereas it was 6·5 months in the German study. In the data reported here overall and for participants with two *SMN2* gene copies, a median endpoint has not yet been reached but the Kaplan-Meier curves diverge from the natural history, suggesting a drug effect. However, it is important that these results be interpreted cautiously, as this was a relatively small open-label study.

Overall, this phase 2 open-label study of intrathecal nusinersen in symptomatic infantile-onset spinal muscular atrophy has shown favourable safety and tolerability, pharmacokinetics, proof-of-concept pharmacodynamics, and an encouraging clinical response. Limitations of this study include the small number of participants, the relatively short duration of follow-up (2–32 months), and the open-label design. Additionally, comparison with natural history case series should be interpreted cautiously. Thus, a large phase 3, randomised, double-blind, sham-controlled study of nusinersen in infantile-onset spinal muscular atrophy is ongoing (NCT02193074). As no safety concerns were identified in this study, the phase 3 study has incorporated a more frequent dosing regimen, especially in the first few months of treatment, when infants with spinal muscular atrophy progress rapidly and are particularly vulnerable. Pre-symptomatic treatment might provide an even greater clinical response; this hypothesis is being examined in an ongoing phase 2 study (NCT02386553). As patients participating in the study still have active disease, combinations of intrathecal nusinersen with other drugs, including drugs that target peripheral tissues, warrant additional investigations. More broadly, the novel evidence for nusinersen mechanistic effects at the mRNA and protein level in participants of this study provides a proof of principle for the use of antisense therapeutics in the treatment of neurological disorders.

Contributors

RSF, CAC, JV, JWD, JM, DCDV, DAN, and KMB designed the trial. RSF, CAC, JV, JWD, JM, and DCDV did the trial. MY and ES monitored safety. FR, GH, and DAN did the pharmacokinetic and pharmacodynamic analyses. SX and KMB did the analysis of the clinical and safety data. RSF, CFB, and KMB interpreted the data and wrote the manuscript. All authors critically revised and approved the final manuscript.

Declaration of interests

RSF reports grants and personal fees from Ionis Pharmaceuticals during the conduct of the study; and grants and advisor fees from Biogen and Roche, outside the submitted work. RSF serves in an advisory capacity to the non-profit organisations, the SMA Foundation, Cure SMA, SMA Reach (UK), and SMA Europe, and also serves on the data safety monitoring board for the AveXis gene transfer study. CAC reports serving as a consultant for Roche Pharmaceuticals, AveXis, and Novartis, and receives grants from the SMA Foundation and the US Department of Defense. JWD reports serving as a consultant for Biogen, grants from Biogen and Ionis Pharmaceuticals, during the conduct of the study; grants from Ionis Pharmaceuticals, Cytokinetics, SMA Foundation, Roche, and Cytokinetics, outside the submitted work; serves as a consultant to AveXis, Cytokinetics, and Ionis Pharmaceuticals; and serves on the advisory board for SMA Foundation. JM reports serving as a consultant for Ionis Pharmaceuticals, and serving on advisory boards for Roche Pharmaceuticals and Biogen. DCDV reports serving as a consultant for, and receiving grants from, Ionis Pharmaceuticals, Biogen, Roche, Ultragenyx, and Sarepta; serves as an advisor to the SMA Foundation, Hope for Children Research Foundation, AveXis, and Glut1 Deficiency Foundation; receives grants from Milestones for Children, Will Foundation, National Institutes of Health, US Department of Defense, and Glut1 Deficiency Foundation; and serves on the data safety monitoring board for Cytokinetics.

MY and KMB were employees of Ionis Pharmaceuticals during the conduct of the study. FR, GH, ES, DAN, SX, and CFB were employees of Ionis Pharmaceuticals during the conduct of the study and are current employees of Ionis Pharmaceuticals. CFB has a patent and is an inventor of several issued and pending patents covering nusinersen. JV declares no competing interests.

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Developmental milestones in type I spinal muscular atrophy

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Abstract

The aim of this retrospective multicentric study was to assess developmental milestones longitudinally in type I SMA infants using the Hammersmith Infant Neurological Examination. Thirty-three type I SMA infants, who classically do not achieve the ability to sit unsupported, were included in the study. Our results confirmed that all patients had a score of 0 out of a scale of 4 on items assessing sitting, rolling, crawling, standing or walking. A score of more than 0 was only achieved in three items: head control (n = 13), kicking (n = 15) and hand grasp (n = 18). In these items, the maximal score achieved was 1 out of a scale of 4, indicating only partial achievement of the milestone. Infants with symptom onset after 6 months of age had longer preservation of a score of 1 when compared to those with onset before 6 months of age. Our results suggest that even when current standards of care are applied, developmental milestones are rarely even partially achieved as part of natural history in type I SMA infants. No infants in this study achieved a major milestone such as rolling over, or sitting independently, which would therefore represent robust outcomes in future interventional trials.

Keywords: Spinal muscular atrophy; Hammersmith Infant Neurological Examination; Outcome measures; Motor milestones

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disorder caused by mutations in the survival of motor neuron 1 (SMN1) telomeric gene, resulting in a reduced amount of functional full-length SMN protein. In children, SMA is classified into 3 phenotypes based on age at onset and maximal motor function achieved [1,2].

Type I SMA is characterized by inability to sit unsupported and by onset within 6 months of age [2,3]. Survival, typically limited to the first year, has increased in the last few decades, due to improvements in the standards of care, with the possibility to opt for respiratory and nutritional support [4,5].

The increased survival and the advent of different therapeutic approaches have highlighted the need for a better definition of the natural history of type I SMA especially as far as the acquisition of functional milestones is concerned, and for identification of appropriate tools to follow the progression of the disease. A few attempts have been made recently to develop clinical tools specifically designed to assess motor function in weak infants, such as the CHOP INTEND [6], or to adapt existing scales, such as the TIMP [7,8]. These scales have helped us to identify the variability of the phenotype and to assess the rapid progression of the disease. Little attempt has been made to monitor developmental milestones in these infants as, by definition, they are unable to sit and only a minority of

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them will acquire the milestones of head control and/or rolling over which are subsequently lost. Other important milestones, such as crawling or standing, are not achieved in type I SMA infants. However, no systematic study has assessed longitudinally the acquisition of motor milestones in type I SMA infants receiving current standards of care.

The aim of this study was to report the longitudinal use of a module capturing developmental milestones in infancy, performed as part of the Hammersmith Infant Neurological Examination (HINE) [9,10], in type I SMA infants. More specifically, we set out to investigate whether developmental milestones are achieved at any stage during the first years of life in these infants and, if achieved, to assess possible changes over time.

2. Materials and methods

This retrospective study was performed by collecting data from different existing large multicentric datasets, including one network based in the United States (the Pediatric Neuromuscular Clinical Research Network for SMA) and one in Italy, in the period between 2010 and October 2014.

All patients had a genetically confirmed diagnosis of SMA with a homozygous deletion of exon 7 in the *SMN1* gene, and a clinically confirmed diagnosis of type I SMA.

The diagnosis of SMA was made by the principal investigator at each site who also classified them according to the Dubowitz's decimal classification [11] and to the recently proposed classification in 3 subtypes [4,12]: type IA presentation at birth with joint contractures and need for respiratory support, or onset of motor and respiratory involvement in the first week; type IB, symptom onset after neonatal period, usually before age 3 months, head control never achieved; and type IC, onset after neonatal period, usually between 3 and 6 months, head control achieved.

Previously identified patients, followed up in our clinics, and newly diagnosed patients were enrolled. All eligible patients were offered participation. We included all infants who had at least two or more assessments over the following two years.

The study was approved by the individual institutional review boards at the participating sites.

Study visits were scheduled at baseline, when first assessed, and, when possible, every 2–3 months until the age of 12 months and every 6 months thereafter, depending on the infant's health and ability to travel.

The HINE [9] is a simple and scorable method for assessing infants between 2 and 24 months of age, including different aspects of neurological examinations as cranial nerves, posture, movements, tone and reflexes. A separate section includes eight selected motor items which document developmental progress (Fig. 1). It provides a summary of motor developmental milestones giving not only the opportunity to record the age at which the various milestones were achieved but also allowing one to

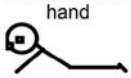
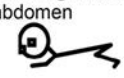
Head control	Unable to maintain head upright normal up to 3m	Wobbles normal up to 4m	Maintained upright all the time normal from 5m		
Sitting	Cannot sit	With support at hips  normal at 4m	Props  normal at 6m	Stable sit  normal at 7-8m	Pivots (rotates)  normal at 9m
Voluntary grasp – note side	No grasp	Uses whole hand	Index finger and thumb but immature grasp	Pincer grasp	
Ability to kick in supine	No kicking	Kicks horizontally but legs do not lift	Upward (vertically)  normal at 3m	Touches leg  normal at 4-5m	Touches toes  normal at 5-6m
Rolling	No rolling	Rolling to side (normal at 4m)	Prone to supine (normal at 6 m)	Supine to prone (normal at 6 m)	
Crawling or bottom shuffling	Does not lift head	On elbow  (normal at 3 m)	On outstretched hand  (normal at 4m)	Crawling flat on abdomen  (normal at 8m)	Crawling on hands and knees  (normal at 10m)
Standing	Does not support weight	Supports weight (normal at 4m)	Stands with support (normal at 7m)	Stands unaided (normal at 12m)	
Walking		Bouncing (normal at 6m)	Cruising (walks holding on) (normal at 12m)	Walking independently (normal by 15m)	

Fig. 1. HINE scoring module illustrating the motor developmental milestones.

quantify intermediate steps leading to the full achievement of the milestone. Each item provides the opportunity to score the level of development on a 5 point scale with 0 as absence of the activity. These milestones were designed according to the gradient of normal maturation, with normative values adapted from Illingworth's work [13]. These items are more granular than the World Health Organization's motor milestones, which capture sitting without support, hands-and-knees crawling, standing with assistance, standing alone, walking with assistance and walking alone [14].

The HINE module, on the other hand, is shorter and includes less items of the motor component of the Bayley scales of infants and toddler development that include many items that are not relevant to type I SMA and is more fatiguing for such fragile infants.

2.1. Statistical analysis

The distribution of scores was plotted for individual infants for each item plotting longitudinal results according to age.

3. Results

Thirty-three infants fulfilled the inclusion criteria and were included in the study. Their age at onset ranged between 1 and 8 months. The age when they were first assessed was between 2 and 9 months.

Seven infants, who were at the severe end of the spectrum of weakness and had early presentation with motor and respiratory involvement within the first week, were classified as 1.1, using the Dubowitz decimal system, or as type IA according to the recent classification. Two other infants, who achieved head control, were at the other end of the spectrum, and were classified as 1.9, or type IC according to the recently proposed classification. In both, onset of signs was around 7–8 months.

Twenty-four infants had the more typical course of type I SMA, and were classified as 1.5, or type IB. In all 24 the onset was after the first week but before the age of 5–6 months. Two of these 24 achieved transient head control.

3.1. Motor developmental milestones

3.1.1. Head control

All 7 infants at the weakest end of the spectrum had scores of 0 on all the assessments.

Among the 24 infants in the main group, 11 had a score of 1 (wobbles) on at least one assessment, 13 had a score of 0 on all assessments. None had a score of 2 or above (Fig. 2).

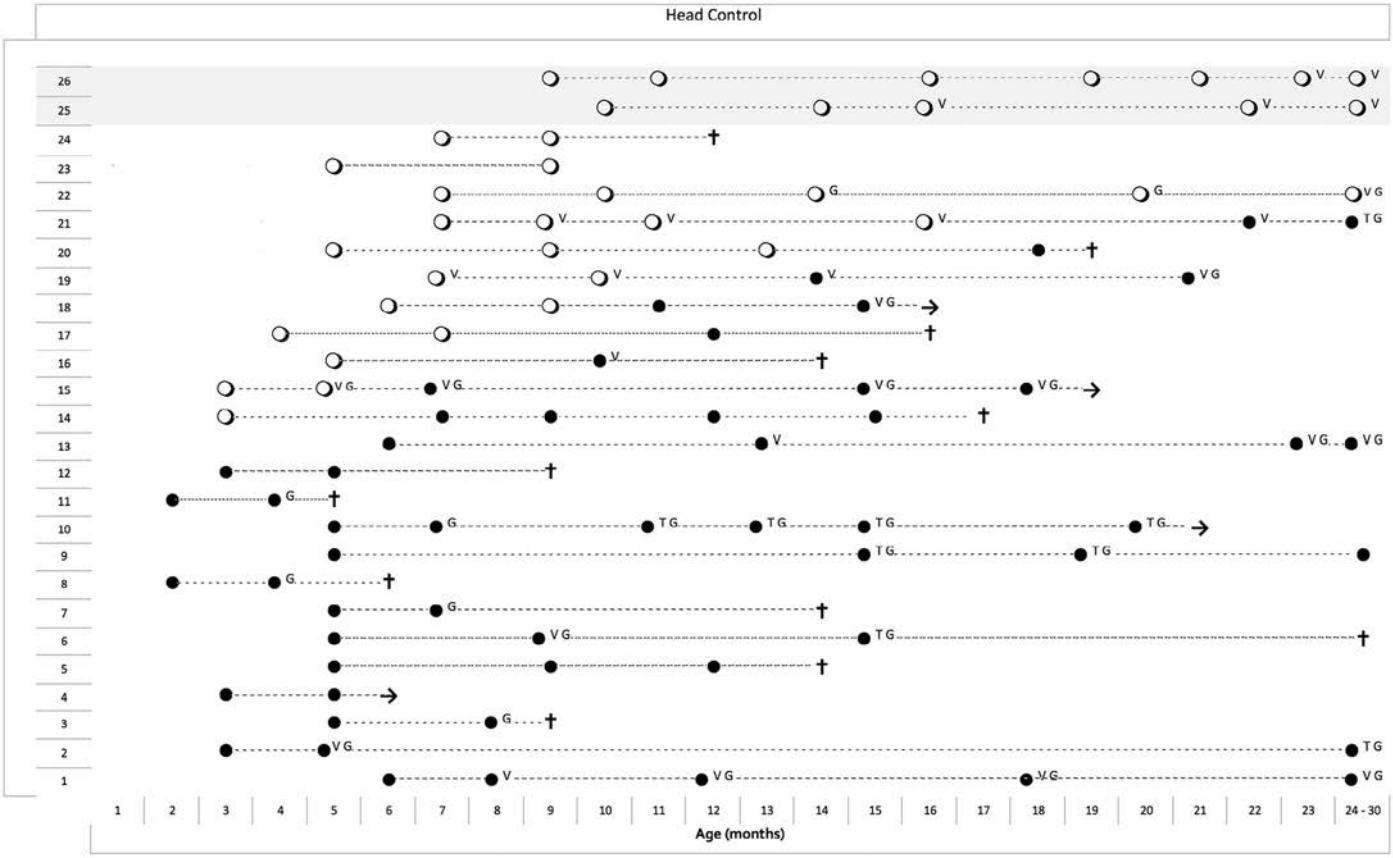


Fig. 2. Longitudinal data of the item assessing the ability to control the head in 24 typical type I SMA infants (from number 1 to 24) and in 2 stronger type I infants with late onset (25–26, shaded). Each line represents the different assessments in the same patient linked by a dotted line. ● = score 0, ○ = score 1, V = ventilation; G = gastrostomy; T = tracheostomy). The seven weakest patients with onset within the first week (type IA or 1.1 according to Dubowitz) were not added to the figure as they all had a score of 0 in all assessments.

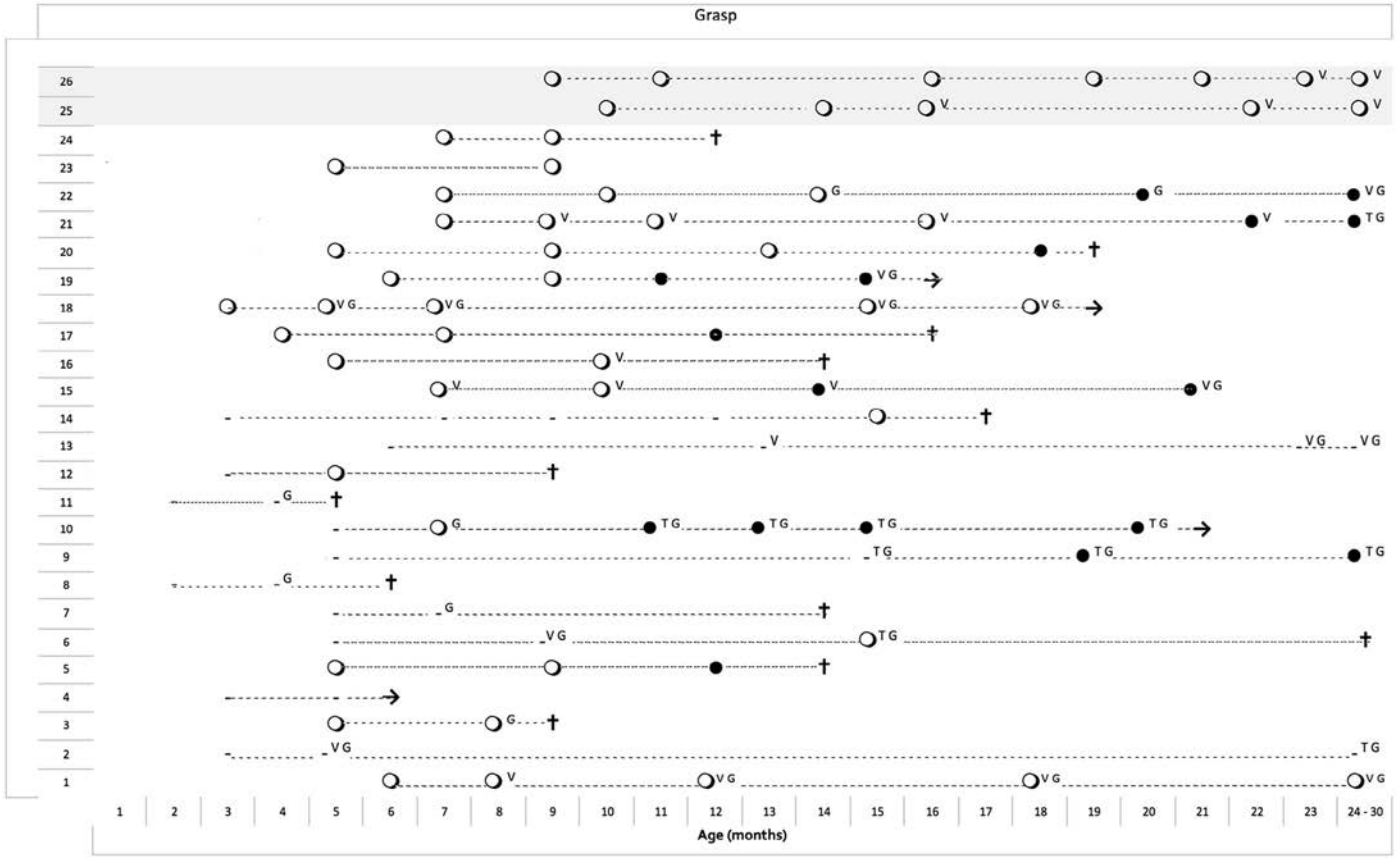


Fig. 3. Longitudinal data of the item assessing voluntary grasp in 24 typical type I SMA infants (from number 1 to 24) and in 2 type I infants with late onset (25–26, shaded). Each line represents the different assessments in the same patient linked by a dotted line. ● = score 0, ○ = score 1, V = ventilation; G = gastrostomy; T = tracheostomy). The seven patients with onset within the first week (type IA or 1.1 according to Dubowitz) were not added to the figure as they all had a score of 0 in all assessments.

Infants who had a score of 0 never achieved higher scores on subsequent assessments.

The 2 stronger infants with late onset had a score of 1 that persisted throughout the assessments over the following 2 years.

3.1.2. *Voluntary grasp*

All 7 infants at the weakest end of the spectrum had a score of 0 on all the assessments.

Among the 24 infants in the main group, 17 had a score of 1 (using whole hand) on at least one assessment, and 7 had a score of 0 on all assessments. None had a score of 2 or above (Fig. 3).

None of the infants who had a score of 0 on one assessment had better scores on the subsequent assessments.

The 2 stronger infants with late onset achieved a score of 1 that persisted throughout the assessments over the following 2 years.

3.1.3. *Ability to kick*

All 7 infants at the weakest end of the spectrum had a score of 0 on all the assessments.

Among the 24 infants in the main group, 13 had a score of 1 (gravity-eliminated movements) on at least one assessment, and 11 had a score of 0 on all assessments. None had scores of 2 or above (Fig. 4). Only one of the infants who had a score of 0 achieved a score of 1 on subsequent assessments. The 2 stronger infants with late onset had a score of 1 that persisted throughout the assessments over the following 2 years.

3.1.4. *Other items*

All infants in this study had a score of 0 on all visits in all the remaining 5 items (sitting, rolling, crawling, standing, walking).

3.1.5. *Gastrostomy and ventilator support*

In the main group, 15 of the 24 infants underwent gastrostomy for swallowing problems and/or failure to thrive, 10 started non-invasive ventilation and 5 had tracheostomy. None had any improvement following the introduction of any of these procedures. In the few patients in whom the scores on individual items were 1 at the time these procedures were introduced, a score of 1 was not always maintained (Figs. 2–4).

4. Discussion

Improvements in standards of care with the more systematic use of optimal respiratory care including non-invasive ventilation and cough augmentation techniques and better management of nutritional issues have resulted in improved survival in type I SMA in the last decade. This has raised the question of whether

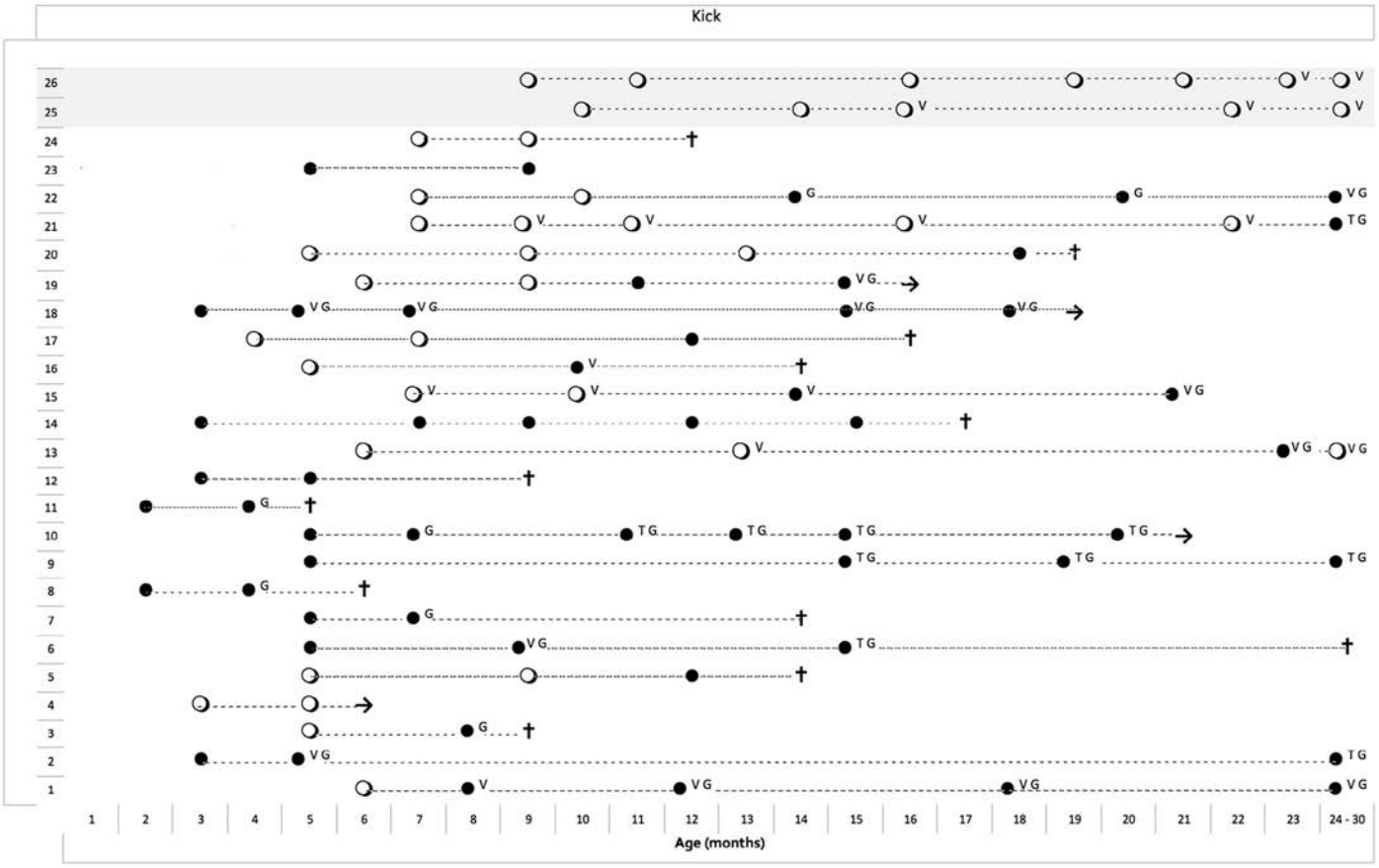


Fig. 4. Longitudinal data of the item assessing ability to kick in 24 typical type I SMA infants (from number 1 to 24) and in 2 type I infants with late onset (25–26, shaded). Each line represents the different assessments in the same patient linked by a dotted line. ● = score 0, ○ = score 1, V = ventilation; G = gastrostomy; T = tracheostomy). The seven patients with onset within the first week (type IA or 1.1 according to Dubowitz) were not added to the figure as they all had a score of 0 in all assessments.

the improved survival is also associated with possible acquisition of motor developmental milestones over time.

In order to capture possible changes in the achievement of motor developmental milestones in infancy, we used a module extracted from the HINE, an assessment of global neurological function and motor developmental milestones in infants that can be used from the age of 2–3 months. The HINE has already been used in several clinical groups [15] and has been validated in typically developing children [9]. Using the developmental module from the HINE we were able to confirm that motor developmental milestones are only rarely acquired in type I SMA infants after the diagnosis is made.

Not surprisingly, infants with the most severe phenotype who also had very early onset of clinical signs never acquired any motor developmental milestones, showing a score of 0 on all of the 8 items at all times.

In contrast, scores higher than 0 could be observed in three of the items in the cohort of infants with onset after the first week of life.

By definition, type I SMA infants are unable to sit unsupported. Our results confirmed that none of the infants achieved independent sitting nor any other of the subsequent milestones, such as crawling or standing in which all patients had a score of 0 at all times. This was also true with the 2 stronger late onset infants who were at the stronger end of the spectrum.

A score higher than 0 was only achieved in head control (n = 13), kicking (n = 15) and hand grasp (n = 18). It is of interest that even in those items, the maximal score achieved was 1 out of a scale of 4, indicating only partial achievement of the milestone.

Partial head control was only achieved in 11 infants and was reported as wobbling. This was transient in the infants in the main cohort and sustained over several assessments in the 2 stronger infants with late onset. None achieved a constant full head control for more than 15 seconds.

Thirteen infants were able to kick horizontally on at least one assessment but legs did not lift with full antigravity movements at any time, including the 2 stronger infants.

Similarly, although the item exploring hand grasp was not specifically designed to capture changes in weak infants but is mainly assessing development of fine motor skills, only a few infants achieved the ability to use the whole hand and only the 2 stronger infants developed better hand grip.

Overall, when present, a score of 1 was found in younger infants on their first assessments and, with only one exception, none showed any improvement from 0 to 1 on subsequent evaluations. Therefore, increased survival does not appear to

increase the possibility of acquiring developmental milestones over time, or to improving their scores even if a full milestone is not achieved. This finding, in fact, is not surprising as several studies have reported progressive loss of motor neurons in both animal models and humans; using neurophysiological biomarkers, like CMAP and MUNE, severe and substantial postnatal progression of motor denervation has been shown in the first months postnatally in type I SMA [16].

When we looked at the possible relationship between ventilatory or nutritional support and developmental milestones, the numbers were too small to allow a meaningful statistical analysis but it was obvious that the introduction of gastrostomy or ventilator support was not consistently associated either with higher scores or with a longer persistence of a score of 1.

This may be explained by the fact that while better standards of care may facilitate well-being and increased survival, the need for ventilation or gastrostomy is, in most cases, a sign that the disease has already progressed and is less likely to be associated with subsequent improvements.

One of the limitations of the study is its retrospective nature; however, data were collected over a limited period of time in centers that were complying with current standards of care. In order to avoid possible bias, the recruitment period was also limited to the time when enrollment for clinical trials had not started in the participating centers as, after that, we would have included only the infants who, for some reason, were thought not to be eligible for clinical trials. The relative small number of patients was justified by the exclusion of infants who had only one assessment and then decided to follow palliative care locally. It is of interest, however, that infants who had only a single assessment were generally performing very poorly and none had a score of 2 (data not shown).

Our results suggest that even with current standards of care, motor developmental milestones are rarely acquired, even partially, as part of natural history in type I SMA infants. Even the stronger ones, who had longer preservation of the scores of 1 when compared to the weaker infants with a more typical course, failed to make any further gains after the first evaluation. In future drug intervention studies, type I SMA infants who achieve motor milestones beyond what is described here can be attributed, with a strong probability, as being due to drug effect and not to enhanced standard of care or prolonged survival. Further longitudinal prospective studies, exploring, in more detail, the correlation with standards of care, SMN2 copy numbers, neurophysiology or biomarkers will help to further elucidate the progression and the variability of the disease.

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Technical standards and guidelines for spinal muscular atrophy testing

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Abstract: Spinal muscular atrophy is a common autosomal recessive neuromuscular disorder caused by mutations in the survival motor neuron (*SMN1*) gene, affecting approximately 1 in 10,000 live births. The disease is characterized by progressive symmetrical muscle weakness resulting from the degeneration and loss of anterior horn cells in the spinal cord and brainstem nuclei. The disease is classified on the basis of age of onset and clinical course. Two almost identical *SMN* genes are present on 5q13: the *SMN1* gene, which is the spinal muscular atrophy-determining gene, and the *SMN2* gene. The homozygous absence of the *SMN1* exon 7 has been observed in the majority of patients and is being used as a reliable and sensitive spinal muscular atrophy diagnostic test. Although *SMN2* produces less full-length transcript than *SMN1*, the number of *SMN2* copies has been shown to modulate the clinical phenotype. Carrier detection relies on the accurate determination of the *SMN1* gene copies. This document follows the outline format of the general Standards and Guidelines for Clinical Laboratories. It is designed to be a checklist for genetic testing professionals who are already familiar with the disease and methods of analysis. ***Genet Med* 2011:13(7):686–694.**

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Disease-specific statements are intended to augment the current general American College of Medical Genetics (ACMG) Standards and Guidelines for Clinical Genetic Laboratories. Individual laboratories are responsible for meeting the CLIA/College of American Pathologists (CAP) quality assurance standards with respect to appropriate sample documentation, assay validation, general proficiency, and quality control measures.

BACKGROUND ON SPINAL MUSCULAR ATROPHY

Gene symbol/chromosome locus

Survival motor neuron 1 (*SMN1*) gene at chromosome 5q11.2-13.3.

OMIM

Spinal muscular atrophy (SMA) type I (253300), SMA type II (253550), and SMA type III (253400).

Brief clinical description

The autosomal recessive disorder proximal SMA is a severe neuromuscular disease characterized by degeneration of alpha motor neurons in the spinal cord, which results in progressive muscle weakness and paralysis. The predominant pathologic feature on autopsy studies of patients with SMA is loss of motor neurons in the ventral horn of the spinal cord and in brainstem motor nuclei. SMA is the second most common fatal autosomal recessive disorder after cystic fibrosis, with an estimated incidence of approximately 1 in 10,000 live births.[1] Childhood SMA is subdivided into three clinical groups on the basis of age of onset and clinical course.[2,3] Type I SMA (Werdnig-Hoffman

disease) is characterized by severe, generalized muscle weakness and hypotonia at birth or within the first 3 months. Death from respiratory failure usually occurs within the first 2 years. Approximately 60–70% of patients with SMA have the type I disease.[4] Type II children are able to sit, although they cannot stand or walk unaided, and typically survive beyond 4 years. The phenotypic variability exceeds that observed in type I patients, ranging from infants who sit transiently and demonstrate severe respiratory insufficiency to children who can sit, crawl, and even stand with support. Prognosis in this group is largely dependent on the degree of respiratory involvement. Type III SMA (Kugelberg-Welander disease) is a milder form, with onset during infancy or youth: patients learn to walk unaided and have prolonged survivals. They comprise a less fragile group than type II patients with respect to respiratory and nutritional vulnerability. Type III SMA is further subdivided into two groups: type IIIa (onset before 3 years of age) and type IIIb (onset at age ≥3 years). Cases presenting with the first symptoms of the disease at the age of 20–30 years are classified as type IV or proximal adult type SMA. The described classification is based on age of onset and clinical course, but it should be recognized that the disorder demonstrates a continuous range of severity. For more information, see the online Gene Reviews profile at http://www.ncbi.nlm.nih.gov/books/NBK1352/.

Mode of inheritance

Inheritance is autosomal recessive, with variable expression.

Gene description/normal gene product

The SMA gene is within a complex region containing multiple repetitive and inverted sequences.[5] The *SMN* gene (Entrez Gene ID number 6606) comprises nine exons with a stop codon present near the end of exon 7.[6] Two inverted *SMN* copies are present: the telomeric or *SMN1* gene, which is the SMA-determining gene and the centromeric or *SMN2* gene. The two *SMN* genes are highly homologous, have equivalent promoters, and only differ at five base pairs.[5,7,8] The base differences are used to differentiate *SMN1* from *SMN2*. The coding sequence of *SMN2* differs from that of *SMN1* by a single nucleotide (840C>T), which does not alter the amino acid but has been shown to be important in splicing. SMA results from a reduction in the amount of the SMN protein, and there is a strong correlation between the disease severity and SMN protein levels.[9,10] The SMN protein is a ubiquitously expressed, highly conserved 294-amino acid polypeptide. The protein is found in both the cytoplasm and nucleus and is concentrated in punctate structures called "gems" in the nucleus. High levels of the protein have been found to exist in the spinal motor neurons, the affected cells in patients with SMA. The protein self-associates into multimeric structures. Biochemically, SMN does not seem to exist within cells in isolation but instead forms part of a large protein complex, the SMN complex. Many of these SMN interacting proteins are components of various ribonuclear protein (RNP) complexes that are involved in distinct aspects of RNA metabolism. The best characterized function of the SMN complex is regulating the assembly of a specific class of RNA-protein complexes, the small nuclear RNPs (snRNPs).[11,12] The snRNPs are a critical component of the spliceosome; a large RNA protein that catalyzes premRNA splicing. SMA may, therefore, be a disorder resulting from aberrant splicing. As the SMN protein is ubiquitously expressed, it remains unknown how a loss of a general housekeeping function (snRNP assembly) causes a selective loss of motor neurons in SMA.[13] The high expression of SMN protein in motor neurons may suggest that the neuronal population is more sensitive to decreases in the SMN protein level. Possibly, the altered splicing of a unique set of premessenger RNAs results in deficient proteins, which are necessary for motor neuron growth and survival. In addition to its role in spliceosomal ribonucleoprotein assembly, SMN may have other functions in motor neurons. A subset of SMN complexes is located in axons and growth cones of motor neurons and may be involved in some aspects of axonal transport and localized translation of specific messenger RNAs.[14,15]

The *SMN1* mutation

Homozygous mutations of the *SMN1* gene cause SMA. Both copies of the *SMN1* gene are absent in approximately 95% of affected patients, whereas the remaining patients have nonsense, frameshift, or missense mutations within the gene.[16] Based on Hardy-Weinberg equilibrium, the remaining patients (with the smaller types of mutations) are virtually all assumed to be hemizygous for the SMN1 deletion. The absence of *SMN1* can occur by deletion, typically a large deletion that includes the whole gene or by conversion to *SMN2*. Although patients with SMA have mutations in *SMN1*, they always carry at least one normal copy of *SMN2*, which is partially functional but unable to fully compensate for the deficiency of the SMN1 protein. The homozygous loss of both genes has not been reported, presumably as a result of lethality.

Genotype/phenotype association

SMN1 exon 7 is absent in the majority of patients independent of the severity of SMA. Several studies have shown that the *SMN2* copy number modifies the severity of the disease.[17–20] The *SMN2* copy number varies from 0 to 3 copies in the normal population, with approximately 10–15% of normal individuals having no *SMN2*. However, patients with a milder phenotype with type II or III SMA have been shown to often have more copies of *SMN2* than type I patients. The majority of patients with the severe type I form have one or two copies of *SMN2*; most patients with type II have three *SMN2* copies; and most patients with type III have three or four *SMN2* copies. Three unaffected family members of patients with SMA, with confirmed *SMN1* homozygous deletions, were shown to have five copies of *SMN2*.[21] These cases not only support the role of *SMN2* modifying the phenotype but they also demonstrate that expression levels consistent with five copies of the *SMN2* genes may be sufficient to compensate for the absence of the *SMN1* gene. This inverse dose-relationship between *SMN2* copy number and disease severity has also been supported by the SMA mouse model.[22,23] The SMA mouse models have not only confirmed the susceptibility of motor neuron degeneration to *SMN* deficiency but have also verified that the degeneration can be prevented by increased *SMN2* dosage. Mice lacking the endogenous mouse *SMN* gene but expressing two copies of the human *SMN2* gene develop severe SMA and die within 1 week of age; however, mice that express multiple copies of *SMN2* do not develop the disease. In addition to the *SMN2* copy number, other modifying factors influence the phenotypic variability of SMA. There are very rare families reported in which markedly different degrees of disease severity are present in affected siblings with the same *SMN2* copy number. These discordant sib pairs, which share the same genetic background around the SMA locus, would indicate that there are other modifier genes outside the SMA region. Differences in splicing factors may allow more full-length expression from the *SMN2* gene and account for some of the variability observed between discordant sibs.[24] It was also found that in some rare families with unaffected *SMN1*-deleted females, the expression of plastin 3 was

higher than in their SMA affected counterparts.[25] Plastin 3 was shown to be important for axonogenesis and, therefore, may act as a protective modifier.

Mutational mechanism

The *SMN1* gene produces full-length transcript, whereas the *SMN2* gene produces predominantly an alternatively spliced transcript (exon 7 deleted) encoding a protein (SMNΔ7) that does not oligomerize efficiently and is unstable.[26,27] The inclusion of exon 7 in *SMN1* transcripts and exclusion of this exon in *SMN2* transcripts are caused by the single nucleotide difference at +6 in *SMN1* exon 7 (c.840C>T). Although the C to T change in *SMN2* exon 7 does not change an amino acid, it does disrupt an exonic splicing enhancer (ESE) or creates an exon silencer element (ESS) that results in the majority of transcripts lacking exon 7.[28,29] The ESEs and ESSs are cis-acting exonic sequences that influence the use of flanking splice sites. ESEs stimulate splicing and are often required for efficient intron removal, whereas ESSs inhibit splicing. Whether it is the loss of an ESE or creation of an ESS, the result is a reduction of full-length transcripts generated from *SMN2*. A single *SMN2* gene produces less functional protein compared with a single *SMN1* gene.[9,10,20–30] Therefore, SMA arises because the *SMN2* gene cannot fully compensate for the lack of functional SMN when *SMN1* is mutated. However, small amounts of full-length transcripts generated by *SMN2* are able to produce a milder type II or III phenotype when the copy number of the *SMN2* gene is increased. SMA is, therefore, caused by low levels of SMN protein, rather than a complete absence of the protein. A recent report described three unrelated patients with SMA who possessed *SMN2* copy numbers that did not correlate with the observed mild clinical phenotypes.[31] A single base substitution in *SMN2*, c.859G>C, was identified in exon 7 in the patients DNA, and it was shown that the substitution created a new ESE element. The new ESE increased the amount of exon 7 inclusion and full-length transcripts generated from *SMN2*, thus resulting in the less severe phenotypes. Therefore, the SMA phenotype may not only be modified by the number of *SMN2* genes but *SMN2* sequence variations can also affect the disease severity. It should, therefore, not be assumed that all *SMN2* genes are equivalent and sequence changes found within the *SMN2* gene must be further investigated for potential positive or negative effects on *SMN2* transcription when there is a lack of correlation between the genotype and phenotype.

Listing of mutations

Although the absence of both copies of the *SMN1* gene is a very reliable and sensitive assay for the molecular diagnosis of SMA, approximately 5% of affected patients have other types of mutations in the *SMN1* gene that will not be detected by homozygous deletion testing. Because of the high deletion frequency and according to the Hardy-Weinberg equilibrium, most of these patients will be compound heterozygotes; with one *SMN1* allele being deleted and the other allele with a point mutation or other types of small mutations. If a patient with SMA possesses only a single copy of *SMN1*, it is likely that the remaining copy contains a more subtle mutation, including nonsense mutations, missense mutations, splice site mutation insertions, and small deletions. Many of the same intragenic mutations have now been reported in unrelated patients.[19,32,33] The most frequently reported mutations are the p.Tyr272Cys (c.815A>G), c.399_402delAGAG, c.770-780dup11, and p.Thr274Ile (c.821C>T). The coding region for *SMN* can be found on ensemble (www.ensemble.org). Proper nomenclature must be used to report any verified sequence mutation (http://www.hgvs.org/mutnomen/).

Table 1 Diagnostic mutation categories

Mutation category	% Affected	Genotype designation
Category 1	95	[0 + 0] or a^2
Category 2	5	[1^d + 0] or 2ad
Category 3	Rare	[1^d + 1^d] or d^2

Ethnic association of SMA

With an incidence of approximately 1 in 10,000 live births and a carrier frequency of 1/40–1/60, SMA is the leading inherited cause of infant mortality. A recent report provides carrier frequencies in several populations including white, Ashkenazi Jewish, African American, Asian, and Hispanic.[34] The lower carrier frequencies found in African Americans and Hispanics would suggest a lower prevalence of SMA in these populations.

GUIDELINES

Definition of normal and mutation category

Alleles

Among normal alleles, a chromosome with one copy of *SMN1* gene is designated as a "1-copy" or "1" allele. A chromosome bearing two copies of *SMN1* gene is designated as a "2-copy" or "2" allele. The most common disease allele bears a deletion (or gene conversion of *SMN1* to *SMN2*) resulting in a loss of exon 7 (Δ7SMN1) is referred to as the "0-copy" or "0" allele. Disease alleles with subtle intragenic point mutations on the *SMN1* gene are referred to as 1^d. The 0, 1, 2, and 1^d alleles are also variably referred to as a, b, c, and d alleles, respectively, in literature.[35] The resulting allele pairings that give rise to the diagnostic and carrier genotypes of SMA are defined later.

Diagnostic

The term "diagnostic" in the context of SMA is characterized by the presence of mutations classified into either of three categories (Table 1). Category 1, which accounts for approximately 95% of affected individuals, is characterized by a homozygous absence of *SMN1* exon 7 (Δ7SMN1) due to deletions or gene conversions of *SMN1* gene to *SMN2* and is designated as the [0 + 0] genotype or a^2.[36] The resulting diagnostic finding is the absence of detectable *SMN1* exon 7 and *SMN1* exon 7 copy number of 0. Category 2, which accounts for approximately 5% of affected individuals, is characterized by compound heterozygosity for a rare intragenic point mutation within the *SMN1* gene on one chromosome and a deletion/gene conversion of *SMN1* exon 7 (Δ7SMN1) on the other chromosome and is designated as the [1^d + 0] genotype or 2ad. The resulting diagnostic finding may be a detectable level of *SMN1* exon 7 and an *SMN1* exon 7 copy number of either 0 or 1 depending on the location of the rare intragenic point mutation within the *SMN1* gene and its ability to interfere with *SMN1* copy number analysis. Category 3, which is very rare and is most likely due to consanguinity, is characterized by subtle intragenic point mutations within the *SMN1* gene on both chromosomes and is designated as the [1^d + 1^d] genotype or d^2. The resulting diagnostic finding may be a detectable level of *SMN1* exon 7 and an *SMN1* exon 7 copy number of 0, 1, or 2 depending on the location of each rare intragenic point mutation within the

SMN1 gene and its ability to interfere with *SMN1* copy number analysis.

Carrier

The term "carrier" in the context of SMA is classified into either of three categories (Table 2). Category 1 is characterized by an *SMN1* exon 7 copy number of 1 and presumes the presence of an *SMN1* deletion/gene conversion on the other chromosome (heterozygous Δ7SMN1) and is designated as the [1 + 0] genotype or 2ab. Category 2 is characterized by a presence of two *SMN1* genes in *cis* on a single chromosome along with a deletion/gene conversion of *SMN1* exon 7 on the opposite chromosome resulting in an *SMN1* exon 7 copy number of 2 and is designated as the [2 + 0] genotype or 2ac. Category 3 is characterized by a subtle intragenic point mutation on one chromosome resulting in an *SMN1* exon 7 copy number of ≥2 and is designated as the [1 + 1^d], and [2 + 1^d] genotypes or 2bd and 2cd, respectively. It must be recognized that other rare carrier genotypes such as [3 + 0] or [3 + 1^d] are likely and are thought to occur at a lower frequency relative to the most common carrier genotypes.[36]

Negative result

A negative test result is characterized by the presence of detectable amounts of *SMN1* exon 7, with an *SMN1* exon 7 copy number of >1, with the presence of subtle intragenic point mutations within the *SMN1* gene having been ruled out. If the presence of subtle intragenic mutations has not been ruled out, a negative test result decreases the likelihood but does not exclude the diagnosis of SMA. Within the context of carrier testing, an *SMN1* copy number of ≥2 is associated with a reduced risk to be a carrier.

TESTING CONSIDERATIONS

As with any genetic testing modality, the required intake information needed to facilitate an accurate result interpretation includes the reason for referral, i.e., diagnostic versus carrier testing, an accurate representation of family history, i.e., additional affected or carrier individuals identified within the family, clinical or diagnostic findings of relevance to SMA, and patient ethnicity. Patient ethnicity helps to provide appropriate risk assessment after a negative carrier testing result in an individual with no family history of SMA.

Diagnostic mutation analysis

Testing by *SMN1* deletion or copy number analysis is indicated for individuals with a suspected diagnosis of SMA, presenting with symptoms of proximal muscle weakness, fasciculations, dysphagia, dysarthria, and absent deep tendon reflexes.

Table 2 Commonly designated carrier mutation categories

Mutation category	*SMN1* exon 7 copy number	Genotype designation
Category 1	1	[1 + 0] or 2ab
Category 2	2	[2 + 0] or 2ac
Category 3[a]	2	[1 + 1^d] or 2bd
Category 3[a]	≥2	[2 + 1^d] or 2cd

[a]*SMN1* exon 7 copy number in Category 3 may depend on the location of the point mutation within the *SMN1* gene and its ability to interfere with copy number analysis.

Sensitivity and specificity

The analytical sensitivity of *SMN1* deletion/copy number analysis (proportion of homozygous Δ7SMN1 among all mutations in diagnostic category 1) is >99% (using the dosage assays described in the guidelines). The clinical sensitivity (proportion of homozygous Δ7SMN1 if 5q13-linked SMA is present) of the diagnostic test is approximately 95%. The remaining 5% of patients fall into either of diagnostic categories 2 or 3 (i.e., the [1^d + 0] and the [1^d + 1^d] genotypes) and represent a source of false-negative diagnostic test results.[3] These are patients with other types of small mutations within the *SMN1* gene and will not be detected by the deletion testing.

Although the absence of both copies of the *SMN1* gene is a very reliable and sensitive assay for the molecular diagnosis of SMA, approximately 5% of affected patients have other types of mutations in the *SMN1* gene that will not be detected by homozygous deletion testing. Because of the high deletion frequency and according to the Hardy-Weinberg equilibrium, most of these patients will be compound heterozygotes; with one *SMN1* allele being deleted and the other allele with a point mutation or other types of small mutations. If a patient with an SMA-like phenotype possesses only a single copy of *SMN1*, it is likely that the remaining copy contains a more subtle mutation, including nonsense mutations, missense mutations, splice site mutation insertions, and small deletions.

Both the analytical specificity (proportion of negative test results if homozygous Δ7SMN1 SMA genotype is not present) and the clinical specificity (proportion of negative test results if 5q13-linked SMA is not present) of *SMN1* deletion/copy number analysis are >99%. Polymorphisms or point mutations under the primer and/or the probe binding regions may influence the analytical and clinical specificity by increasing the false-positive rate depending on the technology used.[37] As a measure of additional quality assurance, follow-up sequencing underneath the primer and probe binding regions on all diagnostic (0 or 1 *SMN1* copy number) results is expected to rule out a false-positive diagnostic finding attributable to this phenomenon and provide a better understanding of the underlying molecular mechanism of the mutation identified.

De novo deletion or gene conversions of paternal origin have been reported to occur at a frequency of 2% of patients with SMA.[38] Carrier testing on both parents of patients with homozygous Δ7SMN1 SMA may provide additional information on the occurrence of de novo deletions. If one of the parents seems to be a noncarrier of SMA (i.e., ≥2 copies of *SMN1*), further carrier testing on both parents of the noncarrier parent (i.e., the grandparents) can be pursued to determine the phase of *SMN1* genes in the noncarrier parent. This helps distinguish a [1 + 1] genotype leading to a de novo deletion in the index case, from a [2 + 0] obligate carrier genotype in the seemingly noncarrier parent. Furthermore, germline mosaicism for *SMN1* deletion/gene conversions has been reported.[6] Although the detection of a de novo mutation in an SMA family substantially lowers the recurrence risk, prenatal diagnosis in subsequent pregnancies should still be considered due to the rare possibility of a recurrent de novo mutation or germ-line mosaicism leading to an affected child.

Carrier testing

Carrier testing for SMA should be offered to asymptomatic individuals with a confirmed or suspected family history of SMA. Given the 1/40–1/60 carrier frequency of SMA, popula-

tion carrier screening has recently been recommended by the ACMG[39] but has not been supported by the American College of Obstetricians and Gynecologists' Committee on Genetics.[40] Carrier frequencies determined from derived allele frequencies should consider all combinations of the most likely allele pairings expected to occur among carriers in the general population, namely [1 + 0], [1 + 1^d], [2 + 0], and [2 + 1^d]. Carrier frequencies derived solely from the observed 1-copy genotype frequencies tend to underestimate the prior risk estimates for carriers of SMA in the general population, thereby leading to inaccuracies in posterior risk estimates after carrier testing. The report by Ogino and Wilson[35] provides Bayesian risk calculations for several situations.

The sensitivity (true positives/true positives + false negatives, or the detection rate) of the carrier test is defined as the proportion of carriers with the heterozygous Δ7SMN1, 1-copy genotype among all SMA carriers (including point mutation carriers and deletion carriers with two copies of the *SMN1* gene) and can be expressed as the ratio [1 + 0]/[1 + 0] + [1 + 1^d] + [2 + 0] + [2 + 1^d]. Here a subset of the denominator, [1 + 1^d] + [2 + 0] + [2 + 1^d], represents a source of false-negative carrier test results. Detection rate varies by ethnicity ranging from 71% in African Americans to 95% in whites.[34] The major contributor to this ethnicity-based variation in detection rate is the occurrence of two (or more) *SMN1* genes in tandem on a single chromosome 5 (i.e., the [2 + 0] Category 2 carrier genotype). The estimated frequency of alleles with two or more copies of SMN1 is 3–8 times more prevalent in African Americans, when compared with other ethnic groups.[34] This translates to a much higher frequency of individuals with the SMA carrier [2 + 0] genotype among African Americans compared with other races. It also has important implications in risk assessment and counseling after carrier screening in individuals of African American ancestry.

As with the diagnostic test, analytical specificity of the carrier test (proportion of negative test results if not a carrier) is >99% if the presence of polymorphisms underneath the primer and/or probe binding sites are ruled out. The negative predictive value, i.e., the proportion of negative tests that correctly identifies noncarriers (true negatives/true negatives + false negatives), is >99% regardless of ethnicity. The positive predictive value, i.e., the proportion of positive tests that correctly identify carriers of 5q13 linked SMA (true positive/true positive + false positives) is >99% if the presence of polymorphisms underneath the primer and/or probe binding sites are ruled out.

A negative test result within the context of carrier screening is defined by an *SMN1* exon 7 copy number ≥2 with a reduced posterior risk for being a carrier. The posterior risk determination takes into consideration the conditional probability of being either a carrier or a noncarrier after an *SMN1* copy number analysis result of ≥2.[36,41] The posterior residual risk for an individual with no family history of SMA to be a carrier following the identification of two copies of *SMN1* exon 7 varies by ethnicity ranging from 1:632 for an individual of white ancestry to 1:121 for an individual of African American ancestry.[34] The residual risk following identification of three copies of SMN1 exon 7 is considerably lower and varies from 1:3500 for an individual of white ancestry to 1:3000 for an individual of African American ancestry.[34] As with other carrier screening tests, patients need to understand that a negative carrier screen reduces but does not eliminate the risk to be a carrier of SMA. Risk assessment calculations using Bayesian analysis are essential for the proper genetic counseling of SMA families.[35]

The laboratory must establish validated, nonoverlapping cutoff values that can accurately and reliably distinguish *SMN1* copy numbers of 0, 1, 2, and ≥3. The accuracy, precision, and confidence of *SMN1* copy number measurements around these established cutoff values should be known to the laboratory. Copy number variations within the genomic internal standard and inefficiency of polymerase chain reaction (PCR) amplification of the internal standard reference gene relative to the *SMN1* gene represent additional sources of false positive or incorrect copy number estimates. Therefore, the copy number of genomic internal standard reference gene should be constant at two copies within the genome, and the PCR amplification efficiency of the *SMN1* gene relative to the chosen internal reference standard gene should be consistent between analyses. Performing replicate copy number measurements with two independent two copy number internal standard reference genes can help assure the accuracy of copy number analysis.

Prenatal testing

Indications for prenatal diagnosis of SMA include a 25% risk for the fetus to be affected (when both carrier parents are identified as a result of family history or following carrier identification by population screening) or the presence of abnormal findings such as decreased fetal movements and contractures in utero or increased NT on fetal ultrasound. Prenatal testing for SMA is performed by direct determination of the homozygous exon 7 deletion. A prerequisite is the previous identification of the homozygous deletion in the index case or positive carrier status in the parents. Testing both parents and the prenatal specimen by the same methodology, in addition to ruling out false-positive results attributed to sequence variants underneath the primer and probe binding sites, facilitates the most accurate interpretation of the prenatal test result. As maternal cell contamination of the fetal specimen can result in a false-negative test result, such contamination must be concurrently ruled out before reporting the prenatal test result. Although the presence of homozygous Δ7SMN1 is consistent with a diagnosis of SMA, the clinical severity, i.e., the type of SMA, cannot be predicted based on these molecular results.

Linkage

In families where a sample from a previous affected child is available, linkage analysis using microsatellite markers flanking the *SMN1* gene may be considered when *SMN1* deletion analysis is negative, and the presence of subtle intragenic mutations tracking within the family is suspected. Another indication for linkage analysis is in distinguishing a [1 + 1] genotype from a [2 + 0] genotype, when two copies of *SMN1* are identified in the parent of an affected child with a homozygous Δ7SMN1. In this latter scenario, linkage analysis can also identify recombination events associated with de novo deletions occurring within the *SMN1* gene.

SMN2 copy number

SMN2 copy number analysis is not routinely performed within the setting of diagnostic or carrier testing for SMA. *SMN2* copy number analysis may be of value within the setting of clinical trials and newborn screening in stratifying patients who are more likely to respond to therapeutic strategies aimed at upregulating the levels of expression of full-length SMN protein from the *SMN2* gene. Within clinical or prenatal diagnostic settings, however, results from *SMN2* copy number analysis if available must be interpreted with caution. Although *SMN2* copy number influences disease severity, *SMN2* sequence variants and other genes have also been implicated in influenc-

ing SMA phenotype.[42] Therefore, *SMN2* copy number results may provide probabilistic information regarding clinical severity for an affected child or fetus but should not be viewed as definitive. Furthermore, an *SMN2* copy number result indicates total *SMN2* copy number for both alleles; therefore, it is not possible to determine *SMN2* phase in unaffected individuals. For indications of carrier testing, *SMN2* copy number determination does not provide information useful for counseling. For example, an *SMN1* deletion carrier ([1 + 0] genotype) may carry three copies of *SMN2,* but it is not possible to determine how many of those *SMN2* copies are in *cis* with the deletion and would, therefore, be passed to offspring. An ability to determine phase is not clinically available at this time but would be necessary to add value to determination of *SMN2* copy number in carrier parents.

METHODOLOGICAL CONSIDERATIONS

Individual US laboratories offering molecular diagnostic and carrier testing for SMA should be in compliance with all federal and state regulations relevant to clinical laboratory operations. This includes meeting all CLIA/CAP quality control requirements. In addition, all laboratories should be active participants in annual CAP SMA proficiency testing challenges. All methodological applications should also be in compliance with the current Standards and Guideline for Clinical Genetics Laboratories developed by the Laboratory Practice Committee of the ACMG. Non-US laboratories should be similarly compliant with their individual countries statutory regulations governing oversight of clinical laboratories.

The absence of detectable *SMN1* in patients with SMA is being used as a reliable and powerful diagnostic test for the majority of patients with SMA. The first diagnostic test for a patient suspected to have SMA should be the *SMN1* gene deletion test. Both copies of the *SMN1* exon 7 are absent in approximately 95% of affected patients, whereas small more subtle mutations have been identified in the remaining affected patients. The molecular diagnosis of the SMA consists of the detection of the absence of exons 7 of the *SMN1* gene. Genetic testing is not only the most rapid and sensitive method to confirm the diagnosis but also the testing allows for further invasive investigations such as electromyography and muscle biopsy to be avoided. *SMN1* dosage testing is used to determine the *SMN1* copy number and detect SMA carriers: carriers will possess one SMN1 copy and noncarriers will have two *SMN1* copies and occasionally have three *SMN1* copies. There are a number of methods being used for the determination of SMN1 copy number, with multiplex ligation-dependent probe amplification (MLPA) and quantitative PCR (qPCR) being the most common. *SMN1* sequencing is used for the identification of the compound heterozygote affected state. All general guidelines for PCR, restriction fragment length polymorphism (RFLP), MLPA, qPCR, and DNA sequencing in the ACMG Standards and Guidelines apply. These technologies have limitations and strengths when applied to diagnostic and/or carrier testing for SMA as detailed later.

RFLP test

The RFLP test is commonly used and allows for the detection of the homozygous deletion of *SMN1* exon 7. It is currently being used as an assay for the diagnosis of SMA in both clinical and prenatal settings.[43] The PCR primer sets first amplify both *SMN1* and *SMN2* exon 7. Although this is a highly repetitive region, the exon 7 base pair difference (840C>T) alters a *Dra*I restriction enzyme site (due to a mismatched primer) and allows one to easily distinguish *SMN1* from *SMN2* on digestion of the PCR products. The absence of the undigested *SMN1* exon 7 product is consistent with the SMA diagnosis. Presence of an undigested product band indicates one or more *SMN1* copies. Another restriction enzyme that can be used in the RFLP assay is *Hinf*I.[19] In the *Hinf*I assay, internal control restriction sites are introduced, which allows for the assessment of complete digestion. Regardless of the enzyme used, the appropriate positive and negative controls should always be included in every assay. Technical advantages of SMA RFLP include (1) reliability of the assay, (2) very robust and minimally sensitive to DNA quality or degradation, and (3) simplicity to set up and operate in a clinical diagnostic laboratory. Technical disadvantages of SMA RFLP include (1) the need to avoid partial digestion problems and (2) DNA sequence variants located under the *SMN1* primer binding sites or enzymatic restriction site that may prevent the primers from annealing properly or proper digestion and might yield a false-positive diagnostic (zero copies) result. Clinical disadvantages of SMA RFLP include the inability to detect carrier status and determine SMN2 copy number.

Multiplex ligation-dependent probe amplification

MLPA is a PCR-based method of quantifying multiple genomic loci in a single reaction. It is based on the ligation of a set of two oligonucleotides probes that have annealed adjacently to a target sequence. Only ligated probes can serve as a template for a subsequent PCR. MLPA analysis of DNA from different individuals should reproducibly generate each of the expected peaks, and their sizes should correspond to those listed for each probe pair. MLPA technology is able to detect copy number of specific genomic loci and, therefore, can be used to test for SMA diagnostic and carrier status in clinical and prenatal settings.[44] Probe sets specific to exon 7 of *SMN1*, *SMN2* (MRC Holland SALSA MLPA KIT P021-A1 SMN Exon 7 probes 1260-L0966 and 1260-L0967), and 20 typically diploid loci located throughout the genome are used in SMA MLPA. After performing the enzymatic reactions, the PCR products are run through a capillary electrophoresis (CE) analyzer. The relative intensities and morphologies of the peaks should be consistent from one sample to the next. When the larger amplification products are weaker than the smaller fragments, this usually indicates that PCR amplification was not optimal or that the DNA sample analyzed might be degraded. Computer-aided scoring is a sensitive method to normalize the peak height or area for each PCR product compared with the 20 endogenous control loci. The median ratios across all the samples for each probe can be used as a reference value. Reference values for a copy number of 2 should approximately 1. A heterozygous deletion should give a ratio of approximately 0.5, whereas an elevated copy number should give a reference value >1.5. Reference values for a homozygous deletion (SMA positive) are close to 0. MLPA technology has several key advantages, including (1) allowance for simultaneous detection of SMN1 and SMN2 copy numbers, therefore, helping differentiate SMA type 1 from SMA types 2 and 3, (2) a high degree of precision for the quantitative detection of three or fewer SMN1 copies, (3) 20 independent control loci are able to be assayed in one reaction, (4) all reactions are performed in a single tube, and (5) probe sets are easy to obtain commercially, (6) a high degree of reproducibility and a large number of samples can be tested simultaneously, and (7) only 20 ng of genomic DNA is required, but 5–6-fold more DNA can be successfully technically validated. MLPA technology has several important limitations including (1) DNA sequence variants located under probe bind-

ing sites of *SMN1* alleles may interfere with probe hybridization and might result in a false-positive carrier (one copy) or false-positive diagnostic (zero copies) result, (2) reactions are sensitive to contaminants but generate uninterpretable results, (3) MLPA cannot yet be used to investigate single cells, which is important for preimplantation genetic diagnosis testing, (4) MLPA is not a suitable method to detect unknown point mutations, (5) MLPA probes are sensitive to small deletions, insertions, and mismatches, and (6) MLPA requires a CE analyzer, which is a higher cost option compared with slab gel electrophoresis for RFLP.

Quantitative PCR

qPCR is a multiplex qPCR that coamplifies (in the same tube) multiple genomic loci to determine gene copy number.[18] The multiplex can consist of competitive coamplification of *SMN1*, *SMN2*, the SMN internal standard, calibration factor, and the calibration factor internal standard. In the competitive PCR method, a known number of copies of a synthetic mutated internal standard are introduced with the patient sample into the PCR mixture. The internal standards are designed to be amplified with the same primer pairs for the SMN1 copy, with efficiencies similar to those of the genomic DNA counterparts and yield PCR products slightly smaller than the *SMN* PCR product. The copy number of *SMN1* is determined by coamplification of *SMN1*, *SMN2*, and the internal standards, and the ratios are quantitated. End point detection of amplification of fluorescently tagged PCR products is done by running the samples through a CE analyzer. The major advantage of this technique is that the internal standard is amplified with the same primers that amplify the target sequence. Thus, the efficiency of the amplification of the patient DNA and the internal standard DNA should be very similar and allow one to accurately determine the gene copy number. Alternatively, real-time detection[45] (as opposed to end point detection) of multiplex PCR reactions using hydrolysis or hybridization fluorescent probes uses other strategies for normalization including (1) standard curve method or (2) comparative threshold method. Using the standard curve method, a standard curve is first constructed from a DNA of known copy number. This curve is then used as a reference standard for extrapolating quantitative information for the *SMN1* copy number. The cycle at which the curve crosses a specified threshold is called the cycle threshold (Ct). Variation introduced due to variable DNA inputs can be corrected by normalizing to the calibration factor. Using the comparative threshold method, the Ct values of the sample of interest are compared with the Ct values of the calibration factor. Ct values of both the calibrator and the sample of interest are normalized to an appropriate endogenous internal control gene. qPCR is able to detect copy number of specific genomic loci and, therefore, can be used to test for SMA diagnostic and carrier status. Key advantages of qPCR include (1) extreme sensitivity, allowing the detection of less than five copies (perhaps only one copy in some cases) of a target sequence, making it possible to analyze small samples such as single cell analysis for the purpose of preimplantation genetic diagnosis, (2) with appropriate internal standards and calculations, mean variation coefficients are 5–10%, allowing reproducible analysis of the gene copy number, (3) all real-time platforms are relatively quick, with some affording high-throughput automation, and (4) real-time platforms are performed in a closed reaction vessel that requires no post-PCR manipulations, thereby minimizing the chances for cross contamination in the laboratory. Important limitations for qPCR techniques include (1) compounds present in certain biological samples or sample collection compounds (i.e., heparin) may inhibit PCR, (2) DNA sequence variants located under the primer binding sites of primers may reduce proper annealing and might result in a false-positive carrier (one copy) or diagnostic (zero copies) result, and (3) improper assay development and incorrect data analysis. Unwarranted conclusions may present the largest limitation of qPCR, and therefore, a robust and extensive validation is warranted to ensure specificity and accuracy of the results. Amplification and melting curves must be visually inspected, whereas independent calculations based on these curves should be double checked for accuracy. Finally, neither the MLPA nor qPCR can determine whether two *SMN1* genes are in *cis* on a single chromosome.

DNA sequencing

Although the absence of both copies of the *SMN1* gene is a very reliable and sensitive assay for the molecular diagnosis of SMA, approximately 5% of affected patients have other types of mutations in the *SMN1* gene that will not be detected by homozygous deletion testing. Because of the high deletion frequency and according to the Hardy-Weinberg equilibrium, most of these patients will be compound heterozygotes; with one *SMN1* allele being deleted and the other allele with a point mutation or other types of small mutations. If a patient with a SMA-like phenotype possesses only a single copy of *SMN1*, it is likely that the remaining copy contains a more subtle mutation, including nonsense mutations, missense mutations, splice site mutation insertions, and small deletions. The development of high-throughput DNA sequencing techniques has made direct DNA sequencing of PCR-amplified genomic DNA a rapid and economical approach to the identification of sequence mutations. As a consequence of the *SMN1* gene being relatively small and given the uniform spectrum of mutations, it is a relatively straightforward procedure to sequence the gene and identify mutations in patients who are negative for the diagnostic deletion test. However, it is necessary to verify that the intragenic mutation has occurred in the *SMN1* gene and not the *SMN2* gene. As an initial screen, primers that do not distinguish between *SMN1* and *SMN2* may be used to amplify each exon for direct DNA sequencing. If variants or mutations are identified, *SMN1*-specific long-range PCR amplification is followed by either direct DNA sequencing of that long-range product or nested PCR sequencing.[46] Important limitations for SMN direct gene sequencing include (1) the requirement of allele-specific sequencing of all variants identified, (2) DNA sequencing does not detect large deletions or insertions, (3) mutations in patients exhibiting mosaicism or chromosomal rearrangements may not be detectable using sequencing technology, and (4) variants of unknown significance.

INTERPRETATIONS

Elements considered essential to the reporting of clinical test results are described in detail in the current ACMG Standards and Guidelines for Clinical Genetics Laboratories. Examples of model laboratory reports are included in the appendix. The following elements must also be included in the reporting of SMA results.

The methodology used to assign the SMA genotype should be clearly stated. All positive results in clinically diagnosed/suspected individuals should state that genetic counseling is indicated, and carrier testing is available for other at-risk family members.

Comments on phenotype, if included, should be abstract rather than case specific. Although the inverse relationship between the *SMN2* copy number and disease severity has been well established,

the report should clearly state that the relationship is not absolute if reporting *SMN2* levels. It is important that couples undergoing carrier screening recognize that the carrier test does not provide genotype/phenotype information. Type I SMA occurs in approximately 60% of the cases, whereas the milder types II and III account for the remaining 40% of the cases.

Alternative diagnosis may be included when two normal copies of the *SMN1* gene have been detected. Other motor neuron disorders should be considered such as SMA with respiratory distress, X-linked SMA, distal muscular atrophy, and juvenile amyotrophic lateral sclerosis.

It is imperative that individuals understand the limitations of the carrier test: two *SMN1* genes in *cis* on the one chromosome 5, presence of rare de novo mutations, and the nondeletion mutations. The issue of these false-negative results must be included on all negative carrier reports. As is true for carrier screening programs, the testing must be voluntary, and assurance of confidentiality is absolutely necessary.

Informed consent and the usual caveats should be addressed including paternity issues, possible diagnostic errors due to sample mix-ups, and genotype errors due the presence of rare polymorphisms.

The following statement must be included on the report: "This test was developed and its performance characteristics determined by this laboratory. It has not been cleared or approved by the US Food and Drug Administration (FDA). The FDA has determined that such clearance or approval is not necessary. This analysis is used for clinical purposes. It should not be regarded as investigational or for research."

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APPENDIX: MODEL LABORATORY REPORTS

Indication

Screening/carrier test/negative family history.

Reported ethnicity

White.

Comment

SMA is an autosomal recessive disease of variable age of onset and severity caused by mutations (most often deletions or gene conversions) in the *SMN1* gene. Molecular testing assesses the number of copies of the *SMN1* gene. Affected individuals have 0 copies of the *SMN1* gene. Individuals with one copy of the *SMN1* gene are predicted to be carriers of SMA. Individuals with two or more copies have a reduced risk to be carriers. This copy number analysis cannot detect individuals who are carriers of SMA as a result of either two (or very rarely three) copies of the *SMN1* gene on one chromosome and the absence of the *SMN1* gene on the other chromosome or small intragenic mutations within the *SMN1* gene. This analysis also will not detect germline mosaicism or mutations in genes other than *SMN1*. Additionally, de novo mutations have been reported in approximately 2% of patients with SMA.

Methods

Specimen DNA is isolated and amplified by real-time PCR for exon 7 of the *SMN1* gene and two reference genes. A mathematical algorithm is used to calculate the number of copies of *SMN1*. Sequencing of the primer and probe binding sites for the *SMN1* real-time PCR reaction is performed on all fetal samples and on samples from individuals with one copy of *SMN1* on carrier testing, to rule out the presence of sequence variants, which could interfere with analysis and interpretation. This test was developed and its performance characteristics determined by this laboratory. It has not been cleared or approved by the FDA. The FDA has determined that such clearance or approval is not necessary. This analysis is used for clinical purposes. It should not be regarded as investigational or for research.

Carrier frequency and risk reductions for individuals with no family history of SMA

Ethnicity	Detection rate[1]	A priori carrier risk[3,4]	Reduced carrier risk for 2 copy result	Reduced carrier risk for 3 copy result
White	94.9%	1:35	1:632	1:3,500
Ashkenazi Jewish	90.2%	1:41	1:350	1:4,000
Asian	92.6%	1:53	1:628	1:5,000
Hispanic	90.6%	1:117	1:1,061	1:11,000
African American	71.1%	1:66	1:121	1:3,000
Mixed ethnicities	For counseling purposes, consider using the ethnic background with the most conservative risk estimates.			

General disclaimer

False-positive or -negative results may occur for reasons that include genetic variants, blood transfusions, bone marrow transplantation, erroneous representation of family relationships, or contamination of a fetal sample with maternal cells.

Example of a negative SMA carrier report

Result

SMN1 copy number: 2 (reduced carrier risk).

Interpretation

This individual has an *SMN1* copy number of 2. This result reduces but does not eliminate the risk to be a carrier of SMA. Ethnic-specific risk reductions based on negative family history and an *SMN1* copy number of 2 are provided in the comment section. Genetic counseling is recommended.

Example of a positive SMA carrier report

Result

SMN copy number: 1 (carrier).

Interpretation

This individual has one copy of *SMN1* and is, therefore, predicted to be a carrier of SMA, a disease of variable age of onset and severity. Genetic counseling is recommended.

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Identification of Proximal Spinal Muscular Atrophy Carriers and Patients by Analysis of SMN^T and SMN^C Gene Copy Number

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Summary

The survival motor neuron (SMN) transcript is encoded by two genes, SMN^T and SMN^C. The autosomal recessive proximal spinal muscular atrophy that maps to 5q12 is caused by mutations in the SMN^T gene. The SMN^T gene can be distinguished from the SMN^C gene by base-pair changes in exons 7 and 8. SMN^T exon 7 is not detected in ~95% of SMA cases due to either deletion or sequence-conversion events. Small mutations in SMN^T now have been identified in some of the remaining nondeletion patients. However, there is no reliable quantitative assay for SMN^T, to distinguish SMA compound heterozygotes from non-5q SMA-like cases (phenocopies) and to accurately determine carrier status. We have developed a quantitative PCR assay for the determination of SMN^T and SMN^C gene-copy number. This report demonstrates how risk estimates for the diagnosis and detection of SMA carriers can be modified by the accurate determination of SMN^T copy number.

Introduction

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disorder resulting in the loss of α motor neurons in the spinal cord. SMA has an estimated incidence of 1/10,000, with a carrier frequency of 1/40–1/60 (Pearn 1980; Melki et al. 1994). The recessive proximal childhood SMAs can be classified clinically into three groups. Type I (Werdnig-Hoffmann) is the most severe form, with onset at <6 mo of age and with death typically at <2 years of age. Type II SMA patients display an intermediate severity, with onset at <18 mo of age and with an inability to walk. Type III (Kugelburg-Welander) individuals are able to walk independently and have a relatively mild phenotype, with onset at >18 mo of age.

All three forms of SMA have been mapped, by linkage analysis, to 5q11.2-q13.3 (Brzustowicz et al. 1990; Melki et al. 1990; Simard et al. 1992; Brahe et al. 1994; Burghes et al. 1994*a;* Wirth et al. 1994; Wirth et al. 1995*a*), to a region that contains multiple copies of genes and markers (Francis et al. 1993; Kleyn et al. 1993; Carpten et al. 1994; Melki et al. 1994; Lefebvre et al. 1995; Roy et al. 1995; Thompson et al. 1995). Analysis of these multicopy markers showed specific alleles associated with SMA, loss of a copy of the marker in ~50% of severe SMA cases, and deletion of all copies of the marker on some SMA chromosomes (Burghes et al. 1994*b;* DiDonato et al. 1994; Melki et al. 1994; Lefebvre et al. 1995; Wirth et al. 1995*b*).

Three cDNAs have been reported that detect deletions in SMA patients (Lefebvre et al. 1995; Roy et al. 1995.; Thompson et al. 1995). Neuronal apoptosis inhibitory protein (NAIP) and XS2G3 are deleted in 50% of type I SMA patients (Roy et al. 1995; Thompson et al. 1995), which most likely represents the extent of the deletion on severe SMA chromosomes (Wirth et al. 1995*b;* Simard et al., in press). The third cDNA, the survival motor neuron, is encoded by SMN^T and SMN^C, two nearly identical genes that can be distinguished by base changes in exons 7 and 8 (Lefebvre et al. 1995; van der Steege et al. 1995). The SMN^T gene is not detectable in ~95% of SMA patients, either because of conversion of sequences in the SMN^T gene to those in the SMN^C gene or as a result of SMN^T gene deletions (Hahnen et al. 1995, 1996; Lefebvre et al. 1995; Rodrigues et al. 1995; van der Steege et al. 1996; Velasco et al. 1996; DiDonato et al. 1997*b*). Several small mutations in the SMN^T gene have been reported in patients without a deleted or sequence-converted SMN^T allele. These mutations include disrupted splicing of exon 7 (Lefebvre et al. 1995), deletion of 4 bp (Bussaglia et al. 1995) or 5 bp (Brahe et al. 1996) in exon 3, an 11-bp duplication in exon 6 (Parsons et al. 1996), and a clustering of missense mutations in exon 6 (Lefebvre et al. 1995; Hahnen et al. 1997; Talbot et al. 1997). These mutations provide strong evidence that SMN^T is the primary SMA-determining gene.

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The absence of detectable SMN^T exons 7 and 8 in SMA patients is being utilized as a powerful diagnostic test for SMA (van der Steege et al. 1995). Although the test has a sensitivity of ~95%, the assay is not quantitative, and it cannot detect individuals with heterozygous deletions of SMN^T. Thus, it is not possible to identify SMA carriers and to distinguish between a non-5q SMA-like patient (in whom both chromosomes contain SMN^T) and a compound heterozygote 5q SMA patient (in whom SMN^T is absent on one chromosome and an unknown alteration in the SMN^T gene is present on the other chromosome). Since SMN^T is homozygously deleted in 95% of 5q SMA cases, then, according to Hardy-Weinburg equilibrium, virtually all the remaining 5q SMA individuals should have a heterozygous deletion. The marker Ag1-CA (C272) lies at the 5′ end of the SMN genes (Burglen et al. 1996) and has been shown to vary, from zero to three copies, on a chromosome (DiDonato et al. 1994; Wirth et al. 1995*b*). This indicates that the copy number of SMN genes also varies on chromosomes. Previous attempts to determine the copy number of the SMN^T gene have measured the ratio of SMN^T to SMN^C; however, this has serious drawbacks.

In this paper, we describe a competitive PCR strategy to determine the SMN^T and SMN^C gene-copy number. The assay uses an exon from the cystic fibrosis transmembrane regulator (CFTR) as a standard to determine the relative dosage of SMN^T and SMN^C and thus avoids bias due to fluctuations in the copy number of SMN^C. We demonstrate that this assay is capable of accurately distinguishing compound heterozygotes from non-5q SMA-like cases. In a type III patient who lacked one copy of SMN^T, we found a novel missense mutation in exon 6. Analysis of normal and carrier individuals by this assay clearly indicates that the copy number of SMN^T and SMN^C varies from zero to at least two per chromosome and that the majority of SMA carriers have a single copy of the SMN^T gene on their normal chromosome. This report demonstrates that it now is possible to directly identify SMA carriers and affected compound heterozygotes by the accurate determination of SMN^T copy number.

Material and Methods

Patient Samples and DNA Isolation

DNA was isolated from peripheral venous blood or lymphoblast cell lines by the salting-out procedure (Miller et al. 1988) or by organic extraction (Sambrook et al. 1989). A total of 76 patients (64 from Ohio State University [OSU], 8 from Hôpital Sainte-Justine, Montreal [HSJ], and 4 from the Hospital for Sick Children, Toronto [HSC]) with a potential diagnosis of SMA were analyzed to identify compound heterozygotes. These patients did not necessarily conform to all criteria defined by the SMA consortium, since most were referred for diagnostic purposes. A small number of the samples were collected for prior genetic studies, which did conform to the diagnostic criteria outlined by the international SMA consortium. We also performed the SMN^T and SMN^C copy-number assay on 54 normal, unselected individuals and on 79 SMA carriers previously characterized by Ag1-CA segregation analysis (DiDonato et al. 1994). Patient 4659 is a 24-year-old male diagnosed with SMA type III, by muscle biopsy, at age 14 years.

Synthesis of Internal Standards

Two internal standards were constructed for the SMN^T and SMN^C copy-number assay. These standards have internal deletions of 20–50 bp, so that they are amplified with the same primer pairs as their genomic counterparts but can be distinguished by size (Celi et al. 1993). Incorporation of equimolar amounts of internal standards in the competitive PCR reaction was used to standardize the amount of input genomic DNA and to monitor the efficiency of the reaction. Genomic DNA was used as a template to generate the in vitro–synthesized CFTR internal standard. The 50-μl PCR reaction contained 200 ng genomic DNA, 3 mM $MgCl_2$, 1 × *Taq* DNA polymerase buffer (USB), 200 μM each dNTP, 30 ng each of CF621F(5′-AGTCACCAAAGCAGTACAGC-3′; Zielenski et al. 1991) and CFTR-IS (5′-GGGCCTGTGCAAGGAAGTGTTAAGCTATTCTCATCTGCATTCCA-3′) primers, and 0.5 U *Taq* DNA polymerase (USB). Diluted plasmid DNA containing a portion of the SMN^T gene was used as a template to generate the in vitro–synthesized SMN internal standard. The 50-μl reaction contained components similar to those in the CFTR internal standard reaction, except that primers R111 (5′-AGACTATCAACTTAATTTCTGATCA-3′; Lefebvre et al. 1995) and SMN-IS (5′-CCTTCCTTCTTTTTGATTTTGTTTATAGCTATATAGACATAGATAGCTA-3′) were used. The reactions were denatured at 95°C for 5 min, then run for 35 cycles at 95°C 1 min, 55°C 2 min, and 72°C 3 min (Ericomp Thermocycler). The products were subcloned into pCR 2.1 (Invitrogen), and plasmid DNA was purified. We found the use of cloned plasmid DNA more reliable than diluted PCR products. The inserts were excised with *Eco*RI and were stored at −70°C.

SMN^T and SMN^C Copy-Number Assay

Two competitive PCR amplifications were performed. In the first reaction, the DNA concentration of each sample was determined by estimation of the number of genome equivalents, according to the method of Sestini et al. (1995). Approximately 200 ng genomic DNA (~60,000 genome equivalents), determined on the basis of spectrophotometric quantitation, was amplified in the

presence of 60,000 CFTR-IS competitor molecules with CF621F and CF621R (5′-GGGCCTGTGCAAGGAAGTGTTA-3′ [Zielenski et al. 1991]) primers. The 25-μl PCR reaction contained 3 mM $MgCl_2$, 1 × *Taq* DNA polymerase buffer (USB), 200 μM each dNTP, 15 ng each CF621 primer, 0.5 U *Taq* DNA polymerase (USB), and 1 μl (60,000 copies) CFTR internal standard. The reaction conditions consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C 1 min, 55°C 2 min, and 72°C 3 min. The products were electrophoresed on an 8% polyacrylamide gel and were stained with ethidium bromide. The band intensities were evaluated, and the target genomic DNA for the SMN-dosage assay was adjusted accordingly.

In the second competitive PCR reaction, R111 and CF621F primers (15 ng each) were end-labeled with 0.1 μl [γ^{32}P]ATP (10 μCi/μl; Amersham) and T4 DNA kinase (Gibco BRL) at 37°C for 20 min. The 25-μl PCR reaction contained 200 ng genomic DNA, 3 mM $MgCl_2$, 1 × *Taq* DNA polymerase buffer (USB), 200 μM each dNTP, end-labeled forward primers, 15 ng each of CF621R and X-7 Dra (5′-CCTTCCTTCTTTTTGATTTTGTTT-3′; van der Steege et al. 1995) primers, 0.5 U *Taq* DNA polymerase (USB), and 1 μl (60,000 copies) each of CFTR and SMN internal standards. The reaction conditions consisted of an initial denaturation at 95°C for 5 min, followed by 16 cycles of 95°C 1 min, 55°C 2 min, and 72°C 3 min. The PCR product (8 μl) was digested with 20 U *Dra*I (New England Biolabs) overnight at 37°C. An SMA-patient sample with a homozygous deletion of SMN^T always was run as a control, to check for complete digestion with *Dra*I. The digested samples were run on a 6% denaturing polyacrylamide gel and were quantitated by autoradiography. Hyperfilm-MP (Amersham) was preflashed with a unit (Amersham) to ensure linearity of film response (Laskey and Mills 1975). The gel was exposed for 16–24 and 48–72 h at −70°C. Densitometry of the bands was performed on a Shimadzu CS-9000. The genomic SMN^T/genomic CFTR and genomic SMN^C/genomic CFTR ratios were determined for all samples. Since there are two copies of CFTR per genome, the SMN/CFTR ratio was used to determine the relative dosage or copy number of SMN^T and SMN^C genes.

The assay was performed at HSJ, with the following modifications. The CF621F and R111 primers were end-labeled with [γ^{33}P]ATP from ICN. The PCR conditions included a hot start with the addition of 1 U *Taq* DNA polymerase (Gibco BRL), followed by 20 cycles of 94°C 1 min, 55°C 1 min, and 72°C 1 min (MJ Research thermocycler). The PCR products were digested with *Dra*I (Gibco BRL). The dried gels were exposed in a phosphor screen for 72 h and were scanned by use of a PhosphorImager SI™ (Molecular Dynamics). Products of each PCR reaction were quantitated by use of the ImageQuant™ software. Thus, the SMN^T and SMN^C copy-number assay was performed independently by two groups and was quantitated by two different methods. Similar ratios were obtained, which demonstrates the versatility of this method.

The quantitative PCR assay to measure SMN^T and SMN^C gene copy number provides clear advantages over existing methods; however, a few technical points should be addressed, since the assay is subject to the potential problems of quantitative PCR. It is important to control for the amount of input genomic DNA, to avoid inaccuracies in the quantitation of copy number (Celi et al. 1994). We observed a small degree of variability in the amplification efficiency of SMN-IS, especially when comparing DNA samples extracted by different methods. Therefore, we recommend the use of normal, carrier, and affected controls prepared by the same extraction method as is used for the samples being tested. Both autoradiography and phosphoimaging were effective and accurate methods to quantitate copy number, but standard precautions, such as preflashing the film and monitoring exposure times, must be taken, to ensure the linearity of film response for autoradiography.

PCR Amplification of SMN for Mutation Detection

PCR primers used for amplification of SMN exons were selected on the basis of published intron/exon boundary sequences (Burglen et al. 1996) and primer sequences kindly provided by Dr. Judith Melki (Hôpital des Enfants Malades, Paris). For samples with one copy of SMN^T, each SMN exon was amplified from genomic DNA and was subjected to SSCP analysis as described elsewhere (Parsons et al. 1996). SMN exon 6 was PCR amplified by use of 30 ng each of primers 541C618 (5′-CTCCCATATGTCCAGATTCTCTTG-3′) and EX63 (5′-AAGAGTAATTTAAGCCTCAGACAG-3′) in a 50-μl reaction mixture containing 1 U *Taq* polymerase (Perkin Elmer), 0.5 mM each dNTP, 3 mM $MgCl_2$, and 1 × PCR buffer (670 mM Tris, 100 mM β-mercaptoethanol, 166 mM ammonium sulfate, 67 mM EDTA, and 0.5 mg BSA/ml). The PCR reaction consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min, with a final extension for 8 min at 72°C.

Heteroduplex Analysis

To allow heteroduplex formation, PCR products were heated to 95°C for 5 min and then incubated at 37°C for 30 min. Fifteen microliters of the PCR product was mixed with 2.5 μl of 6 × MDE™ gel loading buffer and was electrophoresed on a 50-cm vertical, 0.8-mm-thick MDE™ gel (FMC) for 15 h at 1,000 V. The gel was stained in a solution of 0.6 × Tris-borate EDTA con-

taining 1 μg ethidium bromide/ml and was photographed under UV light.

Reverse-Transcriptase–PCR (RT-PCR)

Total RNA was isolated from peripheral blood lymphocytes by use of TRIzol™ Reagent (Gibco BRL). First-strand cDNA synthesis was performed with 2 μg total RNA, oligo(dT), and Superscript II RNase H⁻ Reverse Transcriptase (200 U/μl; Gibco BRL), according to the manufacturer's instructions. The single-stranded cDNAs were PCR amplified by use of 30 ng each of the exon 6 forward primer 541C618 and the exon 8 reverse primer 541C1120 (5′-CTACAACACCCTTCTCACAG-3′), with reaction mixture components and PCR conditions identical to those used for PCR amplification of SMN exon 6.

Subcloning and Sequencing

PCR and RT-PCR amplification products were subcloned into a TA cloning vector (Invitrogen), according to the manufacturer's instructions. Sequencing of DNA purified by use of Wizard Minipreps (Promega) was performed with the dsDNA Cycle Sequencing System (Gibco BRL). Sequencing reaction products were analyzed by use of a 5% denaturing polyacrylamide gel. The mutation was detected on both DNA strands in multiple subclones.

Results

We describe a quantitative PCR-based method to measure the copy number of SMN^T and SMN^C genes in order to detect individuals with a heterozygous deletion of the SMN^T gene. Since homozygous deletions of SMN^T account for ~95% of SMA cases, the detection of a single copy of SMN^T in a patient with clinical features consistent with SMA would support a diagnosis of SMA. Detection of a single copy of SMN^T in an asymptomatic individual would identify that person as an SMA carrier.

To test the reliability and reproducibility of the assay, we measured the SMN^T copy number in a normal individual and in a known SMA carrier determined by haplotype analysis (DiDonato et al. 1994). The samples were amplified in 10 separate PCR reactions, electrophoresed on different gels, and quantitated by densitometry. The values from the densitometric scans and the calculated SMN^T/CFTR and SMN^C/CFTR ratios are shown in table 1. The mean ± SD SMN^T/CFTR and SMN^C/CFTR ratios for the normal individual were 0.68 ± .08 and 0.62 ± .06, respectively, which represents two copies of SMN^T and two copies of SMN^C. The SMA carrier had mean ± SD SMN^T/CFTR and SMN^C/CFTR ratios of 0.28 ± .06 and 0.29 ± .06, respectively, which is consistent with one copy of SMN^T and one copy of SMN^C. (It should be noted that, although we characterized an SMA carrier with one copy of SMN^C, not all carriers have one copy of SMN^C.) There was no overlap in the SMN^T/CFTR or SMN^C/CFTR ratios, within 2 SD of the mean, between the normal and carrier individuals. The described quantitative PCR assay demonstrates high precision between different gel runs and therefore can distinguish reliably between individuals with one and two copies of SMN^T and SMN^C.

We then studied a population of patients referred for SMA diagnostic testing who were not homozygously deleted for SMN^T exons 7 and 8 by either SSCP analysis or the restriction-enzyme assay. Seventy-six nondeletion samples were analyzed by use of the quantitative PCR assay, and the results for those individuals with a single copy of SMN^T are shown in table 2. A heterozygous deletion of SMN^T was detected in 6/76 (~8%) individuals. Heteroduplex or SSCP analysis of the single SMN^T copy present in these patients revealed abnormal bands in 3/6 patients. Mutations have been detected in 2/6 individuals (1 type I SMA [Parsons et al. 1996] and 1 type III patient described below), and the other individual (type I SMA) is currently under investigation. We did not detect abnormal patterns by SSCP or heteroduplex analysis of genomic DNA in 3/6 individuals.

We now describe one of the six patients with a single copy of SMN^T by gene-dosage analysis (patient 4659; table 2 and fig. 1*A* and *B*). SSCP and heteroduplex analysis of SMN exons PCR amplified from genomic DNA in this type III SMA patient demonstrated an abnormal band in exon 6. Sequence analysis of the patient's exon 6 subclones revealed a guanine-to-thymine transversion at nucleotide 818, producing a substitution of isoleucine for serine (S262I) at a conserved residue in the deduced protein (fig. 1*C*). In order to determine whether the variant exon 6 sequence was contained within the telomeric or centromeric copy of SMN, the patient's lymphocyte RNA was amplified by RT-PCR using an exon 6 sense primer (541C618) and an exon 8 antisense primer (541C1120) and then was subcloned and sequenced. The patient's subclones containing SMN^T were identified by restriction-enzyme digestion, and sequence analysis of these clones confirmed that the mutant transcripts were derived from SMN^T. The nucleotide change was not observed in 200 normal chromosomes. These results, in combination with the dosage data demonstrating that the patient possesses only one copy of SMN^T, strongly indicate that this type III SMA patient has two different SMN^T mutations: one SMN^T allele has been deleted, whereas the other contains a missense mutation (S262I) in exon 6.

SMN^T/CFTR and SMN^C/CFTR ratios were determined for 79 SMA carriers and 54 normal individuals, to establish this assay as a valid method to distinguish between SMA carriers with one copy of SMN^T and normal individuals with two copies; for example, figure 2

Table 1

Reproducibility of SMN^T and SMN^C Copy Number

Type	No. from Densitometric Scans for				SMN^T/CFTR[a]	SMN^C/CFTR[a]
	CFTR	CFTR-IS	SMN^T	SMN^C		
Normal	118,967	114,311	71,537	69,543	.60	.58
	107,932	113,891	76,390	73,369	.71	.68
	64,006	73,251	48,313	33,911	.75	.53
	60,206	74,688	32,022	34,595	.53	.57
	54,790	55,420	36,279	31,484	.66	.57
	49,754	48,086	38,716	35,801	.78	.72
	126,412	117,640	85,921	81,091	.68	.64
	96,863	98,561	66,955	64,919	.69	.67
	49,246	37,091	29,185	30,693	.59	.62
	26,518	46,443	19,126	17,042	.72	.64
					(.67 ± .08)	(.62 ± .06)
Carrier	47,802	53,273	14,930	14,214	.31	.30
	52,047	63,934	14,330	12,972	.28	.25
	73,957	74,941	13,279	13,395	.18	.18
	17,460	22,132	3,553	4,064	.20	.23
	63,749	81,768	18,218	16,557	.29	.26
	120,059	102,099	46,225	46,732	.39	.39
	29,175	38,230	8,531	8,325	.29	.29
	29,373	40,148	7,720	9,890	.26	.34
	58,207	62,618	18,274	20,373	.31	.35
	45,177	47,462	15,158	14,638	.34	.32
					(.28 ± .06)	(.29 ± .06)

[a] Data in parentheses are mean ± SD values.

shows the expected results of the SMN^T and SMN^C copy-number assay for normal, carrier, and affected individuals from several families. Two different populations of SMA carriers previously characterized by multicopy markers in the SMA region were analyzed in this study. Fifty-five SMA carriers analyzed at OSU had a mean ± SD SMN^T/CFTR ratio of 0.27 ± 0.07 (one carrier individual had two copies of SMN^T; table 3). Twenty-two French Canadian SMA carriers (from HSJ) had a mean ± SD SMN^T/CFTR ratio of 0.19 ± .03 (one was homozygously deleted for SMN^T). Interestingly, analysis of SMN^C copy number in SMA carriers revealed

Table 2

Detection of SMA Patients with One Copy of SMN^T by Dosage Analysis

Patient[a]	SMA Type	SMN^T/CFTR	SMN^C/CFTR	No. of Telomeric Copies	No. of Centromeric Copies	Mutation
Control	Normal	.58	.55	2	2	. . .
OSU 367	I	.24	.53	1	2	11-bp duplication[b]
OSU 4659	III	.23	.25	1	1	S262I[c]
OSU 284	II/III	.13	.10	1	1	Not detected
OSU 379	III	.33	1.01	1	3	Not detected
HSC[d]	I	.19	.18	1	1	Not detected
FC 72[e]	I	.25	.50	1	2	Abnormal SSCP pattern[f]

[a] Of the 64 patients screened at OSU, the 4 listed here had a heterozygous deletion of the SMN^T gene.
[b] Reported by Parsons et al. (1996).
[c] Novel exon 6 missense mutation dercribed in the Results section.
[d] One of the four HSC samples had a single copy of the SMN^T gene.
[e] One of the eight HSJ nondeletion patients had one copy of the SMN^T gene, by dosage analysis.
[f] L. R. Simard and C. Rochette (unpublished data).

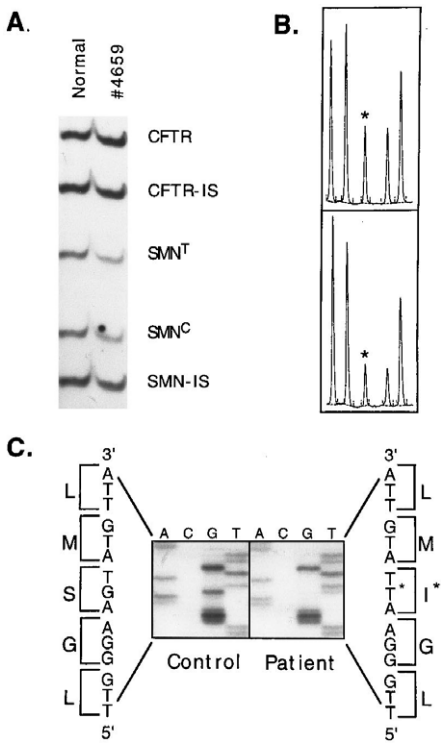

Figure 1 Dosage and sequence analysis of a compound-heterozygote 5q SMA patient. *A,* Autoradiograph of competitive PCR products digested with *Dra*I and run on a 6% denaturing polyacrylamide gel. Equal amounts of genomic DNA were added to each reaction, and the SMN^T and SMN^C bands from patient 4659 were half the intensity that was seen in the normal control. The SMN^T/CFTR ratios of the normal individual and patient 4659 were 0.51 and 0.25, respectively, demonstrating that the patient has half the normal dosage, or one copy of SMN^T. *B,* Densitometric scan of the gel shown in *A*. The order of peaks, from left to right, is CFTR, CFTR-IS, SMN^T, SMN^C, and SMN-IS. An asterisk (*) denotes the SMN^T peak in the normal control (*top panel*) and the patient (*bottom panel*). The area of the patient's SMN^T and SMN^C peaks is half that of the normal control's. *C,* Comparison of nucleotide and deduced amino acid sequences in the normal control's and the patient's SMN^T subclones. Patient 4659 has a G→T transversion that produces a substitution of serine by isoleucine at codon 262. The base change is denoted by an asterisk (*).

that 27/79 (~35%) had three or four copies of SMN^C, indicating that a large number of chromosomes in this population have two copies of SMN^C on one chromosome.

Fifty normal, unselected individuals had a mean ± SD SMN^T/CFTR ratio of 0.66 ± .07 (table 3). One normal individual had one copy of SMN^T, which is consistent with the 1/40–1/60 carrier frequency for SMA in the population. We also found three normal individuals with three copies of SMN^T. SMN^C-dosage analysis in normal individuals revealed that only 1 (1/53, or ~2%) had three copies, 23 had two SMN^C genes, 25 had one SMN^C gene, and 4 had no copies of SMN^C. The results of this analysis clearly demonstrate the limitations of utilizing SMN^C as a standard to determine dosage of SMN^T.

Finally, SMN^T and SMN^C copy number was determined for individuals from several interesting SMA families (Burghes et al. 1994*a;* DiDonato et al. 1997*b*). In one case, an asymptomatic type II/III SMA carrier with a homozygous deletion of SMN^T was shown to have four copies of SMN^C, by the quantitative PCR assay (II.1; fig. 3). However, we observed another asymptomatic carrier deleted for SMN^T who had two SMN^C genes (table 3). We also investigated three cases of haploidentical siblings from SMA families with discordant phenotypes. We found no difference in SMN^T or SMN^C copy numbers (I.4 and I.5; fig. 3).

Discussion

Accurate dosage analysis is necessary in order to identify SMA carriers and to distinguish SMA compound heterozygotes from non-5q SMA-like cases. Both of these diagnostic applications require a method that can differentiate between individuals with one and two copies of the SMN^T gene. We applied a quantitative PCR assay that uses an exon of the CFTR gene as a standard to determine the copy number of the SMN^T and SMN^C genes. The assay also incorporates the use of two internal standards (CFTR-IS and SMN-IS) to monitor the efficiency of the PCR reaction and to ensure that equal amounts of target genomic DNA are added to each tube. Similar quantitative PCR approaches have been used successfully to identify deletions in the insulin-receptor gene (Celi 1994), to detect duplications in Down syndrome patients (Celi 1994), and to quantitate oncogene amplification (Sestini et al. 1994, 1995).

Since this assay uses CFTR as a standard to determine SMN^T and SMN^C copy number, the problems of using the variable SMN^C gene as a standard are avoided. Previous attempts to estimate the copy number of SMN^T and SMN^C have been based on measurement of the SMN^T/SMN^C ratio (Matthijs et al. 1996; Velasco et al. 1996; Schwartz et al. 1997). The copy number of SMN^C was determined in parents of SMA patients in cases in which it was assumed that only one copy of SMN^T was present (Velasco et al. 1996); thus this assay is limited to quantitation of SMN^C in obligate carriers. In another study, a nonradioactive SSCP assay was used to determine SMN^T copy number by means of the SMN^T/SMN^C ratio (Matthijs et al. 1996). An elegant solid minisequencing method was used to determine the SMN^T/SMN^C ratio on normal and SMA chromosomes (Schwartz et al. 1997). However, these methods are not suitable for carrier analysis or detection of compound heterozygotes, because they rely on the ratio of SMN^T/SMN^C without the use of an external standard. This ratio is effected by the variation in SMN^C copy number that occurs in the

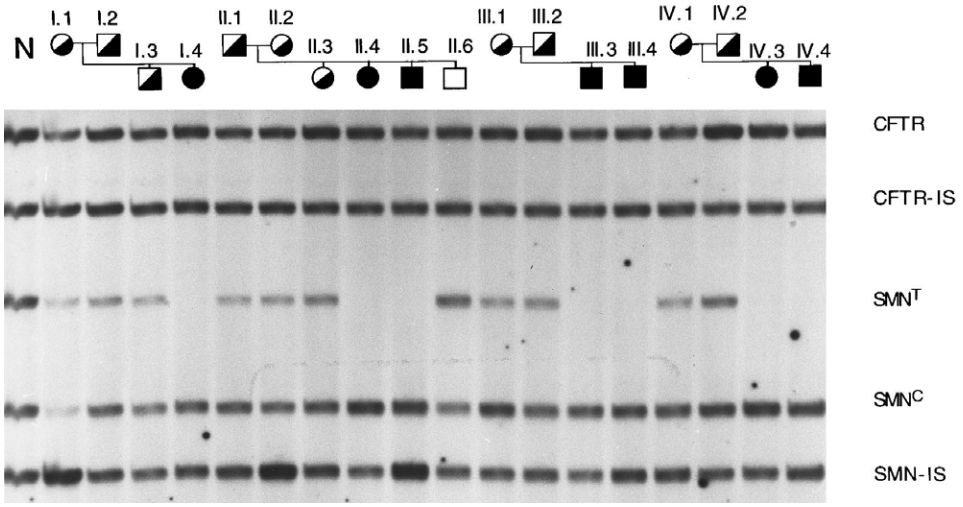

Figure 2 SMN^T-dosage analysis of SMA carriers and their families. All members of these pedigrees were characterized previously by linkage analysis. Families were diagnosed as follows: "I" (type I SMA), "II" (type II/III SMA), "III" (type II SMA), and "IV" (type II SMA). The normal control is represented as "N." SMA carriers are represented as half-blackened boxes, whereas affected SMA patients are represented as completely blackened boxes. The SMN^T/CFTR ratios for SMA carriers are half the ratio for the normal control, whereas affected SMA patients are deleted for SMN^T.

normal population, and it does not account for the possibility of more than one SMN^T gene on a chromosome.

Utilizing the competitive PCR assay, we quantitated the SMN^T copy number in patients with clinical features consistent with SMA without homozygous deletions of SMN^T. We found that a majority (~92%) had normal SMN^T dosage. Although SMA patients with two identical, nondeleted SMN^T alleles were identified in cosanguinous Spanish families (Bussaglia et al. 1995), the estimated frequency of this event is rare in a random population. Although further clinical evaluation may have excluded some of these patients from SMN^T-dosage testing, the results of our analysis clearly indicate that the clinical features of SMA are shared by other neuromuscular disorders. In addition, we did not detect abnormal patterns by SSCP or heteroduplex analysis in 3/6 patients identified with a single copy of SMN^T. Possible explanations include the following: the mutation may be outside of the screened region, there may be a duplication or a deletion of a region other than exon 7, or individuals with one copy of SMN^T may be SMA carriers. We would expect ~2% (1.5/76) of this population to be SMA carriers, and therefore 1 or 2 of these patients could be an SMA carrier with another neuromuscular disorder.

Our assay rapidly identified potential SMA 5q compound heterozygotes and defined a limited population of patients for SMN^T mutation analysis. We identified a novel exon 6 missense mutation in a type III SMA patient with one copy of SMN^T. The serine-to-isoleucine substitution at codon 262 in exon 6 introduces an amino acid with a bulkier side chain and the net loss of a hydroxyl group. The phenotype of this chronic type III SMA patient correlates well with the possibility that he has a combination of a severe (deleted) and a milder (S262I missense mutation) SMN^T allele. This serine residue is conserved in both mouse (DiDonato et al. 1997*a*; Viollet et al. 1997) and *Caenorhabditis elegans* (cosmid C41G7; GenBank accession Z81048) SMN^T proteins. This mutation was identified simultaneously by Hahnen et al. (1997). Talbot et al. (1997) report a clustering of missense mutations in a C-terminal dodecapeptide region, highly conserved in *Saccharomyces pombe* and *C. elegans* homologues, which may be an important binding domain. The S262I missense mutation identified in a type III SMA patient lies just upstream of this region, indicating that the conformation of the region upstream of the dodecapeptide is also important for SMN function.

Since SMA is one of the most common lethal genetic disorders, with a carrier frequency of 1/40–1/60, development of a rapid, direct carrier test would be beneficial to many families. SMA carrier testing presently is being done by linkage analysis and is subject to the potential problems of recombination events, de novo mutations, and the difficulty of obtaining DNA samples from various family members. The advantage of our assay is that it directly measures SMN^T copy number and circumvents these problems. The SMN^T gene-dosage assay identified a single copy of SMN^T in most SMA carriers, except for one that was homozygously deleted and one that had two copies of SMN^T. Possible explanations for this finding include a de novo mutation (Melki et al. 1994, Wirth et al. 1995*b*), somatic mosaicism, or one

Table 3

SMN^T and SMN^C Copy Number in Normal Individuals and SMA Carriers

Type	No. of Individuals	Genotype (SMN^T, SMN^C)	SMN^T/CFTR[a]	SMN^C/CFTR[b]
Normal	3	3, 1	.92 ± .12[c]	.22 ± .06
	1	2, 3	.62	.91
	23	2, 2	.67 ± .08	.64 ± .07
	22	2, 1	.67 ± .06	.27 ± .04
	4	2, 0	.60 ± .10	0
	1	1, 3	.30	1.10
SMA carriers:				
OSU:				
SMA I	1	2, 1	.76	.38
	4	1, 3	.28 ± .09	1.06 ± .13
	6	1, 2	.33 ± .06	.68 ± .09
	5	1, 1	.27 ± .08	.32 ± .09
SMA II	8	1, 3	.26 ± .05	.99 ± .10
	7	1, 2	.23 ± .07	.62 ± .11
	2	1, 1	.29 ± .05	.26 ± .01
SMA II/III	2	1, 3	.29 ± .18	1.09 ± .04
	4	1, 2	.25 ± .10	.63 ± .14
	1	1, 1	.27	.27
SMA III	3	1, 4	.25 ± .06	1.40 ± .09
	6	1, 3	.28 ± .03	1.02 ± .15
	5	1, 2	.31 ± .05	.70 ± .12
	2	1, 1	.28 ± .04	.29 ± .02
HSJ:				
SMA I	2	1, 2	.21 ± .02	.41 ± .08
	2	1, 1	.18 ± 0	.17 ± .02
	1	0, 2	0	.41
SMA II	1	1, 3	.21	.57
	3	1, 2	.20 ± .01	.37 ± .03
SMA III	3	1, 3	.22 ± .02	.62 ± .07
	9	1, 2	.19 ± .04	.41 ± .09
	2	1, 1	.17 ± .01	.18 ± 0

[a] The average for 50 normal individuals with two copies of SMN^T was .66 ± .07; for the OSU sample, the average for 55 carriers with one copy of SMN^T was .27 ± .07, whereas, for the HSJ sample, for 22 carriers with one copy of SMN^T it was .19 ± .03.

[b] SMN^C copy number was calculated for OSU samples by dividing the SMN^C/CFTR ratio by .34 (average SMN^T/CFTR of .27 + 1 SD, as the maximum amplification of one copy of SMN). It should be noted that this method may underestimate SMN^C copy number in some cases, since the SMN^C/SMN^T ratio is not equivalent.

[c] The quantitative PCR assay was repeated in four separate reactions to confirm these results.

chromosome with two SMN^T genes. Since this carrier individual has two affected children with the same haplotype, a de novo mutation is unlikely. Although we cannot distinguish between the latter two possibilities, the case of two SMN^T genes on one chromosome is likely, on the basis of our finding of 3/106 normal chromosomes with two SMN^T genes.

Analysis of SMN^T copy number in 54 normal individuals revealed several interesting points. First, one carrier individual with a single SMN^T gene was identified, which is consistent with the estimated carrier frequency of 1/40–1/60. Second, the marker Ag1-CA lies at the 5′ end of the SMN gene, and this marker varies on chromosomes (DiDonato et al. 1994; Wirth et al. 1995*b*). This would indicate that the copy number of SMN also varies on chromosomes. Quantitation of SMN^T copy number in normal individuals revealed that 3/53 had three copies of SMN^T, indicating that two copies are present on one chromosome. Velasco et al. (1996) previously reported a carrier mother and fetus with two SMN^T genes on one chromosome. Multiple copies of SMN genes on one chromosome may have arisen by either unequal crossing-over or a sequence-conversion event (Hahnen et al. 1996).

The finding of two SMN^T genes on a single chromosome has serious counseling implications, because a carrier individual with two SMN^T genes on one chromosome would be misdiagnosed by SMN^T copy-number

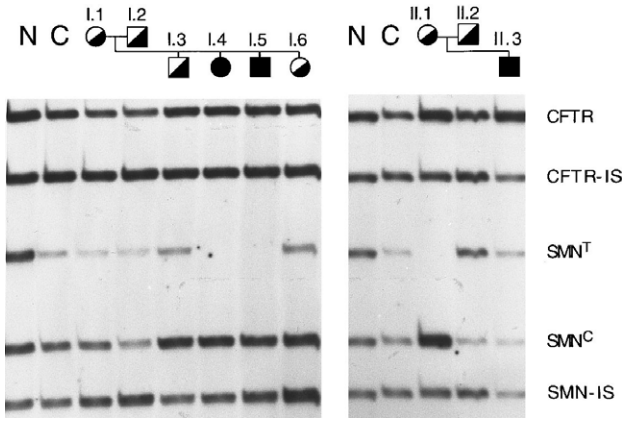

Figure 3 SMN^T- and SMN^C-dosage analysis of two atypical SMA families. Family I (type II SMA) has been described previously, by Burghes et al. (1994*a*), as SMA6. Although linkage analysis demonstrated that individuals I.4 and I.5 are haploidentical, they have remarkably different phenotypes. Individual I.4 had onset at 1 year of age and never walked, whereas individual I.5 was still able to walk at 20 years of age. Individuals I.4 and I.5 have a homozygous deletion of SMN^T and have SMN^C/CFTR ratios of 0.95 and 0.88, respectively. Since both siblings have three copies of SMN^C, the different phenotypes cannot be explained by a difference in SMN^C copy number. Family II (type II/III SMA) represents a case of an asymptomatic carrier parent (II.1) homozygously deleted for SMN^T. The SMN^C/CFTR ratio is consistent with four copies of the SMN^C gene. In table 2, individual II.3 is designated as "OSU 284"; and he currently is being investigated to detect a mutation in the single copy of SMN^T present. Normal and carrier controls are represented as "N" and "C," respectively. Carrier individuals are represented by half-blackened boxes, whereas affected individuals are represented by completely blackened boxes.

analysis. If both a prior probability of 1/40–1/60 of being an SMA carrier in the general population and a conditional probability of ~2% of carrying two SMN^T genes on one chromosome (4/185 chromosomes; i.e., 3/106 normal chromosomes and 1/79 "normal" chromosomes from SMA carriers) are assumed, then a normal SMN^T/CFTR ratio on the basis of gene-dosage analysis would reduce the risk of being an SMA carrier to 1/2,000–1/3,000. Thus, although the finding of normal dosage significantly reduces the risk of being a carrier, our results show that there is still a small recurrence risk of future affected offspring for individuals with normal dosage.

Last, quantitation of SMN^C gene-copy number in normal individuals revealed that 1/53 had three copies, 23/53 had two copies, 25/53 had one copy, and 4/53 were homozygously deleted for SMN^C. The observed genotypes for SMN^C in our normal population were 1.9% (three copies), 43.4% (two copies), 47.2% (one copy), and 7.5% (no copies). The expected genotypes, on the basis of Hardy-Weinburg for a three-allele system, are 2.5% (three copies), 46.9% (two copies), 41.3% (one copy), and 9.3% (no copies). The observed genotype ratios do not differ significantly from the expected genotype ratios ($\chi^2 = 1.6$). It should be noted that this assay does not distinguish between the genotypic groups (1,1) or a single copy of SMN^C on each chromosome from (2,0) or two copies of SMN^C on a single chromosome. Because no normal individuals with four copies of SMN^C were observed, the precise estimation of the frequency of the (2,0) genotype by use of the maximum-likelihood method is not possible. However, the frequency of the (2,0) genotype would be predicted to be negligible. A larger number of individuals will have to be typed to allow accurate assessment of these genotype frequencies.

An interesting finding from these studies is that 27/79 (4/21 type I and 23/58 types II and III) SMA carriers had three or four copies of SMN^C, indicating that there were two copies of SMN^C on one or both chromosomes, whereas only 1/53 (~2%) of the normal individuals had three copies of SMN^C. Previous studies, using the marker Ag1-CA, demonstrated a correlation between the number of copies of Ag1-CA and SMA phenotype (DiDonato et al. 1994; Wirth et al. 1995*b*). Since Ag1-CA lies at the 5′ end of the SMN genes, this implies that type I patients have deleted chromosomes and that type II/III patients have one deleted chromosome and a gene conversion on the other chromosome. The study by Velasco et al. (1996), which demonstrated that three copies of SMN^C predominated in type II/III families is consistent with this interpretation. Therefore, the increase in SMN^C copy number reported here provides evidence to support previous work indicating that a large number of type II/III SMA chromosomes contain gene conversions as opposed to deletions (DiDonato et al. 1994, 1997; Wirth et al. 1995*b;* Hahnen et al. 1996; van der Steege et al. 1996). We currently are preforming a more detailed analysis of gene conversion, using this dosage assay to determine the proportion of gene conversions in different SMA types.

Determination of SMN^C gene-copy number in SMA family members led to some interesting observations. Individual II.3 (fig. 3) previously had been identified as an affected patient who was not homozygously deleted for SMN^T, on the basis of SSCP and restriction-enzyme analysis (DiDonato et al. 1997*b*). These same tests demonstrated that individual II-1, the asymptomatic carrier mother of II-3, was completely deleted for SMN^T. Several reports describe the existence of unaffected individuals with a homozygous deletion of SMN^T (Cobben et al. 1995; Hahnen et al. 1995; Wang et al. 1996). This asymptomatic carrier has four copies of SMN^C, which could suggest that the number of copies of SMN^C in this individual compensates for the lack of SMN^T. However, this situation does not apply to all asymptomatic individuals, since we observed (1) only two SMN^C genes in a second asymptomatic carrier (table 3) and (2) SMA individuals with four copies of SMN^C. In addition, our

group (Burghes et al. 1994*a*) and others (Müller et al. 1992; Rudnik-Schöneborn et al. 1994) have described SMA families in which two sibs have remarkably discordant phenotypes. In the cases analyzed in this study (SMA6 and SMA14 [Burghes et al. 1994*a*] and SMA75 [DiDonato et al. 1997*b*]), no difference in SMN^C copy number was detected between the affected and unaffected/mildly affected individuals (I.4 and I.5; fig. 3), indicating that a change in SMN^C copy number is not the mechanism responsible for discordant phenotypes in these families. Studies at the RNA and protein levels should elucidate whether the critical component in the unaffected/mildly affected individuals is the amount of full-length SMN produced.

In conclusion, we report a powerful, rapid quantitative PCR assay and demonstrate its clinical application for detection of compound-heterozygote 5q SMA patients and SMA carriers. The quantitative SMN^T and SMN^C copy-number assay increases the sensitivity of diagnosis of SMA and allows for direct carrier testing. This assay now can be used to quantitate SMN^C and SMN^T genes in SMA families, to provide insight into the frequency and mechanisms of gene-conversion events.

Acknowledgments

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January 31, 2017

Dr. Jill Jarecki
Chief Scientific Officer, Cure SMA
925 Busse Road
Elk Grove Village, IL, 60007

Dear Dr. Jarecki,

On December 23, 2016, the U.S. Food and Drug Administration approved the first-ever therapy for spinal muscular atrophy (SMA), now called SPINRAZA. Historically, children with the most severe and common type of SMA did not achieve new motor milestones after diagnosis and rarely survived beyond two years of age without permanent respiratory support. Their medical care was typically palliative in nature, aimed at helping them to breathe, cough, and eat. They never achieved motor milestones such as sitting unassisted, standing, and walking.

Ultimately, the approval of this first-ever SMA treatment was based on positive results from multiple clinical trials, including a Phase 3 controlled study evaluating the treatment in infantile-onset SMA children, as well as open label data in pre-symptomatic and symptomatic people likely to develop Type 1, 2 or 3 SMA.

The overall findings of the controlled trial in infantile-onset SMA and the open-label uncontrolled trials support the effectiveness of the treatment across the range of people with SMA and appear to support the early initiation of treatment.

The trials in infants with infantile-onset SMA included symptomatic SMA infants who ranged in age from 30 days to 7 months at the time of first dose, and pre-symptomatic infants, who ranged in age from 8 days to 42 days at the time of first dose.

Clinical Trial Results

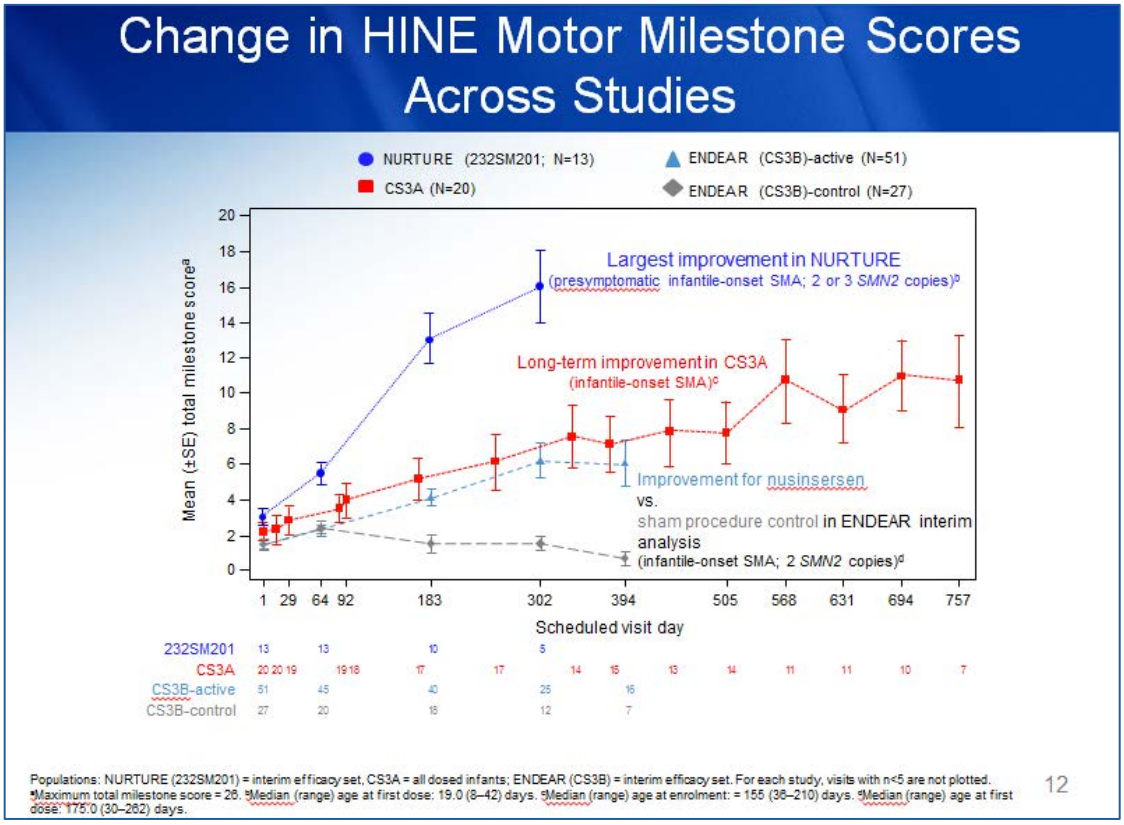
In ENDEAR, a pivotal controlled clinical study of SPINRAZA in infantile-onset SMA babies following symptom onset, babies treated with SPINRAZA achieved and sustained clinically meaningful improvement in motor function compared to untreated babies. In addition, a greater percentage of the babies on SPINRAZA survived without permanent respiratory support compared to untreated babies. Some of the treated babies achieved milestones such as ability to sit unassisted and stand when they would not otherwise be expected to do so; maintained milestones at ages when they would be expected to be lost; and survived without permanent respiratory support to ages unexpected given the natural history of the disease. In ENDEAR, 41% of symptomatic infants who received SPINRAZA achieved new motor milestones compared to 0% who did not receive SPINRAZA. Furthermore, SPINRAZA decreased the risk for death or permanent respiratory support by 47%.[1]

[1] Bertini E, Hwu W-L, Reyna SP, Farwell W, Gheuens S, Sun P, Zhong ZJ, Su J, Schneider E,[4] De Vivo DC, Nusinersen in Pre-symptomatic Infants With Spinal Muscular Atrophy (SMA): Interim Efficacy and Safety Results From the Phase 2 NURTURE Study. Presented at: 21st International Congress of the World Muscle Society; 4-8 October, 2016; Granada, Spain

Babies diagnosed with SMA and treated with SPINRAZA before symptom onset appear to do even better. All of the genetically diagnosed pre-symptomatic SMA infants participating in NURTURE were identified either because they had an older sibling with SMA or through newborn screening pilot programs. Early results of our ongoing open label study of pre-symptomatic infants, NURTURE, show that these infants, who received treatment prior to symptom onset, achieve more significant improvement in motor function when compared to those treated following symptom onset. To date, no pre-symptomatic SMA infant treated with SPINRAZA has died or required permanent respiratory support. Furthermore, infants treated for up to one year have achieved motor milestones such as the ability to sit, stand, and walk in timelines more consistent with normal development than what is observed in the natural history of patients with Type 1 SMA.

Figure 1 features the results from studies of infants with SMA treated with SPINRAZA. It shows that infants treated with SPINRAZA following symptom onset develop more motor milestones than untreated infants, white infants treated prior to symptom onset with SPINRAZA develop more motor milestones than infants treated following symptom onset.

Figure 1[2]



[2] Finkel RS, Kuntz N, Mercuri E, Muntoni F, Chiriboga CA, Darras B, Topaloglu H, Montes J, Su J, Zhong ZJ, Gheuens S, Bennett CF, Schneider E, Farwell W. Primary Efficacy and Safety Results From the Phase 3 ENDEAR Study of Nusinersen in Infants Diagnosed With Spinal Muscular Atrophy (SMA). Presented at: 43rd Annual Congress of the British Paediatric Neurology Association (BPNA); January 11-13, 2017; Cambridge, UK.

In conclusion, it is critical to diagnose babies pre-symptomatically in order to achieve hope of the best possible outcome. Newborn screening is the best way to achieve pre-symptomatic diagnosis in the broadest possible population.

Please feel free to contact me if you have questions about these data.

Regards,

/s/

Wildon R. Farwell, M.D.
Senior Director
Clinical Development

21st International Congress of the World Muscle Society
4-8 October 2016
Granada, Spain

Nusinersen treatment of infantile-onset spinal muscular atrophy (SMA): study design and initial interim efficacy and safety findings from the phase 3 ENDEAR study

Nancy Kuntz

8 October 2016

Kuntz N,[1] Farwell W,[2] Zhong ZJ,[2] Sun P,[2] Gheuens S,[2] Schneider E,[3] and Finkel R[4] on behalf of the ENDEAR study group

[1] Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA; [2] Biogen, Cambridge, MA, USA; [3] Ionis Pharmaceuticals Inc., Carlsbad, CA, USA; [4] Division of Neurology, Department of Pediatrics, Nemours Children's Hospital, Orlando, FL, USA

Disclosures

- NK: reports serving on National Advisory Board for Biogen; outside of the submitted work, NK serves on National Advisory Boards and as consultant for AveXis, Catalyst, Cytokinetics, Marathon, PTC, and Sarepta. NK also serves in an advisory capacity to CureSMA and MGFA
- WF, ZJZ, PS, and SG: full-time employees of Biogen and own stock in Biogen
- ES: full-time employee of Ionis Pharmaceuticals Inc. and owns stock in Ionis Pharmaceuticals Inc
- RF: reports grants and personal fees from Ionis Pharmaceuticals during the conduct of the study; grants and advisor fees from Biogen, grants from Cytokinetics and advisor to Roche, Novartis, and AveXis outside the submitted work. RF serves in an advisory capacity to non-profit organisations: the SMA Foundation, CureSMA, SMA Reach (UK) and SMA Europe, and also serves on the DSMB for the AveXis gene transfer study
- This study was sponsored by Ionis Pharmaceuticals Inc. (Carlsbad, CA, USA) and Biogen (Cambridge, MA, USA)
- Writing and editorial support for the preparation of this presentation was provided by Excel Scientific Solutions (Horsham, UK): funding was provided by Biogen

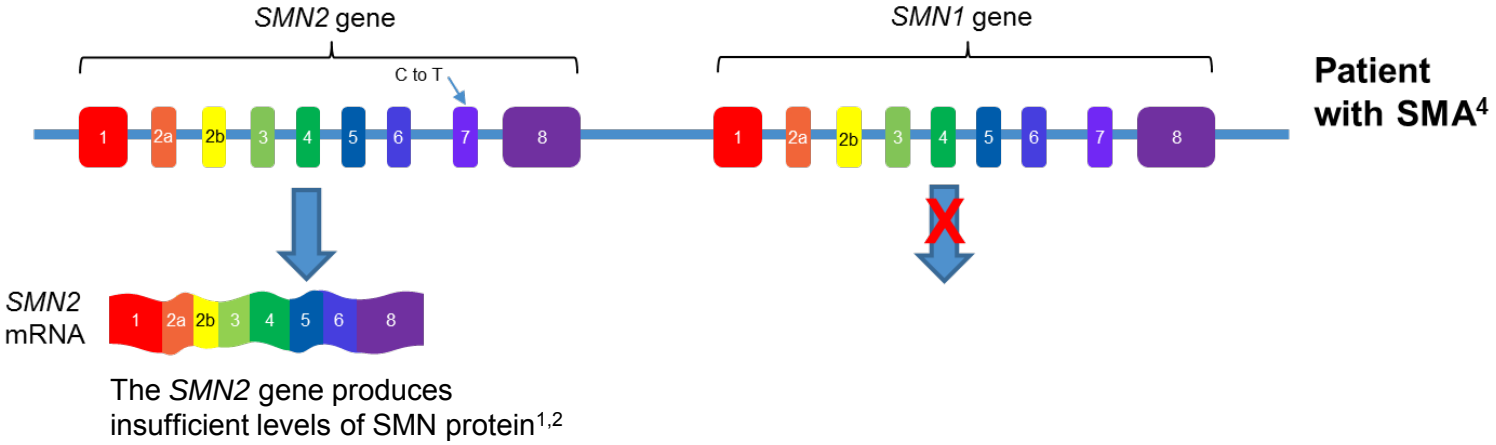
DSMB = data and safety monitoring board

Spinal Muscular Atrophy

SMA is a rare, debilitating, autosomal recessive neuromuscular disorder[1]

- Caused by insufficient levels of SMN protein[2]

SMA subtype	Severity	Age of onset	Natural age of death
Type 1 infantile-onset[3]	Severe	<6 mo	<2 y



mo = months; mRNA = messenger ribonucleic acid; SMA = spinal muscular atrophy; SMN = survival of motor neuron; y = years. 1. Lunn MR *et al. Lancet*. 2008;371:2120-2133; 2. Prior TW. *Curr Opin Pediatr.* 2010;22(6):696-702; 3. Markowitz JA, *et al. Pediatr Neurol* 2012;46:1-124; 4. Chiriboga CA, *et al. Neurology*. 2016;86:890-897

Nusinersen

- Antisense oligonucleotide that increases the amount of full-length *SMN2* mRNA[1]
  - Promotes increased production of functional SMN protein[2,3]

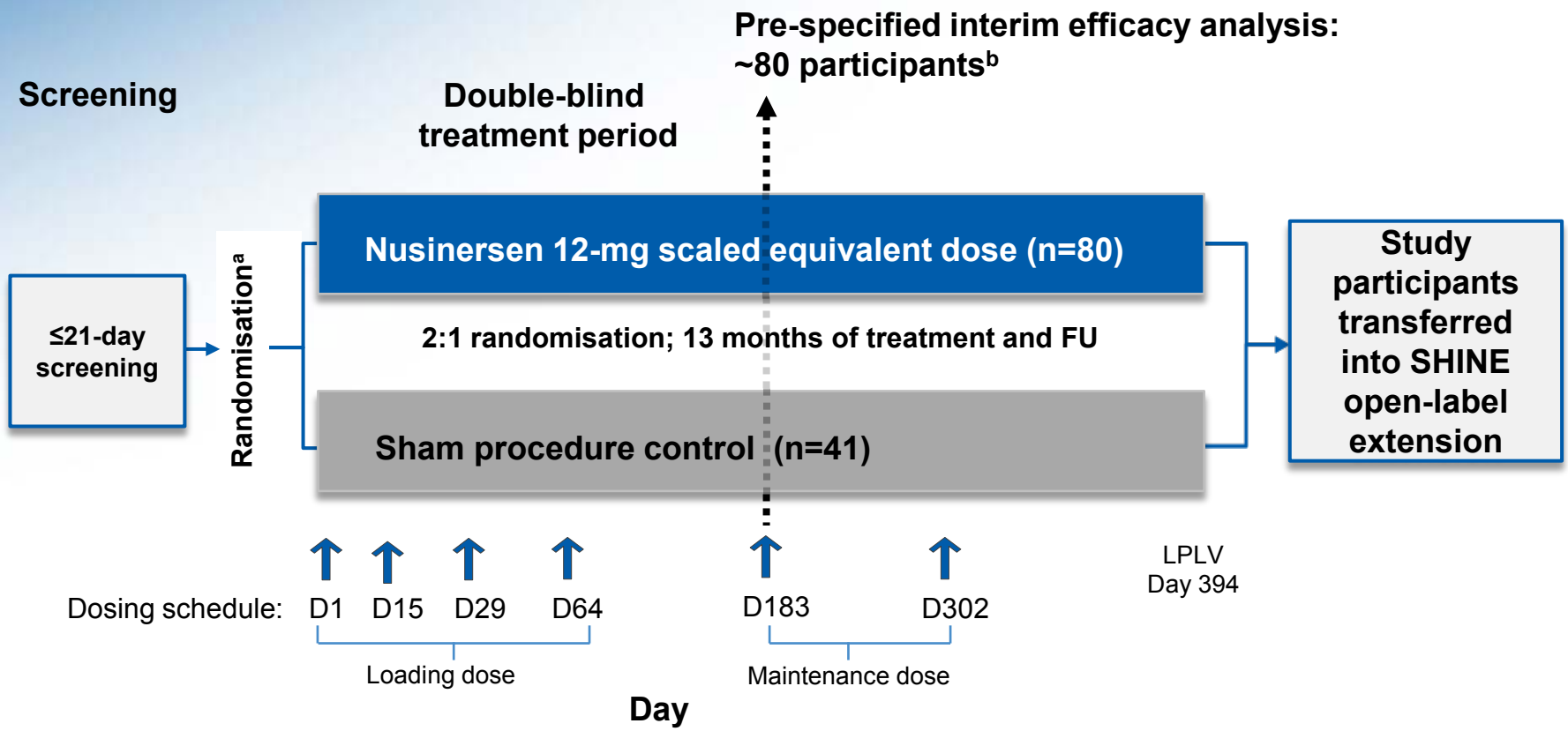
- Phase 2 study (CS3a) interim results[a] in infants with SMA showed promising safety and efficacy[4]
  - No safety or tolerability concerns identified
    - Intrathecal injections were well tolerated
  - Ventilation-free survival of nusinersen-treated infants was divergent from natural history of SMA
  - Achievement of new motor milestones in most treated infants

[a]Interim analysis data cut conducted 26 Jan 2016; 1. Hua Y, *et al. Genes Dev.* 2010;24(15):1634–1644; 2. Passini MA, *et al. Sci Transl Med.* 2011;3(72):72ra18; 3. Darras B, *et al. Neuromusc Disord.* 2014;24(9–10):920; 4. Finkel R, *et al. Neurology.* 2016;86(suppl 16):P5.004

The ENDEAR Study

- ENDEAR is a Phase 3, randomised, double-blind, sham-procedure controlled study to assess the clinical efficacy, safety, and tolerability of intrathecal nusinersen in infants with SMA

ENDEAR Study Design



ITT and Safety population: randomised and received ≥1 dose of study drug

Interim Efficacy population: ITT participants who received nusinersen dose/sham-procedure ≥6 months before cut-off date for interim efficacy analysis, and/or were assessed at any of the Day 183, 302 or 394 visits

D = day; FU = follow-up; ITT = intent to treat; LPLV = last patient last visit; [a]Randomisation was stratified by disease duration during screening (age at screening minus age at symptom onset): ≤12 vs. >12 weeks; [b]Interim efficacy analysis was conducted on 15 June 2016, once ~80 participants had the opportunity to be assessed at Day 183 visit. Final efficacy analysis of ENDEAR will occur when the last surviving participant completes the Day 394 assessment or earlier, as per ethical considerations. Clinicaltrials.gov NCT02193074

Key Inclusion and Exclusion Criteria

Inclusion criteria	Exclusion criteria
Onset of clinical signs and symptoms consistent with SMA at ≤6 months of age	Hypoxemia (oxygen saturation of <96% awake or asleep without ventilation support)
Genetic diagnosis of 5q SMA homozygous gene deletion or mutation or compound heterozygous mutation	Signs or symptoms of SMA present at birth, or within ≤1 week after birth
≤7 months of age at screening[a]	Untreated or treated active infection
2 *SMN2* copies	Previous use of an investigational drug for the treatment of SMA

[a]Additionally, a gestational age of 37–42 weeks was required

ENDEAR Hierarchical Endpoints[a]

- Primary
  - Proportion of motor milestone responders
    - Assessed using modified section 2 of the HINE[1]
    - Interim efficacy analysis conducted once ~80 participants had the opportunity to be assessed at Day 183 visit
  - Time to death or permanent ventilation *[Not tested at the interim efficacy analysis]*
    - Permanent ventilation: tracheostomy or ≥16 hours ventilatory support per day for >21 days

HINE = Hammersmith Infant Neurological Exam. [a]Primary analyses must reach statistical significance before inferential conclusions can be drawn from the remaining secondary and tertiary endpoints; 1. Haataja L, *et al*. *J Pediatr.* 1999;135(2 Pt 1):153–161

ENDEAR Primary Endpoint: Definition of HINE Motor Milestone Responders

Modified section 2 of the HINE[1]

Motor function	Milestone progression score				
	0	1	2	3	4
Voluntary grasp	No grasp	Uses whole hand	Index finger and thumb but immature grasp	Pincer grasp	
Ability to kick (supine)	No kicking	Kick horizontal, legs do not lift	Upward (vertical)	Touches leg	Touches toes
Head control	Unable to maintain upright	Wobbles	All the time upright		
Rolling	No rolling	Rolling to side	Prone to supine	Supine to prone	
Sitting	Cannot sit	Sit with support at hips	Props	Stable sit	Pivots (rotates)
Crawling	Does not lift head	On elbow	On outstretched hand	Crawling flat on abdomen	On hands and knees
Standing	Does not support weight	Supports weight	Stands with support	Stands unaided	
Walking	No walking	Bouncing	Cruising (walks holding on)	Walking independently	

Improvement

Improvement

Improvement: ≥2-point improvement in ability to kick (or maximal score), OR ≥1-point improvement in any other milestone excluding voluntary grasp

Worsening: ≥2-point worsening in ability to kick (or zero score), OR ≥1-point worsening in any other milestone excluding voluntary grasp

Motor Milestone Responder definition[a]: More HINE categories with improvement than worsening

- Participants who die or withdraw are counted as non-responders

[a]Study participants on permanent ventilation will be assessed; 1. Haataja L, *et al*. *J Pediatr.* 1999;135(2 Pt 1):153–161

Other ENDEAR Study Endpoints[a]

Not tested at interim efficacy analysis

- Secondary endpoints include:
  - CHOP-INTEND responders
    - ≥4-point improvement from Baseline in total score from Day 183+
  - Survival rate
  - Participants (%) not requiring permanent ventilation
  - Proportion of CMAP responders (peroneal nerve)
    - Maintenance or increase by ≥1 mV vs. Baseline from Day 183+

- Additional endpoints include:
  - Growth parameters from Baseline
  - Safety and tolerability
  - Pharmacokinetics[b] and immunogenicity

CHOP-INTEND = Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; CMAP = compound muscle action potential;
[a]Primary analyses must reach statistical significance before inferential conclusions are drawn from other endpoints; [b]Pharmacokinetics will be assessed using plasma and cerebrospinal fluid samples

Baseline Demographics: ITT population

- Baseline demographics were balanced except for age and geographic region
  - The nusinersen group was younger than the sham controls group
  - A higher percentage of nusinersen–treated patients were from Asia-Pacific region; more sham-procedure control patients were from Europe

Characteristic	Sham-procedure control (n=41)	Nusinersen (n=80)
Female, n (%)	**24** (59)	**43** (54)
Gestational median age, weeks	**40**	**39**
Median age at screening, days	**190**	**152**
Median age at first dose, days	**205**	**165**
Geographic region, n (%) North America Europe Asia-Pacific	 **22** (54) **17** (41) **2** (5)	 **38** (48) **30** (38) **12** (15)
Ethnicity, n (%) Hispanic or Latin-American White	 **4** (10) **37** (90)	 **12** (15) **68** (85)

Baseline Disease Characteristics: ITT Population

- Disease duration and *SMN2* copy number were similar between groups

Characteristic	Sham-procedure control (n=41)	Nusinersen (n=80)
Age at symptom onset, weeks, n (%) ≤12 weeks >12 weeks	 **32** (78) **9** (22)	 **72** (90) **8** (10)
Median age at symptom onset, weeks	**8.0**	**6.5**
Disease duration, weeks, n (%) ≤12 weeks >12 weeks	 **18** (44) **23** (56)	 **34** (43) **46** (58)
Median disease duration, weeks	**12.7**	**13.1**
Median age of SMA diagnosis, weeks	**20.0**	**11.0**
SMA symptoms, n (%) Hypotonia Developmental motor delay Paradoxical breathing Pneumonia or respiratory symptoms Limb weakness Swallowing or feeding difficulties Other	 **41** (100) **39** (95) **27** (66) **9** (22) **41** (100) **12** (29) **14** (34)	 **80** (100) **71** (89) **71** (89) **28** (35) **79** (99) **41** (51) **20** (25)
Participants requiring ventilation support, n (%)	**6** (15)	**21** (26)
Mean (SD) time on ventilation support at Baseline, h	**6.8** (4.2)	**8.4** (4.3)

ENDEAR Interim Efficacy Analysis Results[a]

- Significant improvement in the proportion of nusinersen–treated motor milestone responders versus sham-procedure control (*P*<.0001)
  - Highly clinically and statistically significant percentage of motor milestone responders

- Interim analysis represents 44.89 patient-years of exposure to nusinersen treatment

[a]The interim efficacy analysis was conducted on 15 June 2016, once ~80 participants had the opportunity to be assessed at Day 183 visit

Summary of Adverse Events (AEs)

- No AEs or serious AEs were considered related to treatment
  - 11% nusinersen–treated versus 15% sham-procedure control participants had AEs possibly related to treatment

	Sham-procedure control n=41	Nusinersen n=80
Any AE, %	93	90
Treatment-related AE,[a] %	0	0
Possibly treatment-related AE, %	15	11
Severe or moderate AE, %	85	78
Severe AE, %	66	55
Serious AE, %	80	70

AE = adverse event. [a]Investigators assessed whether the AE was related to study drug.
A serious AE was any untoward medical occurrence that resulted in death/risk of death, hospitalisation/prolonged hospitalisation, persistent or significant disability/incapacity, or resulted in a congenital anomaly/birth defect. Severe AEs were defined as symptoms causing severe discomfort, incapacitation or significant impact on daily life; participants reporting >1 AE were counted once for total incidence, using the highest severity.

Adverse Event Summary

- Nusinersen was generally well-tolerated
  - Commonly-reported AEs were consistent and age appropriate with those expected in the general population of infants with SMA

	Sham control n=41	Nusinersen n=80
Common AE (>20% in study participants) by MedDRA PT, %		
Pyrexia	54	49
Constipation	22	30
Upper respiratory tract infection	22	25
Respiratory distress	34	24
Pneumonia	15	21
Respiratory failure	34	21
Atelectasis	22	19
Oxygen saturation decreased	22	10
Treatment-emergent AE, %	93	90

MedDRA = Medical Dictionary for Regulatory Activities; PT = preferred term

Conclusions

- ENDEAR is a Phase 3, double-blind, sham-procedure controlled study in infants with SMA
- Nusinersen met the primary endpoint pre-specified for the interim efficacy analysis
  - Clinically and statistically significant percentage of motor milestone responders
  - Acceptable safety profile and well tolerated
- Participants from ENDEAR will be transitioned into the SHINE open-label extension[1]
  - SHINE to enrol all participants with SMA who were previously entered into nusinersen investigational studies
  - Safety, efficacy and tolerability will be assessed
- Final results will be presented at a future medical congress

1. An Open-Label Study (SHINE) for Patients With Spinal Muscular Atrophy (SMA) Who Participated in Studies With IONIS-SMNRx, available at: https://clinicaltrials.gov/ct2/show/NCT02594124?term=NCT0259412

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- The authors thank the ENDEAR study investigators
- The authors also thank all the contributors to the ENDEAR study, including the clinical monitors, study coordinators, physical therapists and laboratory technicians

21st International Congress of the World Muscle Society
4-8 October, 2016
Granada, Spain

Nusinersen in Pre-symptomatic Infants With Spinal Muscular Atrophy (SMA): Interim Efficacy and Safety Results From the Phase 2 NURTURE Study

Enrico Bertini, MD

8 October 2016

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- SPR, WF, SG, PS and ZJZ: employees of and hold stock/stock options in Biogen
- JS and ES: employees of and hold stock/stock options in Ionis Pharmaceuticals, Inc.
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Introduction

- Spinal muscular atrophy (SMA)
  - Rare autosomal recessive neuromuscular disorder[1]
  - Caused by mutations in the *survival motor neuron 1* (*SMN1*) gene[1]
  - Results in SMN protein deficiency[1]
  - A second gene, *SMN2*, produces limited full-length SMN protein[1]
  - *SMN2* copy number correlates with clinical phenotype[1]
- Preclinical data suggest that proactive treatment of pre-symptomatic patients with SMA may lead to improved clinical outcomes[2]
- Nusinersen
  - Antisense oligonucleotide that modifies the splicing of SMN2 precursor mRNA[3]
  - Increases full-length SMN2 mRNA levels
  - Promotes increased production of functional SMN protein[4,5]
  - Safety and tolerability of nusinersen previously demonstrated (study CS3a)[6]

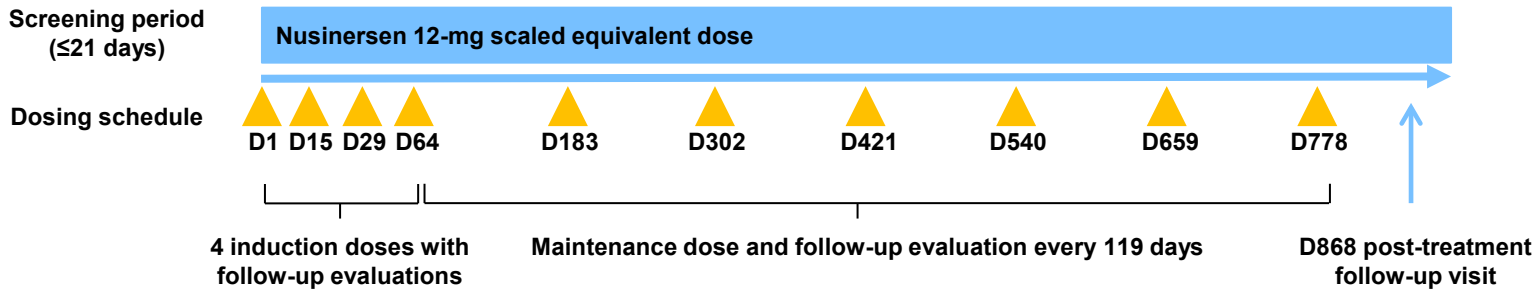
mRNA = messenger RNA. 1. Prior TW. *Curr Opin Pediatr.* 2010;22(6):696-702. 2. Staropoli JF, *et al*. *Genomics.* 2015;105(4):220-228. 3. Hua Y, *et al. Genes Dev.* 2010;24(15):1634-1644. 4. Passini MA, *et al. Sci Transl Med.* 2011;3(72):72ra18. 5. Darras B, *et al. Neuromuscul Disord.* 2014;24(9-10):920. 6. Finkel R, *et al. Neurology.* 2016;86(suppl 16):P5.004.

NURTURE Study Design

- Phase 2, open-label, multicentre, multinational, single-arm study in 10 countries
  - **Objective:** to evaluate the efficacy and safety profile of intrathecal nusinersen in infants with genetically diagnosed and pre-symptomatic SMA
  - **Planned enrolment:** up to 25 infants

Key inclusion criteria:
- Age ≤6 weeks at first dose
- Pre-symptomatic
- Genetic diagnosis of 5q *SMA* gene deletion/ mutation
- Gestational age, 37–42 (34–42 for twins) weeks
- 2 or 3 *SMN2* copies
- Ulnar CMAP amplitude ≥1 mV at Baseline

Key exclusion criteria
- Hypoxemia (O_2 saturation of <96% awake or asleep at sea level)
- Infection during Screening period or ongoing medical condition incompatible with study procedures/ assessments



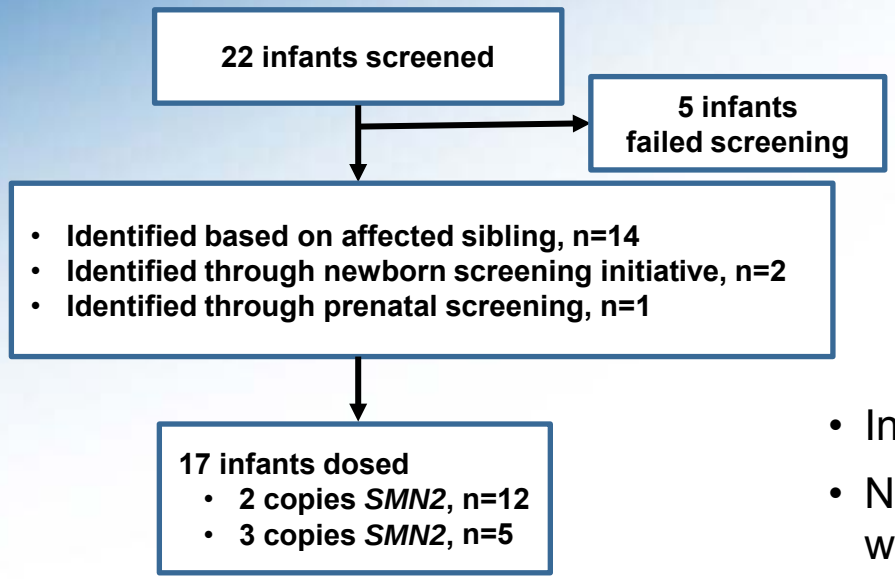
CMAP = compound muscle action potential.

Study Endpoints

- Primary
  - Time to respiratory intervention (invasive or non-invasive ventilation for ≥6 hours/day continuously for ≥7 days or tracheostomy) or death
- Secondary
  - Safety, tolerability and pharmacokinetics
  - Effect on development of SMA by assessing clinical milestones
    - Ability to crawl, stand or walk
  - Motor function milestones
    - Assessed using CHOP INTEND,[1] HINE[2] and WHO[3]
  - Survival (proportion of patients alive)
  - Growth parameters

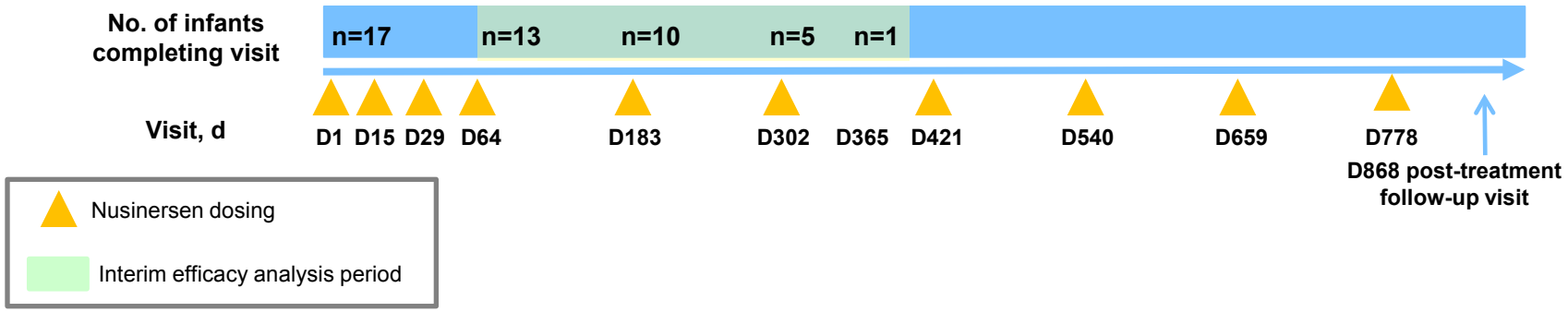
CHOP INTEND = Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; HINE = Hammersmith Infant Neurological Examination; WHO = World Health Organization. 1. Haataja L, *et al*. *J Pediatr.* 1999;135(2 Pt 1):153-161. 2. WHO Multicentre Growth Reference Study Group. *Acta Paediatr Suppl.* 2006;450:86-95. 3. Glanzman AM, *et al*. *Neuromuscul Disord.* 2010;20(3):155-161.

Study Overview: Interim Analysis



- Interim analysis data cutoff date: 8 June 2016
- No infants have discontinued treatment or withdrawn from the study

- Efficacy analyses are based on 13 infants who have reached the first efficacy assessment visit (Day 64, age ~2 months) or longer



Baseline Characteristics

Characteristic	2 *SMN2* copies n=12[a]	3 *SMN2* copies n=5	Total n=17
Age at first dose, d, n (%)			
≤14	5 (42)	1 (20)	6 (35)
>14 to ≤28	5 (42)	2 (40)	7 (41)
>28	2 (17)	2 (40)	4 (24)
Median (range)	17.0 (8–41)	24.0 (12–42)	19.0 (8–42)
Male, n (%)	8 (67)	3 (60)	11 (65)
Female, n (%)	4 (33)	2 (40)	6 (35)
Region, n (%)			
North America	7 (58)	5 (100)	12 (71)
Europe	3 (25)	0	3 (18)
Asia-Pacific	2 (17)	0	2 (12)
Mean CHOP INTEND total score Median (range; n)[b]	48.9 45.0 (39.0–60.0; 9)	53.5 57.0 (40.0–60.0; 4)	50.3 55.0 (39.0–60.0; 13)
Mean HINE total motor milestones Median (range; n)[b]	2.3 3.0 (0–4.0; 9)	4.8 4.5 (3.0–7.0; 4)	3.1 3.0 (0–7.0; 13)
Mean ulnar CMAP amplitude Median (range; n), mV[b]	2.42 2.3 (1.0–4.2; 9)	3.95 4.1 (2.7–4.9; 4)	2.89 3.0 (1.0–4.9; 13)
Mean peroneal CMAP amplitude Median (range; n), mV[b]	2.76 2.8 (1.1–4.2; 7)	4.35 4.2 (4.0–5.0; 4)	3.34 3.4 (1.1–5.0; 11)

NURTURE study interim analysis data cutoff date: 8 June 2016. [a]Included 1 set of twins each with 2 copies of *SMN2*. [b]Based on efficacy set of patients who completed Day 64 visit or longer (n=13).

Primary Endpoint: Time to Death or Respiratory Failure[a]

- At the time of the interim analysis, infants had been enrolled for up to ~13 months
- All infants were still alive
- No infants have required invasive ventilation or tracheostomy
- No infants have required non-invasive ventilation for ≥6 hours/day continuously for ≥7 days

NURTURE study interim analysis data cutoff date: 8 June 2016. [a]Respiratory failure was defined as invasive or non-invasive ventilation for ≥6 hours/day continuously for ≥7 days or tracheostomy.

Summary of HINE Motor Milestone[1] Achievements

Milestone	Total no. of infants achieving milestone n=13[a]	2 copies of *SMN2* n=9	3 copies of *SMN2* n=4
Head control (Full)	9	5	4
Sitting (Independent: stable, pivot)	5	4	1
Standing (Stands with support, unaided)	3	2	1
Walking (Cruising, walking)	1	1	–

1. Haataja L, *et al*. *J Pediatr*. 1999;135(2 Pt 1):153-161. NURTURE study interim analysis data cutoff date: 8 June 2016. [a]Efficacy analyses are based on 13 infants who have reached the first efficacy assessment visit (Day 64, age ~2 months) or longer.

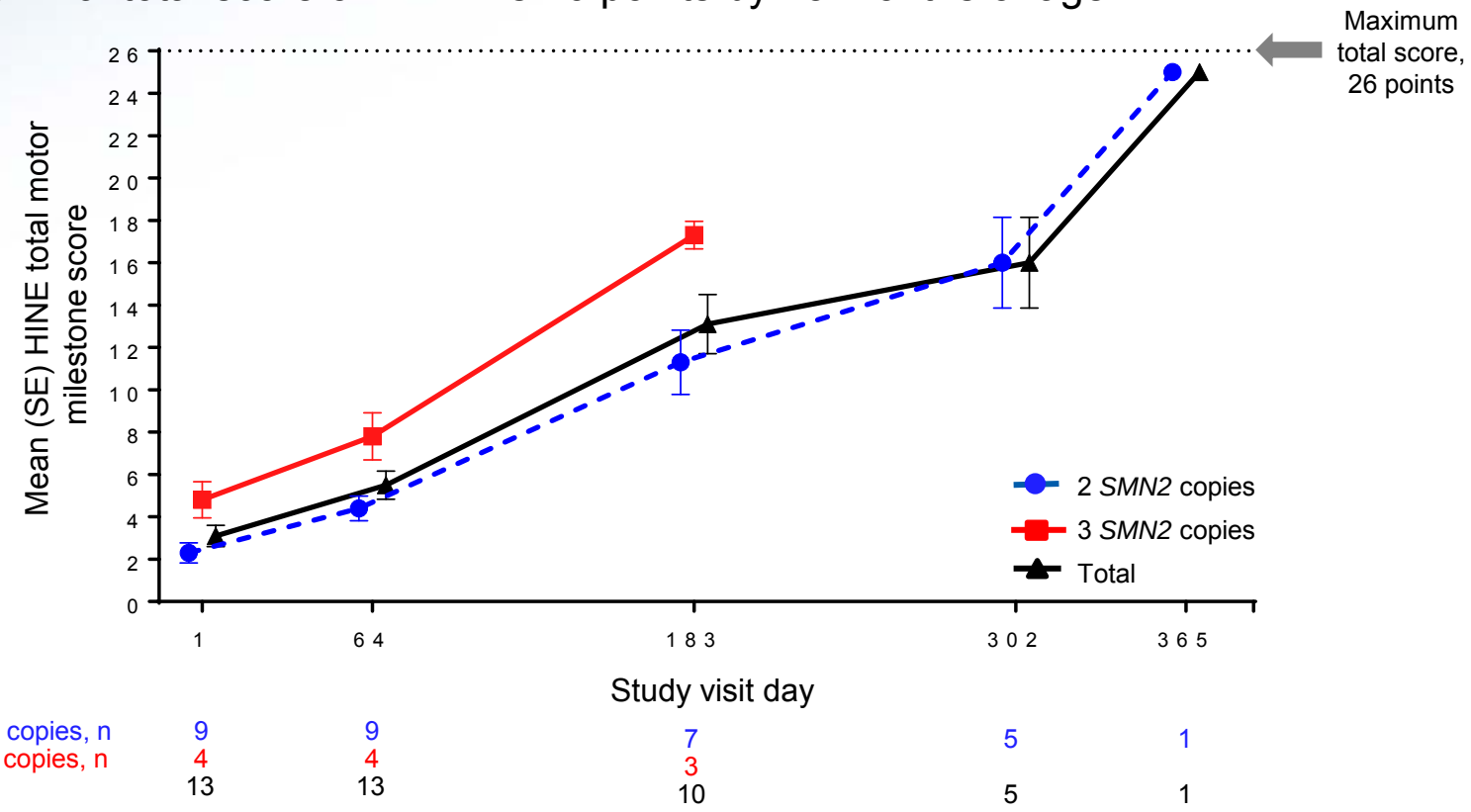
Age-Appropriate Motor Milestone Development Based on HINE

Milestone attainment in nusinersen-treated infants	Motor milestone	Expected age of attainment in healthy infant[1]
8/10 infants Age ≥5 mo	 Full head control	Age 5 mo
5/7 infants Age ≥7 mo	 Independent sitting	Age 7 mo
1/1 infant Age ≥11 mo	 Walking with support (cruising)	Age 11 mo
1/1 infant Age ≥12 mo	 Standing unaided[a]	Age 12 mo

1. Haataja L, *et al*. *J Pediatr*. 1999;135(2 Pt 1):153-161. NURTURE study interim analysis data cutoff date: 8 June 2016. [a]Two infants <8 months of age were standing with support (expected age of attainment: 8 months of age[1]).

Mean HINE Total Motor Milestone Score Over Time

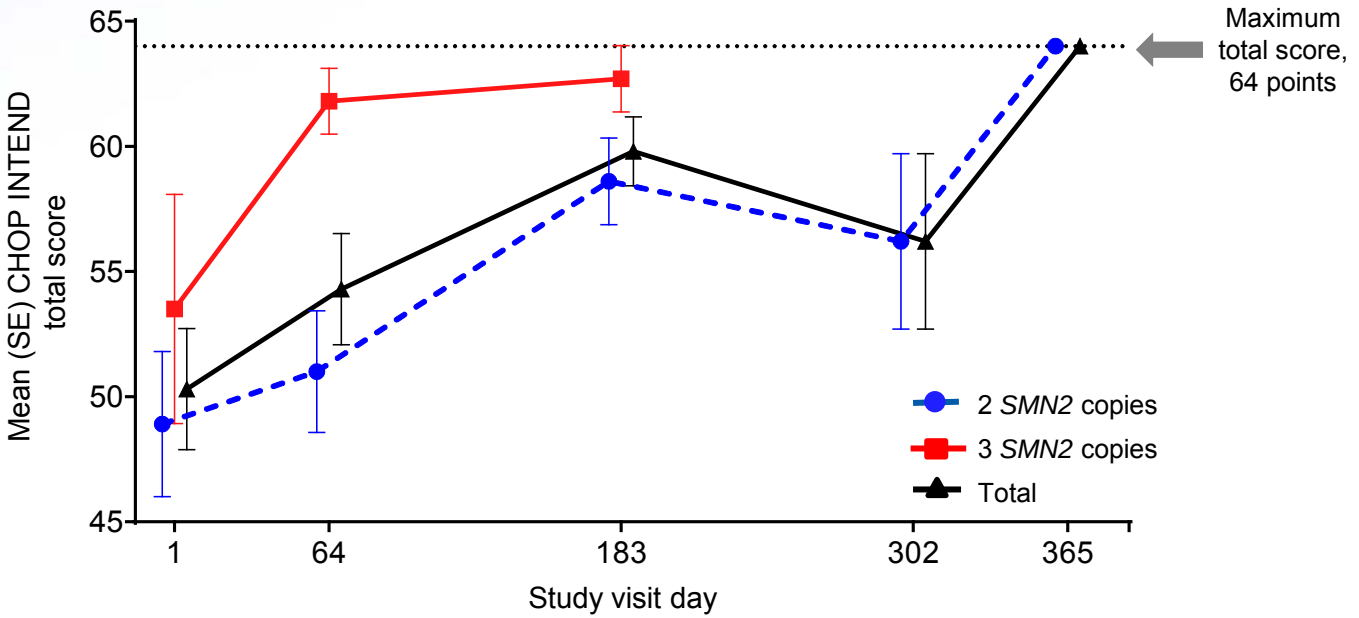
- In general, all enrolled infants demonstrated increased motor milestone scores from Baseline to last evaluation
  - Milestone gain followed a similar trajectory for infants with 2 and 3 copies of the *SMN2* gene
  - Maximal total score on HINE is 26 points by 15 months of age



NURTURE study interim analysis data cutoff date: 8 June 2016.

Mean CHOP INTEND Total Score Over Time

- 10/13 (77%) infants achieved increases (range, 4–20 points)
- 3/13 (23%) experienced decreases (range, 2–3 points)
- Baseline median (range) CHOP INTEND total score was 55.0 (39–60) points in the total efficacy population
  - CHOP INTEND total scores in infants with SMA ≤6 months of age from a natural history study ranged from 10–52 points[1]

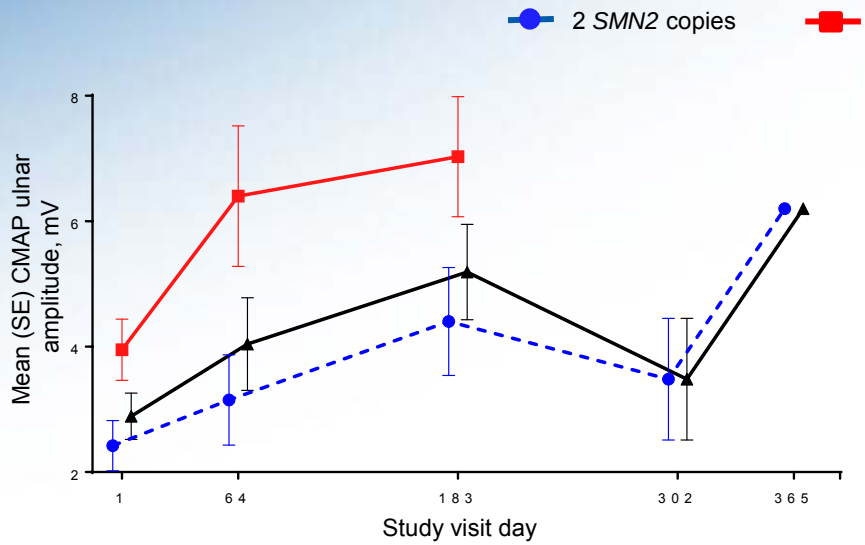


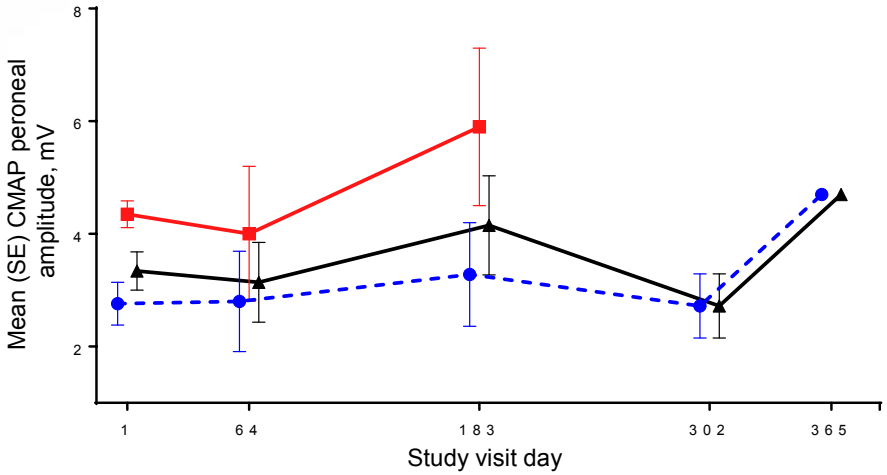
2 *SMN2* copies, n	9	9	7	5	1
3 *SMN2* copies, n	4	4	3		
Total, n	13	13	10	5	1

1. Kolb SJ, *et al*; NeuroNEXT Clinical Trial Network and on behalf of the NN101 SMA Biomarker Investigators. *Ann Clin Transl Neurol*. 2016;3(2):132-145. NURTURE study interim analysis data cutoff date: 8 June 2016.

Mean Ulnar and Peroneal Nerve CMAP Amplitude Over Time

- Overall, mean CMAP amplitude appears to be increasing





2 *SMN2* copies, n	9	8	7	5	1	7	5	4	5	1
3 *SMN2* copies, n	4	3	3			4	2	2		
Total, n	13	11	10	5	1	11	7	6	5	1

Age	CMAP amplitude in healthy infants, mV[1]	
	Ulnar nerve	Peroneal nerve
Neonate	1.6–7.0	1.8–4.0
1–6 mo	2.5–7.4	1.6–8.0
7–12 mo	3.2–10.0	2.3–6.0

1. Miller RG, Kuntz NL. *J Child Neurol*. 1986;1(1):19-26. NURTURE study interim analysis data cutoff date: 8 June 2016.

Growth Parameters

- The majority of infants gained weight over time, consistent with normal development
- Four of 10 infants met the criteria for growth failure at Day 183[a]
  - Three of the 4 infants continued to gain weight over time
  - One infant had a percutaneous gastric tube inserted to assist with feeding

NURTURE study interim analysis data cutoff date: 8 June 2016. [a]Growth failure was defined as weight for age below the fifth percentile (based on WHO growth charts) or a decreased growth velocity resulting in weight for age failing ≥2 major percentiles over a 6-month period. The 4 infants were determined to have growth failure because their weight for age decreased by ≥2 major percentiles over a 6-month period.

Summary of Safety

- Five (29%) infants experienced an SAE. There were no SAEs considered related to study drug
- No severe AEs were reported
- Three (18%) infants experienced AEs considered by the investigator to be possibly related to study drug.
  - Muscular weakness and weight-bearing difficulty (n=1), hyperreflexia and tachycardia (n=1) and increased ALT and AST and pyrexia (n=1)
  - All AEs considered by the investigator to be possibly related to study drug resolved during study follow-up
- No infants experienced AEs that led to discontinuation of study drug or withdrawal from the study
- The lumbar puncture procedure was generally well tolerated
- No clinically significant adverse changes in laboratory or neurological examinations considered related to nusinersen

AE = adverse event; ALT = alanine aminotransferase; AST = aspartate aminotransferase; SAE = serious adverse event. NURTURE study interim analysis data cutoff date: 8 June 2016.

Conclusions

- Findings from the NURTURE interim analysis show that all the pre-symptomatic infants with SMA treated with nusinersen are alive without requiring chronic respiratory support and are exhibiting improvements in function and motor milestones
- Most infants are achieving motor milestone and growth parameter gains generally consistent with normal development
- The majority of infants are gaining weight
- To date, no new safety concerns have been identified

NURTURE study interim analysis data cutoff date: 8 June 2016.

Acknowledgements

- The authors thank the patients who are participating in this study and their parents/guardians and family members, without whom this effort cannot succeed
- The authors also thank the people who are contributing to this study, including the study site principal investigators, clinical monitors, study coordinators, physical therapists and laboratory technicians

Back up

Modified Section 2 of the HINE Scoring and Normal Age of Achievement[a]

Motor function	Milestone progression score				
	0	1	2	3	4
Voluntary grasp	No grasp	Uses whole hand	Index finger and thumb but immature grasp	Pincer grasp	
Ability to kick (supine)	No kicking	Kick horizontal, legs do not lift	Upward (vertical); 3 months	Touches leg; 4–5 months	Touches toes; 5–6 months
Head control	Unable to maintain upright; <3 months	Wobbles; 4 months	All the time upright; 5 months		
Rolling	No rolling	Rolling to side; 4 months	Prone to supine; 6 months	Supine to prone; 7 months	
Sitting	Cannot sit	Sit with support at hips; 4 months	Props; 6 months	Stable sit; 7 months	Pivots (rotates); 10 months
Crawling	Does not lift head	On elbow; 3 months	On outstretched hand; 4–5 months	Crawling flat on abdomen; 8 months	On hands and knees; 10 months
Standing	Does not support weight	Supports weight; 4–5 months	Stands with support; 8 months	Stands unaided; 12 months	
Walking	No walking	Bouncing; 6 months	Cruising (walks holding on); 11 months	Walking independently; 15 months	

Overall maximum total score = 26
(higher score indicates milestone attained)

Haataja L, *et al*. *J Pediatr.* 1999;135(2 Pt 1):153-161. [a]Ages (months) are considered to be the normal age of achievement in individuals without SMA.

43rd Annual Congress of the British Paediatric Neurology Association
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Primary Efficacy and Safety Results From the Phase 3 ENDEAR Study of Nusinersen in Infants Diagnosed With Spinal Muscular Atrophy (SMA)

Richard S. Finkel, MD

13 January 2017

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Disclosures

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Introduction

- Spinal muscular atrophy (SMA)
  - SMA is a rare, debilitating, autosomal recessive neuromuscular disorder[1]
  - Caused by insufficient levels of SMN protein[2]
- Nusinersen: an antisense oligonucleotide
  - Modulates splicing of *SMN2* pre-mRNA to promote inclusion of exon 7
  - Increases the amount of full-length *SMN2* mRNA[3]
  - Promotes increased production of full-length SMN protein[4,5]
- Phase 2 study (CS3A) interim results[a] in infants with SMA[6]
  - Demonstrated target engagement in motor neurons, increase in full-length *SMN2* mRNA and SMN protein levels
  - Showed promising safety and efficacy
  - No safety or tolerability concerns identified
    - Intrathecal injections were well tolerated
  - Ventilation-free survival of nusinersen-treated infants was divergent from natural history of SMA
  - Achievement of new motor milestones in most treated infants

mRNA = messenger RNA; SMN = survival of motor neuron. [a]Interim analysis data cut conducted 26 January 2016. 1. Lunn MR, Wang CH. *Lancet*. 2008;371(9630):2120-2133. 2. Prior TW. *Curr Opin Pediatr.* 2010;22(6):696-702. 3. Hua Y, *et al. Genes Dev.* 2010;24(15):1634-1644. 4. Passini MA, *et al. Sci Transl Med.* 2011;3(72):72ra18. 5. Darras B, *et al. Neuromusc Disord.* 2014;24(9-10):920. 6. Finkel RS, *et al. Lancet.* 2016; 388(10063):3017-3026.

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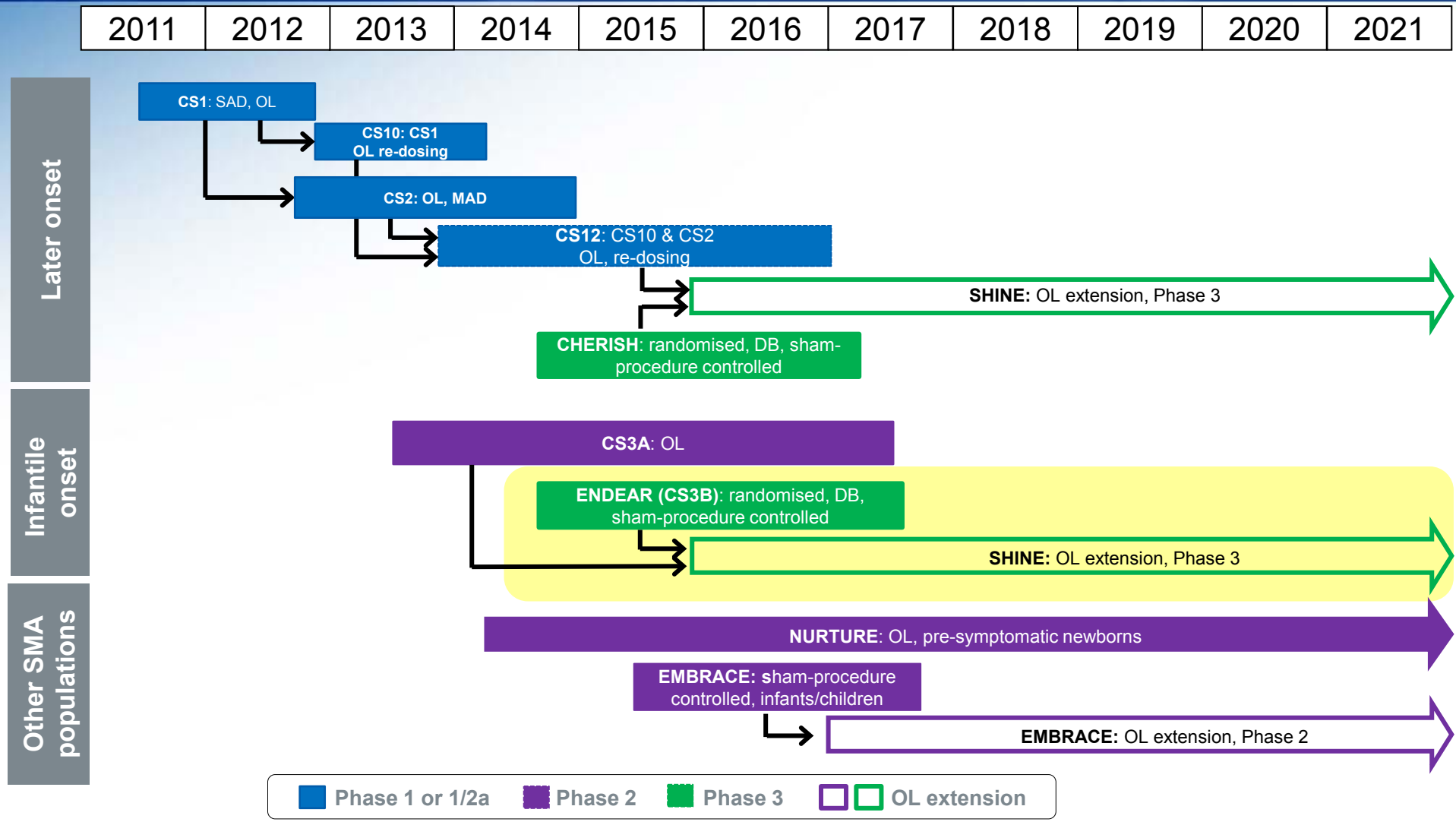
Nusinersen Clinical Development Program



DB = double-blind; OL = open-label; MAD = multiple ascending dose; SAD = single ascending dose.

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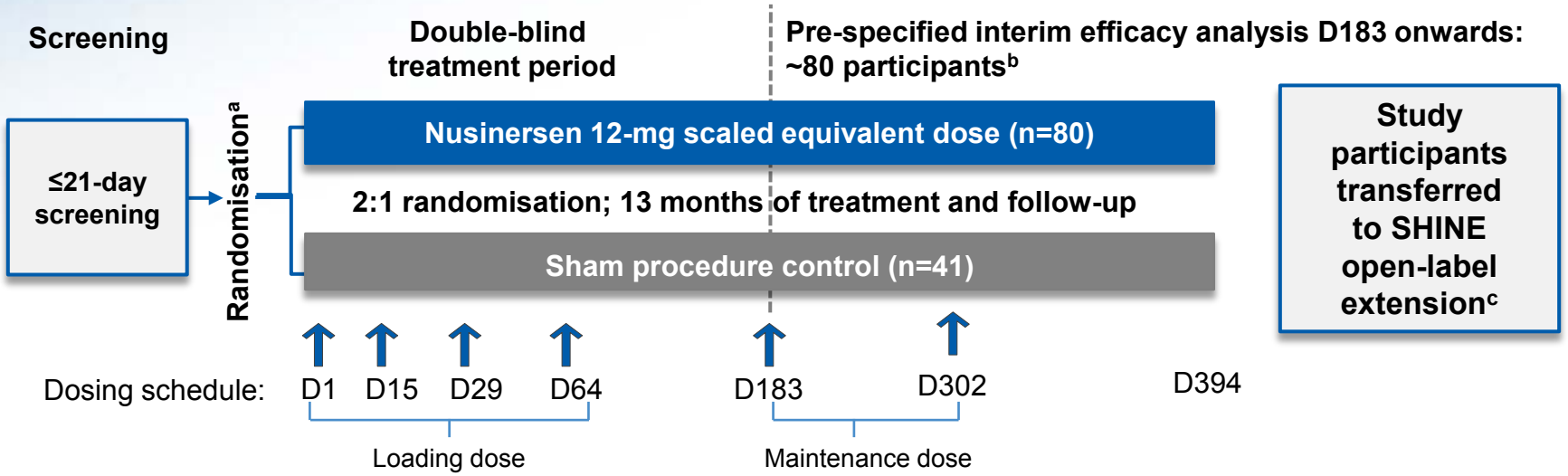
ENDEAR Study Design

- Phase 3, randomised, double-blind, sham-procedure controlled study to assess the clinical efficacy, safety and tolerability of intrathecal nusinersen in infants with SMA
  - Key eligibility criteria: genetic diagnosis of SMA, 2 copies of the *SMN2* gene, onset of SMA symptoms at age ≤6 months and age ≤7 months with no hypoxemia at screening



- **ITT and safety population:** randomised and received ≥1 dose of study drug
- **Interim efficacy set (IES):** ITT participants who received nusinersen dose/sham procedure control ≥6 months before cutoff date for interim efficacy analysis and/or were assessed at any of the Day 183, 302 or 394 visits

ITT = intention-to-treat. [a]Randomisation was stratified by disease duration during screening (age at screening minus age at symptom onset): ≤12 vs. >12 weeks. [b]Interim efficacy analysis was conducted on 15 June 2016, once ~80 participants had the opportunity to be assessed at the Day 183 visit. [c]All infants completing the end of study visit for ENDEAR had the opportunity to enrol in SHINE. ClinicalTrials.gov, NCT02193074.

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ENDEAR Hierarchical Endpoints

- Primary endpoints[a]
  - Proportion of motor milestone responders (IES population)
    - Assessed from Day 183 onwards using modified section 2 of the HINE[1]
    - Interim efficacy analysis conducted once ~80 participants had the opportunity to be assessed at the Day 183 visit
      - » Only endpoint with formal statistical testing at interim
  - Event-free survival, i.e., time to death or permanent ventilation (ITT population at end of study)
    - Permanent ventilation: tracheostomy or ≥16 hours ventilatory support per day for >21 days
    - Events adjudicated by a blinded, central, independent EAC
- Secondary endpoints[a]
  - CHOP INTEND responders
    - ≥4-point improvement from Baseline in total score from Day 183 onwards
  - Survival rate
  - Participants (%) not requiring permanent ventilation
  - Proportion of CMAP responders (peroneal nerve)
    - Maintenance or increase by ≥1 mV vs. Baseline from Day 183 onwards
  - Time to death or permanent ventilation in the subgroups of participants below the study median disease duration
  - Time to death or permanent ventilation in the subgroups of participants above the study median disease duration

CHOP INTEND = Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders; CMAP = compound muscle action potential; EAC = endpoint adjudication committee; HINE = Hammersmith Infant Neurological Exam; IES = interim efficacy set. [a]Primary analyses must reach statistical significance before inferential conclusions can be drawn from the remaining secondary and tertiary endpoints. 1. Haataja L, *et al*. *J Pediatr.* 1999;135(2 pt 1):153-161.

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ENDEAR Primary Endpoint: Definition of HINE Motor Milestone Responders

Modified section 2 of the HINE[1]

Motor function	Milestone progression score				
	0	1	2	3	4
Voluntary grasp	No grasp	Uses whole hand	Index finger and thumb but immature grasp	Pincer grasp	
Ability to kick (supine)	No kicking	Kick horizontal, legs do not lift	Upward (vertical)	Touches leg	Touches toes
Head control	Unable to maintain upright	Wobbles	All the time upright		
Rolling	No rolling	Rolling to side	Prone to supine	Supine to prone	
Sitting	Cannot sit	Sit with support at hips	Props	Stable sit	Pivots (rotates)
Crawling	Does not lift head	On elbow	On outstretched hand	Crawling flat on abdomen	On hands and knees
Standing	Does not support weight	Supports weight	Stands with support	Stands unaided	
Walking	No walking	Bouncing	Cruising (walks holding on)	Walking independently	

Improvement

Improvement

Improvement: ≥2-point improvement in ability to kick (or maximal score), or ≥1-point improvement in any other milestone, excluding voluntary grasp

Worsening: ≥2-point worsening in ability to kick (or zero score), or ≥1-point worsening in any other milestone, excluding voluntary grasp

- **Motor milestone responder definition[a]:** more HINE categories with improvement than worsening
  - Participants who die or withdraw are counted as non-responders

[a]Study participants on permanent ventilation will be assessed. 1. Haataja L, *et al*. *J Pediatr.* 1999;135(2 pt 1):153-161.

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Baseline Disease Characteristics: ITT Population

Characteristic	Sham procedure control n=41	Nusinersen n=80
Female, n (%)	24 **(59)**	43 **(54)**
Median age at first dose, d	205	165
Median age at symptom onset, wk	8.0	6.5
Median age at SMA diagnosis, wk	20.0	11.0
Median disease duration, wk	12.7	13.1
SMA symptoms, n (%)		
Hypotonia	41 **(100)**	80 **(100)**
Developmental motor delay	39 **(95)**	71 **(89)**
Paradoxical breathing	27 **(66)**	71 **(89)**
Pneumonia or respiratory symptoms	9 **(22)**	28 **(35)**
Limb weakness	41 **(100)**	79 **(99)**
Swallowing or feeding difficulties	12 **(29)**	41 **(51)**
Other	14 **(34)**	20 **(25)**
Participants requiring ventilation support, n (%)	6 **(15)**	21 **(26)**

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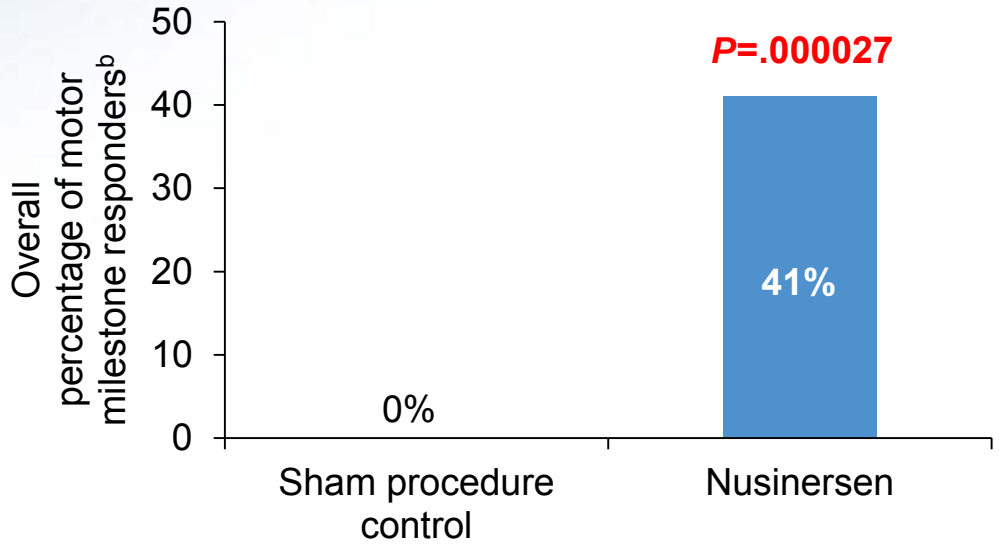
Motor Milestone Responders: IES Population

- Highly clinically and statistically significant percentage of motor milestone responders[a]



Motor milestone responders, %	Sham procedure control	Nusinersen	*P* value
6-month visit (n=78)	7	39	.0032
10-month visit (n=55)	0	47	.0002
13-month visit (n=34)	0	43	.0135

[a]The interim efficacy analysis was conducted on 15 June 2016, once ~80 participants had the opportunity to be assessed at the Day 183 visit. [b]n=78.

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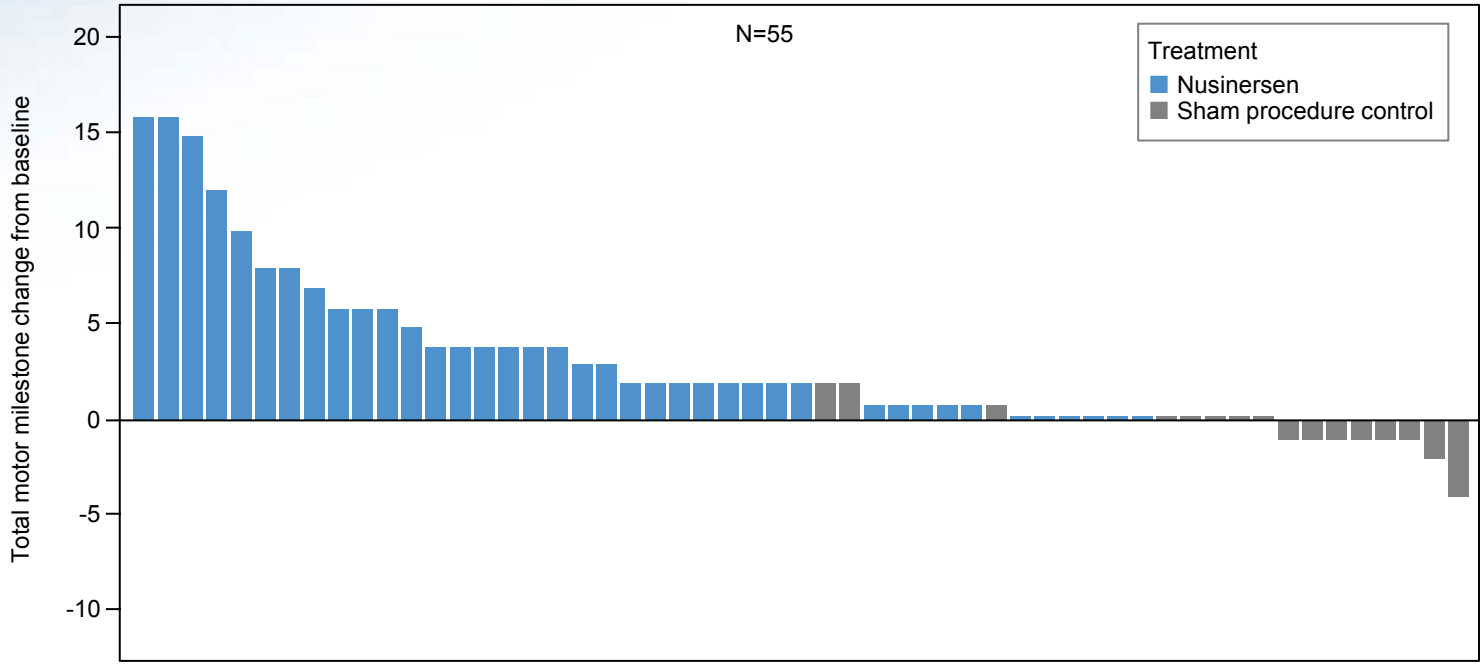
Improvement in Total Motor Milestone Score (HINE Section 2): IES Population

- Infants treated with nusinersen had greater improvement in total motor milestone score[a] vs. sham procedure control



[a]Total motor milestone change from baseline to later of Day 183, 302, 394. Shortest bars indicate zero value. Of the 78 infants in the interim efficacy set, 21 died (nusinersen, n=11; sham procedure control, n=10) and 2 withdrew for a reason other than death (nusinersen, n=1; sham procedure control, n=1) and were not included in this analysis.

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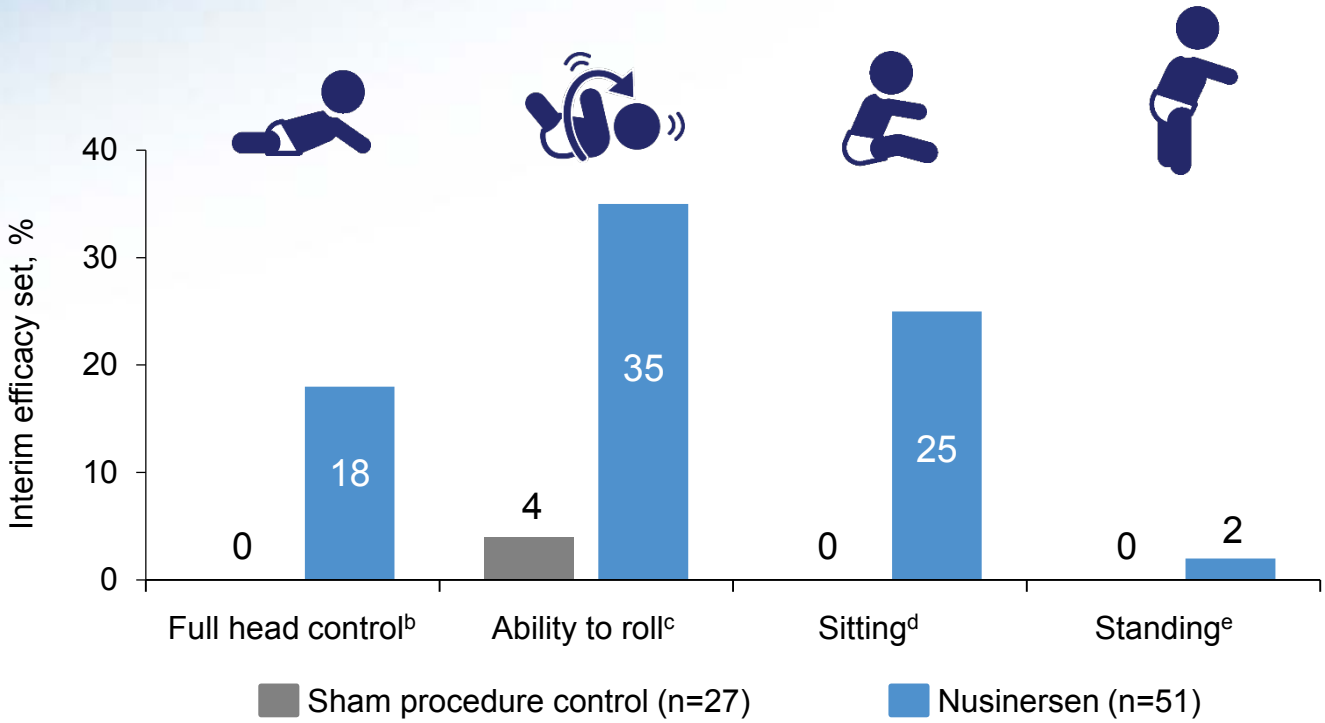
Quality of Motor Responses: IES Population

- Infants on nusinersen achieved motor milestones unexpected for individuals with SMA Type I[a]



Nusinersen-treated infants achieved:
- Supine to prone rolling: 10%
- Stable sit: 6%
- Pivots (rotate) sit: 4%

[a]HINE motor milestone achievement in infants at the later of Days 183, 302 and 394.[b]Full head control was defined as all the time upright (HINE score = 2). [c]Rolling includes HINE score categories: rolling to the side, prone to supine and supine to prone. [d]Sitting includes HINE score categories: sits with support at hips, props, stable sit and pivots (rotates). [e]Standing includes HINE score categories: stands with support and stands unaided.

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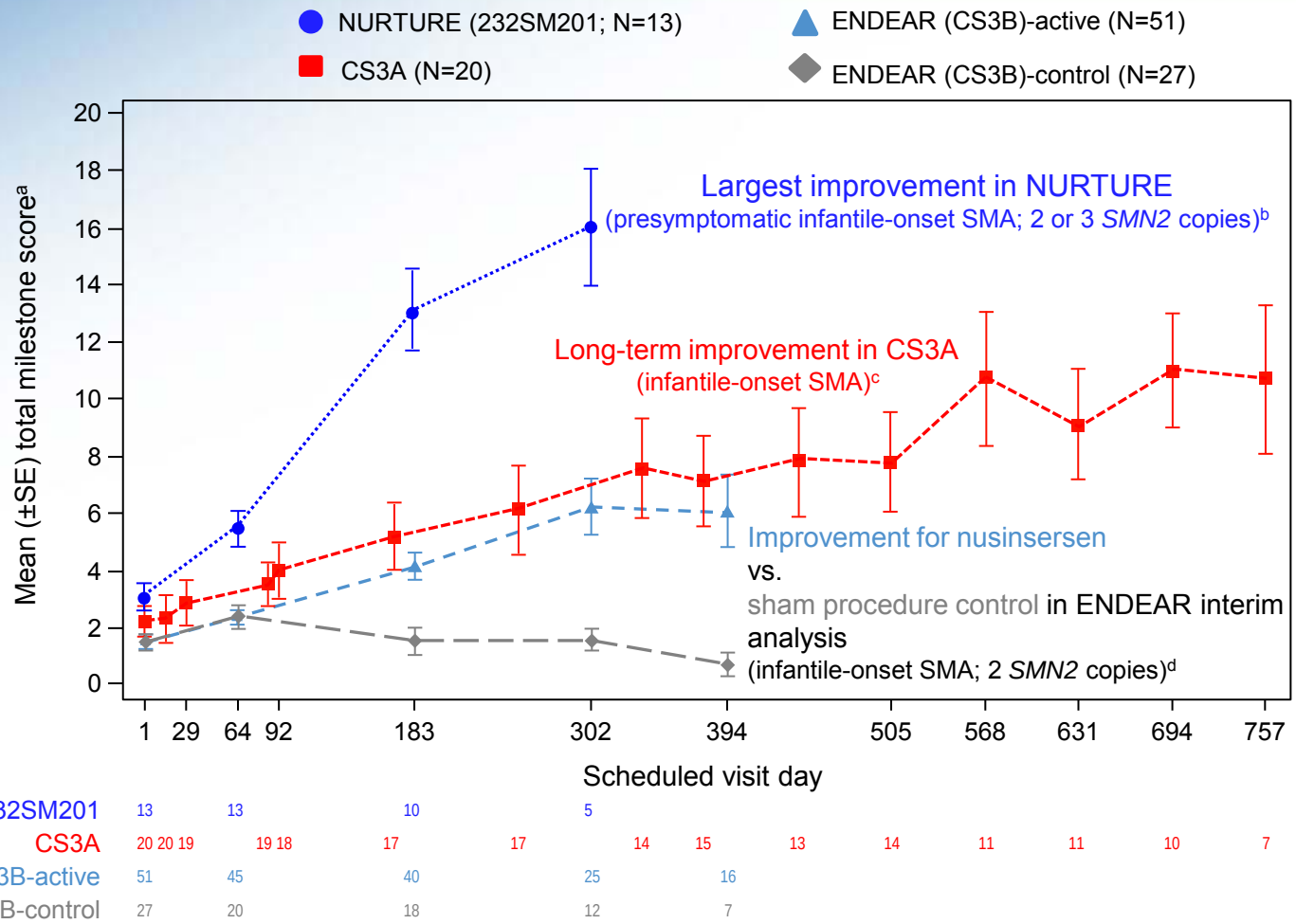
Change in HINE Motor Milestone Scores Across Studies



Populations: NURTURE (232SM201) = interim efficacy set, CS3A = all dosed infants; ENDEAR (CS3B) = interim efficacy set. For each study, visits with n<5 are not plotted.
[a]Maximum total milestone score = 26. [b]Median (range) age at first dose: 19.0 (8–42) days. [c]Median (range) age at enrolment: = 155 (36–210) days. [d]Median (range) age at first dose: 175.0 (30–262) days.

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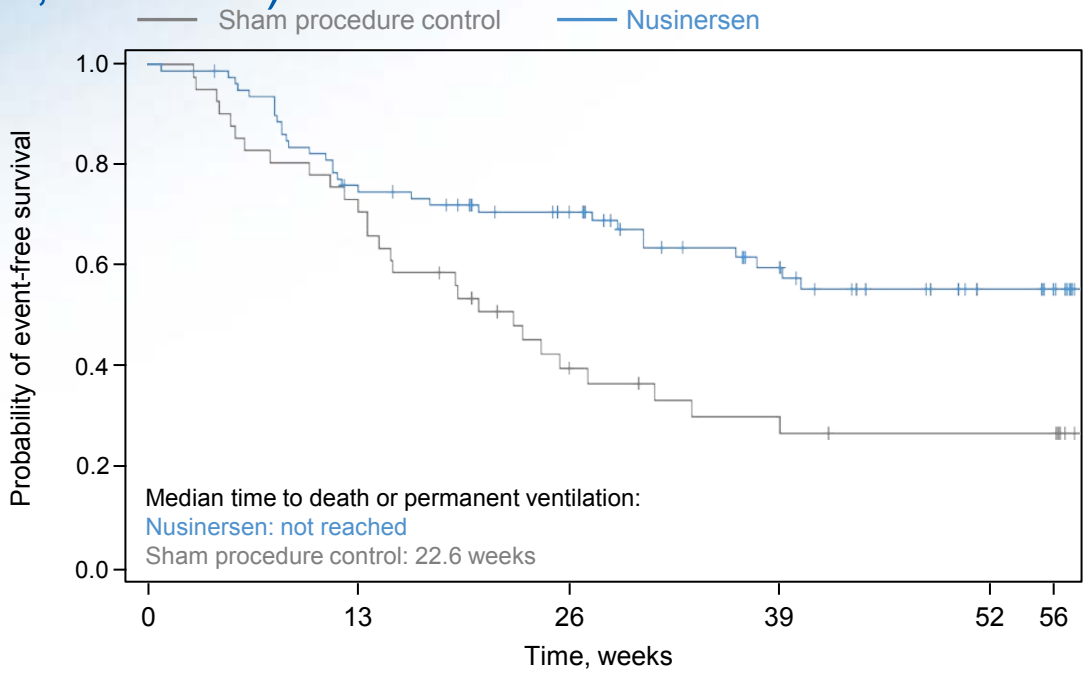
Event-Free Survival: ITT Population at End of Study

- Significantly greater event-free survival[a] in nusinersen-treated infants (HR, 0.53; *P*=.0046)



Nusinersen: 61%

Sham procedure control: 32%

Outcome	Sham procedure control	Nusinersen
Death or permanent ventilation, n (%)	28 (68%)	31 (39%)
Alive and no permanent ventilation, n (%)	13 (32%)	49 (61%)

[a]Event-free survival = time to death or permanent ventilation (permanent ventilation was defined as tracheostomy or ≥16 hours ventilatory support per day for >21 days in the absence of acute reversible event in the determination of an independent endpoint adjudication committee). HR = hazard ratio.

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AE Summary: End of Study Analysis

- No AEs or serious AEs were considered related to treatment by the investigator
- All AEs that led to discontinuation were AEs with fatal outcomes

AE, n (%)	Sham procedure control n=41	Nusinersen n=80
Any AE	40 **(98)**	77 **(96)**
AEs leading to discontinuation	16 **(39)**	13 **(16)**
Treatment-related AE[a]	0	0
Possibly treatment-related AE[a]	6 **(15)**	9 **(11)**
Severe AE	33 **(80)**	45 **(56)**
Serious AE	39 **(95)**	61 **(76)**
Serious AE with fatal outcome	16 **(39)**	13 **(16)**
Respiratory, thoracic and mediastinal disorders	12 **(29)**	7 **(9)**
Cardiac disorders	3 **(7)**	2 **(3)**
General disorders	1 **(2)**	2 **(3)**
Nervous system disorders	0	2 **(3)**

AE = adverse event. [a]Investigators assessed whether the AE was related to study drug. A serious AE was any untoward medical occurrence that resulted in death/risk of death, hospitalisation/prolonged hospitalisation, persistent or significant disability/incapacity or that resulted in a congenital anomaly/birth defect. Severe AEs were defined as symptoms causing severe discomfort, incapacitation or significant impact on daily life; participants reporting >1 AE were counted once for total incidence, using the highest severity.

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AE Summary: End of Study Analysis (cont)

AE, n (%)	Sham procedure control n=41	Nusinersen n=80
Common AEs (≥20% in study participants)		
Pyrexia	24 **(59)**	45 **(56)**
Constipation	9 **(22)**	28 **(35)**
Upper respiratory tract infection	9 **(22)**	24 **(30)**
Pneumonia	7 **(17)**	23 **(29)**
Respiratory distress	12 **(29)**	21 **(26)**
Respiratory failure	16 **(39)**	20 **(25)**
Atelectasis	12 **(29)**	18 **(23)**
Vomiting	8 **(20)**	14 **(18)**
Acute respiratory failure	10 **(24)**	11 **(14)**
Gastroesophageal reflux disease	8 **(20)**	10 **(13)**
Oxygen saturation decreased	10 **(24)**	10 **(13)**
Cough	8 **(20)**	9 **(11)**
Dysphagia	9 **(22)**	9 **(11)**
Most frequent serious AEs (≥10% in either treatment group)		
Respiratory distress	8 **(20)**	21 **(26)**
Respiratory failure	16 **(39)**	20 **(25)**
Pneumonia	5 **(12)**	19 **(24)**
Atelectasis	4 **(10)**	14 **(18)**
Acute respiratory failure	9 **(22)**	11 **(14)**
Pneumonia aspiration	5 **(12)**	8 **(10)**
Cardiorespiratory arrest	5 **(12)**	5 **(6)**
Respiratory arrest	4 **(10)**	5 **(6)**
Viral upper respiratory tract infection	6 **(15)**	3 **(4)**
Bronchial secretion retention	5 **(12)**	1 **(1)**

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Conclusions

- Nusinersen-treated infants demonstrated a clinically and statistically significantly greater percentage of motor milestone responders vs. sham procedure control
- Nusinersen-treated infants demonstrated a statistically significant increase in event-free survival vs. sham procedure control
- Nusinersen was well tolerated and no safety concerns were identified
  - Commonly reported AEs were consistent with those expected in the general population of infants with SMA

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Conclusions (cont)

- The overall findings of the controlled trial in infantile-onset SMA and the open-label uncontrolled trials support the effectiveness of nusinersen across the range of patients with SMA and appear to support the early initiation of treatment with nusinersen
- Participants from ENDEAR have been transitioned to the SHINE open-label extension[1]
  - SHINE is enrolling all participants with SMA who were previously entered into and completed nusinersen investigational studies
  - Safety, efficacy and tolerability will be assessed

1. ClinicalTrials.gov, NCT02594124.

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Acknowledgements

- The authors thank the patients who are participating in this study and their parents/guardians and family members, without whom this effort cannot succeed
- The authors thank the ENDEAR study investigators
- The authors also thank all the contributors to the ENDEAR study, including the clinical monitors, study coordinators, physical therapists, pharmacists and laboratory technicians
- Patient advocacy groups assisted in promoting awareness of these studies

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January 24th 2017

Jill Jarecki, PhD
Chief Scientific Officer
Cure SMA

Dear Dr. Jarecki:

We are writing to confirm the interim clinical data that was presented at the WMS meeting in Granada, Spain on October 8, 2016. These data are from an open-label, Phase 1 clinical trial of 15 patients with spinal muscular atrophy (SMA) Type 1, all with genetically confirmed bi-allelic deletions of the SMN1 gene and two copies of the SMN2 gene. Enrollment was completed in December 2015 with 3 patients in Cohort 1 (low dose) and 12 patients in Cohort 2 (proposed therapeutic dose). A summary of the reported data is as follows:

As of September 15th, 2016, AVXS-101 appeared to have a favorable safety profile and is well tolerated. Four patients experienced treatment related elevation in serum transaminase levels which were clinically asymptomatic and resolved with prednisolone treatment.

Observed increases in motor function appear to be dose dependent, with the low dose cohort increasing an average of 9.0 points from an average baseline CHOP INTEND score of 16.3 points and the high dose cohort increasing an average of 24.8 points from an average baseline CHOP INTEND score of 28.2 points. Comparative natural history data for similar patients with SMA Type 1 has shown that none have been observed scoring above 40 points by 6 months of age, with one transient exception. In this study, 11 out of 12 patients in the high dose cohort reached a CHOP INTEND score ≥40 points, 9 out of 12 patients reached a CHOP INTEND score ≥50 points, and 3 out of 12 patients reached a CHOP INTEND score ≥60 points. In addition, all but one patient in the high dose cohort gained a milestone:

- 11 out of 12 patients could sit with assistance
- 8 out of 12 could sit unassisted, including one patient who achieved the milestone after September 15th
- 7 of 12 patients could roll

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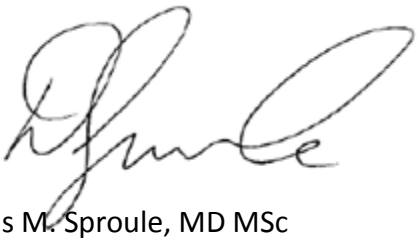
- 2 of 12 were walking independently; these two patients each achieved earlier and important developmental milestones such as crawling, standing with support, standing alone and walking with support.

All patients in Cohort 2 (proposed therapeutic dose) were alive and event free. The median age at last follow-up for Cohort 2 is 17.3 months, with the oldest patient at 27.4 months. Natural history data (Finkel et al 2014) shows a 25% survival rate at 13.6 months.

Sincerely,



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AVXS-101 Clinical Update

Including Motor Milestones as Presented at the World Muscle Society, October 8, 2016

October 10, 2016

Disclaimers

Forward-Looking Statements

This presentation contains forward-looking statements, including: statements about: the timing, progress and results of preclinical studies and clinical trials for AVXS-101, including statements regarding the timing of initiation and completion of studies or trials and related preparatory work, the period during which the results of the trials will become available and our research and development programs. These statements involve substantial known and unknown risks, uncertainties and other factors that may cause our actual results, levels of activity, performance or achievements to be materially different from the information expressed or implied by these forward-looking statements. We may not actually achieve the plans, intentions or expectations disclosed in our forward-looking statements, and you should not place undue reliance on our forward-looking statements. Actual results or events could differ materially from the plans, intentions and expectations disclosed in the forward-looking statements we make. The forward-looking statements in this presentation represent our views as of the date of this presentation. We anticipate that subsequent events and developments will cause our views to change. However, while we may elect to update these forward-looking statements at some point in the future, we have no current intention of doing so except to the extent required by applicable law. You should, therefore, not rely on these forward-looking statements as representing our views as of any date subsequent to the date of this presentation.

Overview of SMA

SMA is a devastating orphan disease – with no current FDA-approved treatments – that results in motor neuron loss and progressive weakness; it is the most common genetic cause of infant death

- Incidence: ~1 in 10,000 live births
- Caused by reduced SMN (survival motor neuron) protein levels from loss of/defective SMN1 gene
- SMA divided into sub-categories, Type 1- 4, with Type 1 being most severe
  - Severity correlates with # of copies of SMN2 backup gene
- Current treatments limited to palliative care

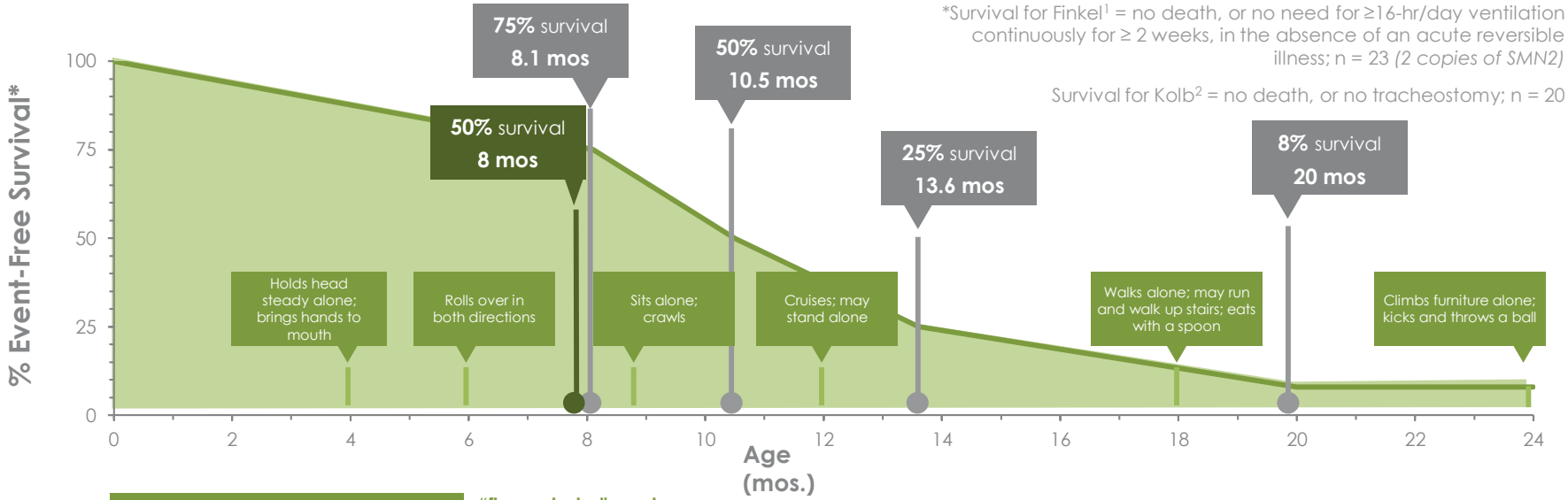
SMA Types: A Devastating Disease

	TYPE 1	TYPE 2	TYPE 3	TYPE 4
SMN2 Copy Number	Two	Three or Four	Three or Four	Four to Eight
Onset	Before 6 Months	6-18 Months	Early childhood to early adulthood (juvenile)	Adulthood (20s-30s) usually after 30
Incidence per Live Birth	Approximately 60%	Approximately 27%	Approximately 13%	Uncommon; limited information available
Developmental Milestones	• Will never be able to sit without support • Difficulty breathing & swallowing • Can't crawl/will never walk	• Will never be able to walk or stand without support	• Stand alone and walk but may lose ability to walk in 30s-40s	• Stand alone and walk but may lose ability to walk in 30s-40s (Same as Type 3)
Survival	• <10% Event free* by two years of age	• 68% alive at age 25	• Normal	• Normal

*Event = Death or ≥16-hr/day ventilation continuously for ≥ 2 wks, in the absence of an acute reversible illness

Natural History of SMA Type 1

More than 90% of SMA Type 1 patients will not survive or will need permanent ventilation support by age 2



Onset of SMA Type 1 by 6 months

Symptoms may present

"floppy baby" syndrome
muscle weakness (legs more than arms)
poor head control
belly breathing
bulbar muscle weakness (weak cry, difficulty swallowing, aspiration)
will never sit unsupported
loss of motor function:

- **NeuroNEXT -- CHOP INTEND decrease of 10.5 points/yr.**
- **PNCR -- CHOP INTEND decrease of 1.27 points/yr.**

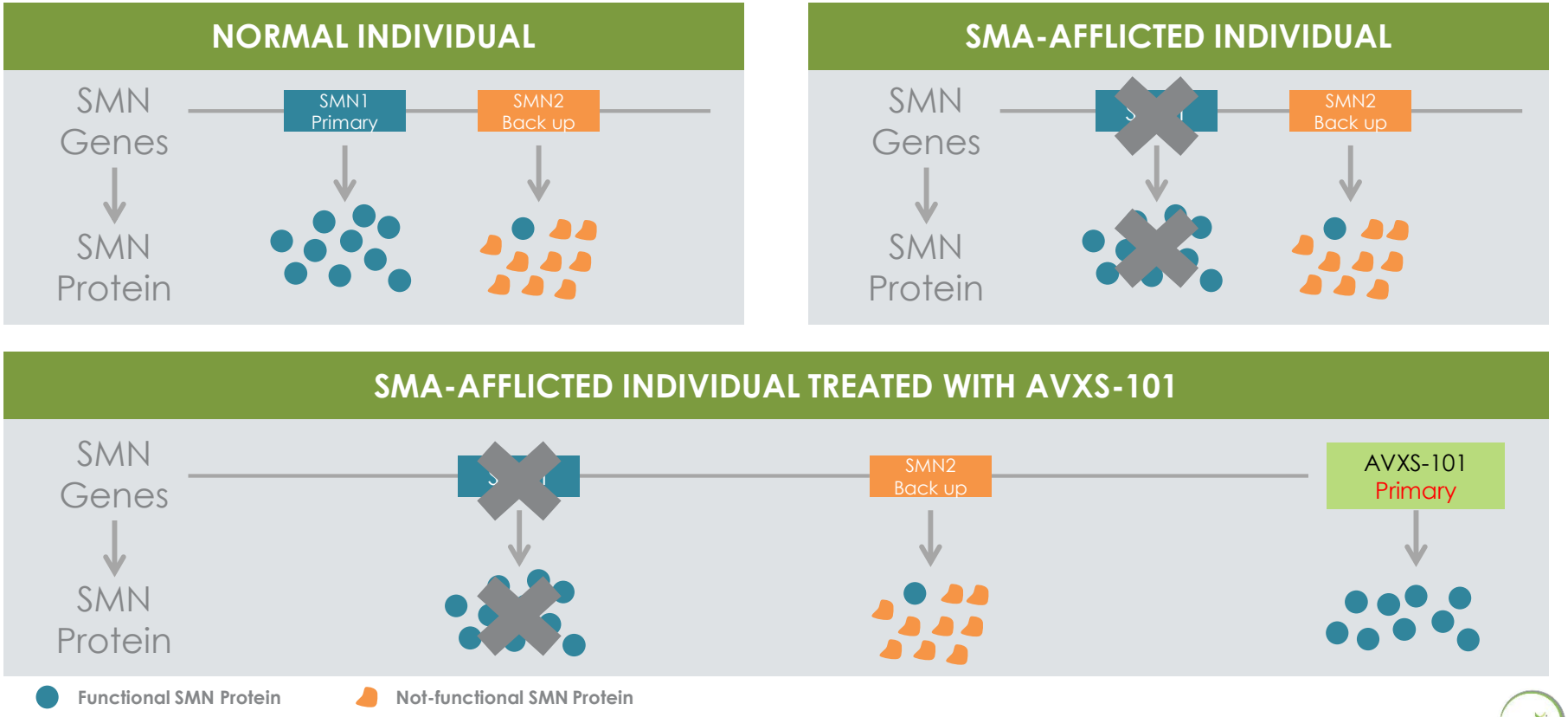
Milestone for a healthy infant

SMA Type 1 survival rates per Finkel[1]

SMA Type 1 survival rate per Kolb[2]

1. PNCR (Finkel)
2. NeuroNEXT (Kolb)

AVXS-101 Targets the Primary SMN Gene

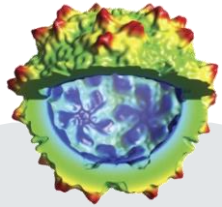


Our Solution: AVXS-101

An Innovative Treatment Approach for SMA

Gene therapy is the right approach for SMA: Monogenic mutation that drives the pathology

Recombinant AAV9 Capsid Shell



scAAV ITR	Continuous Promoter	Human SMN Transgene	scAAV ITR

KEY COMPONENTS	PURPOSE
Recombinant AAV9 Capsid Shell	• Ability to deliver across the blood brain barrier (BBB) and into the spinal cord - Avoids the need for intrathecal delivery when treating infants • Non-replicating virus does not modify the existing DNA of the patient.
scAAV ITR (Self-complementary DNA technology)	• Enables rapid onset of effect which is key in a quickly deteriorating population
Continuous Promoter	• Activates the transgene to allow for continuous and sustained SMN expression
Human SMN Transgene	• Full copy of a stable, functioning SMN gene that is introduced into the cell's nucleus

Rendering adapted from DiMattia et al. Structural Insight into the Unique Properties of Adeno-Associated Virus Serotype 9. J. *Virol*. June 2012.

Phase 1 Trial Design

TRIAL OVERVIEW

Study Site
Nationwide Children's
"When your child needs a hospital, everything matters."

Principal Investigator
Jerry R. Mendell, M.D.

Trial Design
Open-label, dose-escalation

Route of Administration
One-time intravenous infusion through peripheral limb vein
Prednisolone 1 mg/kg 1 day Pre-GT

KEY ENROLLMENT CRITERIA

Inclusion

- 9 months of age / 6 months of age[1] and younger at day of vector infusion with SMA Type 1 as defined by the following features:
  - **Bi-allelic SMN1 gene mutations (deletion or point mutations)**
  - **2 copies of SMN2**
  - **Onset of disease at birth to 6 months of age**
  - Hypotonia by clinical evaluation with delay in motor skills, poor head control, round shoulder posture and hypermobility of joints

Exclusion

- Active viral infection (includes HIV or serology positive for hepatitis B or C)
- Use of invasive ventilatory support (tracheotomy with positive pressure)* or pulse oximetry <95% saturation
- Patients with Anti-AAV9 antibody titers >1:50 as determined by ELISA binding immunoassay
- Abnormal laboratory values considered to be clinically significant
- **Patients with the c.859G>C mutation in SMN2 exon 7 (predicted mild phenotype)[2]**

*Patients may be put on non-invasive ventilatory support (BiPAP) for <16 hours/day at discretion of their physician or study staff
Clinicaltrials.gov Identifier = NCT02122952
[1] Inclusion criteria was 9 months of age and younger for the first nine patients. 6 months of age and younger for the last six patients.
[2] Exclusion criteria related to c.859G>C was for the last six patients.

OBJECTIVES

Primary

- Safety and Tolerability

Secondary

- **Time from birth until death or time to ≥16-hour ventilation continuously for ≥2 weeks** in the absence of an acute reversible illness or perioperatively
- **Video confirmed achievement of ability to sit unassisted**

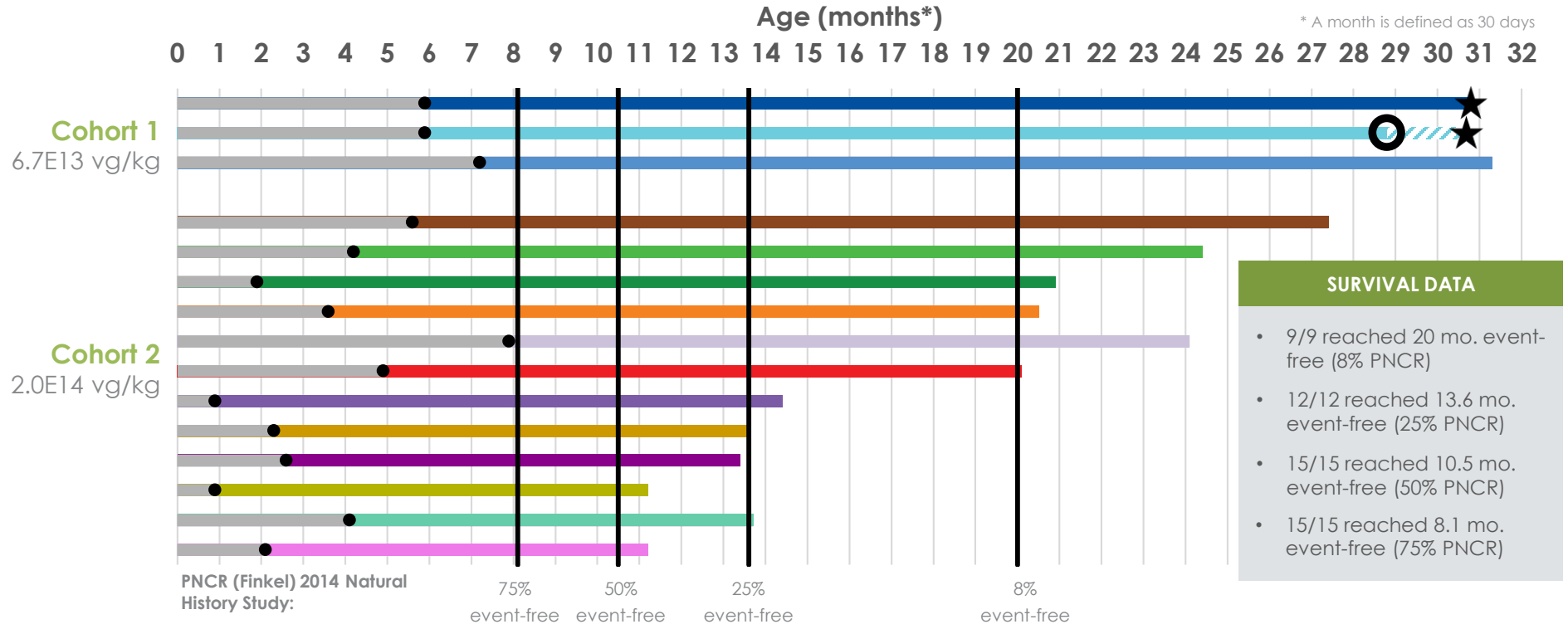
Additional

- CHOP INTEND
- Bayley Motor Scales of Infant/Toddler development – Gross Motor

Exploratory

- Ability Captured Through Interactive Video Evaluation-mini (**ACTIVE-mini**)
- Comp Motor Action Pot (**CMAP**)
- Motor Unit Number (**MUNE**)
- Electrical Impedance Myography (**EIM**)

Event-Free Survival Data – Ongoing Phase 1 Trial



SURVIVAL DATA

- 9/9 reached 20 mo. event-free (8% PNCR)
- 12/12 reached 13.6 mo. event-free (25% PNCR)
- 15/15 reached 10.5 mo. event-free (50% PNCR)
- 15/15 reached 8.1 mo. event-free (75% PNCR)

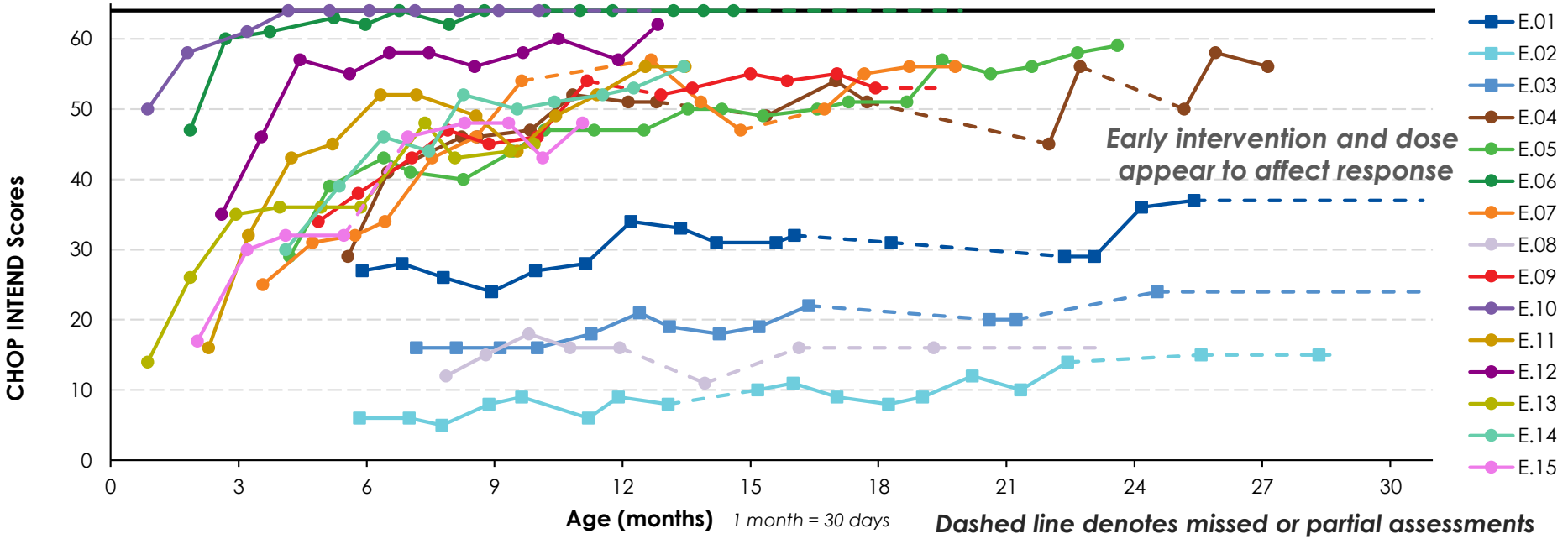
Age at Last Follow-up

Cohort 1*:	30.8 months (median)	30.3 months (mean)
Cohort 2:	17.3 months (median)	17.9 months (mean)

**reflects E.01's age at Last Trial Visit, E.02's age at Pulmonary Event*

● Day of Gene Transfer ★ Last Trial Visit – Age Fixed ○ Pulmonary Event – Age Fixed

CHOP INTEND vs. Age



COHORT 1 (n=3)
Baseline Age (months): 5.9 [median], 6.3 [mean]
Current Age* (months): 30.8 [median], 30.3 [mean]
Mean CHOP INTEND Increase: 9.0 points

** reflects E.01's age at Last Trial Visit, E.02's age at Pulmonary Event*

COHORT 2 (n=12)
Baseline Age (months): 3.1 [median], 3.4 [mean]
Current Age (months): 17.3 [median], 17.9 [mean]
Mean CHOP INTEND Increase**: 24.8 points

*** Calculation includes 2 patients who achieved max score*

Safety Data – Ongoing Phase 1 Trial

AVXS-101 appears to have a favorable safety profile and appears to be generally well-tolerated in patients studied to date

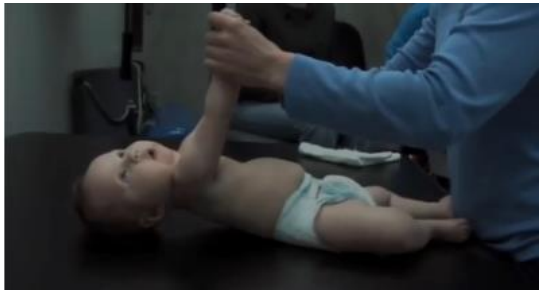
SAFETY AND TOLERABILITY OBSERVATIONS

- **No new treatment-related SAEs or AEs observed**
- As previously reported, a total of 5 treatment-related AEs in 4 patients have been observed to date
  - Treatment-related SAEs and AEs were **clinically asymptomatic** elevated liver function enzymes (LFEs) **resolved with prednisolone treatment***
  - 2 were SAEs experienced by 2 patients (SAEs defined on the basis of laboratory tests showing elevated LFEs)
  - 3 were AEs experienced by 2 patients
- A total 118 AEs (34 SAEs and 84 non-serious AEs) have been reported

*No drug-induced liver injury (DILI) as defined by Hy's Law

Children with SMA Type 1 Never Sit Unassisted

The Natural History of SMA Type 1 is marked by the inability to achieve or maintain developmental milestones



Disease Characteristics

- Disease onset <6 months
- Hypotonia and weakness
- Bulbar muscle weakness
- Difficulty breathing and swallowing
- Inexorable progression to nutritional failure
- Inexorable progression to respiratory failure

Developmental Milestone Prognosis

- Progressive decline in motor function soon after birth
- Rapid loss of any early milestones (e.g. head control, hands to mouth)
- Will never be able to sit unassisted
- Will never be able to roll
- Will never be able to crawl, stand, or walk

Motor Milestone Achievement in Proposed Therapeutic Dose (Interim Data)

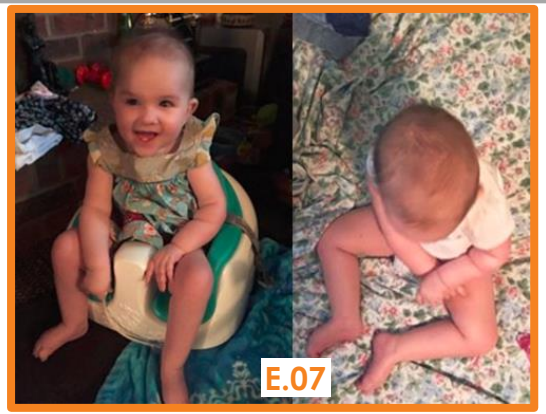
Cohort 2 2.0e14 vg/kg	Age at GT (mos)	Motor Milestone Achievement					
		Brings hand to mouth	Head control	Rolls over (partial)	Rolls over (complete)	Sitting with assistance	Sitting unassisted
E.04	6	✔	✔	✔		✔	✔
E.05	4	✔	✔	✔	✔	✔	✔
E.06	2	✔	✔	✔	✔	✔	✔
E.07	4	✔	✔	✔		✔	✔
E.08	8	✔					
E.09	5	✔	✔	✔	✔	✔	✔
E.10	1	✔	✔	✔	✔	✔	✔
E.11	2	✔	✔	✔		✔	✔
E.12	3	✔	✔	✔	✔	✔	✔
E.13	1	✔	✔	✔	✔	✔	
E.14	4	✔	✔	✔	✔	✔	
E.15	2	✔	✔			✔	

- **7 patients are feeding themselves**
- **5 patients are speaking (1 bilingual)**
- **2 patients are crawling**
- **4 patients are standing with support**
- **2 patients are standing alone**
- **2 patients are walking independently**

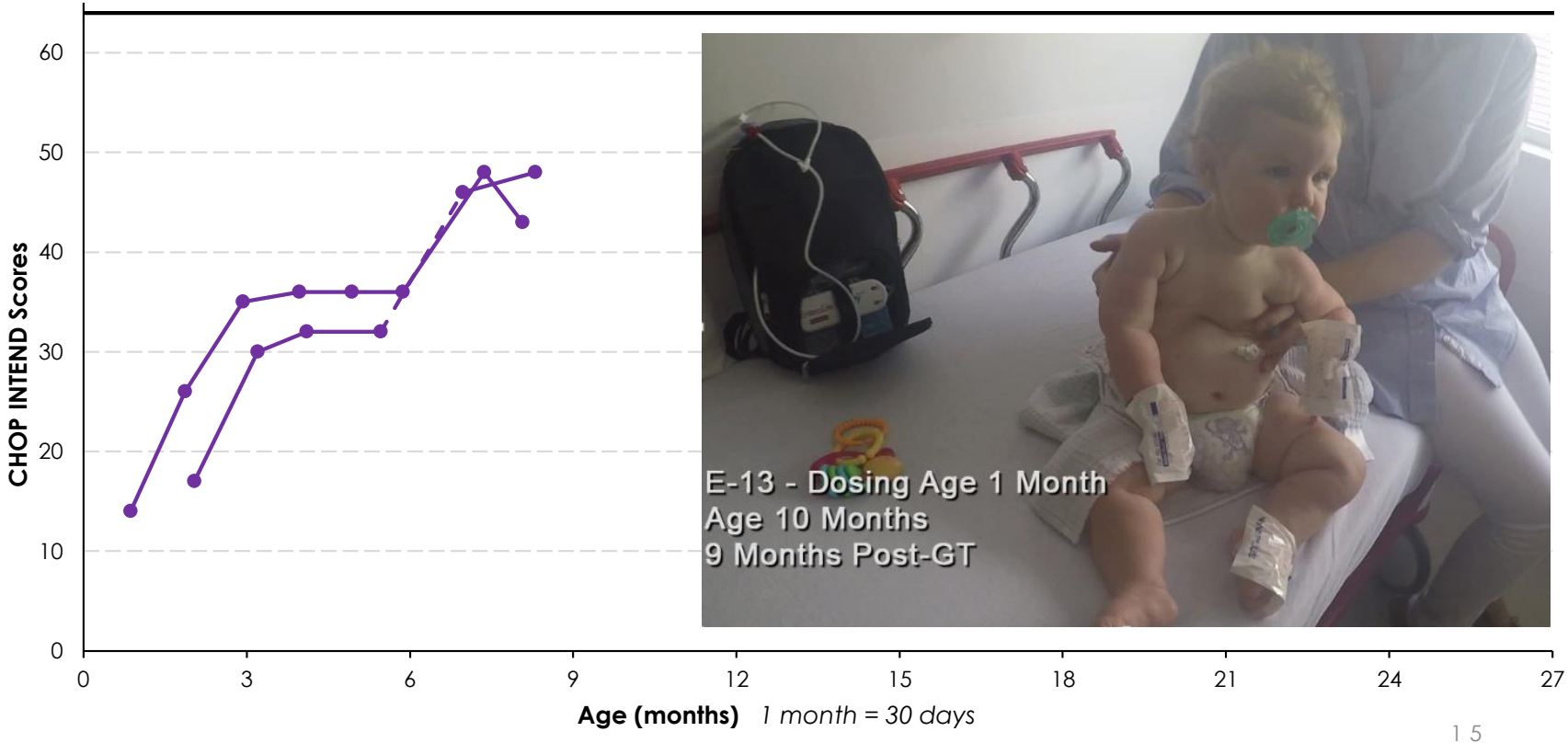
E.11 sitting unassisted and standing with support, E.10 walking independently, E.12 standing with support, and E.15 feeding self were confirmed with video evidence after September 15, 2016.

Two-thirds of Patients in Cohort 2 are Sitting Unassisted

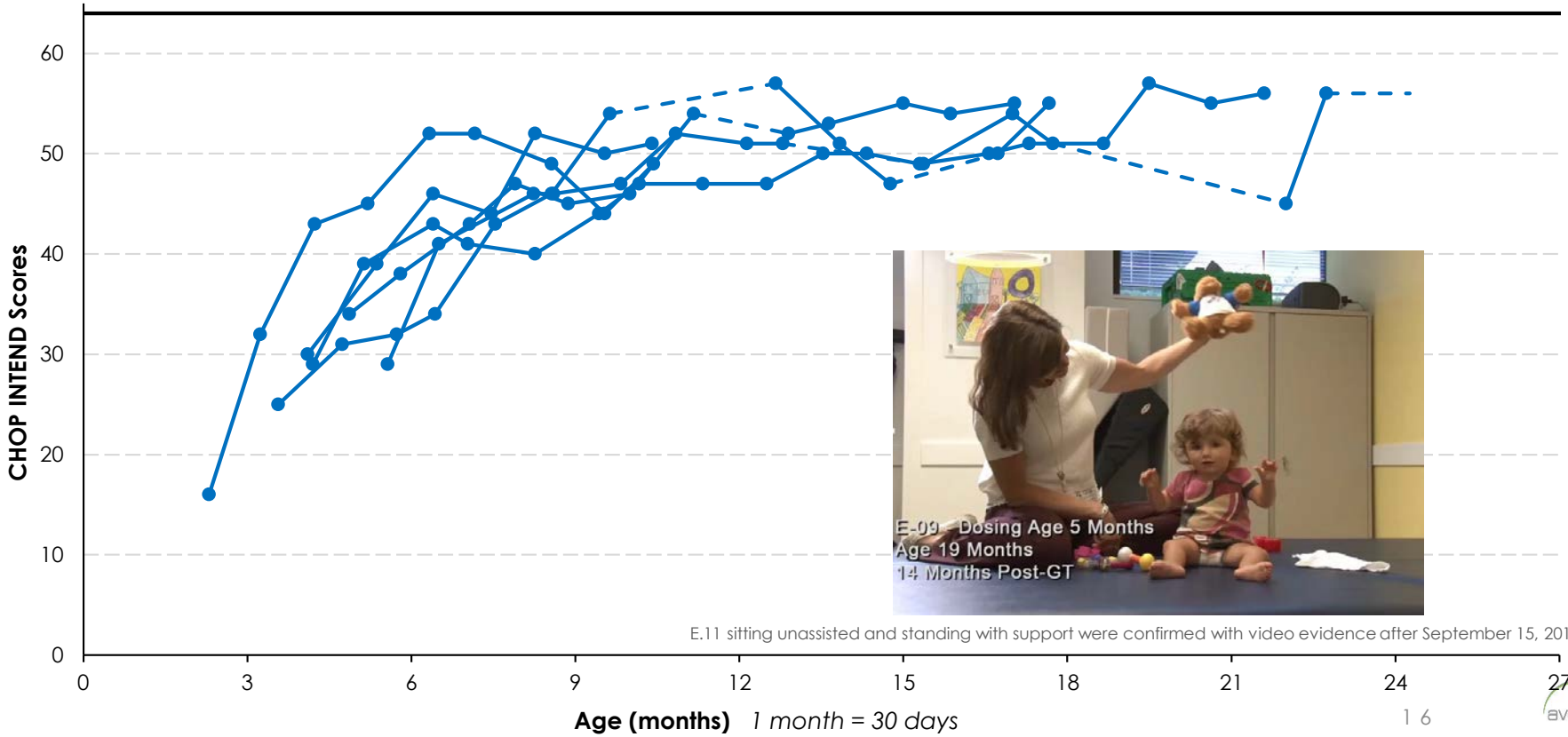
Inability to Sit is the Hallmark Motor Milestone of SMA Type 1



Patients with CHOP INTEND ≥ 40

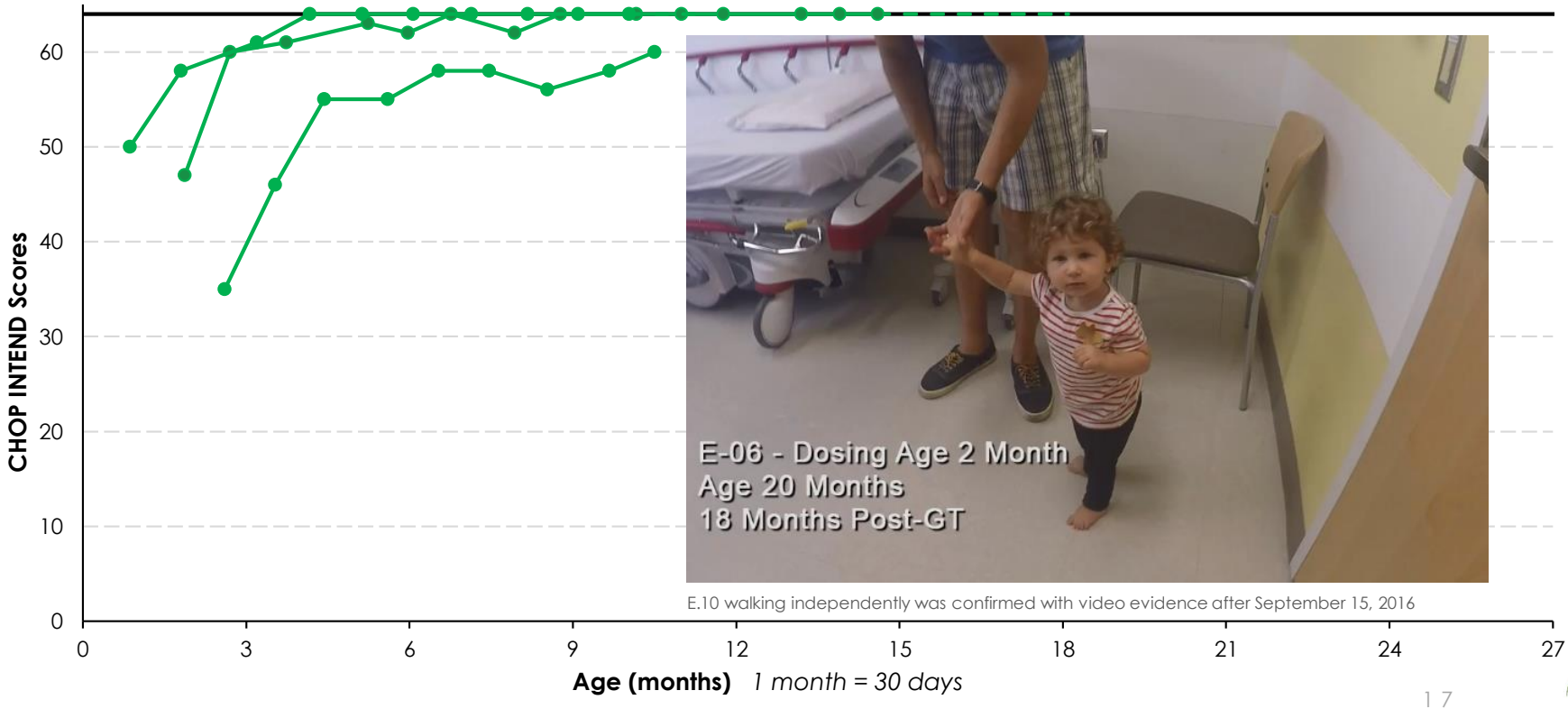


Patients with CHOP INTEND ≥ 50



E.11 sitting unassisted and standing with support were confirmed with video evidence after September 15, 2016

Patients with CHOP INTEND ≥ 60



E.10 walking independently was confirmed with video evidence after September 15, 2016

Summary: Ongoing Phase 1 Data

- AVXS-101 appears to have a favorable safety profile and appears to be generally well tolerated
  - A total 118 AEs (34 SAEs and 84 non-serious AEs) have been reported
  - No additional treatment-related AEs have been observed beyond the previously reported five elevated LFEs experienced by 4 patients, two of which were Grade 4 SAEs. All were resolved following prednisolone treatment.

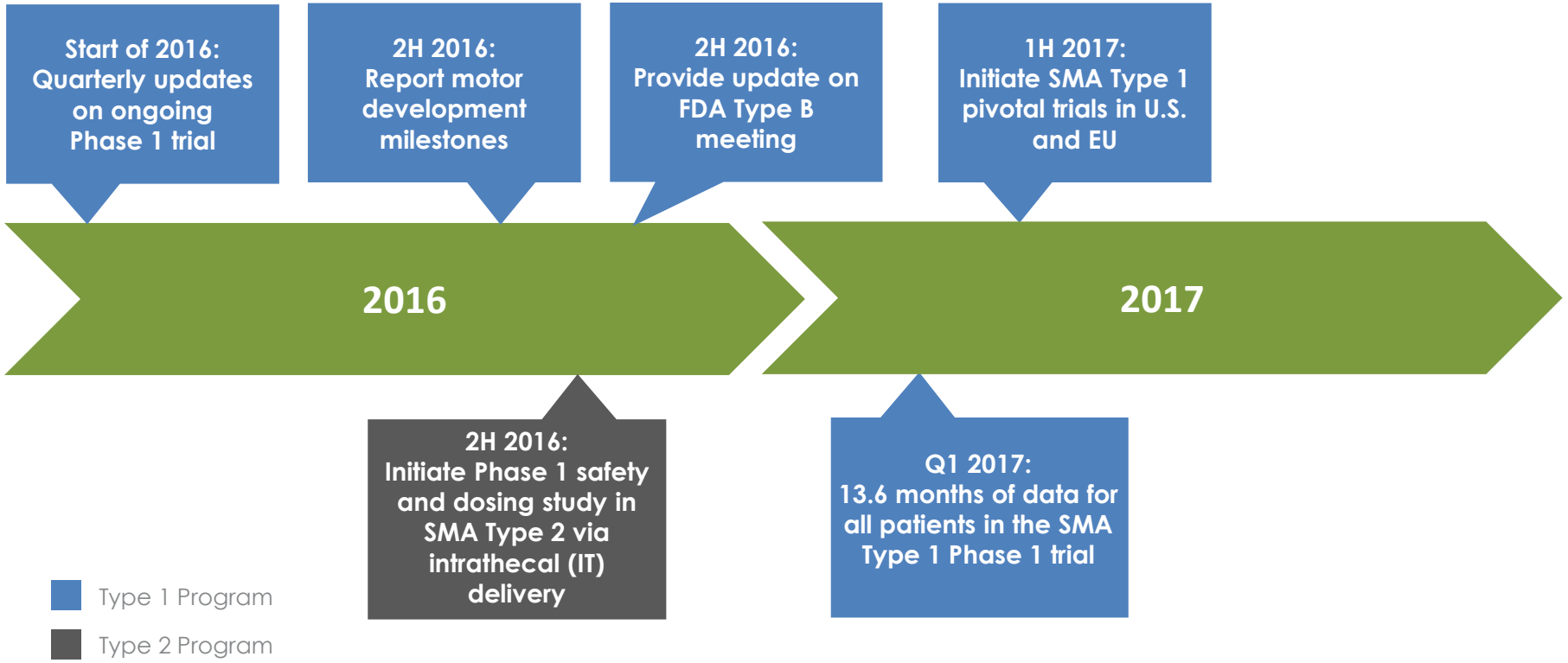
- Significant and sustained increases in motor function appear to be dose-dependent
  - Cohort 1 (n=3) has increased an average of 9.0 points from an average baseline CHOP INTEND score of 16.3 points.
  - Cohort 2 (n=12) has increased an average of 24.8 points from an average baseline CHOP INTEND score of 28.2 points.
    - Finkel 2014 observed no SMA Type 1 patients scoring above 40 points (1 transient exception)
    - 11 out of 12 patients in Cohort 2 reached CHOP INTEND ≥40 points
    - 9 out of 12 patients in Cohort 2 reached CHOP INTEND ≥50 points
    - 3 out of 12 patients in Cohort 2 reached CHOP INTEND ≥60 points

- All patients in Cohort 2 (except E.08) have achieved at least one motor development milestone
  - 11 out of 12 patients can sit with assistance and 8 out of 12 can sit unassisted†
  - 7 of 12 patients can roll over completely; 7 of 12 are feeding themselves; 5 of 12 are speaking; 4 of 12 are standing with support
  - 2 patients who are now walking independently† have also each achieved crawling, standing with support, standing alone, and walking with support

- AVXS-101 administration has resulted in marked and positive impact on the disease course of SMA Type 1.
  - The median age at last follow-up of all 15 patients is 20.5 months, with the oldest patient at 31.3 months of age.* One patient in Cohort 1 reached Pulmonary Event at 28.8 months of age but has since returned below the 16 hrs/day event threshold.
  - All patients in Cohort 2 are alive and event-free. The median age at last follow-up for Cohort 2 is 17.3 months, with the oldest patient at 27.4 months of age.

**E.01's age is calculated as of Last Trial Visit (May 2016); E.02's age is calculated at Pulmonary Event (July 2016)*
†A patient in this group was confirmed to have achieved this milestone with video evidence after Sept 15, 2016

Clinical Development Milestones



Question and Answer Session

October 10, 2016

Thank You

February 3, 2017

Dr. Jill Jarecki
Chief Scientific Officer, Cure SMA
925 Busse Road
Elk Grove Village, IL, 60007

Dear Dr. Jarecki,

I am writing to provide my support for your nomination of Spinal Muscular Atrophy (SMA) to the Recommended Uniform Screening Panel and confirm the data provided in the nomination form describing the Taiwanese newborn screening pilot are accurate.

From November 2014 through September 2016, I led a pilot newborn screen at the National Taiwan Hospital in Taipei, Taiwan where 120,267 newborns were screened for Spinal Muscular Atrophy (SMA).

The pilot screening utilized a real-time PCR TaqMan® single nucleotide polymorphism (SNP) genotyping assay on a StepOnePlus™ RT-PCR 96-well System (Applied Biosystems). The assay targets a SNP in SMN1 intron 7 to distinguish SMN1 from SMN2. DNA was extracted from 3-mm dried blood spot punches to detect homozygous deletions in SMN1 intron 7. We implemented a second tier assay during this screen using digital droplet PCR (ddPCR) to exclude false positives and to measure SMN2 copy number.

This screening paradigm was validated by testing the dried blood spot samples of 2937 anonymous newborns and 9 DNA samples with known SMN1 and SMN2 copy numbers. Provided that DNA can be extracted from a given blood spot, the screen is valid. Quality controls in each 96-well plate included a water blank, a filter paper blank, and 3 DNA samples with known SMN1:SMN2 copy numbers, 0:2 (affected), 1:2 (carrier), and 2:2 (normal). The presence of DNA is assessed by ensuring amplification of the internal control gene. The detection rate for the assay is 100%; thus, all specimens with homozygous deletions of SMN1 have screened positive with this method. However, this screening method does not detect point mutations in the SMN1 gene, which are present in around 5% of SMA. It does detect a hybrid SMN1 allele present in the Taiwanese population resulting in identification of some false positives which is why the second tier assay was implemented. This test is intended as a laboratory-developed test and is not FDA-approved.

The pilot screen conducted on 120,267 newborns in Taiwan detected 15 positives by the primary test, 8 of which were ruled as false positives by second tier testing. The number of false negatives is unknown. The seven infants who screened positive by the first and second tier tests were molecularly confirmed to have a homozygous SMN1 deletion by MLPA assay.

Table 1 below provides current information regarding the seven positive cases detected.

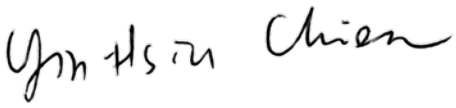
Table 1. Infants screened positive for SMA during the Taiwanese newborn screening pilot study.

Patient Number	Clinical Follow up
1	Normal at 25 months of age
2	Family refused further contact. Sibling has SMA.
3	Onset of SMA at 13 months of age
4	Respiratory failure at birth and death at 3 months of age
5	Onset of SMA at 2 months of age
6	Enrolled in Biogen NUTURE trial for pre-symptomatic infants
7	Enrolled in Biogen NUTURE trial for pre-symptomatic infants

We are currently in the process of submitting these data for publication in a peer-reviewed journal.

Please feel free to contact me if you have questions about these data.

Regards,



Yin-Hsiu (Nancy) Chien, M.D., Ph.D

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January 25, 2017

Dear Members of the Advisory Committee on Heritable Disorders in Newborns and Children,

We are providing this letter as additional support for the proposal to include spinal muscular atrophy on the recommended uniform screening panel. We have conducted a successful, consented pilot study of newborn screening for spinal muscular atrophy in New York State for the last 12 months. We enrolled a total of 3705 babies from three hospitals in the New York Presbyterian healthcare system in New York City. Testing was performed at the Wadsworth Center, Albany, New York in addition to routine newborn screening.

Molecular genetic testing was performed to validate the assay which is a custom TaqMan real-time polymerase chain reaction (PCR) assay targeting the SMN1 exon 7 deletion and a fragment of RNaseP (used as an internal control gene) run on a real-time PCR platform. Of the 3705 babies screened, 3484 (94.04%) had a normal screening result, 57 (1.538%) were carriers, and 1 (0.027%) infant was homozygous for the SMN1 exon 7 deletion. The incidence of SMA in our study population was 1 in 3705. The carrier frequency in this cohort was 1 in 65. Results of this testing were made available along with the routine newborn screening report.

We had no problems with sample failures, false positives, or known false negatives. We believe this system is robust, accurate, and scalable to newborn screening programs around the country. We would be glad to provide any expertise to help you evaluate this application for the addition of spinal muscular atrophy to the recommended uniform screening panel.

Sincerely,

Wendy K. Chung, MD PhD
Kennedy Family Associate Professor of Pediatrics and Medicine
Columbia University

Denise Kay, PhD
Research Scientist, Newborn Screening Program
Wadsworth Center

Michele Caggana, Sc.D., FACMG
Director, Newborn Screening Program
Wadsworth Center

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Linh.Hoang@perkinelmer.cor

February 3, 2017

To Whom It May Concern:

This letter is to confirm that the data from PerkinElmer presented in the SMA nomination form submitted by Cure SMA is data that was generated from a prototype assay under development at PerkinElmer. The assay under development is a 5-plex real-time PCR assay detecting and quantifying SMN1 and SMN2, TREC and KREC and using the RNase P target as an internal control. We are currently testing this assay in an R&D study on de-identified putative normal newborn dried blood spot samples as well as approximately 70 SMN1 and SMN2 characterized reference samples. We plan to test up to 3,000 samples.

We fully support the nomination of SMA by the Cure SMA foundation.

Sincerely,

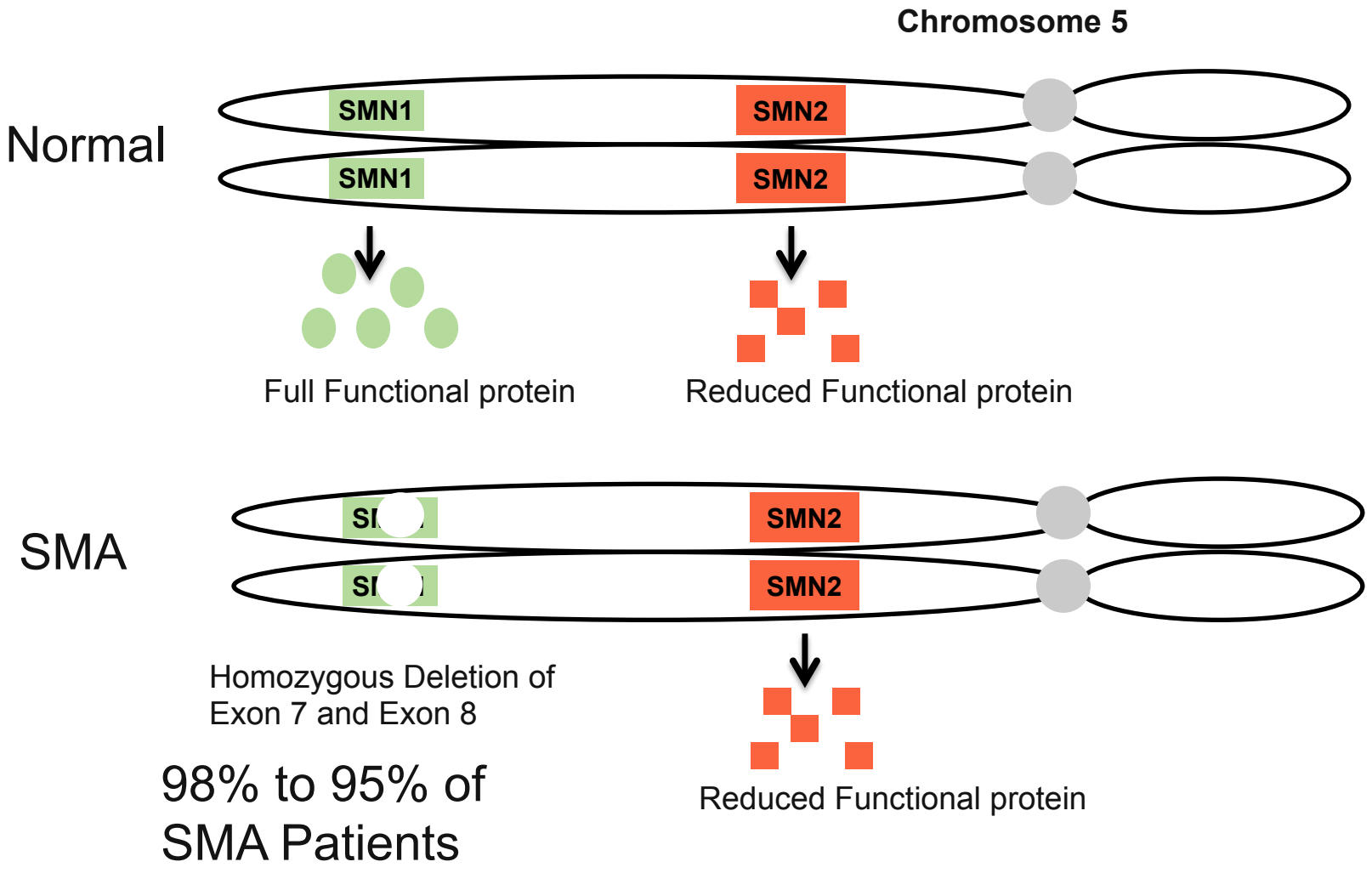
Linh Hoang
Vice President Neonatal Screening

Preliminary Data from Prototype 5-plex real-time PCR Assay

PerkinElmer®
For the Better

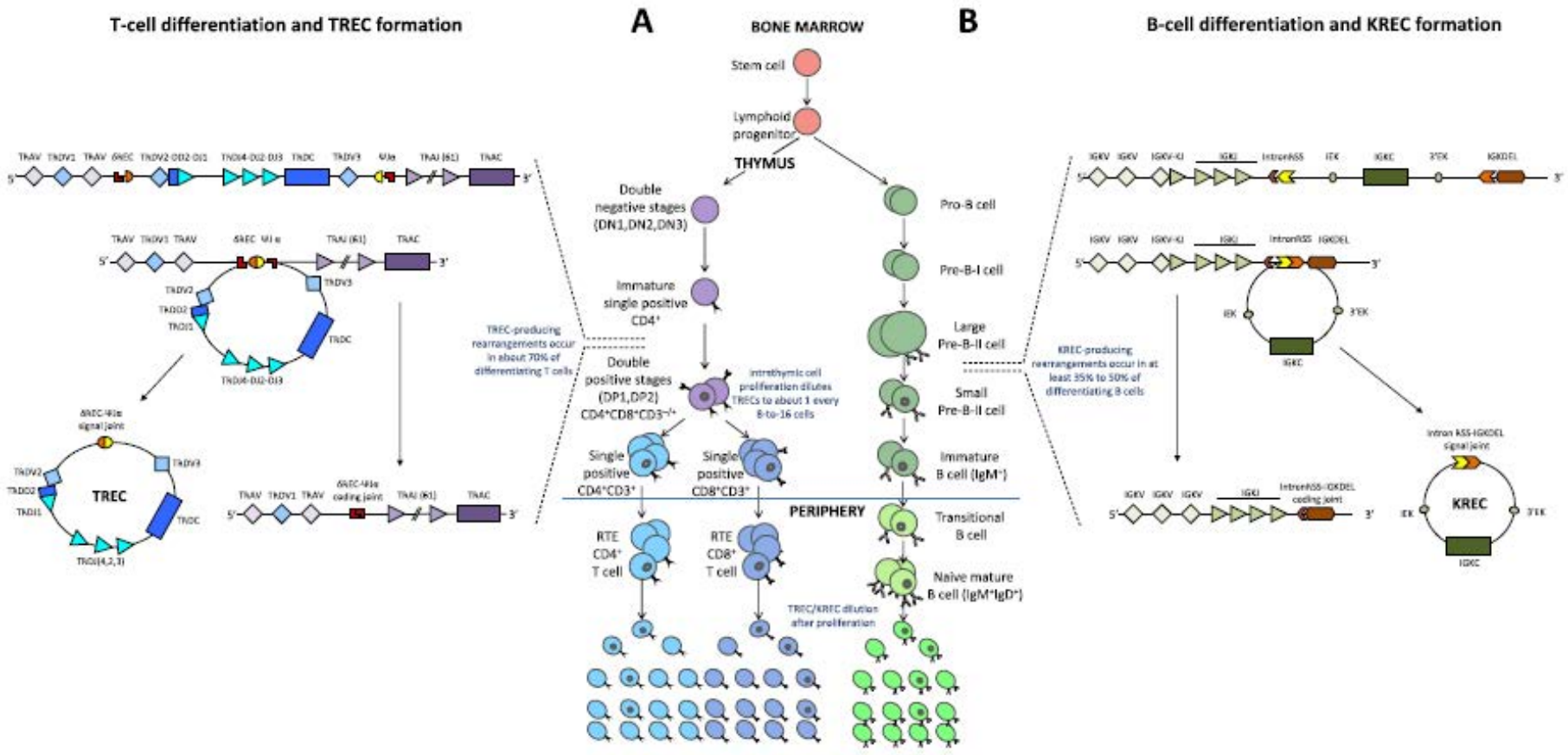
February, 2017

Genomics of the SMA Disorder



2-5% of SMA patients are the result of mis-sense mutation or gene conversion events from SMN2

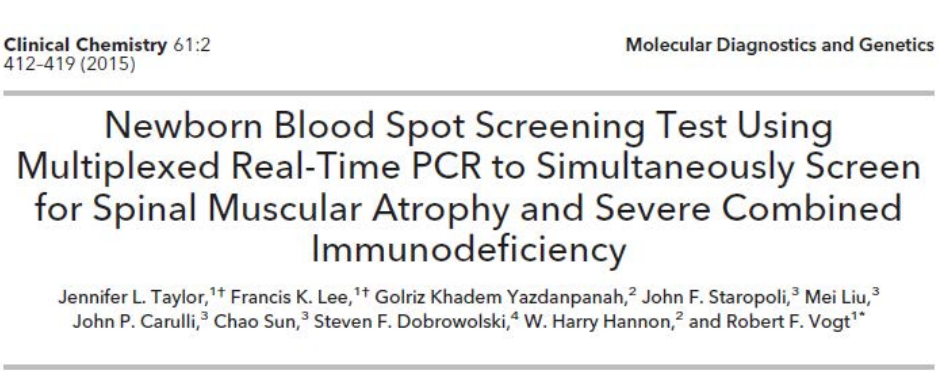
TREC and KREC molecules are byproduct of T-cell and B-cell Maturation



Serana, et al. J. Trans. Med.; 11:119, (2013)

Roots of the PCR assay: first we reproduced published works

- Taylor et al: TREC + SMN1 + RNAse P (2015, CDC)
- Mensen et al: TREC + KREC (2013, Charité – Universitätsmedizin Berlin)

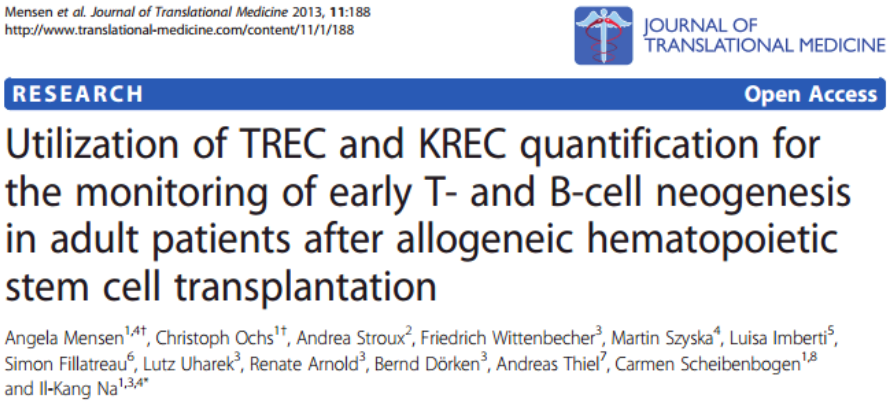


Clinical Chemistry 61:2
412-419 (2015)

Molecular Diagnostics and Genetics

Newborn Blood Spot Screening Test Using Multiplexed Real-Time PCR to Simultaneously Screen for Spinal Muscular Atrophy and Severe Combined Immunodeficiency

Jennifer L. Taylor,[1†] Francis K. Lee,[1†] Golriz Khadem Yazdanpanah,[2] John F. Staropoli,[3] Mei Liu,[3] John P. Carulli,[3] Chao Sun,[3] Steven F. Dobrowolski,[4] W. Harry Hannon,[2] and Robert F. Vogt[1*]



Mensen et al. Journal of Translational Medicine 2013, 11:188
http://www.translational-medicine.com/content/11/1/188

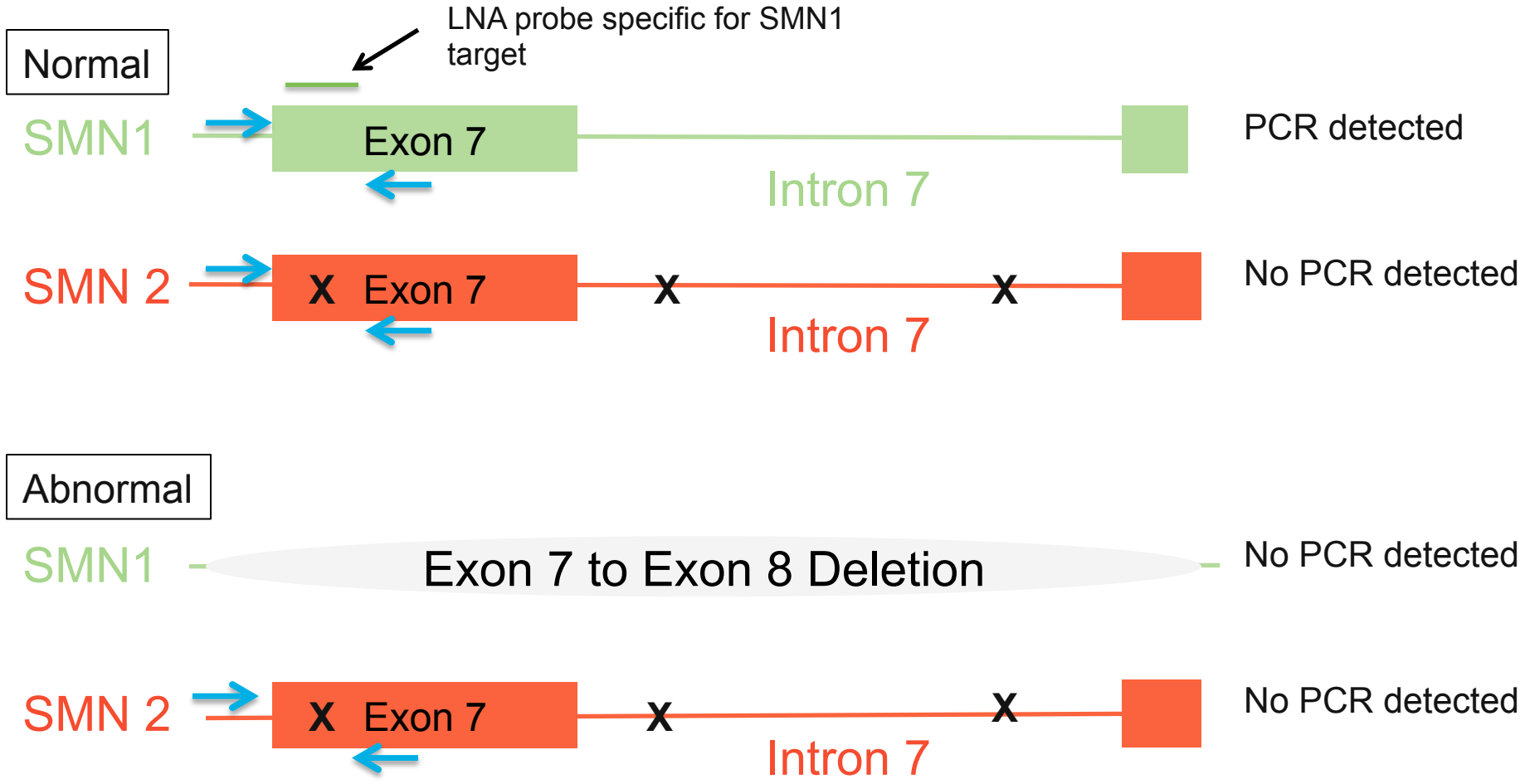
JOURNAL OF TRANSLATIONAL MEDICINE

RESEARCH Open Access

Utilization of TREC and KREC quantification for the monitoring of early T- and B-cell neogenesis in adult patients after allogeneic hematopoietic stem cell transplantation

Angela Mensen[1,4†], Christoph Ochs[1†], Andrea Stroux[2], Friedrich Wittenbecher[3], Martin Szyska[4], Luisa Imberti[5], Simon Fillatreau[6], Lutz Uharek[3], Renate Arnold[3], Bernd Dörken[3], Andreas Thiel[7], Carmen Scheibenbogen[1,8] and Il-Kang Na[1,3,4*]

Assay to target single base mutation in Exon 7

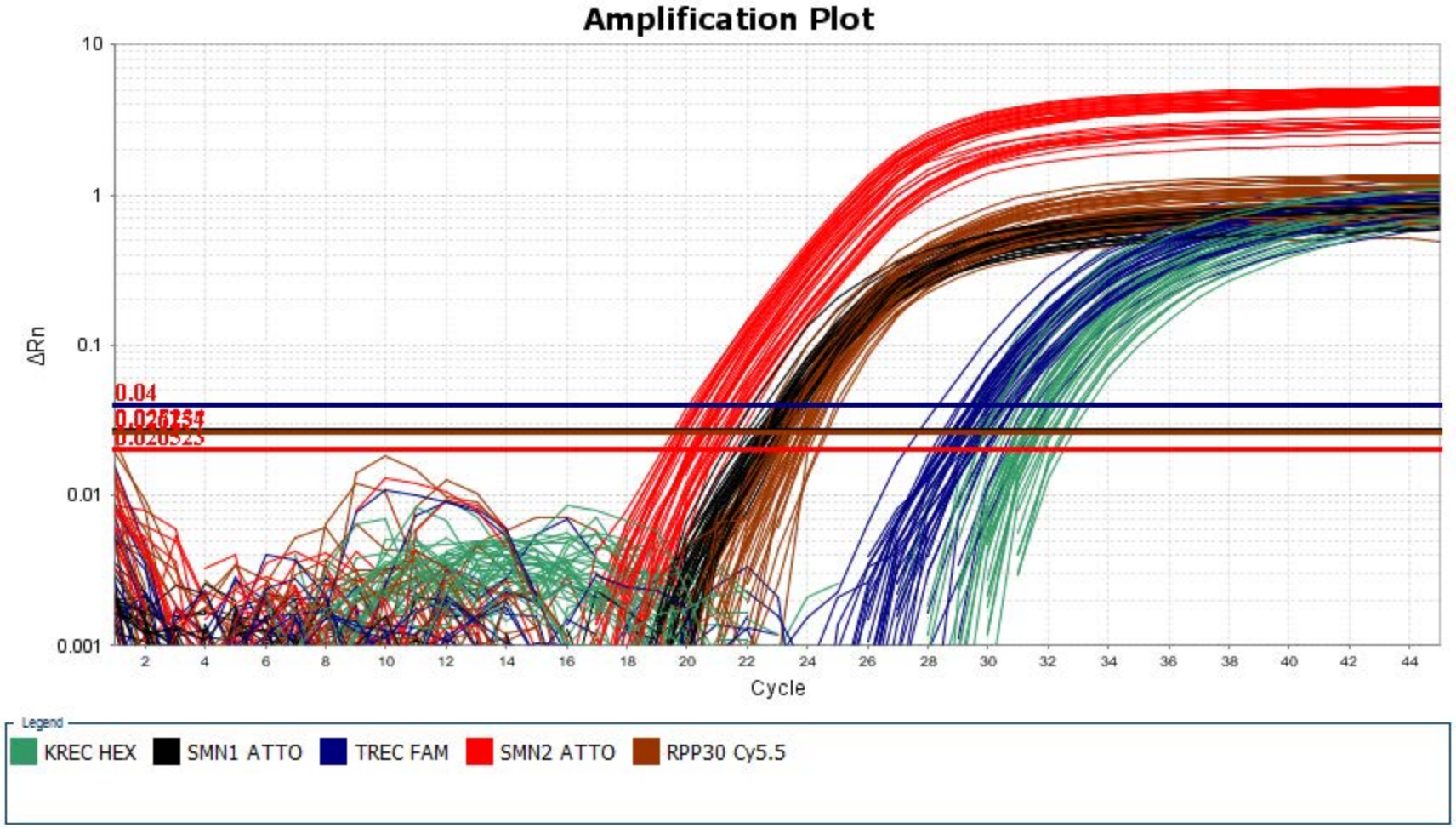


 Homozygous deletion of SMN1 Exon 7 will be detected by absence of PCR product.

The prototype 5-plex assay

Locus	Fluorophore	Function
SMN1	ATTO 550	– detects deletion of exon 7, SMA
SMN2	ATTO 647N	– quantitates SMN2 copy number
TREC	FAM	– very low level indicates SCID
KREC	HEX	– very low level indicates XLA
RNase P	Cy5.5	– internal control + reference gene for copy number calculation
	ROX	– reference dye, improves qPCR robustness

5-plex prototype assay on DNA from newborn DBS

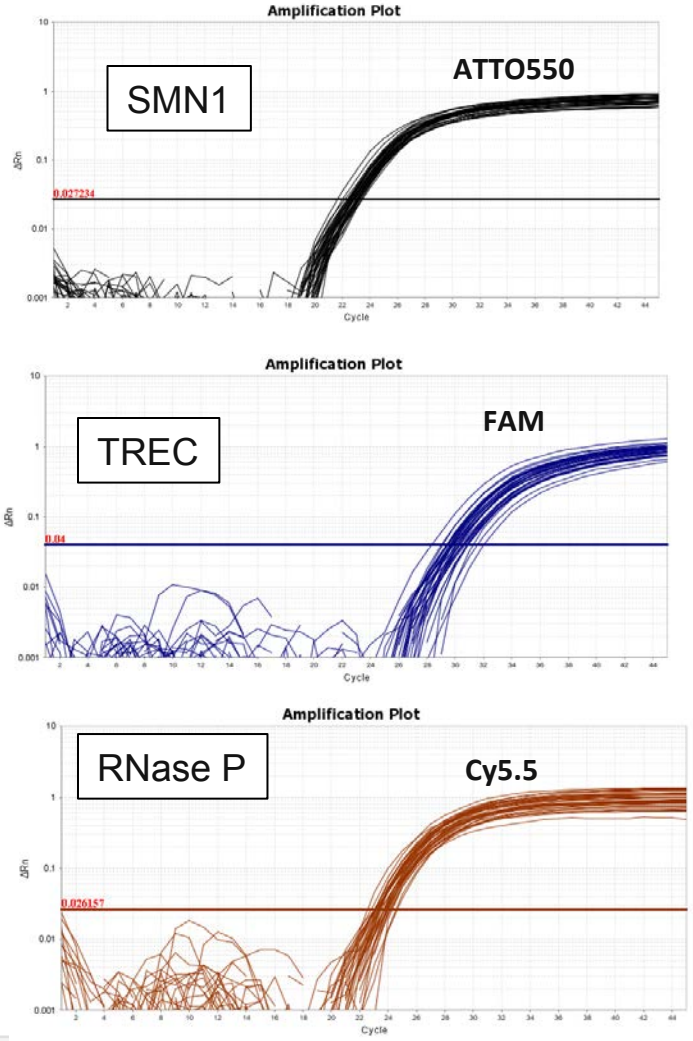


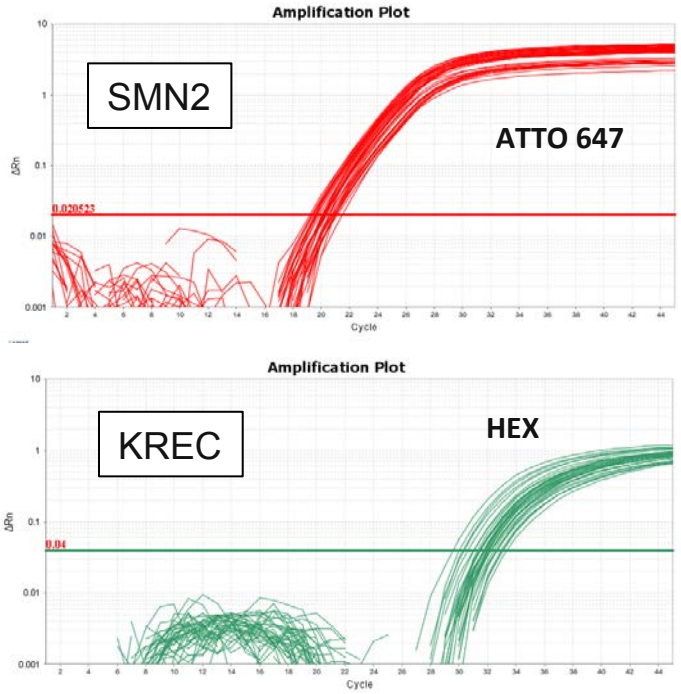
TREC and KREC are not genomic and are less abundant

 One plate, 75 de-identified newborn DBS samples + 21 reference samples

PerkinElmer

5-plex prototype assay on DNA from newborn DBS

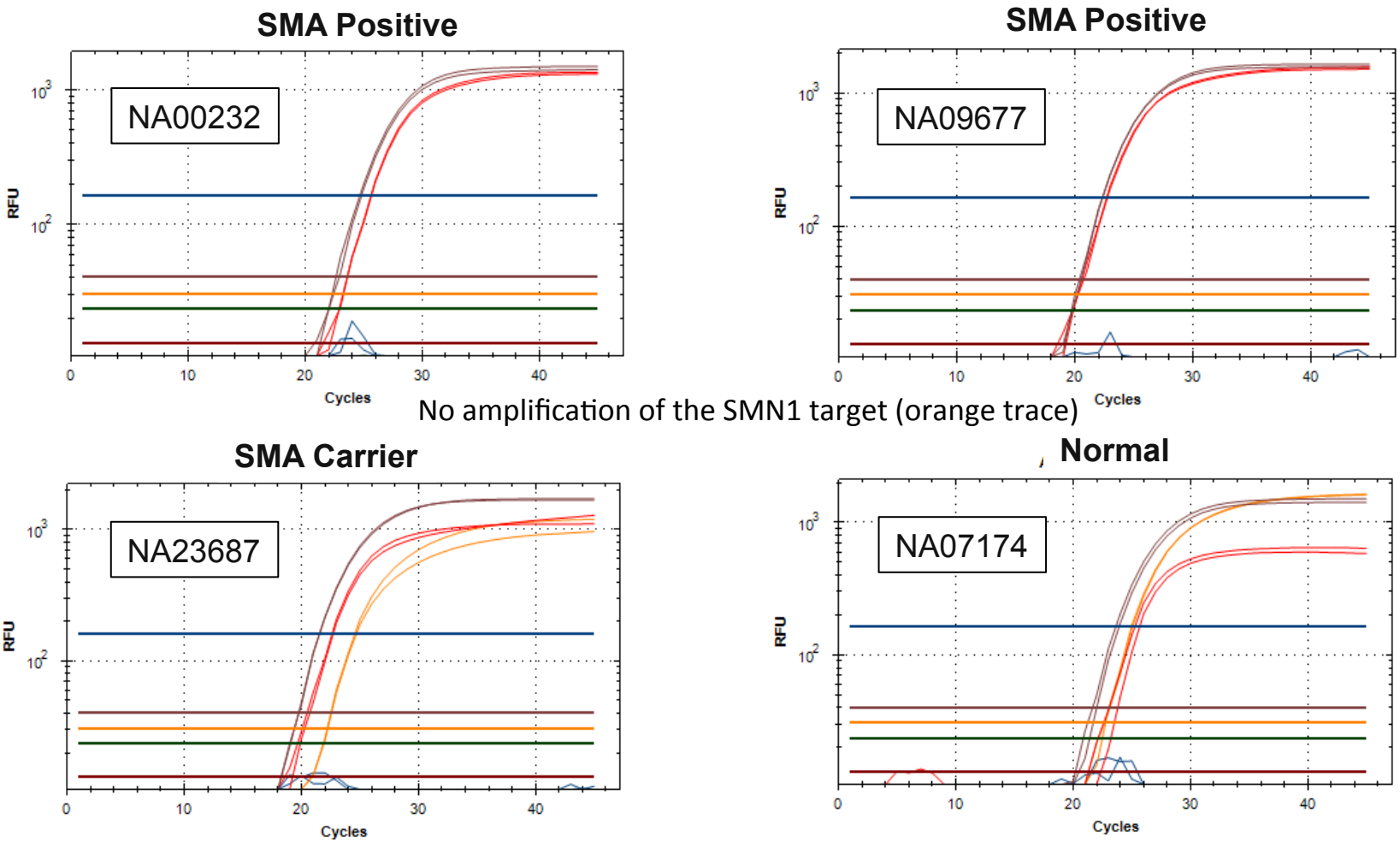




Note that the less abundant TREC & KREC reach the threshold about 4 cycles later than the targets in genomic DNA

PerkinElmer

5-plex run on Coriell reference DNA samples



No amplification of the SMN1 target (orange trace)

Carrier SMN1 crosses threshold around cycle 22, normal at about 21

Coriell cell lines have no TRECs and no KRECs

TREC/FAM KREC/HEX SMN1/ATTO 550 SMN2/ATTO 647 RPP30/Cy5.5

PerkinElmer

SMN1 copy number confirmed in the 5-plex assay on characterized reference samples

The copy number is calculate using an ΔΔCt algorithim * using the Rnase P amplification as a reference gene

Sample	Run	Copies (Biogen)	Copies (PKI)
SMA 110	a	0	0
	b	0	0
SMA 111	a	0	0
	b	0	0
SMA 112	a	0	0
	b	0	0
SMA 113	a	0	0
	b	0	0
SMA 165	a	0	0
	b	0	0
SMA166	a	0	0
	b	0	0
SMA 167	a	0	0
	b	0	0

Sample	Run	Copies (Biogen)	Copies (PKI)
SMA 106	a	1	1
	b	1	1
SMA 107	a	1	1
	b	1	1
SMA 108	a	1	1
	b	1	1
SMA 119	a	1	1
	b	1	1
SMA 120	a	1	1
	b	1	1
SMA121	a	1	1
	b	1	1

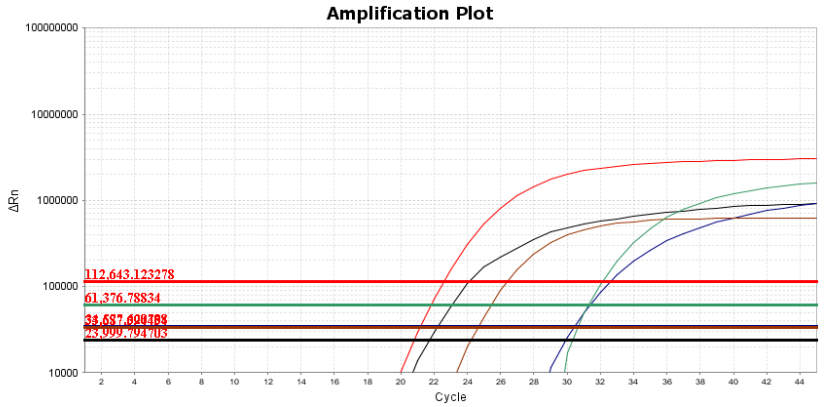
Sample	Run	Copies (Biogen)	Copies (PKI)
SMA 174	a	3	3
	b	3	3
SMA 175	a	3	3
	b	3	4
SMA 176	a	3	3
	b	3	4
SMA 177	a	3	3
	b	3	3
SMA 178	a	3	3
	b	3	3

Reference samples SMN1 numbers characterized by digital PCR and generously provided for this project by Biogen

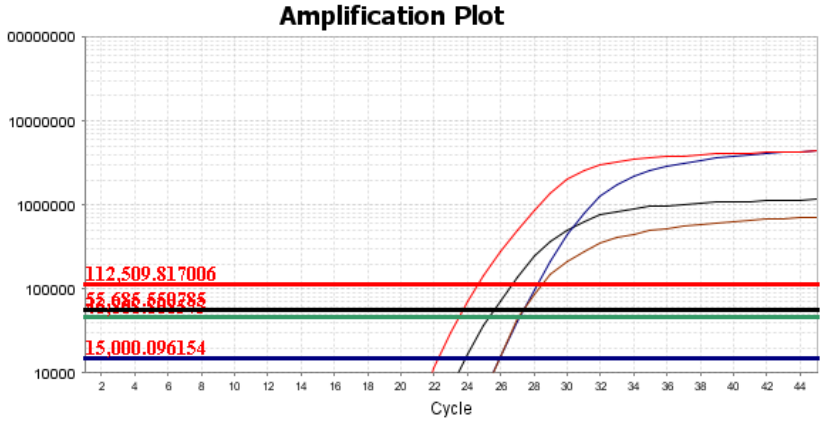
* Rao, et al. Biostat. Bioinforma. Biomath. 3 (3), pp 71-85 (2013)

Control DBS for TREC and KREC from the CDC

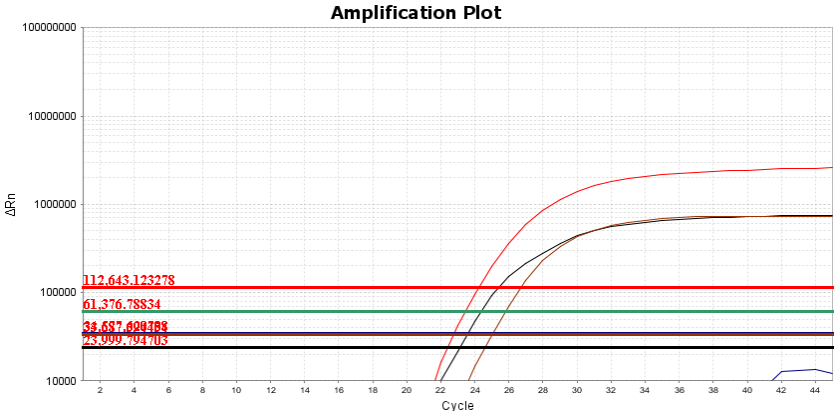
Cord Blood, Normal



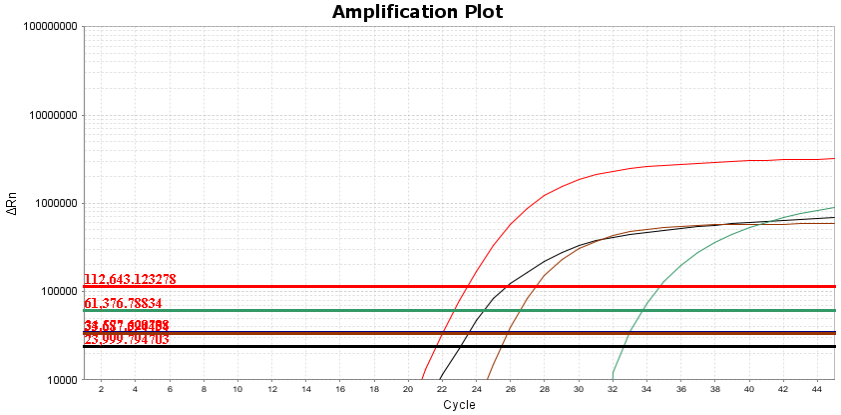
KREC minus construct



TREC, KREC minus construct



TREC minus construct



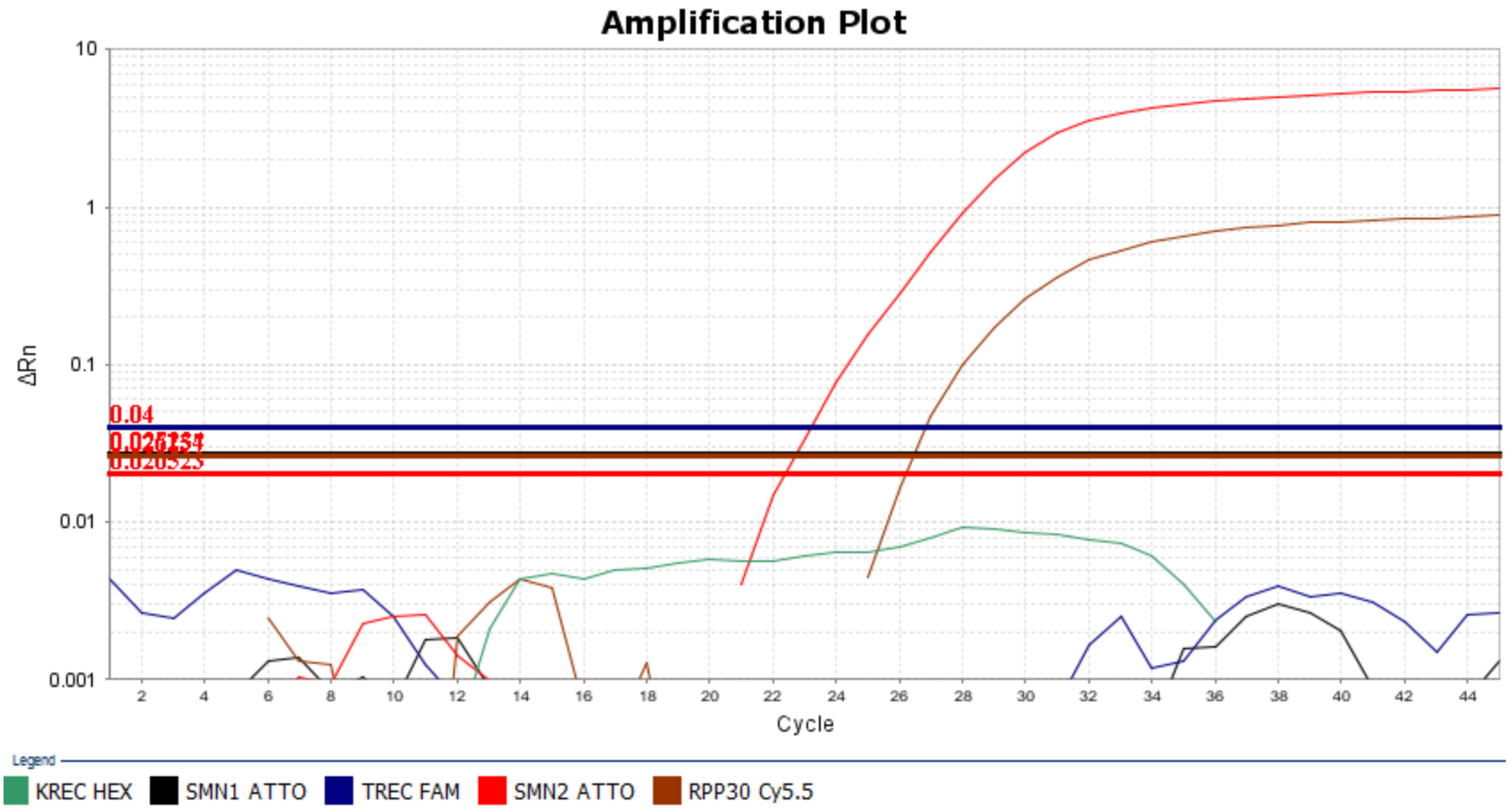
DBS controls generously provided by Francis Lee (CDC)

KREC HEX | SMN1 ATTO | TREC FAM | SMN2 ATTO | RPP30 Cy5.5

PerkinElmer

Prototype PerkinElmer DBS controls

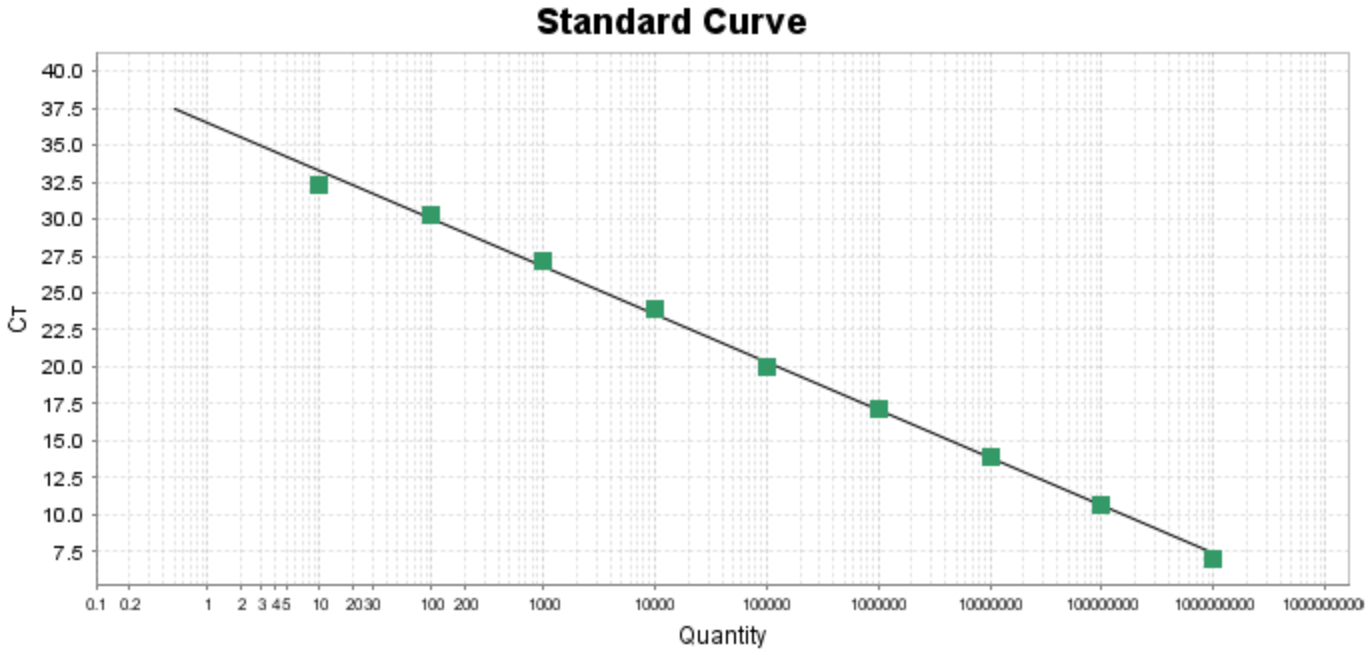
SMA-positive Coriell cell line spiked into red blood cells



 No TREC & KREC because it's a cell line. SMN1 no amplification.

PerkinElmer

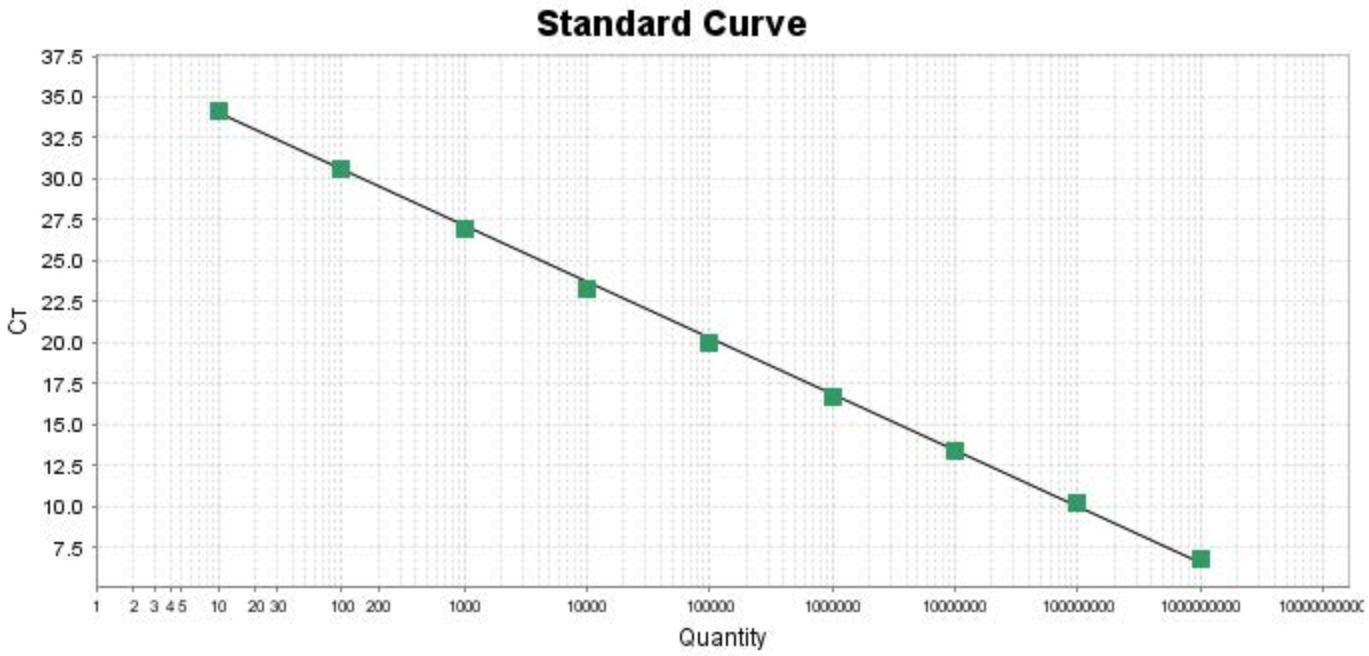
Dynamic Range: TREC



Target: TREC FAM Slope: -3.238 Y-Inter: 36.526 R^2: 0.996 Eff%: 103.621 Error: 0.022

PerkinElmer

Dynamic Range: SMN1

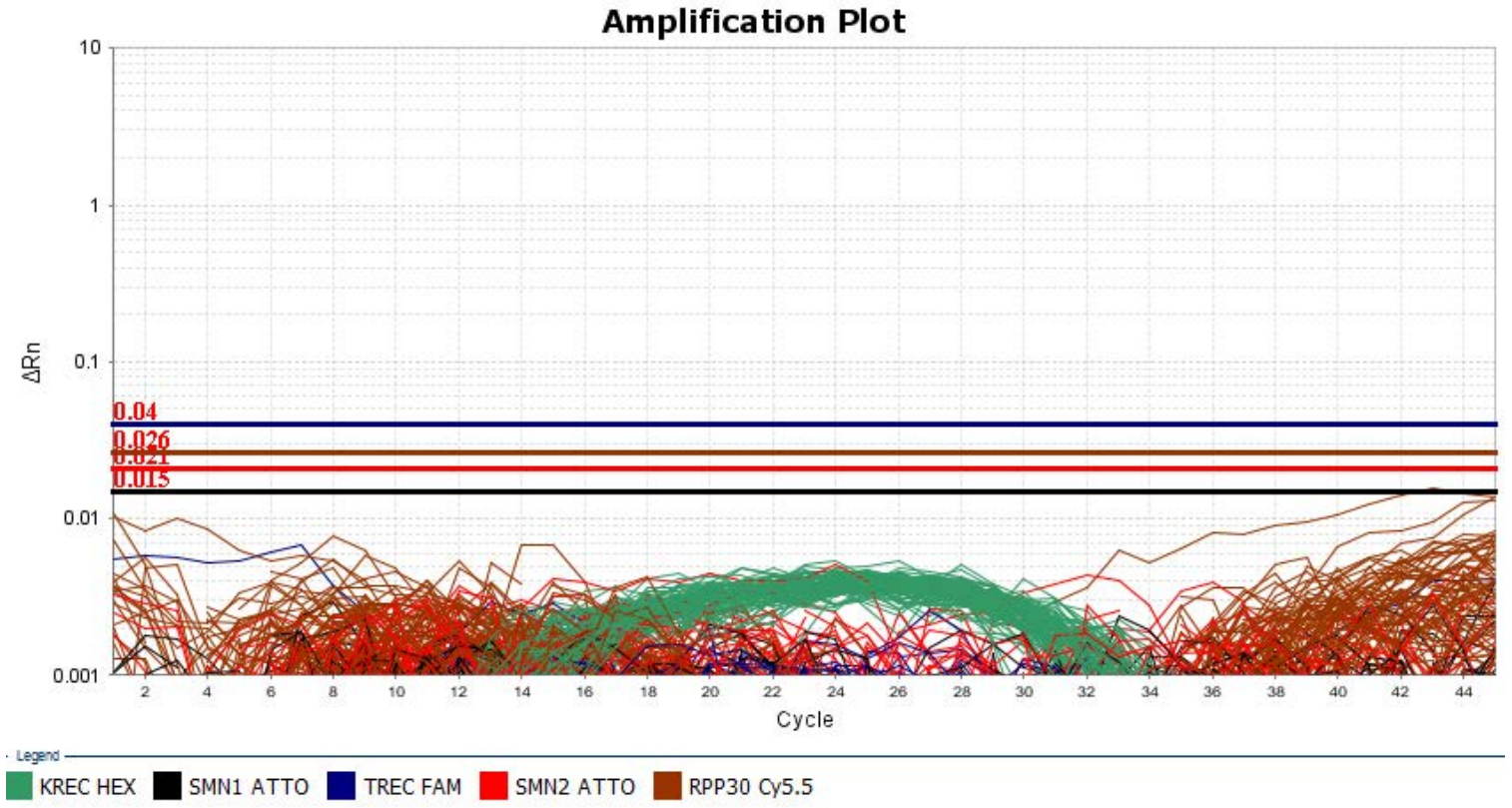


Target: SMN1 ATTO Slope: -3.44 Y-Inter: 37.489 R^2: 0.996 Eff%: 95.294 Error: 0.026

PerkinElmer

Analytical Sensitivity; Limit of the Blank

- Plate with 12-point Standard Curve, 3 DBS and 81 NTC (4uL of elution buffer added)



The LOB is 0. there was no amplification of the blanks after 45 cycles

PerkinElmer

Ongoing PerkinElmer R&D study

Study Specifics

- Testing the 5-Plex assay on de-identified newborn DBS
- Including Biogen samples of known SMN1 and 2 copy number
- Testing prototype PerkinElmer DBS controls
- Number of newborn samples to test: at least 3,000
- DNA obtained by alkaline buffer extraction method

First pass results Summary to date

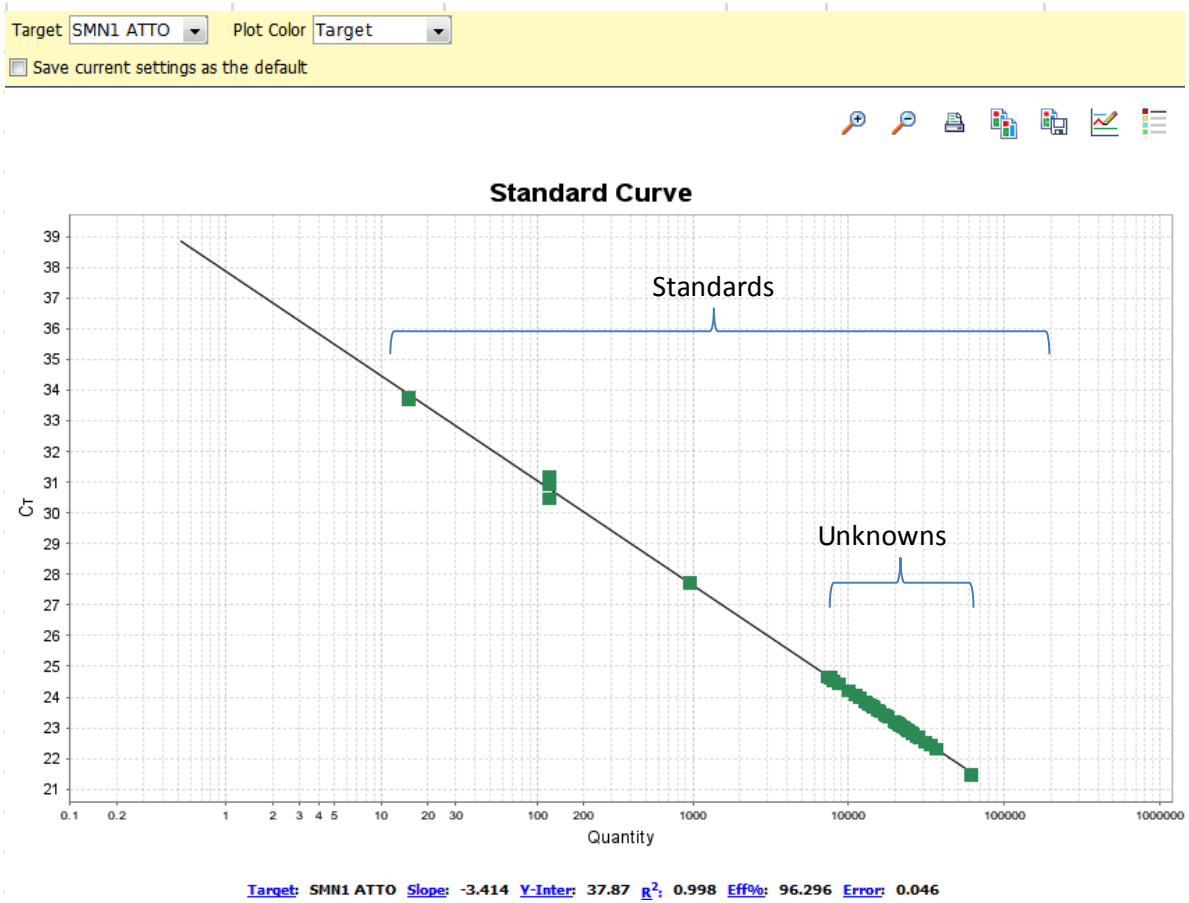
- Completed 1080 newborn samples, 15 96-well PCR plates
- There were 3/1080 "No Results" due to very poor or no amplification in all 5 PCR targets
- There were 0/1080 SMN1 "0 copies"; all samples had an SMN1 copy number of 1 or greater
- There were 0/1080 TREC or KREC "0 copies" samples
- The cutoffs for repeat testing would be established in a larger pilot study

Typical Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 2	Std 3	Std 4	Std 5	Std 1	Std 2	Std 3	Std 4	Std 5	Std 4	Std 5
B	CB	SL	G4	147	CC	BC 148		B1 175	B2 176	148	149	150
C	151	152	153	154	155	156	157	158	159	160	161	162
D	163	164	165	166	167	168	169	170	171	172	173	174
E	175	176	177	178	179	180	181	182	183	184	185	186
F	187	188	189	190	191	192	193	194	195	196	197	198
G	199	200	201	202	203	204	205	206	207	208	209	210
H	211	212	213	214	215	216	217	218	219	PC	NTC	NTC

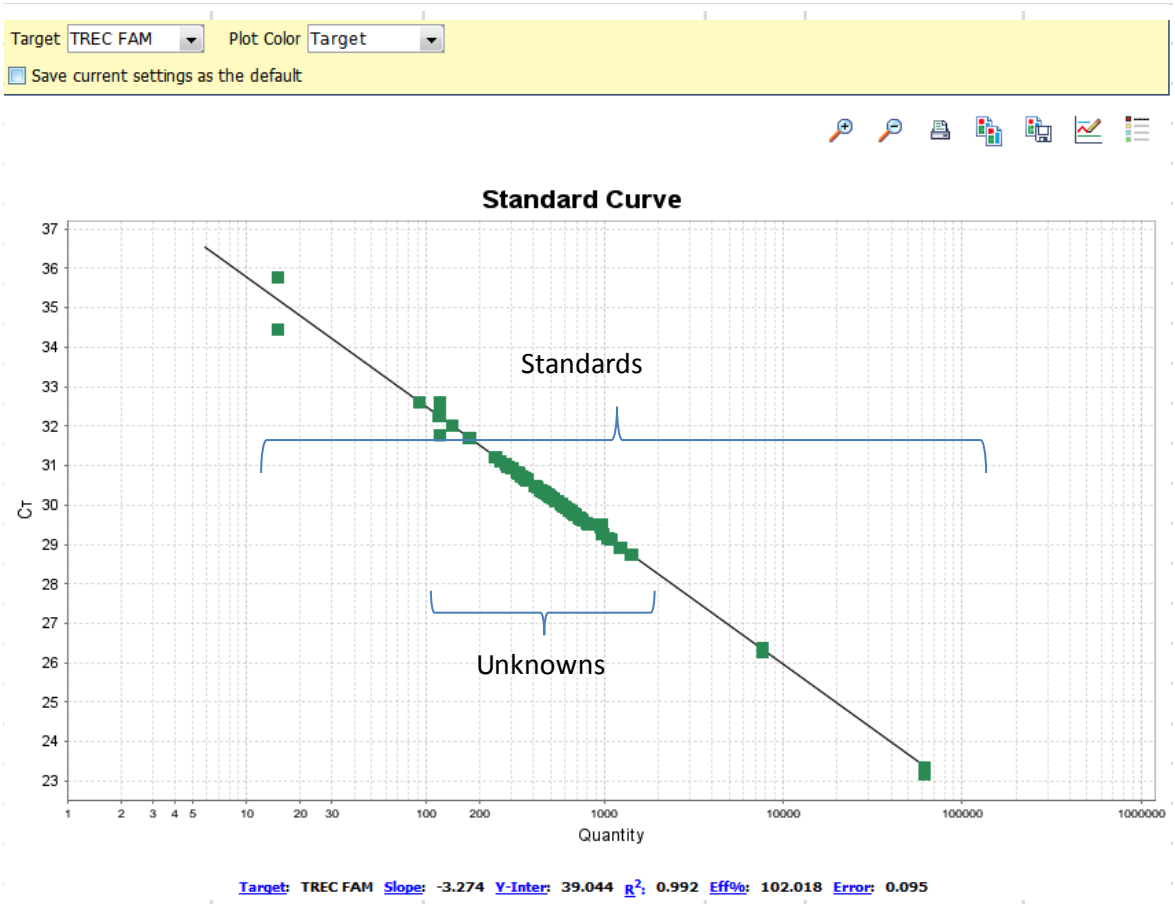
CB	Cord Blood
SL	SCIDs like (older person)
G4	TREC, SMN1 neg gBlock control
G5	KREC, SMN2, RPP neg gBlock control
CC	Coriell SMA cells ID 23689
BC	Biogen control (SMA-146; SMA-147; SMA-148; SMA-149); In duplicates (same control twice/plate)
BC	Biogen control (SMA-146; SMA-147; SMA-148; SMA-149); In duplicates (same control twice/plate)
B1	Biogen sample (anything expect the four above)
B2	Biogen sample (anything expect the four above)
PC	Process control
NTC	Non-templated control

Typical Standard Curve, SMN1 Target



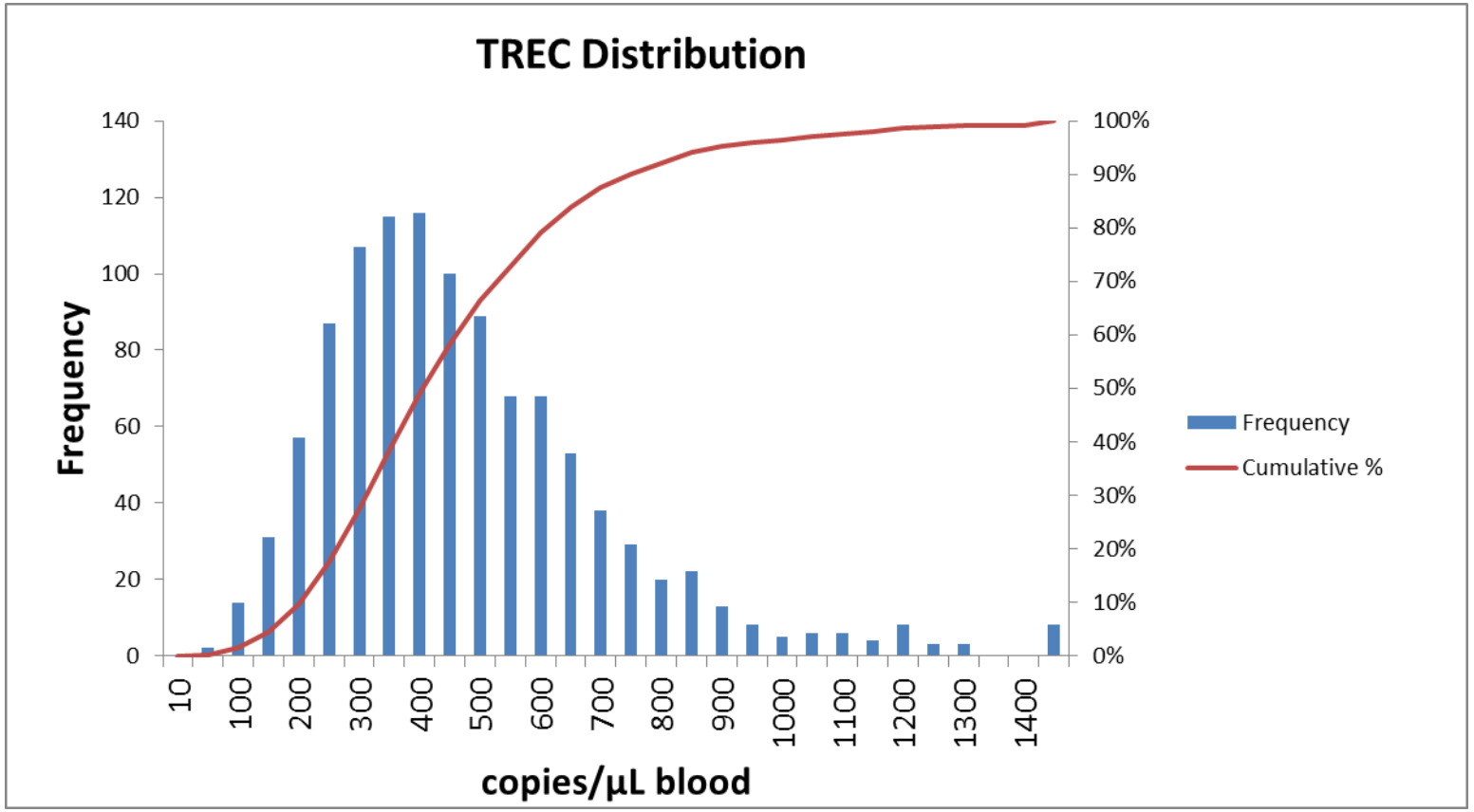
PerkinElmer

Typical Standard Curve, TREC target



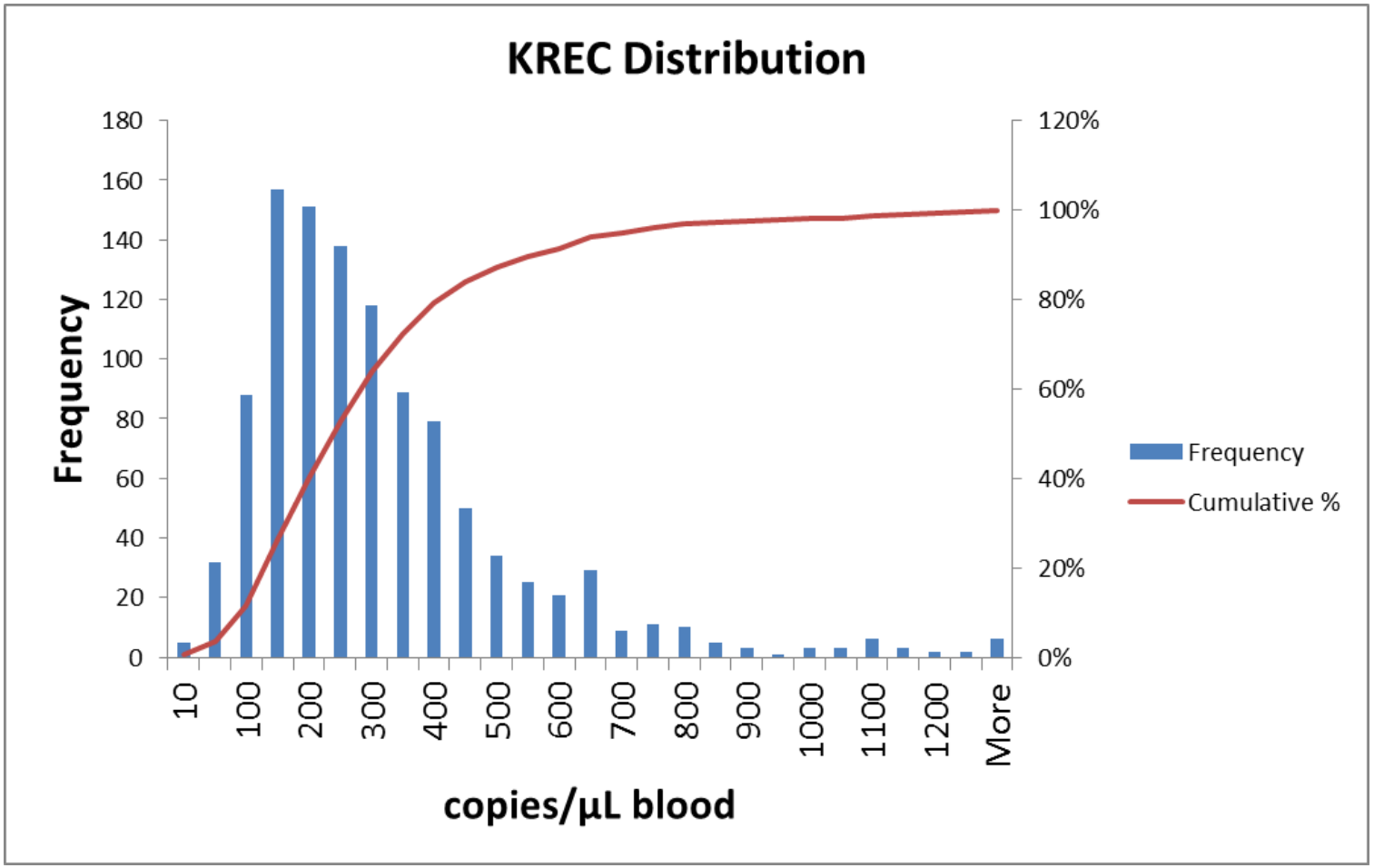
PerkinElmer

TREC molecules detected from DBS samples



PerkinElmer

KREC molecules detected in DBS samples



PerkinElmer

Climbing Higher

Conflict of Interest Disclosure Form

Note: A potential or actual conflict of interest exists when commitments and obligations are likely to be compromised by the nominator(s)'other material interests, or relationships (especially economic), particularly if those interests or commitments are not disclosed.

This Conflict of Interest Form should indicate whether the nominator(s) has an economic interest in, or acts as an officer or a director of, any outside entity whose financial interests would reasonably appear to be affected by the addition of the nominated condition to the newborn screening panel. The nominator(s) should also disclose any personal, business, or volunteer affiliations that may give rise to a real or apparent conflict of interest. Relevant Federally and organizationally established regulations and guidelines in financial conflicts must be abided by. Individuals with a conflict of interest should refrain from nominating a condition for screening.

Date: 02/21/2017

Name: Kenneth Hobby

Position: President

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☑ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: _____

Date: 02/21/2017

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Date: 02/22/2017

Name: Valerie A. Cwik, M.D.

Position: EVP - Chief Medical and Scientific Officer

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☑ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Valerie A. Cwik

Date: 02/22/2017

Conflict of Interest Disclosure Form

Note: A potential or actual conflict of interest exists when commitments and obligations are likely to be compromised by the nominator(s)'other material interests, or relationships (especially economic), particularly if those interests or commitments are not disclosed.

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Date: 02/15/2017

Name: Jill Jarecki

Position: Chief Scientific Officer, Cure SMA

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☑ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Jill Jarecki

Date: 02/15/2017

Conflict of Interest Disclosure Form

Note: A potential or actual conflict of interest exists when commitments and obligations are likely to be compromised by the nominator(s)'other material interests, or relationships (especially economic), particularly if those interests or commitments are not disclosed.

This Conflict of Interest Form should indicate whether the nominator(s) has an economic interest in, or *acts as an officer or a director of, any outside entity whose financial interests would reasonably appear* to be affected by the addition of the nominated condition to the newborn screening panel. The nominator(s) should also disclose any personal, business, or volunteer affiliations that may give rise to a real or apparent conflict of interest. Relevant Federally and organizationally established regulations and guidelines in financial conflicts must be abided by. Individuals with a conflict of interest should refrain from nominating a condition for screening.

Date: 02/17/2017

Name: Jacqueline Glascock

Position: Scientific Program Manager, Cure SMA

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

X I have no conflict of interest to report.

_____ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Jacqueline Glascock

Date: 02/17/2017

Conflict of Interest Disclosure Form

Note: A potential or actual conflict of interest exists when commitments and obligations are likely to be compromised by the nominator(s)'other material interests, or relationships (especially economic), particularly if those interests or commitments are not disclosed.

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Date: 02/17/2017

Name: Mary Schroth

Position: Medical Director, Cure SMA

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☐ I have no conflict of interest to report.

☑ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. Biogen, Member, DSMBs

2. AveXis, Member, Advisory Board

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Mary Schroth

Date: 02/17/2017

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Date: February 17, 2017

Name: Spencer Perlman

Position: Cure SMA Board Member

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☒ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: _____

Date: 2/17/17

Conflict of Interest Disclosure Form

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Date: 02/17/2017

Name: Corey Braastad

Position: VP & GM Genomics, Covance Drug Discovery

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☐ I have no conflict of interest to report.

☑ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. Covance is wholly owned by Laboratory Corporation of America Holdings, Inc (LabCo

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Corey Braastad

Date: 02/17/2017

Conflict of Interest Disclosure Form

Note: A potential or actual conflict of interest exists when commitments and obligations are likely to be compromised by the nominator(s)'other material interests, or relationships (especially economic), particularly if those interests or commitments are not disclosed.

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Date: 02/19/2017

Name: Katherine Klinger

Position: Global Head Translational Sciences, Sanofi
Cure SMA BOD/committee

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☐ I have no conflict of interest to report.

☒ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. I am employed by Sanofi. The Genzyme division markets therapies for rare diseases, and could in the future address SMA
2.
3.

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Katherine W. Klinger

Date: 02/19/2017

Conflict of Interest Disclosure Form

Note: A potential or actual conflict of interest exists when commitments and obligations are likely to be compromised by the nominator(s)'other material interests, or relationships (especially economic), particularly if those interests or commitments are not disclosed.

This Conflict of Interest Form should indicate whether the nominator(s) has an economic interest in, or acts as an officer or a director of, any outside entity whose financial interests would reasonably appear to be affected by the addition of the nominated condition to the newborn screening panel. The nominator(s) should also disclose any personal, business, or volunteer affiliations that may give rise to a real or apparent conflict of interest. Relevant Federally and organizationally established regulations and guidelines in financial conflicts must be abided by. Individuals with a conflict of interest should refrain from nominating a condition for screening.

Date: 02/15/2017

Name: Douglas Kerr

Position: Vice President, R and D, Shire Pharmaceuticals

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☑ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Douglas Kerr

Date: 02/15/2017

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Date: 02/17/2017

Name: Amanda Haidet-Phillips

Position: Scientific Program Officer, Muscular Dystrophy Assc

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

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☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

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I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Amanda Haidet-Phillips

Date: 02/17/2017

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Date: 02/21/2017

Name: Kristin Stephenson

Position: Vice President, Policy & Advocacy, MDA

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☑ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Kristin Stephenson

Date: 02/21/2017

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Date: 02/01/2017

Name: R. Rodney Howell, M. D.

Position: Professor of Pediatrics, Miller School of Medicine, U

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☐ I have no conflict of interest to report.

☑ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. Chairman of the Board, Muscular Dystrophy Association

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: R Rodney Howell

Date: 02/01/2017

Conflict of Interest Disclosure Form

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Date: 2/15/2017

Name: Dr. Thomas Crawford

Position: Pediatric Neurology

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☒ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: _____

Date: 15 Feb 2017

Conflict of Interest Disclosure Form

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Date: 02/17/2017

Name: John W. Day

Position: Professor of Neurology and Pediatrics, Stanford Uni

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☑ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

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I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: John W. Day

Date: 02/17/2017

Conflict of Interest Disclosure Form

Note: A potential or actual conflict of interest exists when commitments and obligations are likely to be compromised by the nominator(s)'other material interests, or relationships (especially economic), particularly if those interests or commitments are not disclosed.

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Date: 2/17/17

Name: Thomas W. Prior

Position: Professor of Pathology
Ohio State University

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

☒ I have no conflict of interest to report.

☐ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

1. _____

2. _____

3. _____

I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: Thomas W. Prior

Date: 2/17/17

Conflict of Interest Disclosure Form

Note: A potential or actual conflict of interest exists when commitments and obligations are likely to be compromised by the nominator(s)'other material interests, or relationships (especially economic), particularly if those interests or commitments are not disclosed.

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Date: February 17, 2017

Name: Jacinda Sampson MD PhD

Position: Associate Professor of Neurology

Please describe below any relationships, transactions, positions you hold (volunteer or otherwise), or circumstances that you believe could contribute to a conflict of interest:

X I have no conflict of interest to report.

_____ I have the following conflict of interest to report (please specify other nonprofit and for-profit boards you (and your spouse) sit on, any for-profit businesses for which you or an immediate family member are an officer or director, or a majority shareholder, and the name of your employer and any businesses you or a family member own:

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I hereby certify that the information set forth above is true and complete to the best of my knowledge.

Signature: _____

Date: 2/17/2017