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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

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 ($1.4 \pm 2.2$ mV) was significantly reduced compared to controls ($5.5 \pm 2.0$ 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, SMN$\Delta$7, 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

**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 |

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.

## 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 $1 \times 10^7$ 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
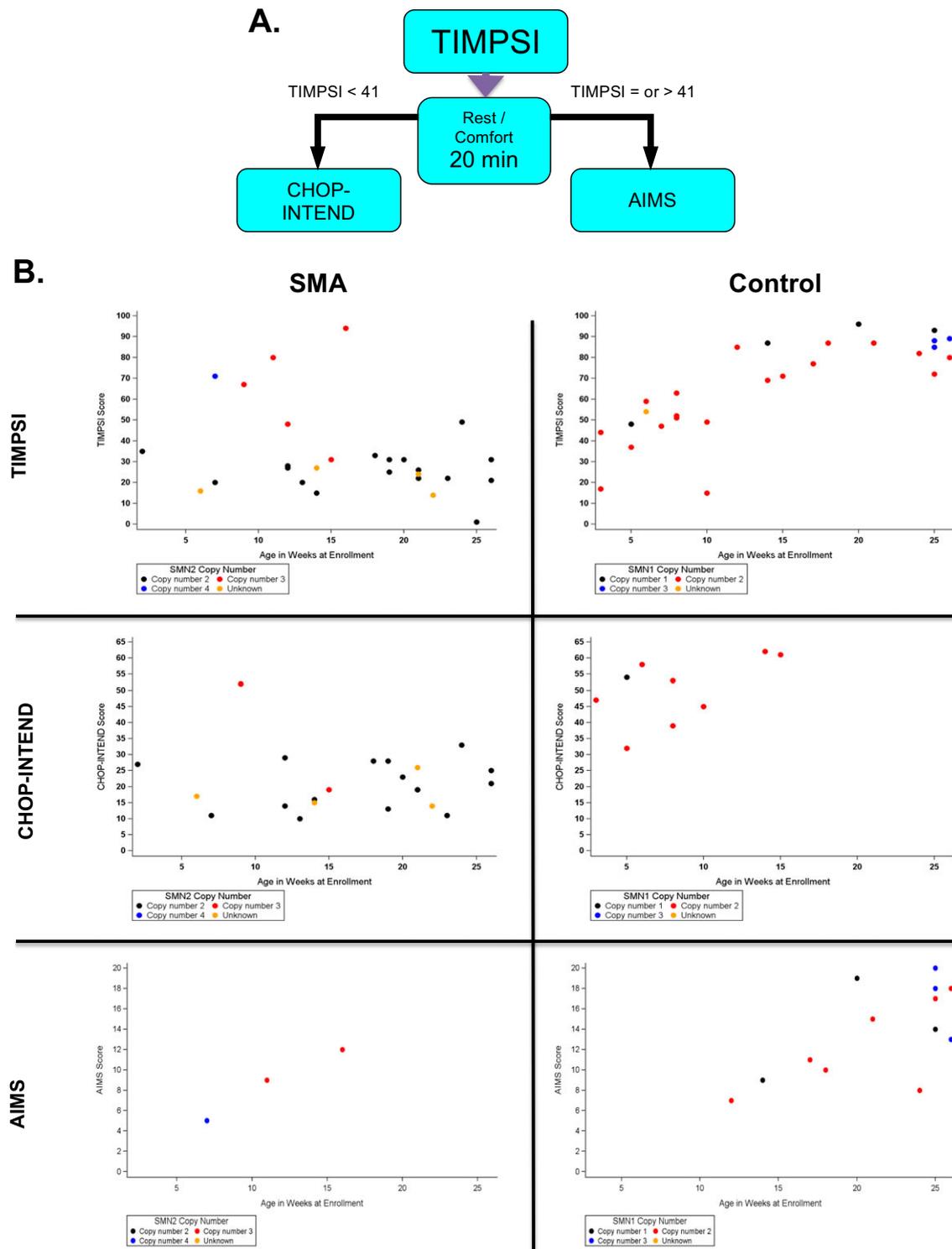
**Figure 1.** Motor function assessments in SMA and healthy infants in the first 6 months of life. (A) Motor function testing paradigm. All infants were tested using the TIMPSI. After the TIMPSI, a mandatory rest period of 20 minutes was followed by either the CHOP-INTEND or AIMS assessment. Infants 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.

## SMA

## Control



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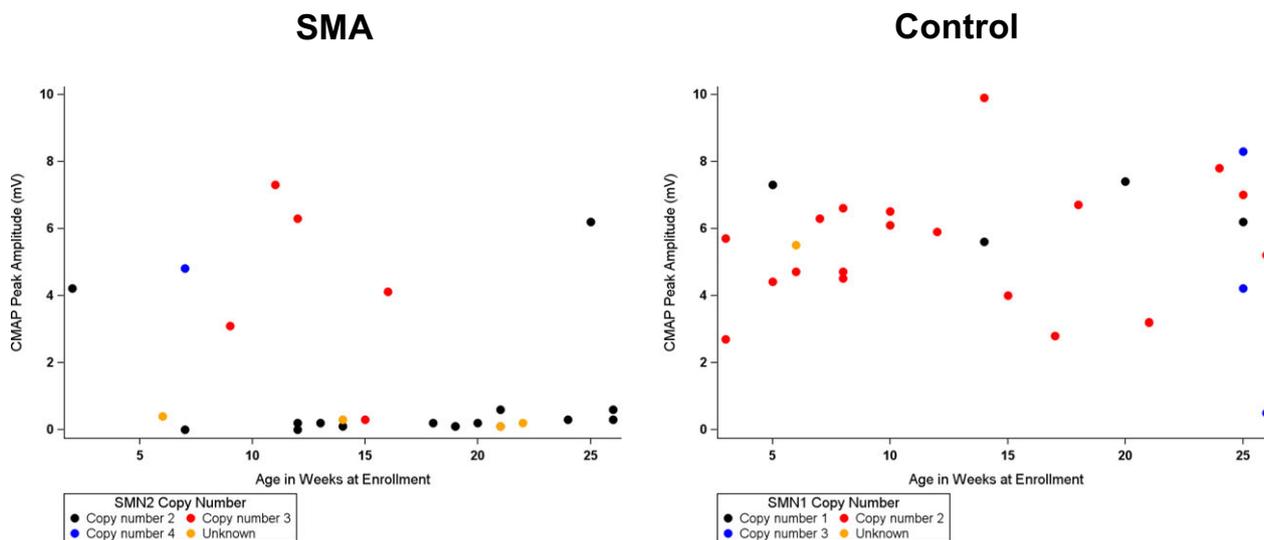
**Table 3.** Baseline electrical impedance myography results in SMA and healthy control infants.

|  | 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
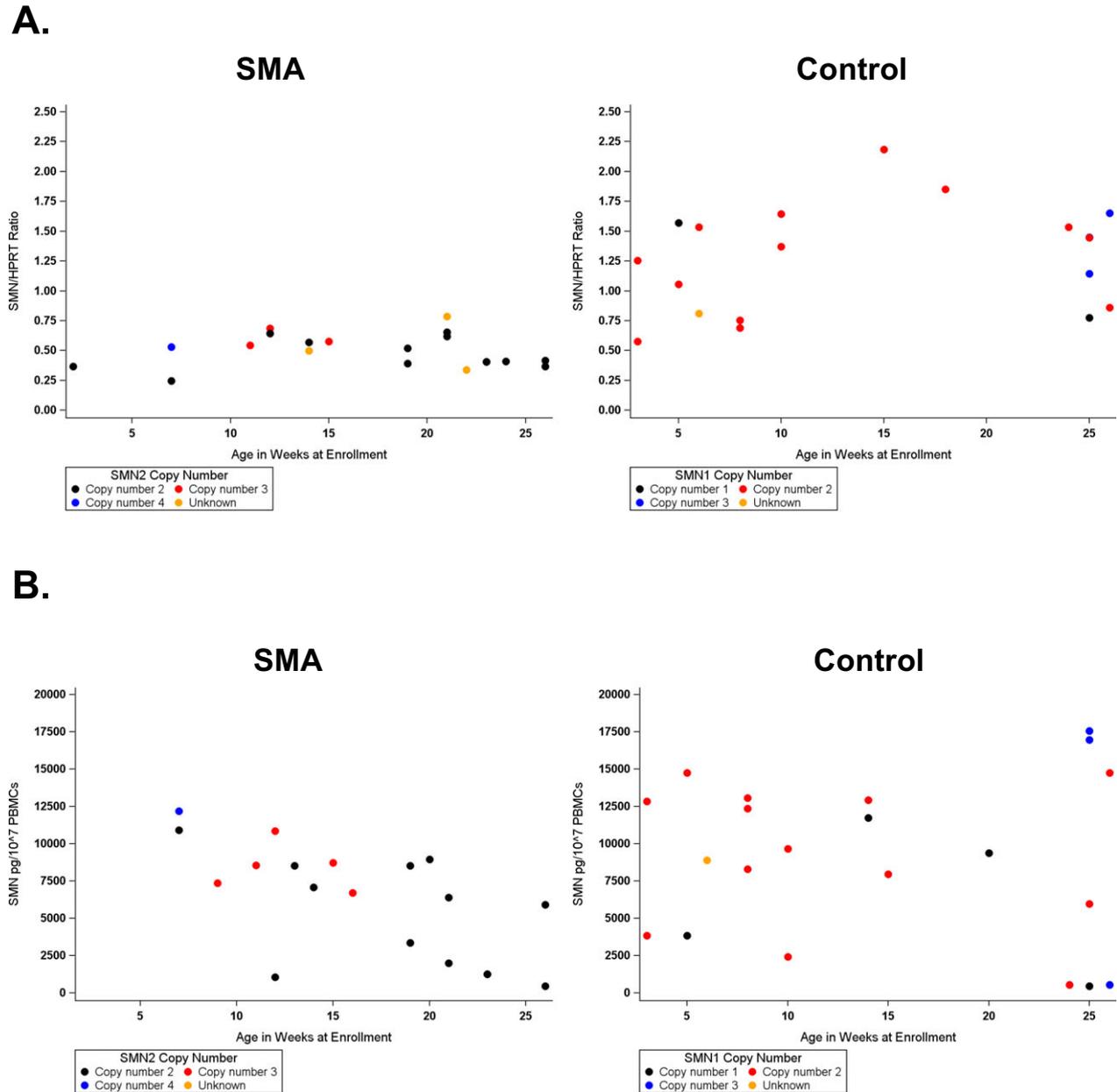
## A.



## B.



**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) ($\mu$g/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) ($\mu$g/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) ($\mu$g/mL)** | **14.62 (8.54)** | **12.24 (6.78)** | **20.64 (13.17)** | **0.1075** | **0.0227** |
| Complement factor H-related protein 1 (CFHR1) ($\mu$g/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 ($\mu$g/mL) | 648.4 (187.2) | 653.2 (163.7) | 622.0 (141.5) | 0.6241 | 0.5638 |
| Fibulin-1C (Fib-1C) ($\mu$g/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) ($\mu$g/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) ($\mu$g/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 ($\mu$g/mL)** | **7.39 (1.62)** | **6.78 (1.23)** | **9.06 (3.20)** | **0.0493** | **0.0078** |
| Thrombospondin-4 (TSP4) ($\mu$g/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 Neuro-NEXT 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.