Utility of Survival Motor Neuron ELISA for Spinal Muscular Atrophy clinical and preclinical analyses

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Introduction

Background:
As new SMN-targeting drugs evolve into preclinical drug candidates and ultimately enter clinical trials, it becomes imperative to utilize an SMN protein measurement tool that is relatively high throughput, quantitative, and manufactured using standardized processes to maximize accuracy and reproducibility of results. In order to do so, the SMA Foundation has develop an immunoassay (ELISA) capable of accurately measuring SMN protein levels in blood and other tissue samples in SMA animal models and patients. Using ELISA will help in validating SMN protein as a biomarker in SMA patients.

Objective:
To develop an immunoassay capable of accurately measuring SMN protein levels in blood and other tissue samples from SMA animal models and patients as a tool for validating SMN protein as a biomarker in SMA.

Strategy:
A sandwich enzyme-linked immunosorbert assay (ELISA) was developed and validated for measuring SMN protein in human PBMCs, SMA patient fibroblasts and muscle tissue homogenates. Protocols for detection and extraction of SMN from various SMA mouse tissues were also developed.

Results:
The ELISA's sensitivity for human SMN is 50pg/mL. Initial analysis reveals that PBMCs yield enough protein to quantify SMN in blood samples with volumes less than 1mL. SMA Type I patients' PBMCs show ~ 90% reduction of SMN protein compared to healthy adults. Using the ELISA, SMN is quantifiable in non-SMA human muscle and SMA fibroblasts. The ELISA can also reliably quantify SMN protein in mouse PBMCs as well as in other tissues (muscle, brain, and spinal cord) from the delta7 model.

Discussion and Next Steps:
This SMN ELISA enables reliable, quantitative and rapid quantification of SMN protein in healthy human and SMA patient PBMCs, muscle and fibroblasts. SMN can also be detected in several tissues from mouse models of SMA, as well as in wildtype mouse tissues, and is highly divergent across tissues. This assay has general applicability for preclinical and clinical research, and will be used to develop optimized protocols for SMN measurement in human PBMCs (**please see poster #23A**).

Methods

Reagents and ELISA protocol

Recombinant human SMN1 was generated from full-length cDNA expressed in bacterial expression vectors and purified for use as a standard in the ELISA. The capture antibody Sigma anti-SMN clone 2B1 from Enzo was coated at 100uL onto Costar Stripwell at 3.5μg/mL. After overnight incubation at room temperature, the plate was blocked for 5 hours with 1% BSA in PBS. Cell lysate samples and recombinant hSMN1 or HeLa cell lysate standards were loaded at 100uL per well. Standards were diluted in 2-fold dilutions or from 50-3200pg/mL. Samples were incubated for one hour at room temperature, washed and then incubated with a detection antibody from Proteintech at 2ug/mL for one hour at room temperature. After washing a peroxidase conjugated goat anti-rabbit IgG from Jackson Labs was applied at 50ng/mL to the plate and incubated for 30 minutes at room temperature. After washing, plates were developed with TMB substrate for 30 minutes incubation at room temperature and the reaction stopped with 1N HCl acid. Plates were then read on a spectrophotometer at 450nm. Plates were sealed and gently shaken during all incubations, dilutions of sample and standard is done in assay buffer (1% BSA, 0.1% Tween-20 in PBS).
All error bars represent standard errors except for Figure 1A.

Cell lysis

A cell count with a hemocytometer was always performed immediately prior to lyses for accurate count of viable cells, which was used for determining volume of cell lysis suspension. PBMCs were thawed in a 37°C water bath and resuspended in ER4 lysis buffer with inhibitors at 106-108 cells/mL. The cell suspension was gently vortexed and placed on ice for 30 minutes. The cell lysate was transferred to a 1.5 mL centrifuge tube and was clarified by centrifugation for 10 minutes at 14,000 RCF, 4°C. The supernatant was transferred to a clean vial and either assayed immediately or stored at -70°C until use.

Assay Details

Table 1: SMN ELISA characteristics

rhSMN Standard Curve	Dynamic range Reproducibility Sensitivity limit	3200-50pg/mL <11% CV 50pg/mL
Freeze Thaw	Thaw 1 recovery Thaw 2 recovery Thaw 3 recovery	102% 112% 79%
PBMC Dilution Linearity	1:4 1:8 1:16 1:32	88-106% (98%) 93-109% (102%) 95-112% (105%) 100%
PBMC SMN Spike Recovery (1:4 PBMC dilution)	267pg/mL spike 667pg/mL spike 1667pg/mL spike	88-116% (100%) 95-137% (105%) 79-131% (99%)
Minimum Sample Dilutions	Human PBMCs Human Muscle Mouse PBMCs Mouse Brain, Fat, Heart, Liver, Muscle, Skin, Spinal Cord	1:4 1:5 1:2 1:5-1:10

Acknowledgments

Suzanne Forrest, Cynthia Joyce, Kathleen McCarthy, Sergey Paushkin and Sarah Przedborski of the SMA Foundation; Dr. Kathy Swoboda, Benjamin Chisum, and Kelly Murphy and from the University of Utah Department of Neurology,; Dr. Wendy Chung, Jiancheng Guo, and Patricia Lanzano of Columbia University Medical Center; Sylvie Ramboz, Kim Cirillo, Bassem El-Khodor, Monica Patry and Judy Johnson Watson from PsychoGenics; Lexicon Pharmaceuticals, Inc.; Charlotte Sumner, Tara Martinez, and James Van Meerbeke of Johns Hopkins University, Rajeev Sivasankaran and Cheng Song of Novartis; Heather Plasterer and Sean Jennings of Repligen.

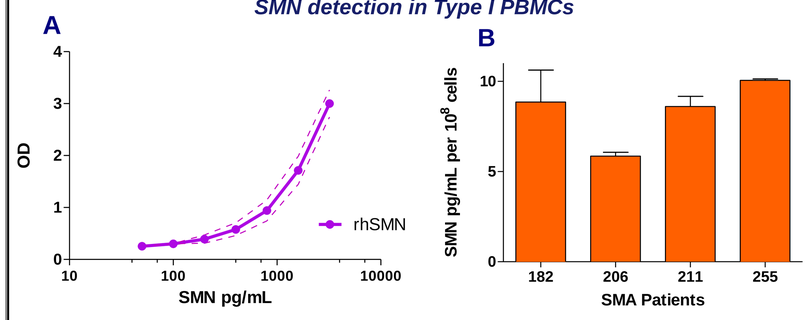
All work was funded by the SMA Foundation.

Results

ELISA standard curve, measurement of SMN in Type 1 PBMCs

The recombinant hSMN standard curve data developed from N=6 curves were highly reproducible with standard deviations of about ±0.23 OD unit variations (Figure 1A). The dotted lines surrounding the dose-response curve represent 2 standard deviations. SMA Type I patient samples (N=4) were tested in the SMN ELISA (Figure 1B). SMN protein signal was detected in all samples, with an average of 8.32 pg SMN protein per 10^6 cells. Based on the average of 70.2 pg SMN protein per 10^6 cells calculated for adult normal donor PBMCs, the amount of SMN protein in PBMCs of Type I SMA patients in this patient cohort is 88% less than normal.

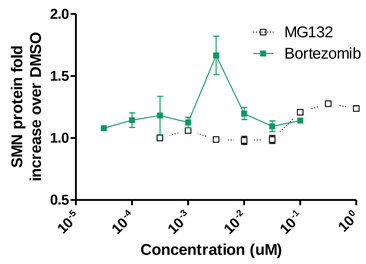
Figure 1: rhSMN standard curve and SMN detection in Type I PBMCs



Detection of SMN protein dose-response in SMA fibroblasts

SMA Type I fibroblasts (line 3813) were treated for 24h with the proteasome inhibitors MG132 or bortezomib (N=2 each). A: Peak SMN protein upregulation of 27% was observed with 0.3μM of MG132 (EC50=0.68uM) and a 67% increase was seen with 3nM bortezomib (EC50=10nM). B: Cell viability decreased to 50-75% at doses near and above doses that increased SMN protein levels.

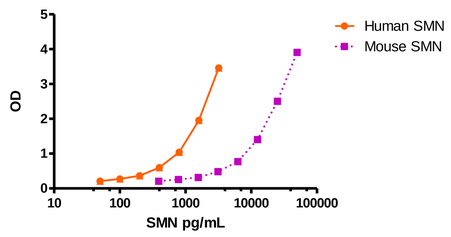
Figure 2: SMA patient fibroblast SMN protein responses



Human SMN detected more sensitively than mouse Smn

Full-length recombinant mouse and human SMN proteins were tested in the ELISA at a range of concentrations (390-50000 pg/mL and 50-3200 pg/mL respectively). The human SMN was detected with 10-fold greater signal at all points than the mouse SMN. The relationship between the mouse to human dose-response curve was linear and values were comparable across equivalent points in the dilution curves.

Figure 3: Comparison of human and mouse SMN reactance



SMN ELISA detects protein in human muscle

Human muscle samples (N=4) were acquired from Asterand (Detroit, MI) and analyzed in the SMN ELISA and also in a desmin ELISA as a muscle-specific control from USCN Life Science (#E90373Hu, Burlington, NC). There were 4 muscle samples collected post-mortem from donors aged 37-97 years that died of coronary heart disease or accidental head trauma. Samples were homogenized using a polytron and the resulting lysates were tested in the SMN ELISA and a desmin ELISA.

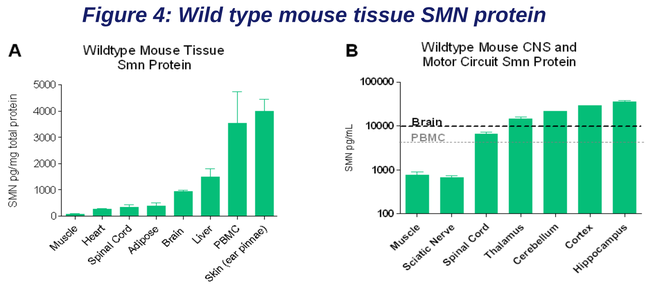
Table 2: SMN protein in human muscle

	High fat content	Total protein (mg/mL)	SMN (pg/mL)	Desmin (ng/mL)	SMN (pg/mg) total protein norm.	SMN (pg/ng) Desmin norm.
6181A1	Yes	4.53	454	18.5	501	24,500
7103A1	Yes	1.36	810	1.39	2980	583,000
9834B1	No	6.92	2230	8.48	1620	263,000
9846A1	No	5.94	693	9.24	582	75,000

SMN ELISA detects protein in several wildtype mouse tissues

Smn levels varied by as much as 50-fold across tissue types in adult FVB mice (14 weeks old). A: SMN levels by tissue were distributed on a basis loosely ordered by having lesser to greater dividing cell populations. Smn levels for tissues are represented as Smn pg/mg total protein. PBMCs are represented as Smn pg/mg total protein in the lysis buffer extract. On a per cell basis the average Smn level was 67.2pg/10^6 PBMCs. B: SMN levels in different parts of the brain and motor circuit are presented. The level of SMN in sciatic nerve and muscle is similar, while the levels in the hippocampus was nearly 3x that of the thalamus and 50x that of the muscle and nerve. SMN was expressed as pg/mL to represent SMN levels across tissues on the same basis. Error bars are expressed as standard deviations.

Figure 4: Wild type mouse tissue SMN protein

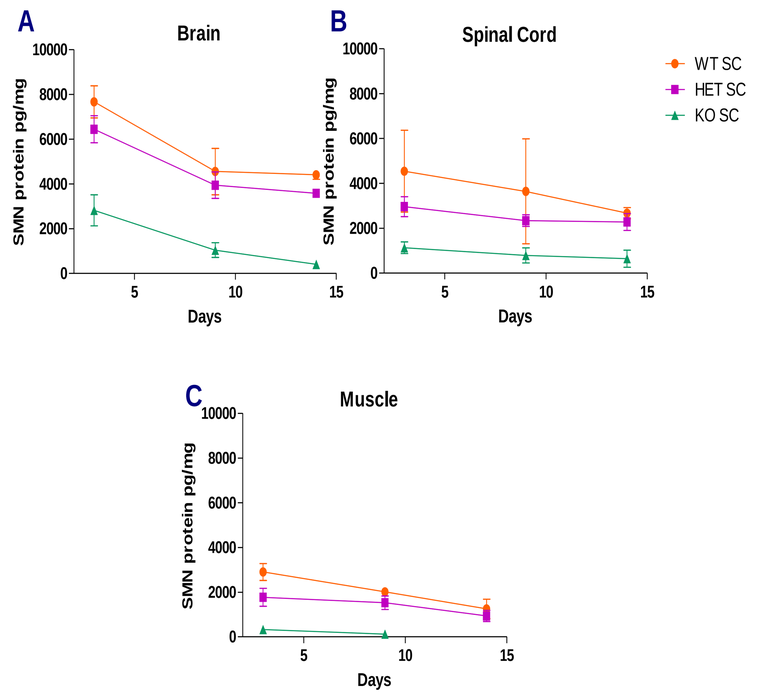


Results

SMN protein in WT, HET and KO Delta7 mice

Brain, muscle and spinal cord tissues were collected from postnatal day 3, 9 and 14 Delta7 severe phenotype mice across genotypes (Stock #5025 from Jackson Labs, N=6 per group). While tissue levels in brain homogenates (Figure 5A) were higher than both spinal cord (Figure 5B) and muscle (Figure 5C) across all genotypes, the ratio of brain to spinal cord, and brain to muscle in the homozygous KO mice was 1.4-fold and 12.2-fold, respectively. The decline of SMN levels over time between muscle and spinal cord tissues was relatively similar across genotypes; however, the reduction seen for brain SMN was more precipitous dropping ~40% between P3 and P9.

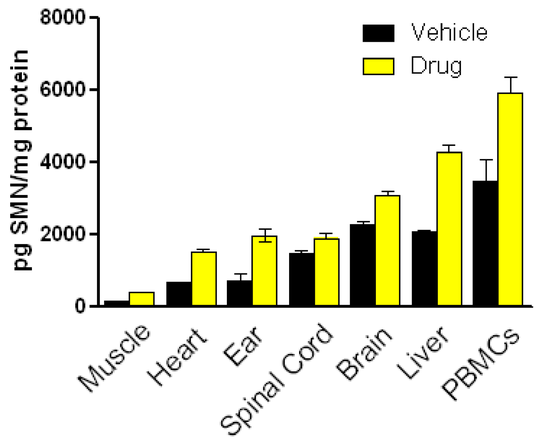
Figure 5: Delta7 tissue SMN protein time course



Drug-induced SMN protein levels changes in mild SMA mice: relationship between PBMC and other tissues

A mild mouse model of spinal muscular atrophy (C/C line, #8604 from Jackson Labs) was used to assess the effects of drugs on SMN protein levels in several tissue extracts (Figure 6A-H). Treatment was administered by oral gavage for 10 days, and then samples collected 1 hr after the final dose on Day 10. Drug treatment induced SMN protein increases from ~25% (spinal cord) to ~200% (liver, PBMCs). Vehicle N=5, Drug N=7.

Figure 6: Drug-induced SMN protein levels across tissues in a mild SMA mouse model



Correlations between SMN response in PBMCs and other tissues

Pearson's correlations were done on the SMN values between the tissue in the C/C mouse dosing experiment. The analysis revealed that there were multiple relationships between the tissues with and without treatment, and in combination (Table 3). In particular, PBMC SMN levels correlated significantly to several tissues except for the spinal cord. Surprisingly brain and spinal cord levels did not correlate, possibly due to tissue collection artifacts. The liver SMN levels correlated to a high degree to all other tissues' SMN levels.

Table 3: Correlation of SMN levels across tissues

	Brain	Spinal Cord	Liver	Heart	Muscle	Ear
PBMC	**R=0.91 p<0.001**	R=0.22 P=0.50	**R=0.78 p=0.003**	**R=0.84 p=0.006**	**R=0.72 p=0.009**	**R=0.83 p=0.001**
Brain		R=0.34 p=0.27	**R=0.82 p=0.001**	**R=0.89 p<0.001**	**R=0.85 p<0.001**	**R=0.81 p=0.002**
Spinal Cord			**R=0.61 p=0.03**	R=0.50 p=0.10	**R=0.69 p=0.014**	**R=0.42 p=0.17**
Liver				**R=0.92 p<0.001**	**R=0.95 p<0.001**	**R=0.84 p=0.007**
Heart					**R=0.94 p<0.001**	**R=0.74 p=0.006**
Muscle						**R=0.76 p=0.004**