Optimization of peripheral blood mononuclear cell processing for SMN protein signal analysis

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Abstract

SMN protein levels in peripheral blood mononuclear cells (PBMCs) have potential to become a key pharmacodynamic (PD) marker of drug efficacy in SMA clinical trials that utilize SMN-targeted approaches. Detailed analysis of variability and factors affecting SMN protein levels in PBMCs are required to allow interpretation of SMN signals in trials. Factors like sample processing delays, subject age, meals, and respiratory infections were evaluated for their ability to impact SMN protein levels in PBMCs. A best practice protocol for SMN ELISA analysis of PBMCs was developed based on these studies as well as prior experience.

A series of studies focused on measuring SMN signals in the same healthy individuals over time, with pre-processing delays, over the course of recovery from a respiratory infection. Another study, SMAF-001, was conducted with SMA patients and carriers were conducted at a Phase 1 clinical study unit. In healthy volunteers there were up to 10 fold differences in SMN protein levels between individuals, and in the same individuals there was up to 4-fold differences when samples were examined at T=0, 6h (after a carbohydrate-rich meal), 4h, 7d and 30d. Interestingly, protein concentrations were twice as high in the CD14+ PBMCs, though SMN levels among the subtypes did not significantly differ. Processing delays also produced wide differences in PBMC SMN protein concentrations; 48-72h delays gave signals up to 200-fold lower than the samples processed in 24h or less. Two individuals with acute respiratory infections were evaluated at different points up to 30d after the onset of symptoms. Their SMN levels varied in association with their disease state and recovery, with SMN levels peaking at 2-fold 7 days after symptom onset. SMA carriers and patients (aged 1.4-60 years) submitted PBMC samples for evaluation (Study SMAF-001). There was a trend for lower SMN levels in SMA patients by type, however levels were lower across ages, with similar decreases for SMA carriers and patients as a group or separated by disease status and type.

Overview of Studies

Study (1-3 were in the 2011 poster)	Subjects	Results
1-2: Processing Delay to 24h	N=4 healthy adults	• Delays and freezing reduces cell yield/viability • SMN levels increase in CPT tubes with delay
3: CPT vs EDTA tubes	N=4 healthy adults	• EDTA tubes produce higher, SMN signal stable through 48h
4 Processing delay to 72h	N=2 healthy adults	• Processing delays >48h can destroy SMN signal
5: Intra-individual SMN variability across time	N=6 healthy adults T=0h, 6h, 24h, 7d, 1mo PBMC subtype fractionations Impact of meals on SMN signal	• Variability up to 2x in same individual over timepoints • SMN in PBMC subtypes can vary up to 5x
6: Impact of illness on SMN	N=2 adults with colds T=0h, 6h, 24h, 7d, 1mo PBMC subtype fractionations	• Shifts in PBMC subpopulations can drive SMN signal down
7: SMAF-001	N=12 SMA patients 1-4.60yo N=15 Parent carriers 25-57yo	• SMN signal robust, stable in storage • Signal is lower in older subjects

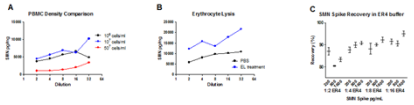
PBMC lysis cell density and impact of red cell lysis

This analysis explored the best SMN protein signal based on different PBMC lysate cell densities and whether erythrocyte lysis impacts the signal. A cell density of 10^6 PBMCs per mL lysis buffer improved signal by up to 6x signal and lysing red blood cells also protein also improved signal levels by 0.3x

PBMC Lysate densities and erythrocyte lysis
Samples were analyzed as per SMN ELISA kit instructions except where described below. Frozen PBMCs were provided by AllCells. Counted PBMCs were centrifuged at 1500 rpm for 10min at 4°C, and samples of 10^6, 10^7 and 50^7 cells/mL and samples were treated with PBS or erythrocyte lysis (EL) buffer for 5 min. Samples were lysed in a minimum of 100uL of ER4 lysis buffer (provided in the SMN ELISA kit) with protease inhibitors. Lysates were clarified by centrifugation at 14,000 rpm and frozen at –80°C until SMN assay.

Survival Motor Neuron (SMN) Protein Analysis
SMN protein was evaluated using an SMN ELISA assay kit (Enzo Life Sciences) and manufacturer's directions. SMN protein standards were assayed when diluted 1:2, 1:4, 1:8, 1:16 and 1:32 into assay buffer or ER4 provided by the kit.

Figure 1: Impact of PBMC Lysate Density, Red Cell Lysis and ER4 on SMN Signal



STUDY 4: Processing delays to 72h

Study 4 determined the impact of PBMC isolation delay on SMN protein signal in conditions analogous to collection of samples in a multisite clinical trial with a central processing center. Processing delays reduced SMN signal up to 200x in 72h.

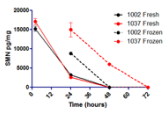
Blood Collection and PBMC Isolation
Whole blood was drawn into sodium citrate tubes and left upright at room temperature for 2, 24, 48 or 72h to simulate delays in processing due to shipment. After the delay, the whole blood was diluted 1:2 using room temperature PBS. Diluted blood was layered onto 3 mL of Lymphoprep and centrifuged at 2500 rpm at 20°C for 20min. PBMC's were harvested from the interface. Ice cold PBS was added to a final volume of 15 ml and mixed by inversion. Cells were collected by centrifugation at 1500 rpm at 4°C for 10min. Supernatant was removed and cells re-suspended in 5 ml of ice cold erythrocyte lysis buffer (EL) and incubated on ice for 5min. Cells were collected by centrifugation at 1500 rpm set at 4°C for 10min. The cells were re-suspended in 5 ml of PBS and counted with a hemocytometer. Cell viability was monitored with Trypan blue.

PBMC Lysis
Counted PBMCs were centrifuged at 1500 rpm for 10 minutes at 4°C, and the resulting cells lysed using Extraction Reagent 4 (ER4 supplied with the Enzo SMN ELISA kit) containing protease inhibitors at a final cell density of 10^7 cells/ml in a minimum of 100 µL of ER4 buffer. Lysates were clarified by centrifugation at 14,000 rpm and frozen at -80°C until SMN assay.

Cryopreservation of PBMCs
One half of the PBMCs isolated from sodium citrate collection tubes were frozen in fetal calf serum containing 15% DMSO. Cells were re-suspended in 500 µl of fetal calf serum containing 15% DMSO and frozen slowly at -20°C for one hour and the frozen. Samples transferred to a -80°C freezer and stored frozen for 1 month prior to thawing.

Soluble Protein Analysis
Total protein in clarified PBMC lysate supernatant was evaluated in a BCA protein assay kit (Pierce) and manufacturer's instructions.

Figure 2: PBMC SMN Signal in Citrate Tubes Isolated After Delays



Studies 5-7 used EDTA tube whole blood collection with lymphoprep PBMC isolation after a 24h delay, ER4 at 1:8 in the SMN standard and 1x10^7 PBMCs/ml in ER4 lysis

STUDY 5:Intra-individual SMN variability across time

Study 5 assessed the extent of the significant variability of SMN signal over longitudinal collections of the same individuals and to evaluate the contribution to signal variability by PBMC subtypes
Study 5 was performed with 6 individuals who provided blood at t=0h, 6h, 24h, 7d and 30d. At T=3h, subjects were fed a heavy pasta meal in an effort to stimulate PBMCs. On Day 7, the samples were also fractionated to isolate CD4, D8, CD14, CD19 and CD56 subtypes with distributions and functions described below.

Figure 3: Interindividual PBMC SMN Varies Up to 20x and Intraindividual SMN Varies Up to 6x Across Time

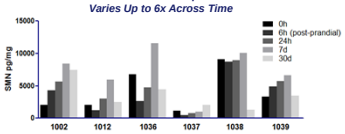
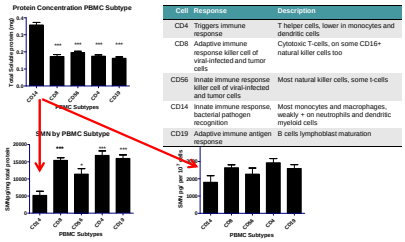


Figure 4: SMN in PBMC Subtypes Is Impacted Significantly by Normalization, and Disproportionately Affected by the CD14+ Cell Population



Cell	Response	Description
CD4	Triggers immune response	T helper cells, lower in monocytes and dendritic cells
CD8	Adaptive immune response killer cell of viral-infected and tumor cells	Cytotoxic T cells, on some CD16+ natural killer cells too
CD56	Innate immune response killer cell of viral-infected and tumor cells	Most natural killer cells, some t-cells
CD14	Innate immune response, bacterial pathogen recognition	Most monocytes and macrophages, weakly + on neutrophils and dendritic myeloid cells
CD19	Adaptive immune antigen response	B cells lymphoblast maturation

STUDY 6: Impact of illness on SMN

Study 6 determines the degree to which SMN protein is affected by respiratory infections and which PBMC subtypes drives this effect

In Study 5 it was noted that subjects #1002 and #1036 had highly variable SMN levels. The subjects were re-collected at a subsequent timepoint developing signs and symptoms of an initial or recurrent respiratory infection. Samples were collected at Day 0, Day 7, and Day 30, and Day 76 !2-3 days after onset of fever, cough, sore throat. PBMCs were isolated at each timepoint for SMN protein analysis with additional fractionation and SMN protein measurements of the PBMC subtypes at Day 7. While PBMC subtypes fluctuate by up to 5x, variability in the CD8 population is more limited (by ~0.3x) and could be useful for normalizing the total SMN signal.

In addition, cells from Day 76 were also assessed in DNA quantitation assays and compared to manual cell counts (Figure 6). A Cyquant cell proliferation dye assay was used per manufacturer instructions and read with a fluorescent plate reader. DNA quantification versus cell counting may provide a more reliable method for normalizing SMN.

Figure 5: SMN PBMC Subtypes in Subjects with Respiratory Infections; lesser variability in CD8 population

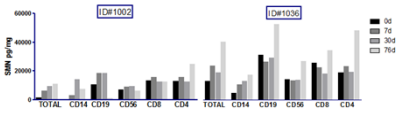
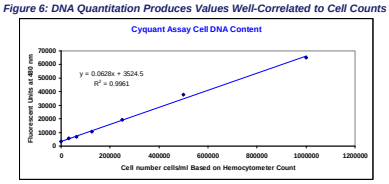


Figure 6: DNA Quantitation Produces Values Well-Correlated to Cell Counts



STUDY 7: PBMC signals in SMA patients

Study 7 (SMAF-001) evaluated the SMN signal and yield in PBMCs from SMA patients and carriers. While the sample size was too small to draw conclusions, age and medication may impact SMN levels in PBMCs.
In Study 7, blood was collected and processed to PBMCs from N=15 SMA patients and N=12 carriers, on an IRB-approved protocol. Each subject self-identified as a patient or carrier, and was seen for a single visit. Subjects provided information on their type, age of onset, current motor function (ambulatory or non-ambulatory), highest motor function achieved (sitting, rolling, crawling, standing, walking), age, and medications for the past 7 days. PBMCs samples were processed and SMN signal measured after 24h delay. Individual aliquots of the PBMC lysates were created for analysis at 48, 7d, 1mo, 3mo and 6mo after initial collection and processing to determine lysate signal stability.

There was trend towards decreased SMN in older subjects (Figure 8). Some SMA subjects were on Valproic acid (N=4 ages 1-4, while others (N=4, ages 22-60) were on drugs that have known effects on blood cells (atenolol, flonase, singulair, zyrtec) had a tendency towards lower values as well.

SMAF-001 Demographics

Characteristic	Age (yrs)	N
Type 2		7
Females	1-50	6
Males	1.4	1
Type 3		5
Females	2- 60	1
Males	4	4
Carrier		15
Females	25-57	9
Males	32-48	6
SMA Sitting	1.4-60	4
SMA Crawling/standing	1-13	2
SMA Walking	2-4	6

Figure 7: PBMCs Count and SMN Levels by SMA Type

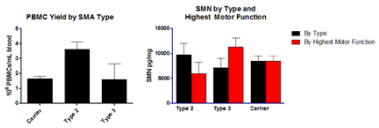
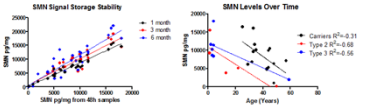


Figure 8: SMN Signal Stability and Cross-Sectional SMN levels over Age



Conclusions

Changes in the PBMC lysate density and use of lysis buffer with the SMN protein standard yielded improvements in the protein signal by the SMN ELISA.

Several factors affect the levels of SMN protein in PBMCs, including age, infections and processing times. Normalization to PBMC subpopulation may mitigate the variability to some degree. Delays of 48-72h in isolating PBMCs reduced SMN levels significantly, down to undetectable levels. PBMCs collected from individuals experiencing symptoms of respiratory Infections had levels which varied from -20 to +2-fold from baselines across subcellular populations, depending on time since symptom onset though CD8+ populations were less variable. DNA quantification by kit also may reduce any variability or imprecision due to manual cell counting. Finally, PBMC SMN levels appeared to decrease in a cross-sectional manner with age (R~-0.50, p<0.01); as this could impact interpretation of clinical trials, measuring SMN protein levels longitudinally is worthy of further investigation.

These results have guided the generation of a best practice protocol for PBMC SMN ELISA analysis and highlight important concepts for the interpretation of SMN signal:

- **Optimized isolation of PBMCS with EDTA tubes for whole blood collection followed by isolation on a lymphoprep gradient in 24h or less**
- **Extraction of SMN with 10^7 cells/mL ER4 reagent and use of 1:8 ER4 in dilution of SMN standard**
- **Careful determination of accessory clinical information e.g. respiratory infections help to interpret SMN data**
- **Consideration for the impact of age and drugs that have effects on blood in interpreting SMN signal in PBMCs**

Acknowledgments

We have many people to thank for their contributions to this work: The generous SMA patients and parents, and healthy volunteers who contributed samples for these studies, and The Families of SMA and their local chapters in Michigan who provided invaluable help in recruiting for Study SMAF-001. All work was funded by the SMA Foundation.