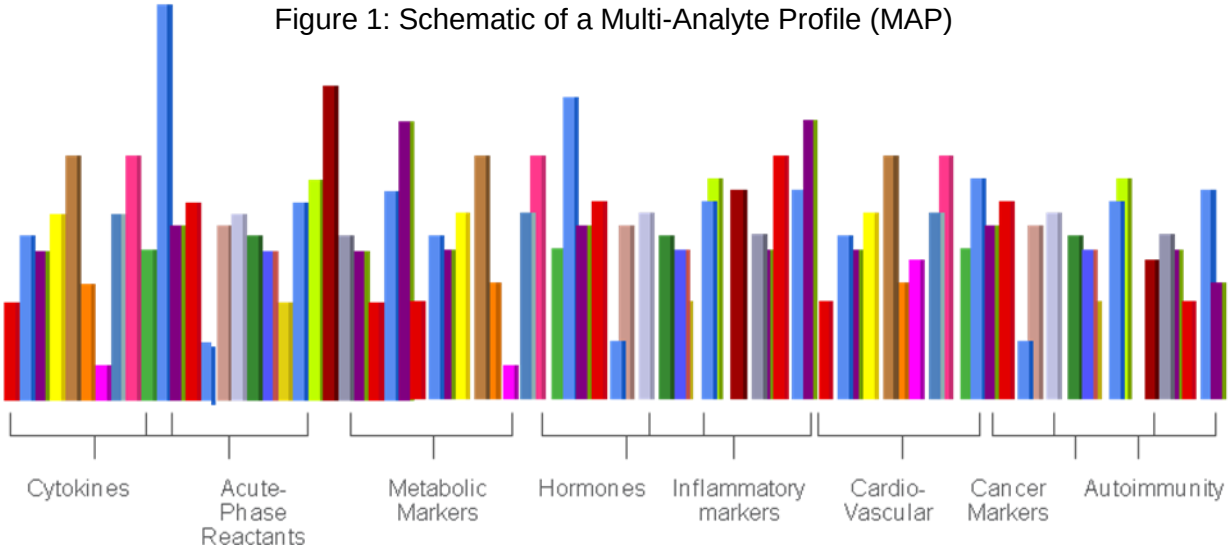
Background

The Spinal Muscular Atrophy (SMA) Foundation and Myriad Rules-Based Medicine, Inc. (Myriad RBM) are collaborating on a program to develop a panel of plasma protein biomarkers for SMA using Myriad RBM's Multi-Analyte Profiling (MAP) technology platform. In this collaboration, Myriad RBM is helping to discover new biomarkers for SMA and also confirming plasma protein biomarker candidates previously identified from the multicenter Biomarkers for SMA (BforSMA) clinical study, sponsored by the SMA Foundation and conducted by the Biomarkers for SMA Study Group in conjunction with BG Medicine (Waltham, MA). The identification of such biomarkers may help to assess drug efficacy and shorten the duration of clinical trials of SMA therapies.

In the initial stage of the collaboration, the SMA Foundation provided BforSMA plasma samples to Myriad RBM for processing on their multiplexed immunoassay panel DiscoveryMAP® 250+ (Figure 1), which measures hundreds of biochemical markers in a very small sample volume. Candidate biomarkers were identified that significantly differentiated between disease and control groups and that correlated with SMA disease severity. The SMA Foundation and Myriad RBM are continuing to analyze these results and plan to create a specific panel of biomarker assays for use in clinical trials exploring new treatments for SMA. In particular, access to the custom SMA MAP panel and the data from prior analyses with the BforSMA and other SMA sample collections are being made available for NEXT SMA Biomarker applicants with an interest in using the panels in their proposal.

Figure 1: Schematic of a Multi-Analyte Profile (MAP)



Biomarkers and the Potential Use of the Custom SMA MAP Panel

A biomarker is any measurement that can describe a biological state or process. There are several types of biomarkers, which largely fall into 3 categories: diagnostic markers, prognostic markers and response markers. Diagnostic markers are those that can define a disease state, like the presence of SMA in an individual. Prognostic markers are those that are indicative of a disease process and relate to the natural history of disease, in the absence of any therapeutic interventions. Response biomarkers are those that inform on a change in status due to the application of a therapeutic intervention. Pharmacologic markers are an important subcategory of response biomarkers that relate to changes due to drug activity, e.g. an increase in SMN

protein or transcript in response to treatment with an SMN-upregulating compound. Surrogate markers are another type of response biomarker that relates to an actual clinical endpoint. Surrogate markers require significant qualification and often years of drug trials before they can be used to represent clinical changes.

The custom SMA MAP panel was developed initially from analysis of samples from SMA patients and control subjects in a single-visit study at a time when no prescription medications were being taken. Further confirmation was done using samples from patients that were on medications (including putative SMN-enhancing drugs) at two timepoints 12 months apart. In both cases, the biosamples that informed the identification and development of the SMA MAP came from SMA patients with different severities of disease. Thus, the markers identified here may be of value for qualification as prognostic markers. Furthermore, as several of the markers in the custom panel are members and modifiers of Receptor Tyrosine Kinase/AKT and Transforming Growth Factor canonical pathways that have been implicated as SMN modifiers or expression regulators, a subset of the markers may also have value as SMN pharmacodynamics markers (1-3).

Custom SMA MAP Panel Overview

An effort was undertaken to identify a marker or panel of markers in plasma from a wide range of SMA patients that segregates with measures of clinical severity. Initial hits were identified using samples from the BforSMA clinical study (Crawford et al submitted for publication). Markers were identified as hits for their association with disease severity as determined by motor function as measured by the Modified Hammersmith Functional Motor Scale (MHFMS). Data was generated from the BforSMA panels across two different discovery studies or campaigns. The first campaign was a **LC/MS** iTRAQ analysis done by BG Medicine. The second campaign used the human **DiscoveryMAP® v 1.0** and **OncologyMAP® v 1.0** immunoassay panels described above. In these two platforms, the BforSMA samples were used to identify 161 potential SMA plasma protein biomarkers. From the complete hitlist, the top 35 hits were selected with a combination of statistical analyses and assay performance evaluation. These 35 hits represent the SMA MAP analytes, a custom panel of SMA biomarkers which will be confirmed using a new SMA plasma sample set derived from the Pediatric Neuromuscular Clinical Research Network's (PNCR) SMA Natural History Study (NHS). A manuscript describing these results is being prepared for publication. All of the data for the BforSMA study will be released in a public database (http://neuinfo.org/BforSMA). The confirmation of the panel in the PNCR SMA samples will include analysis against different Hammersmith scales and extend to other motor function scales, pulmonary function, quality of life, electrophysiology, nutritional status and measures of SMN protein and transcript. These data will be available in July 2011.

Statistical Analysis

The LC/MS analyte data were analyzed using univariate and multivariate regression methods. Age and gender were controlled for in the analysis. Hits were identified by significant p-values whereby the lowest p-values were the strongest hits. The MAPs were analyzed using ANOVA, t-test, Fisher's Exact test, Pearson's correlation and by multivariate regression analysis (linear, lasso, random forest). Analytes that had a high number of missing values (greater than 40% below limit of detection) were excluded from the analyses. Analytes whose values were driven by a few outliers generally fell out of the hitlist using these analyses. The false discovery rate (FDR) was controlled by the Benjamin Hochberg method and the FDR q-value cutoff was set at 0.10. Further multivariate analyses were performed on both the LC/MS and MAP data to identify the best hits from all three discovery campaigns (Figure 2). The ability of top hits to predict SMA function scores is represented in Figure 3.

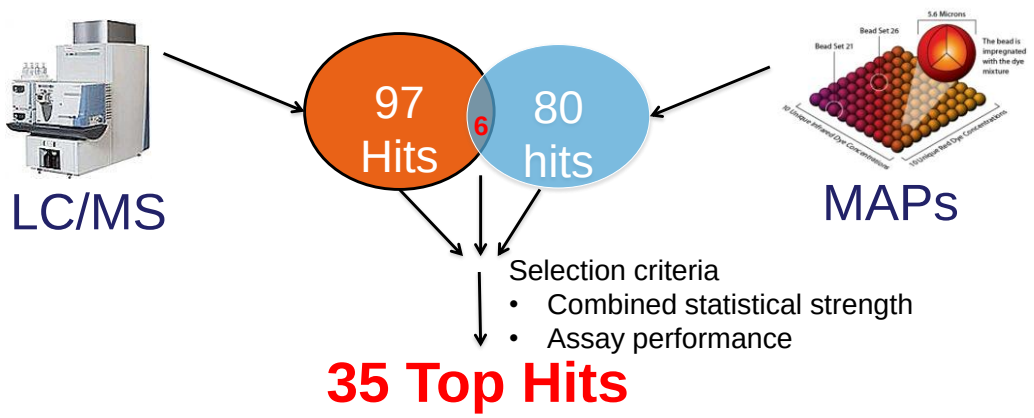


Figure 2: SMA plasma protein biomarker discovery plan

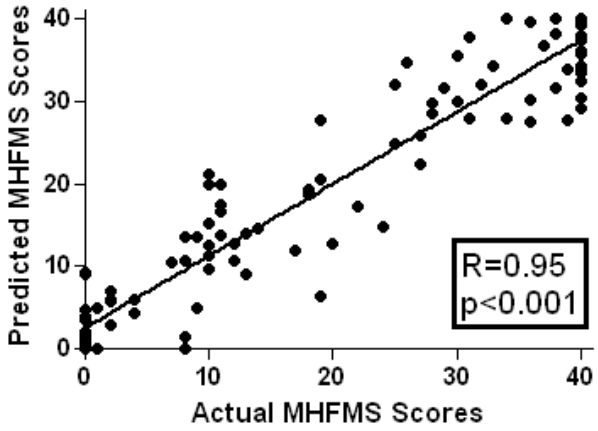


Figure 3: Correlations between actual MHFMS scores and scores predicted by a panel of SMA plasma biomarker

BforSMA Samples

Samples used in the first analysis were plasma samples collected in the BforSMA Study. This study was a multi-center, pilot study enrolling 130 subjects, age 2 to 12 years from 18 academic pediatric neuromuscular clinics. Each subject was seen for a single visit, during which an assessment of functional ability, pulmonary status and nutritional status was performed. No therapeutic intervention occurred.

In the BforSMA study, three groups of SMA patients and one cohort of control children were enrolled according to the classifications below:

• Children with **type I** SMA (n=17)
• Children with **type II** SMA (n=49)
• Children with **type III** SMA (n=42)
• Control children (n=22)

129 plasma samples were collected from the SMA patients and matched control subjects. These samples were used in ensuing LC/MS and MAP platform biomarker analyses.

PNCR NHS Samples

The assays are being further developed in the context of the analysis of a set of samples obtained from the Pediatric Neuromuscular Clinical Research Network (PNCR) SMA Natural History Study (NHS) from Columbia University, Boston Children's Hospital and Children's Hospital of Philadelphia. This study was a multisite, longitudinal prospective study enrolling 101 patients age 4 months to 45 years from three academic pediatric neuromuscular clinics. Subjects were seen at 0, 6, 12, 18, 24, 30 and 36 month visits, during which assessments using multiple motor scales and tests were made; pulmonary status, strength, nerve/muscle physiology and nutritional status assessments were also performed. No therapeutic intervention occurred.

Three SMA groups from the PNCR NHS were enrolled according to the classifications below:

- Subjects with **type I** SMA (n=27)
- Subjects with **type II** SMA (n=40)
- Subjects with **type III** SMA (n=34)

158 plasma samples from the NHS are used in the SMA MAP confirmation analysis, including N=36 Type Is, N=65 Type IIs and N=57 Type IIIs samples from the 0 (N=100) and 12mo (N=58) visits, aged from 3 months to 40 years.

SMA MAP Panel

The 35 top hits were identified and selected for their statistical value and assay performance for use in a new SMA MAP panel with both LC/MS and DiscoveryMAP and OncologyMAP analytes. New immunoassays were created for the top 9 LC/MS analyte hits: Tetranectin (CLEC3B) Tenascin XB, CD93, Cartilage Oligomeric Protein, Lumican, Cadherin13, Dipeptidyl peptidase 4, Peptidase D, and Thrombospondin-4, and the remaining 27 hits came from the DiscoveryMAP (N=15) and OncologyMAP (N=11) analyses. A table of the hits is provided below.

#	Protein	Description	Top 9 LC/MS?	DiscoveryMAP?	OncologyMAP?
1	MDC	Macrophage-Derived Chemokine	No	Yes	N/A
2	MIP1B	Macrophage Inflammatory Protein 1 beta	No	Yes	N/A
3	PPY	Pancreatic Polypeptide	No	Yes	N/A
4	AXL	AXL Receptor Tyrosine Kinase	No	Yes	N/A
5	HCC4	Chemokine CC4; C-C motif chemokine 16	No	Yes	N/A
6	ANGPT2	Angiopoeitin2	No	Yes	N/A
7	MMP7	Matrix Metalloproteinase 7	No	Yes	N/A
8	APOA4	Apoliprotein A IV	No	Yes	N/A
9	HER-2	Human Epidermal Growth Factor Receptor 2	No	N/A	Yes
10	SPP1	Osteopontin	No	N/A	Yes
11	KLK5	Kallikrein 5	No	N/A	No
12	PLGF	Placental Growth Factor	No	Yes	N/A
13	LEP	Leptin	No	Yes	Yes

14	ENG	Endoglin	No	N/A	Yes
15	TNFRI	Tumor Necrosis Factor Receptor I	No	N/A	No
16	GLO1	Glyoxalase 1; Lactoylglutathione lyase	No	N/A	Yes
17	ERBB3	Receptor Tyrosine Protein Kinase erbB	No	N/A	No
18	CDH13	Cadherin 13	Yes	N/A	N/A
19	TNXB	Tenascin X Isoform B	Yes	N/A	N/A
20	IGFBP6	Insulin-like Growth Factor Binding Protein 6	No	N/A	Yes
21	MB	Myoglobin	No	Yes	N/A
22	CD93	CD93; Complement Component 1, Q Receptor	Yes	N/A	N/A
23	COMP	Cartilage Oligomeric Protein	Yes	N/A	N/A
24	DPP4	dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	Yes	N/A	N/A
25	LUM	Lumican	Yes	N/A	N/A
26	PEPD	Peptidase D	Yes	N/A	N/A
27	THSB4	Thrombospondin 4	Yes	N/A	N/A
28	CTSD	Cathepsin D	No	N/A	Yes
29	FBLN1	Fibulin 1C	No	N/A	No
30	CLEC3B	C-type lectin domain family 3, member B; Tetranectin	Yes	N/A	Yes
31	YKL-40	YKL-40; Chitinase-3-like protein 1	No	N/A	Yes
32	CRP	C-reactive protein, pentyraxin-related	No	Yes	N/A
33	APCS	Serum Amyloid P Component	No	Yes	N/A
34	CFH	Complement Factor H	No	Yes	N/A
35	ASHG	Alpha-2-HS-glycoprotein; Fetuin-A	No	Yes	N/A

SMA MAP Panel and Data Availability

The PNCR NHS samples were analyzed in June 2011 and protein values will be available in mid-July. Statistical analysis of the panel results against multiple outcome measures alone and in concert with SMN protein and transcript measures will be made available by late July. The 35 marker SMA MAP panel is available to others for testing in August 2011. The SMA Foundation will continue with another project to streamline the panel further, which will decreasethe sample volume requirement and reduce the cost for testing each sample..

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Appendix: Multi-Analyte Profile (MAP) Technology

Rules-Based Medicine ("RBM") is an Austin, Texas based biotechnology company started in 1999 as a research and development project and as a wholly owned subsidiary of the Luminex Corporation. RBM completed a spinout transaction from Luminex in September 2002. In May, 2011, Myriad Genetics, Inc. completed the acquisition of RBM, which now operates under the name Myriad RBM, Inc. as a wholly-owned subsidiary of Myriad Genetics, Inc.

By leveraging experience and expertise with the powerful Luminex xMAP® technology platform, RBM has developed species-specific MAPS that measure hundreds of biochemical markers in a very small sample volume. Luminex technology performs up to 100 multiplexed, microsphere-based assays in a single reaction vessel by combining optical classification schemes, biochemical assays, flow cytometry and advanced digital signal processing hardware and software (Figure 4). Multiplexing is accomplished by assigning each analyte-specific assay a microsphere set labeled with a unique fluorescence signature (Figure 45A). Assay-specific capture reagent (e.g., antibody) is conjugated covalently to each unique set of microspheres. The assay-specific capture reagent on each individual microsphere binds the analyte of interest.

RBM MAPs provide the diagnostic researcher an unprecedented ability to discover biomarker patterns within very small sample volumes using a fast, accurate and cost-effective method. MAPs offer additional advantages in that they are developed fully, validated and available for immediate use. Once the biomarker patterns for a specific aim are discovered and validated, the biomarkers themselves can be adapted to many other detection platforms. Currently, RBM's MAPs measure markers of cancer, infectious disease, autoimmunity, cardiovascular risk, as well as hormones, cytokines/chemokines, acute phase reactants, clotting proteins, growth factors, tissue modeling factors and other typical plasma proteins. This massive screening approach is employed by pharmaceutical and medical research communities to identify combinations, levels, or absences of proteins associated with various disease states or drug treatments.

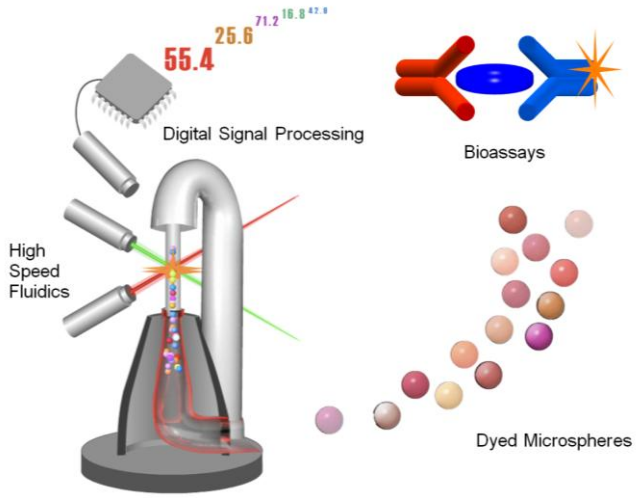


Figure 4: Schematic of Luminex Technology

A cocktail of assay-specific, biotinylated detecting reagents is reacted with the microsphere mixture, followed by a streptavidin-labeled fluorescent “reporter” molecule. Similar to a flow cytometer, as each individual microsphere passes through a series of excitation beams, it is analyzed for size, encoded fluorescence signature and the amount of fluorescence generated in proportion to the analyte.

A minimum of one hundred individual microspheres from each unique set are analyzed and the median value of the analyte-specific, fluorescence is logged. Using internal controls of known quantity, sensitive and quantitative results are achieved with precision enhanced by the analysis of one hundred microspheres per data point. Before Luminex xMAP®, the technology was not available to cost-effectively quantify large numbers of biomarkers in a very small sample volume.

To attain 100 distinct microsphere signatures, two fluorescent dyes, red and far red, are mixed in various combinations using ten intensity levels of each dye (i.e., 10 x 10). Each batch or set of microspheres is encoded with a fluorescent signature by impregnating the microspheres with one of these dye combinations (Figure 5B). After the encoding process, an assay-specific capture reagent (i.e., antigens, antibodies, receptors, peptides, enzyme substrates, etc.) is conjugated covalently to each unique set of microspheres (Figure 6).

Covalent attachment of the capture reagent to the microspheres is achieved with standard carbodiimide chemistry using carboxyl functional groups located on the surface of each 5.6 μm microsphere and primary amines within the capture reagent. Coupling chemistry is performed on large numbers of individual microspheres (10^7-10^9 microspheres/mL) simultaneously within each unique set, resulting in low microsphere-to-microsphere variability.

After optimizing the parameters of each assay separately, MAPs are performed by mixing up to 100 different sets of the microspheres in a single well of a 96- or 384-format microtiter plate. A small sample volume (10 μL - 20 μL) is added to the well and allowed to react with the microspheres. The assay-specific capture reagent on each individual microsphere binds the analyte of interest. A cocktail of assay-specific, biotinylated detecting reagents (e.g., antigens, antibodies, ligands, etc.), is reacted with the microsphere mixture, followed by a streptavidin-labeled fluorescent “reporter” molecule (typically phycoerythrin). Because the microspheres are in suspension, the assay kinetics are near solution-phase. Finally, the multiplex is washed to remove unbound detecting reagents.

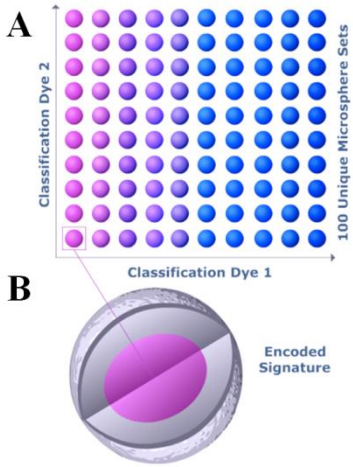


Figure 5. Schematic of one hundred unique microsphere sets. Each set contains a specific ratio of two fluorescent dyes on the interior of the microsphere, distinguishing the set from other sets in the array.

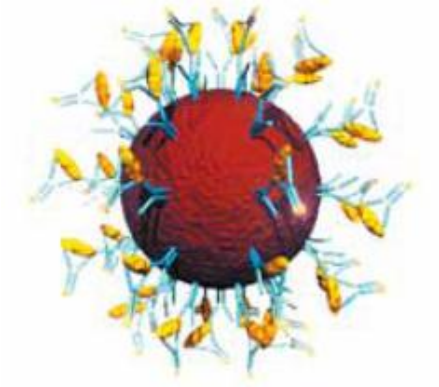
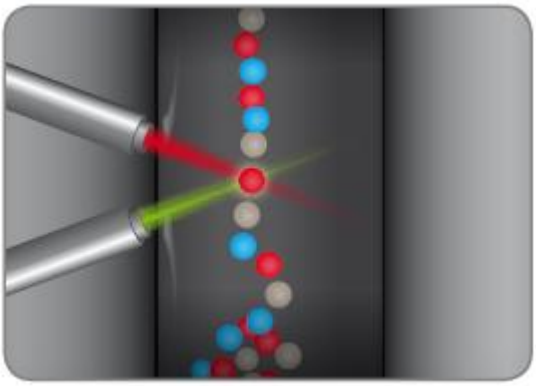


Figure 6. Schematic of a capture-sandwich immunoassay performed on the surface of each individual microsphere. Capture antibodies are conjugated to the surface of the microsphere. The capture antibodies react with specific antigens (yellow) present in the sample. Detecting antibodies, labeled with a fluorescent reporter molecule, bind in proportion to the captured antigen.

After washing, the mixture of microspheres is analyzed using the Luminex 100™ instrument. Similar to a flow cytometer, the instrument uses hydrodynamic focusing to pass the microspheres in single file through two laser beams (Figure 7). As each individual microsphere passes through the excitation beams, it is analyzed for size, encoded fluorescence signature and the amount of fluorescence generated in proportion to the analyte. Microsphere size, determined by measuring the 90-degree light scatter as the microspheres pass through a red diode laser (633 nm), is used to eliminate microsphere aggregates from the analysis. While in the red excitation beam, the encoded red and far red dyes are excited and the resulting fluorescence signature (ratio 660 nm / 720 nm) is filtered, measured using avalanche photodiodes, and classified to a microsphere set. Since each microsphere is encoded with a unique signature, the classification identifies the analyte being measured on that individual microsphere ~~(Figure 5C).~~ As the microsphere passes through a green diode-pumped solid state laser (532 nm), a fluorescence "reporter" signal (580 nm) is generated in proportion to the analyte concentration, filtered and measured using a photomultiplier tube (Figure 7).

Figure 7. Multiplexed assays are analyzed in a flow device that interrogates each microsphere individually as it passes through the path of a red and green laser. The green laser excites the assay reporter molecule on the surface of each microsphere and quantitates the resulting fluorescence in proportion to the amount of analyte present in the sample. The red laser excites the dyes encoded within each microsphere, leading to instant microsphere classification.



Data acquisition, analysis and reporting are performed in real-time on all microsphere sets included in the MAP. A minimum of one hundred individual microspheres from each unique set are analyzed and the median value of the analyte-specific, or "reporter," fluorescence is logged. Using internal controls of known quantity, sensitive and quantitative results are achieved with precision enhanced by the analysis of one hundred microspheres per data point. The quantitative data generated by the MAPs can be mined for distinctive patterns of biomarkers that indicate disease, drug toxicity or efficacy, or some other useful biochemical phenotype.

References

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