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Mass Spectrometric Discovery and Selective Reaction Monitoring (SRM) of Putative Protein Biomarker Candidates in First Trimester Trisomy 21 Maternal Serum

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The accurate diagnosis of Trisomy 21 requires invasive procedures that carry a risk of miscarriage. The current state-of-the-art maternal serum screening tests measure levels of PAPP-A, free bhCG, AFP, and uE3 in various combinations with a maximum sensitivity of 60−75% and a false positive rate of 5%. There is currently an unmet need for noninvasive screening tests with high selectivity that can detect pregnancies at risk, preferably within the first trimester. The aim of this study was to apply proteomics and mass spectrometry techniques for the discovery of new putative biomarkers for Trisomy 21 in first trimester maternal serum coupled with the immediate development of quantitative selective reaction monitoring (SRM) assays. The results of the novel workflow were 2-fold: (1) we identified a list of differentially expressed proteins in Trisomy21 vs Normal samples, including PAPP-A, and (2) we developed a multiplexed, high-throughput SRM assay for verification of 12 new putative markers identified in the discovery experiments. To narrow down the initial large list of differentially expressed candidates resulting from the discovery experiments, we incorporated receiver operating characteristic (ROC) curve algorithms early in the data analysis process. We believe this approach provides a substantial advantage in sifting through the large and complex data typically obtained from discovery experiments. The workflow efficiently mined information derived from high-resolution LC−MS/MS discovery data for the seamless construction of rapid, targeted assays that were performed on unfractionated serum digests. The SRM assay lower limit of detection (LLOD) for the target peptides in a background of digested serum matrix was approximately 250−500 attomoles on column and the limit of accurate quantitation (LOQ) was approximately 1−5 femtomoles on column. The assay error as determined by coefficient of variation at LOQ and above ranged from 0 to 16%. The workflow developed in this study bridges the gap between proteomic biomarker discovery and translation into a clinical research environment. Specifically, for Trisomy 21, the described multiplexed SRM assay provides a vehicle for high-throughput verification of these, and potentially other, peptide candidates on larger sample cohorts.

Keywords: biomarker • discovery • Trisomy 21 • SRM assay • mass spectrometry • proteomics

Introduction

Women over 35 years old constitute about 15% of pregnancies in most developed countries worldwide. The risk for many chromosomal defects in fetal development increases with maternal age. Down's Syndrome (DS) occurs at a rate of 1 in 600−800 pregnancies and prompts most prenatal diagnoses that involve invasive procedures such as amniocentesis or chorionic villous sampling. These procedures carry a risk of miscarriage and therefore are only applied to women in high risk groups.[1,2] Over the past several years, there has been an effort to develop less invasive screening methods that can identify aneuploidy early in the pregnancy. Maternal blood tests that measure serum protein markers associated with DS such as free b-hCG, PAPP-A, alpha fetoprotein and inhibin A, combined with the measurement of nuchal translucency, have garnered increased acceptance for first trimester screening[3] but the sensitivity and specificity of these markers could still be improved. Previous proteomic studies have identified a variety of other putative serum markers.[4−6] In the most recent study, the authors employed a combination of immunodepletion of high-abundance proteins coupled with quantitative mass

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spectrometric analysis using an isobaric labeling (iTRAQ) technology.[6] Immunodepletion was used to decrease the dynamic range of the proteins in plasma, in the hope of uncovering lower abundance putative markers. Although the sample size was small (6 samples from Trisomy 21 and 6 samples from normal pregnancies), the results of this study identified over 200 proteins with altered concentration in DS versus euploid plasma samples. Despite the immunodepletion, many of the differentially expressed proteins were relatively high-abundance proteins including several apolipoproteins, transferrin and complement component proteins. There is some overlap in the putative biomarkers discovered in these studies but relatively little consistency in expression ratios across the different techniques employed including 2-D DIGE, MALDI Mass spectrometry and shotgun proteomics. Partly, this discrepancy may be due to the fact that all these techniques except iTRAQ are not truly quantitative. In addition, some of the studies incorporated second trimester as well as first trimester samples, and the relative abundances of proteins may shift between these stages.[4,5] The results from these proteomic studies highlight the complexity of DS and the challenge of finding new markers with the requisite specificity, although the unmet need remains urgent. In the current study, we set out to bridge the gap between biomarker discovery and verification. Our goal was to expand upon the previous discovery studies and to develop a mechanism for ranking new putative biomarkers, thus facilitating their incorporation into higher-throughput, statistically rigorous verification studies. To achieve this goal, we coupled high-resolution LC−MS/MS with novel, label-free differential analysis software[7] to analyze a cohort of maternal blood samples from first trimester DS and normal pregnancies. Bioinformatic analysis was carried out using chromatographic alignment, global intensity-based feature extraction and aggregate protein identification. We did not perform sample immunodepletion as this added complexity and expense and likely would not be practical in a clinical research environment. As in the previous proteomic studies, we identified a large list of proteins with associated expression ratios and Pvalues, including PAPP-A. Several of the differentially expressed proteins in our study were in common with the previously published reports. Unfortunately, differential expression ratios do not provide any guide as to the potential "usefulness" of putative markers to differentiate sample classes. Therefore, we incorporated a novel algorithm for receiver operating characteristic (ROC) curve analysis as a filtering strategy to test the classification power of the initial list of differentially expressed proteins. The area under the curve (AUC) of ROC curves is typically used to calculate the sensitivity and specificity of diagnostic markers. To our knowledge, the application of ROC curves early in the process of biomarker discovery is a novel approach. In the current study, this strategy allowed much more efficient and rapid stratification and ranking of putative markers than using ratio and Pvalue alone. Upon the basis of the ROC AUC and other factors, such as relative protein abundance and literature references, we selected a subset of proteins for further development into SRM assays.[8,9] Interestingly, PAPP-A had a lower AUC (0.59) than all of the new putative markers we selected. Novel SRM development software facilitated the efficient selection of signature peptides from the targeted proteins and the resulting SRM analyses monitored predicted precursor and fragment transition ion pairs. Heavy-isotope internal standards were used for relative target peptide quantification and the SRM assays were then applied to the original cohort samples to verify the putative biomarker abundance ratios observed in the discovery experiments. Future experiments will test the SRM marker panel on other sample cohorts. The described workflow demonstrates an approach that may, in general, be applied to translate biomarker discovery studies into candidate verification assays.

Experimental Procedures

Clinical Serum Samples. Maternal serum samples from Trisomy 21 and normal first trimester pregnancies were provided by the Fetal Medicine Foundation and collected from study participants with full consent and approval. At the time of sample collection, the majority of the women were found to be high risk based on the FMF risk calculation for Trisomy 21 and were therefore classified as Trisomy 21. Diagnosis of Trisomy 21 was confirmed after birth. Blood samples were collected into red-top tubes (BD Vacutainer REF 367694 Z) that did not contain anticoagulant. The average sample volume was 7.0 mL. Tubes were labeled and kept at 4 °C less than 8 h prior to centrifuging. Samples were centrifuged at 1500 rpm for 15 min at 4 °C. At the end of the spin, the tubes were taken out carefully and placed on a rack. The separated sera were gently aspirated with a pipet, transferred to microfuge tubes, and frozen. Samples were transported frozen and were thawed immediately before processing. Twenty-four samples from Trisomy 21 patients and 21 samples from healthy, matched control patients were processed in a random order to prevent systematic errors and variations from experiment to experiment.

Sample Processing. Trypsin Digestion, Reduction/Alkylation and Desalting. Serum samples (25 μL) were thawed on ice and mixed with 100 μL of 8 M Guanidine HCl 150 mM Tris-HCl 10 mM DTT pH 8.5. Samples were incubated at 37 °C for 1 h. The denatured samples were then alkylated with 45 mM Iodoacetic acid (500 mM stock concentration in 1 M Ammonium Bicarbonate) in the dark for 1 h at room temperature. Residual alkylation agent was then reacted with 15 mM DTT. Samples are then diluted with 25 mM TrisHCl 5 mM CaCl2 to 2.5 mL, then added to a glass vial of trypsin(Pierce, 20ug, in 250uL of 25 mM acetic acid). Samples were allowed to digest overnight. Digested samples were desalted with HyperSep−96 C18 solid phase extraction media (Thermo Fisher Scientific). The HyperSep C18 resin was conditioned before use with *n*-propanol, then 0.25% TFA (v/v) water. Samples were then loaded on the resin and washed with 0.25% TFA/water (v/v) and eluted with 75% (v/v) acetonitrile in 0.1% (v/v) formic acid.

High Resolution LC−MS/MS. High resolution LC−MS/MS analysis was carried out on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a Surveyor MS pump and a Thermo Micro AS auto sampler. Samples, 10 μL, in 5% (v/v) acetonitrile 0.1% (v/v) formic acid were injected onto a 75 μm × 25 cm fused silica capillary column packed with Hypersil Gold C18AQ 5 μm media (Thermo Fisher Scientific), in a linear 250 nL/min gradient of 5% (v/v) acetonitrile, 0.1% (v/v) formic acid to 30% (v/v) acetonitrile, 0.1% (v/v) formic acid over the course of 180 min with a total run length of 240 min. The LTQ-Orbitrap was run in a top 5 configuration at 60K resolution for a full scan, with monoisotopic precursor selection enabled, and +1, and unassigned charge state rejected. The analysis was carried out with CID and HCD fragmentation modes.

Label-Free Differential Analysis, Protein Identification and Receiver Operating Characteristic (ROC) Analysis. Label-free differential analysis, protein identification and peaklist generation were performed using the SIEVE v.1.2.1 (Thermo Fisher Scientific) algorithm[7] incorporating the SEQUEST v.28 search engine,[10] Percolator[11] and a novel algorithm for classification incorporating ROC curves.[12] The iterative workflow included chromatographic alignment, global intensity-based feature extraction and aggregate protein identification assignment. Chromatographic alignment was based upon pairwise MS full scan comparison of all experimental MS runs with respect to a chosen reference MS run. Subsequent to chromatographic alignment, potentially interesting features were exposed based upon high-intensity peaks found in the aligned collective data set. Individually, these peaks defined frames, *ie* well-defined rectangular regions in the full scan (M/Z vs retention time) plane. Reconstructed ion chromatograms were calculated for each frame to assess relative expression ratios and supporting statistics. After framing, MS2 fragment scans associated with each frame were processed with SEQUEST[10] and peptide quality scores were derived by processing against decoy shuffled databases using Percolator.[11]

Peptides assessed with less than 2% estimated false discovery rate were retained and a consensus protein report was constructed by statistically aggregating frame information to construct peptides and peptide information to build proteins (see Supplementary Table 1 for the complete list of identified proteins). The ROC curves were created by calculating the true positive (TP) rate (sensitivity) and the false positive (FP) rate (100-specificity) to differentiate the two populations. For the ROC plots in this study, the FPs and TPs were tabulated as the criteria threshold was swept across both of the curves. The cutoff point or threshold used to discriminate the two populations was used to construct the ROC curves.

SEQUEST search parameters included the following:

1. Allowed missed cleavages: 3
2. Fixed modifications: 1 (C)
3. Variable modifications: 1 (M)
4. Mass tolerance for precursor ions: 25 ppm
5. Mass tolerance for fragment ions: 0.5 Da
6. Database searched: ipi human v361 processed database
7. Number of protein entries in the database actually searched: 82 806
8. Cut off score value for accepting individual MS/MS spectra: 2% FDR
9. Justification of the threshold: Percolator recommended value

Individual MS/MS spectra with ion assignments for single peptide identifications are provided in Supplementary Data File 1 (Supporting Information).

Upon the basis of the MS1 peak areas from the various LC−MS/MS runs, SIEVE identified proteins that discriminated the biological groups. For this implementation, the true positive and the false positive rates at any chosen threshold value of corresponding protein abundance were plotted to generate the ROC curve. The area under the curve (AUC) was then used to classify the candidate peptides. While many other statistical tools are commonly used to identify putative biomarkers,[13] we find that AUC is particularly robust in a large study such as this one where a high degree of biological variance is evident. In these cases, values such as expression ratios and *p*-values of the *t* test are highly sensitive to outliers.

SRM Assays. SRM assays were developed on a Vantage triple quadrupole mass spectrometer, Surveyor MS pump, CTC PAL Autosampler and an IonMax Source equipped with a high flow metal needle (Thermo Fisher Scientific). Reverse phase separations were carried out in a 9 min linear gradient from 5 to 30% B, with a total run time of 15 min (Solvent A = Water 0.2% FA, Solvent B = ACN 0.2% FA). The flow rate during the linear gradient was set to 160 μL/min. The total injection volume was 20 μL for all samples and points on the curve. A 50 mm $\times$1 mm 1.9 μm Hypersil Gold column was run at a temperature of 50 °C. There were three technical replicates per sample.

Calibration Curve Generation. Calibration curves were created with a pool of all samples as a background matrix. Each point on the calibration curve (and every sample analyzed) included 100 fmol of heavy labeled peptides. The amount of background matrix on column was 30 μg for each point on the calibration curve, as well as in all analyzed samples. In addition, all samples were brought up in a solution of 200 μg/mL of glucagon in 97% Water 3% ACN and 0.2% FA to minimize binding to plastic surfaces.

Choice of Peptides and Transitions. The obtained retention time information from the discovery MS experiments was imported into Pinpoint to build a preliminary scheduled SRM method for optimization. To accomplish this, individual instrument parameters such as collision energy, tube lens, dwell time and predicted retention times were automatically tested for every transition. After multiple iterations, the optimized, (i.e., highest intensity signal and least overlap with other transitions), list of peptides and transitions were finalized and one proteotypic peptide[15] and several fragment transitions were chosen for each protein, (see Supplementary Table 2 for a list of all peptides and transitions, Supporting Information), for a total of 12 peptides, both light and heavy-labeled, totaling 191 transitions. Figure 6a shows the distribution of these transitions over the gradient illustrating the benefit of scheduling. This technology ensured high quantitative data quality because no more than 64 transitions are monitored across any part of the gradient.

The decision to limit the number of peptides from each protein to one was based on two factors: (1) multiple peptides might quantify different isoforms of the same protein thereby producing conflicting results and our goal was to monitor the most common isoform (and hence the most abundant peptide), and (2) the choice of a nonredundant peptide sequence and its characterization using a synthetic standard with multiple, coeluting transitions ensured the verification of peptide identity (eliminating interferences) and thus its validity as a surrogate for the target protein.

Pinpoint software (Thermo Fisher Scientific) was used for targeted protein quantitation (Figure 1). Peptides were identified by coeluting light and heavy-labeled transitions in the chromatographic separation. For additional verification and elimination of interferences, the transition ratios were confirmed using discovery spectra. Time alignment and relative quantification of the transitions were performed with Pinpoint. All clinical samples were assayed in triplicate.

t-SRM (Scheduling of SRMs). Transitions were scheduled based on the retention time observed in the discovery experiments. In the first iteration, a window of $\pm$1 min (around the mapped discovery retention time, see Figure 3), was used to schedule the various transitions. In the next iteration, a window of $\pm$0.5 min (around the observed retention time) was used to schedule the transitions. The choice of the window size was

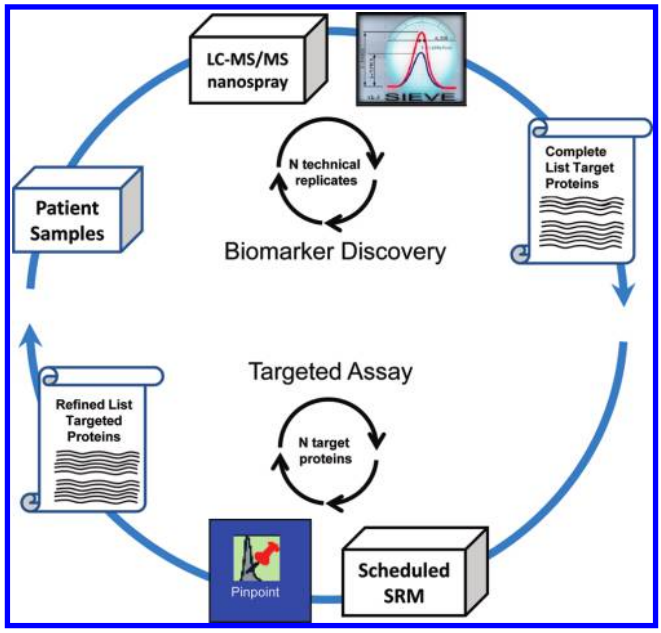

Figure 1. Discovery-driven SRM Assay workflow.Samples are analyzed with high-resolution LC−MS/MS and SIEVE software to generate a database of differentially expressed proteins. The SIEVE database is then mined by Pinpoint software and a subset list of target proteins is chosen for SRM assay development. Iterative, scheduled SRM assays are run on a triple quadrupole MS to optimize the assay. Clinical samples are then interrogated with the optimized, multiplexed SRM assays.

based upon the error rates that are typically seen in the two steps. Over the course of multiple iterative serum runs, the retention times varied by less than 10 s or <2% of the gradient length. The corresponding start and end times for the various transitions are stated in Supplementary Table 2 (Supporting Information).

Light and Heavy-Labeled Peptides. Light and heavy versions of each target peptide were synthesized (Thermo Fisher Scientific, Ulm Germany). Heavy peptides had identical sequences to the light peptides, but the C-terminal Lysine or Arginine was fully labeled (>98.5%) with ^{13}C or ^{15}N,[14] see Supplementary Table 2 for peptide sequences (Supporting Information). The peptides were high purity (>97%) allowing us to confidently characterize their ionization, elution and fragmentation characteristics, therefore facilitating optimization of the SRM assay.

Ingenuity Pathway Analysis. Protein interactions and pathways were analyzed using the Ingenuity Pathway Analysis (IPA) software and database (Ingenuity Systems, Redwood City, CA). Ingenuity's knowledge base is created by manual curation of the scientific literature supported by experimental results that are structured into an ontological relational database. We analyzed a data set of 378 proteins generated from the differential expression discovery analysis using SIEVE software. The data set was imported directly into IPA for a Core analysis. For IPA network analysis, direct interactions between the molecules were analyzed.

Results

Figure 1 illustrates a mass spectrometry-driven workflow linking unbiased biomarker discovery with the development of targeted assays. This strategy facilitates the high-throughput verification of large lists of putative biomarkers in an efficient and rapid manner. Inherent to the success of the workflow is the similarity of peptide ion fragmentation behavior in trap and triple quadrupole instruments. Figure 2 demonstrates the correspondence between fragment ion intensities in CID and fragmentation spectra generated an LTQ linear ion trap and triple quadrupole mass spectrometers, respectively. This correspondence holds true for different triple quadrupole instruments such as TSQ Quantum (Figure 2) and Vantage (used in this study) because the instruments are identical with respect to Q1, Q2 and Q3 and the high energy collision dissociation (HCD) mode. Therefore, fragmentation is expected to be identical. As is evident from the figure, major fragment ion intensities are closely correlated allowing the data generated in discovery experiments with SIEVE software to be mined for the optimization of SRM assay development using Pinpoint software. Although algorithms can be used to predict unique peptides and SRM transitions (in the absence of empirical discovery data), this hypothetical approach provides no assurance that the peptides will actually be detected in the sample. Therefore, the optimal approach, in our hands, was to utilize MS/MS spectra from LC MS/MS discovery experiments to enhance the design of effective SRM assays for putative biomarker verification. Furthermore, development of a comprehensive SRM method for multiple peptides and transitions required efficient scheduling of peptides and transitions across the LC gradient. In order to extrapolate elution behavior of peptides from the discovery LC workflow to the targeted LC workflow, 10 heavy peptides were spiked into samples and run on both platforms (discovery hybrid trap and targeted triple quadrupole, see methods section). The observed retention times obtained with both systems were used to generate a retention time plot comparing the linearity of the discovery LC versus the targeted LC workflows (Figure 3). The high R^2 value (0.93) demonstrated the strong correlation between the two workflows thereby ensuring the feasibility of predictive scheduling.

Analysis of High Resolution MS2 Data by SIEVE. Individual samples were processed and subjected to nanospray high-resolution LC−MS/MS and the resulting RAW mass spectral files were analyzed with SIEVE as described in the methods.

In order to generate a "short list" of putative biomarkers for further verification and development into SRM assays the ROC plots and AUC values for every peptide created by SIEVE were evaluated. Figure 4 represents a screen capture of the protein report for one such differentially expressed protein, Serum Amyloid 4 (SA4). Figure 4, top panel, shows the integrated intensities for the peptide EALQGVGDMGR in each of the MS raw files. Blue represents Trisomy 21 samples and red represents normal samples. The LC−MS/MS analysis identified 9 individual peptides for SA4 protein, Figure 4, bottom panel. Average protein ratios were calculated using variance weighted averaging of each individual peptide measurement. Figure 4, bottom panel, shows a whisker plot of the ratios calculated for the 9 peptides from SA4. The average weighted ratio of all peptides from SA4, Trisomy 21 over Normal, was 0.61 with a calculated area-under-ROC curve of 0.71 (Table 1), indicating that it was underexpressed in the Trisomy 21 samples and had potential to differentiate the two groups. Using this approach, all RAW files were analyzed and a list of differentially expressed proteins was generated (see Supplementary Table 1 for the complete list of differentially expressed proteins and peptides, Supporting Information).

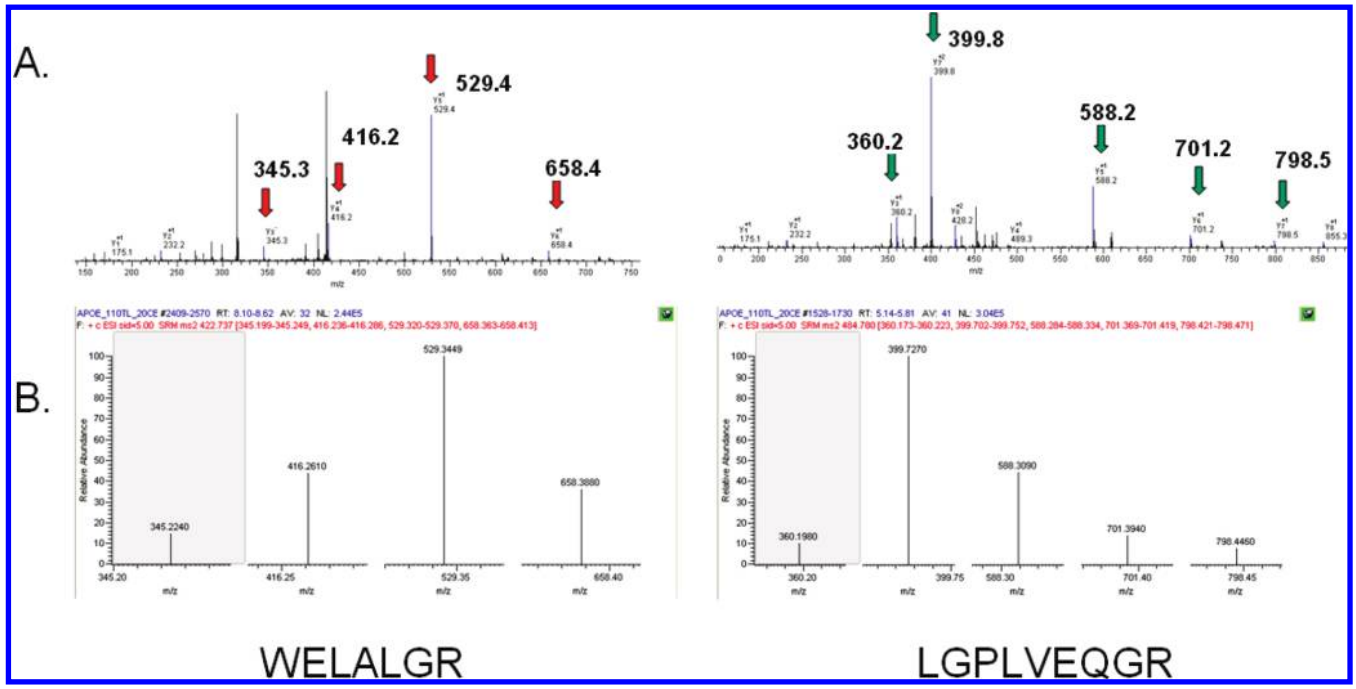

Figure 2. Comparative product ion spectra for two human apolipoprotein E peptides in +2 charge state. (A) LTQ via on-resonance CID. (B) SRM on the TSQ Quantum Ultra. The Quantum Ultra (this figure) and Vantage triple quadrupole (used in the study) instruments are identical with respect to Q1, Q2 and Q3. The high energy collision dissociation (HCD) fragmentation is therefore expected to be identical. A high degree of correspondence between the two platforms is evident in the intensities of the most abundant fragment ions.

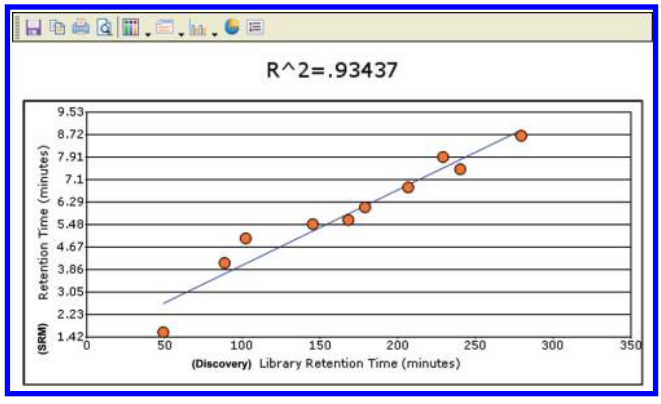

Figure 3. LC retention time plot for 10 heavy peptides comparing the discovery (LTQ Orbitrap XL hybrid trap) and targeted (TSQ Vantage triple quadrupole) platforms. Peptides were chosen based upon their predicted elution times across the gradient. The Discovery gradient was 180 min in length and the SRM gradient was 9 min in length. The observed linear correlation between the two LC workflows was 0.93.

Most of the differentially expressed proteins that were identified, (with FDR of 2% or less), were underexpressed. Potential systematic baseline bias in the individual peptide ratio measurements was excluded by examining the ratio of all monoisotopic/C_{12} peaks in the entire full scan data set. In this case, the overall ratio was determined to be 0.99 ± 2.2. PAPP-A protein was one of the proteins identified and the Trisomy 21 over normal expression ratio was 0.69. The underexpression of PAPP-A in the Trisomy 21 vs normal samples in this cohort is consistent with its expression (underexpression) in first trimester DS screening applications. The ROC AUC for PAPP-A was 0.59 indicating that it had relatively weak classification power in this cohort and significantly lower classification power than many other proteins. Our intent was to discover and evaluate new putative markers therefore we did not include PAPP-A for further evaluation in our study. From the complete protein list, we selected 12 proteins for further development into multiplexed SRM assays (Table 1) using the following criteria:

1. AUC curve values > 0.60. This filter included only proteins that had the potential to discriminate Trisomy 21 from normal samples. Many of the differentially expressed proteins had low (<50) AUC values; the highest was serum amyloid 4 with a value of 0.71 (Table 1).

2. High-abundance proteins. This emphasized proteins that could be robustly identified by SRM assays in serum digests without fractionation, depletion or further enrichment, thereby ensuring a simple assay that could be easily developed and potentially translated into a clinical research environment.

3. Literature references. Novel protein biomarkers that had either been previously reported to be involved with Trisomy 21 or other pregnancy-related diseases. We chose not to focus on markers (PAPP-A, fbHCG, etc) that are already in routine clinical use.

With respect to the determination of what qualified as an acceptable differentially expressed target protein, we believe that ratio is less important than classification power; hence, our choice of ROC AUC as a more stringent distinguishing criteria. Also, because discovery experiments are so time and labor intensive, we chose to run a larger set of biological replicates (versus technical replicates) in our discovery experiments to gain a better understanding of the biological variance. Indeed the point of developing the described workflow was to employ the high-throughput SRM assays to verify the putative list of target biomarkers initially identified in the discovery experiments. Based upon our previous experience with the described discovery platform, our coefficient of variation was typically less than 15%.[7]

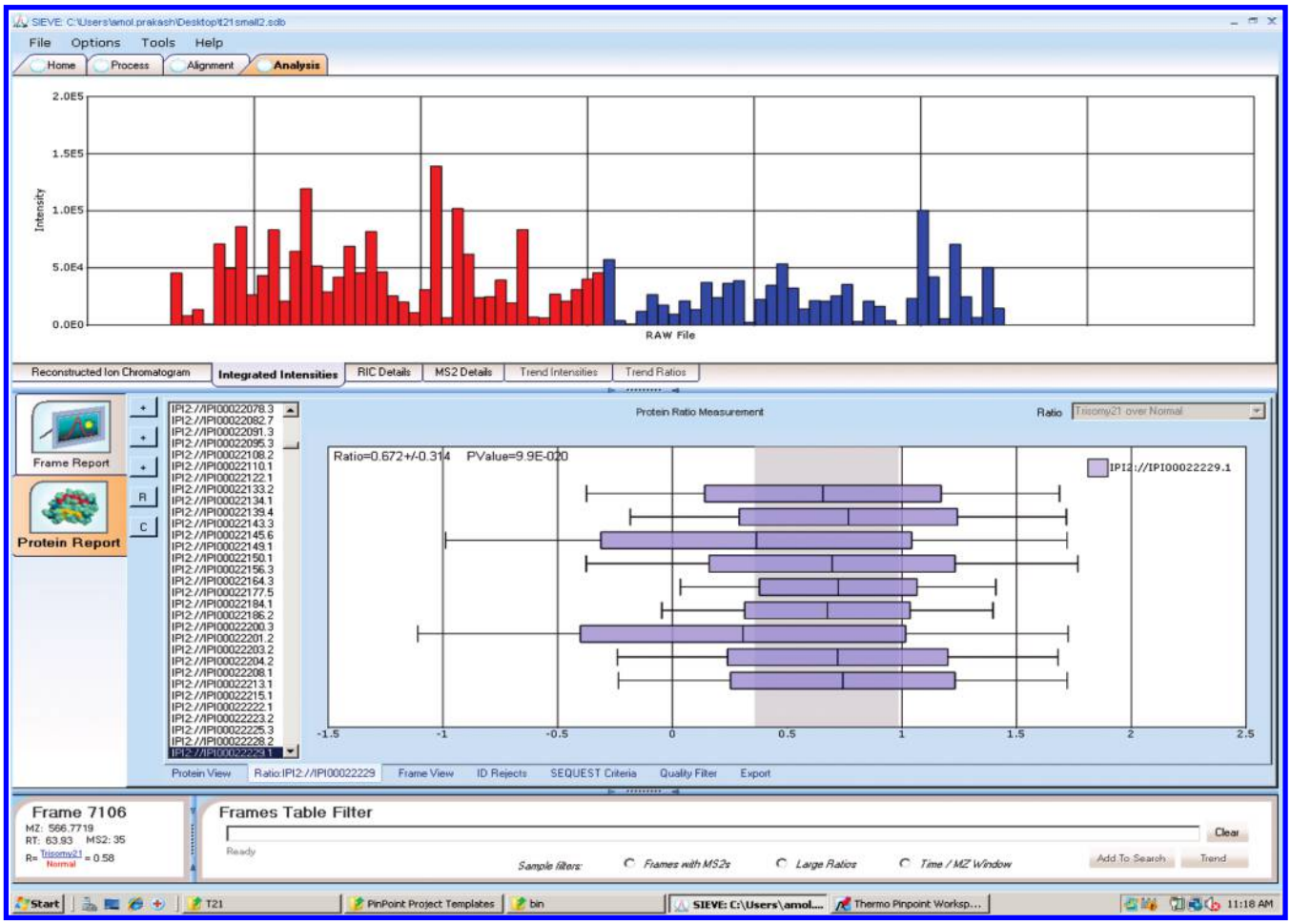

Figure 4. Label-free quantitative analysis using SIEVE. Protein report view of differential expression analysis. (Top panel) Integrated intensity graph for Serum Amyloid 4 (SA4) peptide EALQGVGGDMGR in all raw files, Red = Normal, Blue = Trisomy 21. (Bottom panel) Whisker plot of Normal/Trisomy 21 ratios for all peptides identified in the data set from SA4. The average ratio for all the peptides is 0.67 $\pm$ 0.314 with a *p*-value of 9.9 $\times$ 10^{-20}.

Table 1. Expression Ratios of Biomarker Candidates from SIEVE and Pinpoint Analyses

protein ID	SIEVE T21/N average ratio all peptides FDR 0.02	SIEVE area-under ROC curve	SIEVE number of peptides	Pinpoint T21/N ratio SRM target peptide
NP_001630.1| serum amyloid P component precursor [*Homo sapiens*] [mass = 25 387]	0.74	0.66	1	0.71
NP_002855.1| pregnancy-zone protein [*Homo sapiens*] [mass = 163 835]	0.53	0.61	2	0.76
NP_000005.2| alpha-2-macroglobulin precursor [*Homo sapiens*] [mass = 163 291]	0.65	0.62	28	0.76
NP_001634.1| apolipoprotein A-II preproprotein [*Homo sapiens*] [mass = 11 175]	0.68	0.63	2	0.76
NP_001636.1| apolipoprotein C−I precursor [*Homo sapiens*] [mass = 9332]	0.66	0.66	2	0.66
NP_000437.3| paraoxonase 1 [*Homo sapiens*] [mass = 39 731]	0.63	0.67	1	0.71
NP_000030.1| apolipoprotein A-I preproprotein [*Homo sapiens*] [mass = 30 778]	0.68	0.67	16	0.76
NP_000375.2| apolipoprotein B [*Homo sapiens*] [mass = 515 527]	0.67	0.62	8	0.76
NP_000031.1| apolipoprotein C−III precursor [*Homo sapiens*] [mass = 10 852]	0.72	0.66	1	0.66
NP_000403.1| histidine-rich glycoprotein precursor [*Homo sapiens*] [mass = 59 578]	0.34	0.65	1	0.71
NP_006503.1| serum amyloid A4; constitutive [*Homo sapiens*] [mass = 14 807]	0.61	0.71	2	0.58
NP_000474.2| apolipoprotein C−II [*Homo sapiens*] [mass = 11 284]	0.60	0.66	1	0.58

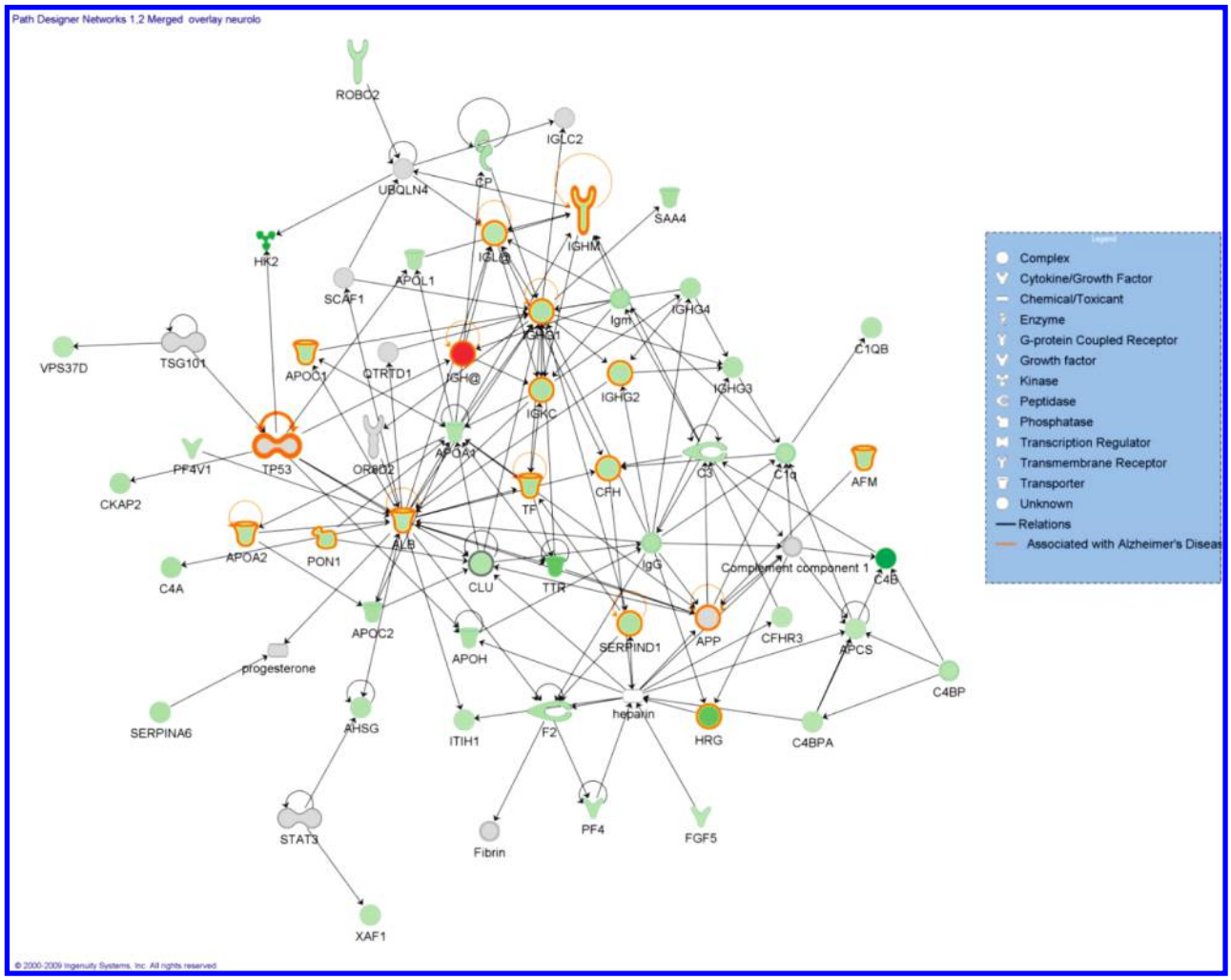

Figure 5. IPA pathway analysis of SIEVE data set. The SIEVE differential expression data set (Supplemental Table 1, Supporting Information) was imported into IPA pathway analysis program. The top 2 scoring networks identified by IPA (Antigen Presentation/Cell-mediated Immune Response/Humoral Immune Response, Network score 63, and Cardiovascular System Development, Network score 47) were merged to illustrate the molecular interactions. Only direct interactions are shown. Green molecules had Trisomy21/Normal ratios that were <1 and red molecules had ratios >1. Gray molecules were not observed in the data set. Orange highlighted molecules are associated with Alzheimer's disease.

As is evident in Table 1, all the proteins we selected as candidates were decreased in Trisomy 21 vs normal samples.

Analysis of Quantitative Differential Expression Discovery Data by Ingenuity Pathways Analysis (IPA). To determine the pathways of biological importance that were correlated with the differentially expressed proteins, the entire data set was analyzed with IPA. The top three networks identified by IPA analysis were (a) Antigen Presentation/Cell-mediated Immune Response/Humoral Immune Response, (b) Cardiovascular System Development, and (c) Function/Cellular Development/Cellular Growth and Proliferation and Lipid Metabolism/Small Molecule Biochemistry, with IPA Network scores of 63, 47, and 28, respectively. Figure 5 shows a representation of the top 2 IPA merged networks. The IPA analysis identified neurological and inflammatory disease as the top disorders, with antigen presentation, lipid metabolism and small molecule biochemistry as the top molecular, cellular and physiological system development functions. These data are consistent with the characterization of Down's Syndrome as a disorder with broad and diverse effects due to dysregulation of multiple genes and pathways.

Development of SRM Targeted Assays. The SIEVE discovery MS/MS database was imported into Pinpoint software in order to develop a multiplexed method for simultaneous quantitative SRM of the selected subset of 12 differentially expressed proteins (Table 1).

Figure 6b shows a screen capture from Pinpoint software illustrating quantitative SRM results for peptide TGISPLALIK from Apolipoprotein B (Apo B) precursor. The top panel indicates the parent and fragment ion transition masses for the light and heavy versions of the peptide. A total of five individual fragment transitions were monitored for light and heavy TGISPLALIK. (For a complete list of all parent and fragment transitions monitored in the experiment, see Supplementary Table 2, Supporting Information). The bottom panel demonstrates that all transitions from the light and heavy versions of the peptide coeluted as expected. In addition, the fragment ion ratios for light and heavy transitions were consistent confirming peptide identity across all samples.

Calibration curves were generated in a background of serum matrix for all target heavy peptides using $1/x$ weighting. An example for Apolipoprotein CIII heavy peptide GWVTDGFSS-

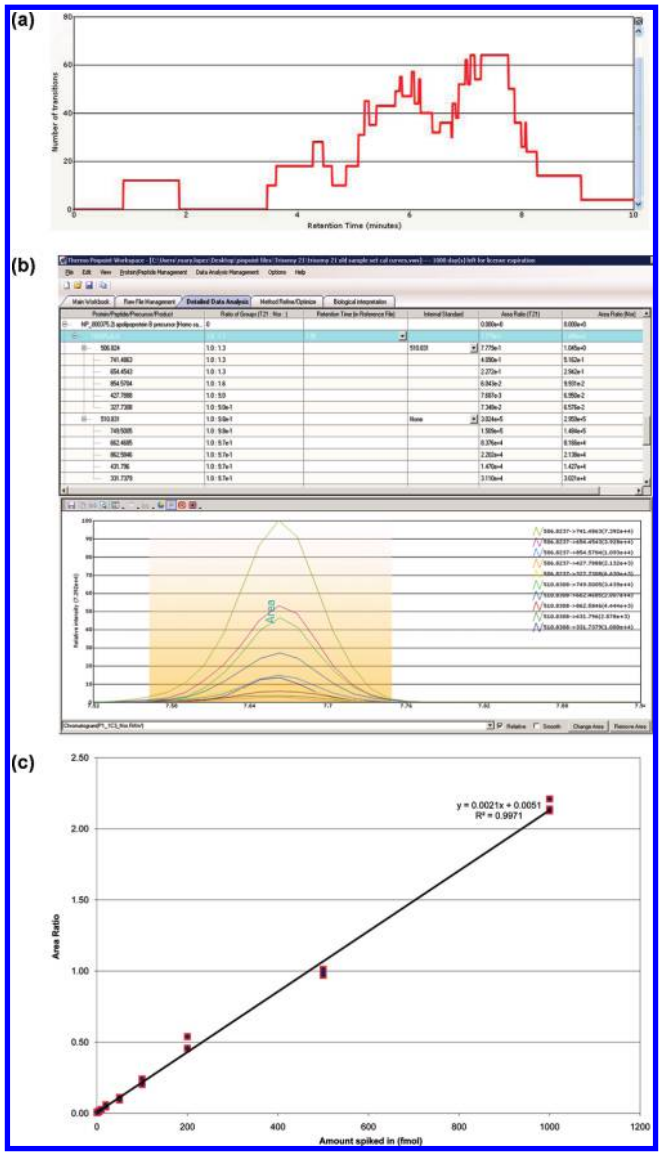

Figure 6. (A) Development of SRM assay for target proteins. Plot of the number of transitions scheduled for acquisition on the TSQ Vantage across the length of the gradient. Transition scheduling limits MS scanning to a window centered on predicted peptide elution. In this example, the method will allow active scanning of only 64 of 206 total transitions at any given time during the gradient. Scheduling increases dwell time and maximizes quantitative accuracy. (B) Development of SRM assay for target proteins. Pinpoint analysis of SRM assay data for Apolipoprotein B peptide TGISPLALIK. (Top panel) Protein, peptide and transition information including retention time, signal (normalized to heavy peptide), and signal ratio between Trisomy 21 and Normal samples. (Bottom panel) Overlay of chromatograms of peptides TGISPLALIK and TGISPLALIK[HeavyK] in a normal sample. The peaks for the heavy synthetic (parent ion 510.8308) and light endogenous (parent ion 506.8237) peptide coeluted exactly as is clearly illustrated by the individual fragment ion transition traces. (C) Development of SRM assay for target proteins. Calibration curve of Apolipoprotein CIII synthetic peptide GWVTDGFSSLK[HeavyK] in a background of 30 μg raw serum digest. The 8 point curve measured concentrations from 250 attomoles to 500 femtomoles on column, in triplicate. LLOD was estimated at 250−500 attomoles and LOQ was calculated to be 1−5 femtomoles. The linear correlation coefficient was 0.99 and the CV for points above the LOQ ranged from 0−20%.

LK[Heavy K] is shown in Figure 6c. The lower limit of detection (LLOD) for this target peptide in serum matrix was approximately 250 attomoles on column and the limit of accurate quantification (LOQ) was approximately 500 attomoles −1 femtomole on column. LOQ was calculated from the standard curve where coefficient of variation was less than or equal to 20%. LOD was calculated from the lowest point on the curve where the peptide was detected. These numbers were typical for all the peptides monitored. The assay error as determined by CV (coefficient of variation) of technical replicate points was on average less than 10%. SRM interferences were ruled out based upon fragment ion ratios. The ion ratios of multiple transitions must be consistent across different samples and from heavy to light peptides. Table 1 shows the quantitative SRM expression ratios and AUC values of the target peptides in Trisomy 21 vs normal samples. The differential expression results from the SRM experiments were, for the most part, consistent with the SIEVE data. All the target proteins had ratios that were <1, that is, they were underexpressed in the Trisomy 21 samples. SRM expression values ranged from 0.58 to 0.76.

Discussion

The goals of this study were primarily 2-fold: to integrate unbiased biomarker discovery with hypothesis-driven, targeted assay development in a seamless manner, and application of this workflow to DS in an effort to facilitate the development of a method for noninvasive, early detection. Mass spectrometry has typically been the technology of choice in the search for proteomic biomarkers, but the translation of this information into high-throughput assays that facilitate the verification of putative markers has not been straightforward. SRM-based assays provide a powerful vehicle for the verification and implementation of putative biomarker information derived from high resolution LC−MS/MS experiments because of their selectivity, sensitivity, robustness and speed.[16] However, bridging the gap between the typically long lists of putative biomarkers in discovery experiments with the capability to run hundreds or thousands of samples using quantitative SRM assays has been hampered because of the lack of an objective and efficient mechanism for ranking the best candidates. In this report, we describe the development and application of bioinformatic algorithms, including a novel application of ROC curves allowing efficient mining of LC−MS/MS discovery data with subsequent integration into SRM methods, thus paving the way for future biomarker verification with larger clinical cohorts.

The workflow described herein was applied to a small cohort of maternal serum samples from Trisomy 21 and normal pregnancies. Because the definitive diagnosis for DS requires invasive procedures that incur significant risk, there is a need for the development of new, noninvasive assays that can detect this disorder and preferably, within the first trimester.[1] The unmet need for early markers of DS remains critical due to the lack of specificity of the currently available blood tests (2−5% false positive rate). As a result of our discovery analysis, we identified over 300 differentially expressed proteins, including PAPP-A which is currently used as a screening marker for DS (Supplementary Table 1, Supporting Information). Most of the differentially expressed proteins that we identified (with a FDR of 0.02 or less) were underexpressed in Trisomy 21 vs normal samples. From the initial list, we identified a group of 12 proteins that fulfilled our criteria for relatively high abundance and promising AUC values (Table 1). PAPP-A was not

included in the list because our intent was to focus on new putative markers, and also because its ROC AUC of 0.59 indicated it had relatively weak classification power for T21 vs normal in this sample cohort. The final target list included 12 underexpressed proteins. It is important to note that biological variation in the data was very great and standard deviations between biological samples ranged from 50−100%. As described above, we monitored only one peptide from each protein in the SRM experiments. This approach was based upon the assumption that the abundance of the target peptide was representative of total protein abundance (assuming trypsin digestion was complete) but this assumption may not necessarily be accurate for every protein. Also, we do not know if the target proteins exist only in intact form (as opposed to multiple truncated forms.[17] Therefore, it is difficult to conclude whether any observed differences in discovery vs SRM expression ratios were due to the number of peptides being monitored or the large biological variation in the data. Repeating the SRM experiment with a larger sample size may resolve this question.

Using the described methods, we were successful in developing a robust, 15 min multiplexed SRM assay for the target proteins that provided fully quantitative information without the need for any further depletion or enrichment from serum digests. IPA pathway analysis of the LC-MS/MS discovery data mapped the putative biomarkers to several metabolic pathways, most notably immune response, lipid metabolism and neurological disorders.

Several of the presumed biomarkers that we identified in this study have also been identified in previous studies utilizing proteomic techniques. Most recently,[6] isobaric labeling technology was used on depleted plasma to perform biomarker discovery on 6 first trimester DS and normal maternal plasma samples. This study identified several underexpressed biomarkers in common with the present study including apolipoprotein A1 and alpha-2-macroglobulin. In contrast to the present study, however, pregnancy zone protein and serum amyloid P were found to be overexpressed. In a prior study,[4] the authors searched for biomarkers in first and second trimester maternal serum from Trisomy 21 and normal patient samples using a variety of methods including 2D DIGE, MUDPIT, LC/LC−MS/MS and MALDI TOF-MS profiling. Nine proteins were identified as potential biomarkers in common with the present study including various apolipoproteins, serum amyloid A, and alpha-2-macroglobulin. A more recent study[5] focused on the application of 2D-DIGE to find putative biomarkers in second trimester maternal plasma. That study also identified proteins in common with the current study including histidine rich glycoprotein (HRG) and serum amyloid P. None of the previously published proteomic studies identified PAPP-A in the analyzed samples. Clearly, it is evident that there is a large degree of variability in the expression ratios reported in MS-based proteomic studies of DS related biomarkers. This may be due to many factors including sample size, preparation, and analytical approaches, most of which are not rigorously quantitative. The relative lack of consistency in proteomic discovery experiments emphasizes the need for more standardized methods that allow the processing of larger numbers of samples. In addition, the ability to quickly stratify candidates and verify with orthogonal methods would add a higher level of confidence to the results of discovery experiments. Major advantages of the approach described in the current study are the powerful combination of ROC analysis of label-free LC−MS/MS discovery results coupled with the concomitant development of targeted SRM assays. This allows rapid and accurate quantification in undepleted serum digests thus facilitating high-throughput verification or elimination of putative biomarkers.

With respect to the biology of DS, the broad representation of apolipoproteins in the differentially expressed protein list from the current study supports a hypothesis of dysregulation of lipid metabolism in DS. Numerous studies[18−22] have documented the central role of lipid metabolism and apolipoproteins in DS and also in Alzheimer's Disease where there is copious evidence of similar pathology and potentially similar mechanisms.[23,24] Figure 5 illustrates the significant overlap of proteins involved in Alzheimer's disease with the proteins from our discovery data set identified in the top 2 IPA Networks.

In conclusion, the workflow outlined in this report presents a seamless bridge between proteomic discovery experiments and the development of high-throughput, targeted assays suitable for biomarker verification in a clinical research environment. The practical application of this approach resulted in the discovery of putative proteomic biomarkers for Trisomy 21 in first trimester maternal serum with the subsequent development of a quantitative, multiplexed high-throughput SRM assay. This assay may, in future studies, be applied to other clinical samples for marker verification and further development. Ultimately, much larger clinical cohorts will be required to establish prevalence and incidence ranges in population based-studies with an ultimate goal of increasing the sensitivity and especially the selectivity of screening methods for DS.

Abbreviations: LC, liquid chromatography; MS/MS, tandem mass spectrometry; SPE, solid phase extraction; ESI, electrospray ionization; ACN, acetonitrile; *m*/*z*, mass to charge ratio; SRM, selective reaction monitoring, APO, apolipoprotein; DS, Down's Syndrome; AD, Alzheimer's Disease; LLOD, lower limit of detection; LOQ, limit of quantitation; FDR, false discovery rate; FPR, false positive rate, ROC, receiver operating characteristic; PAPP-A, pregnancy associated plasma protein; bhCG, beta human chorionic gonadotropin; AFP, alphafetoprotein; uE3, unconjugated estriol; FMF, familial Mediterranean fever.

Supporting Information Available: Thirteen putative markers were chosen from the initial SIEVE discovery data set based upon relative abundance and AUC. Optimized SRM assays were developed for single peptides from the target proteins and the entire clinical sample set was interrogated in triplicate. The corresponding ratios for the SIEVE and Pinpoint analyses are presented in the table. Supplementary Table 1. Label-free, differential analysis and protein identification. Analyses were performed using the SIEVE algorithm (9) incorporating SEQUEST (10) and Percolator (11). RAW files from all clinical samples were analyzed. Supplementary Table 2. Peptides and transitions that were incorporated into the SRM assays for the targeted proteins. Thirteen proteins were chosen as putative biomarker targets from the SIEVE database. The SIEVE database was imported into Pinpoint and SRM assays were developed and optimized using the illustrated peptides and transitions. Supplementary Data 1. Single peptide-based protein identifications. Individual MS/MS spectra and ion assignments for all single peptide identifications are provided in this data file. This material is available free of charge via the Internet at http://pubs.acs.org.

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