Abstract T-012

Proteomic Discovery of Serum Biomarkers Predictive of Preterm Birth

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Proteomic Discovery of Serum Biomarkers Predictive of Preterm Birth

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Abstract

Introduction: The etiology of preterm birth (PTB) includes factors such as infection, placental hemorrhage and stress. Existing tests have poor performance and are limited in utility, especially in nulliparous patients. Thus, there is a compelling need to identify biomarkers of PTB to enable interventions for patients at risk.

Methods: The Proteomic Assessment of Preterm Risk clinical trial (11 sites) enrolled 5500 patients representative of the US population. We employed a 2-phase discovery strategy (Fig 1). First, candidate protein biomarkers were assembled by both hypothesis dependent database query and a first-pass shotgun proteomics discovery effort. Second, a single MRM-MS assay was developed to quantify over 200 candidate biomarkers in serum samples from approximately 90 PTB cases and 180 controls. Univariate p-values and AUROC values were calculated. Multivariate classifier performance using panels of significant analytes was assessed by a bootstrap method with LASSO and Random Forest models.

Figure 1

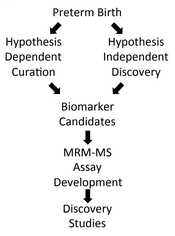


Table 1

Functional Category	%	P-Value
Immunity and defense	38	<0.001
Complement-mediated immunity	10	<0.001
Proteolysis	22	<0.001
Macrophage-mediated immunity	10	<0.01
Lipid and fatty acid transport	8	<0.01
Cytokine/chemokine signaling	10	<0.01
Ligand-mediated signaling	12	<0.05
Cell communication	20	<0.05
Blood clotting	6	<0.05

Results: Univariate analysis of the MRM-MS discovery studies identified analytes amongst multiple functional pathways of relevance to PTB, with immune response highly represented (Table 1, DAVID Software). Multivariate classifiers demonstrated a highly statistically significant separation of cases and controls in these training models.

Conclusions: Our strategy for identification of biomarkers enabled greater predictive power than obtained from clinical characteristics alone and revealed functional pathways important to the etiology of PTB. Once classifiers have been finalized, their performance will be assessed in follow-up validation studies.

Introduction

- PTB is multifactorial with etiologies that include: infection, inflammation, placental hemorrhage, uterine distention and stress[1].
- Existing tests have poor performance and are limited in utility, especially in nulliparous patients.
- There is a compelling need to identify biomarkers of PTB to enable interventions for patients at risk.
- The multi-etiologies of PTB indicate that a prerequisite of good test performance will be the coverage of multiple biological pathways.
- We used both hypothesis dependent and independent approaches to identify candidate analytes of broad biological relevance.
- We report the development of an MS-based highly multiplexed assay and its use in the discovery of biomarkers of PTB.

Methods

- The Proteomic Assessment of Preterm Risk clinical trial serum samples were used for assay development and biomarker discovery.
- We employed a 2-phase discovery strategy.
- First, candidate protein biomarkers were assembled by both hypothesis dependent database query and a first-pass shotgun proteomics discovery effort.
- Second, a single MRM-MS assay was developed to quantify over 200 candidate biomarkers in serum samples from approximately 90 PTB cases and 180 controls.
- Univariate p-values and area under the receiver operating characteristic curve values were calculated.
- Multivariate classifier performance using panels of significant analytes was assessed by a bootstrap method with LASSO and Random Forest models.

Proteomic Assessment of Preterm Birth (PAPR) Clinical Trial

- Purpose: The purpose of this study is to to collect and store blood samples that will be utilized to develop a multimarker test to predict preterm delivery.
- Condition: Preterm Birth
- Primary Outcome Measures: Spontaneous Preterm Birth
- Inclusion Criteria:

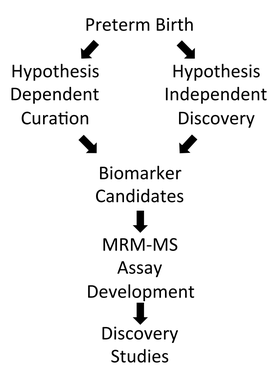
  -Subject is 18 years or older.
  -Subject has a singleton pregnancy.
  -Subject is able to provide consent.

- Exclusion Criteria:

  -Subject is pregnant with more than one fetus.
  -There is a known or suspected fetal anomaly.

ClinicalTrials.gov Identifier: NCT01371019

Discovery Strategy



Hypothesis Dependent Curation

Search Strategy

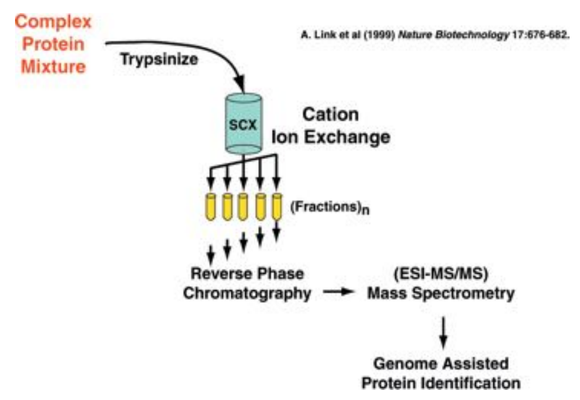
- Key word searches were conducted using publicly available databases.
- Keywords included relevant clinical terms (e.g. preterm birth), their synonyms and related terms and other conditions in maternal fetal medicine (e.g. preeclampsia, intrauterine growth restriction, etc.).
- Clinical search terms were combined with terms such as “biomarker”, “proteomics”, “genomics”, “blood”, “serum”, “plasma”, etc.
- Candidate proteins were extracted and mapped to the best RefSeq and UniProt identifiers. When possible relevant splice variants were identified.
- Candidate proteotypic tryptic peptides and their corresponding transitions were selected using proprietary and public databases.

Hypothesis Independent Discovery

Process workflow

- Serum samples were depleted of the 14 highest abundance proteins using the Agilent Human 14 Multiple Affinity Removal Column (MARS-14), digested with trypsin and subjected to MudPIT[2] analysis using an LTQ-Orbitrap.

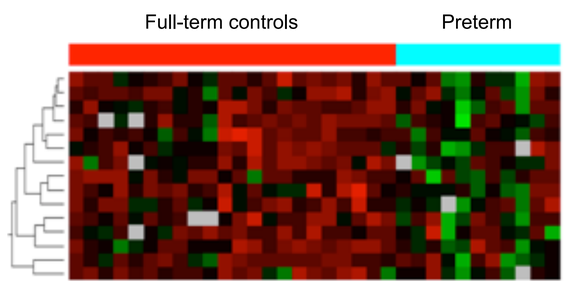
MudPIT



- Relative quantification of tryptic peptides was determined by spectral counts using X! Tandem[3].
- Univariate analysis was used to elect proteins for incorporation into the MRM-MS assay.

Results

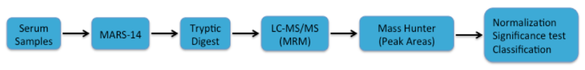
- Heat map for top 15 shotgun hits showing different levels of expression between PTB samples and full term controls.



- A single highly multiplexed MRM-MS assay was built combining all transitions, from both discovery efforts, corresponding to over 240 proteins.
- Measurability and early discovery studies using PAPR samples were used to identify the most robust peptides/transitions to each protein.
- The resulting Advanced Discovery MRM-MS assay was used in the studies described here.

MRM-MS Assay Development

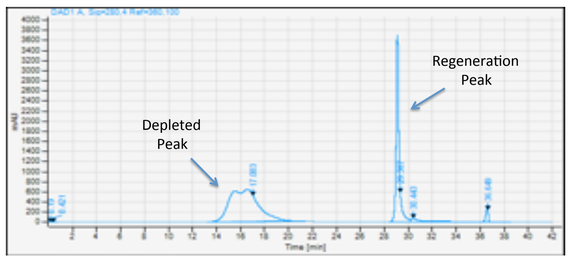
Process workflow



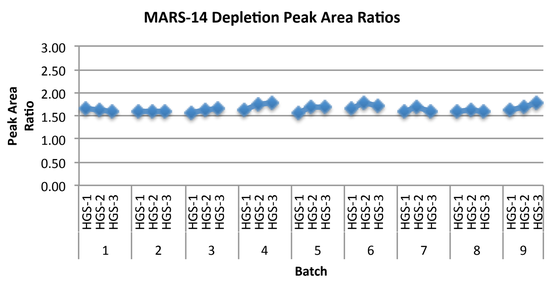
- The workflow described above was developed to evaluate PAPR serum samples in a 96 well format.
- Each step in the process was optimized to reduce variability and improve throughput.
- Serum samples were immunoaffinity-depleted of the 14 highest abundance proteins (MARS-14), digested with trypsin and subjected to LC-MS/MS quantification by MRM.
- Peak areas were determined using Agilent’s Mass Hunter™ software.
- Data was corrected for run-order and batch effects and used for significance testing and univariate and multivariate classification.

Process Performance

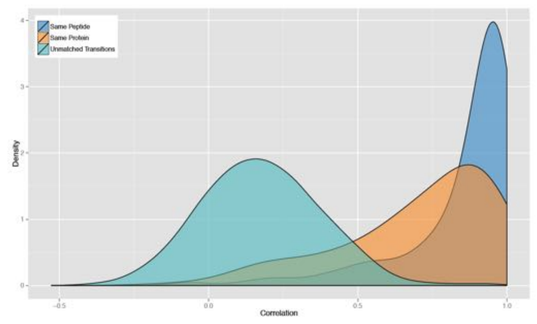
- Mars-14 Depletion Chromatogram.



- Graph showing reproducibility of MARS-14 depletion.



- Graph showing that transitions to the same peptide and peptides to the same protein were highly correlated, indicative of a robust assay.



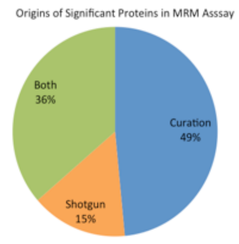
- Variability for the MARS-14 depletion and LC-MS/MS steps were very low.
- Process optimization included sample volumes, buffer compositions, digest and desalting parameters, instrument specific parameters and LC-MS/MS transition QC metrics.
- This resulted in a process with relatively low overall analytical variability.

Process Step	CV%
MARS-14 Depletion	3.9
LC-MS/MS (Internal standards)	8.4

- Corrections for sample run-order and batch reduced variability further.
- Advanced MRM-MS assay specifications:
  - >900 transitions
  - >450 peptides
  - >200 proteins

Discovery Study Results

- Proteins found to distinguish full-term controls and PTB cases by univariate analysis came from both curation and discovery contributions to the MRM assay.



- Univariate analysis of the MRM-MS discovery studies identified analytes amongst multiple pathways and functional categories of relevance to PTB[1,3-8], with immune response highly represented (DAVID).

Functional Category	%	Enrichment P-Value
Immunity and defense	38	<0.001
Complement-mediated immunity	10	<0.001
Proteolysis	22	<0.001
Macrophage-mediated immunity	10	<0.01
Lipid and fatty acid transport	8	<0.01
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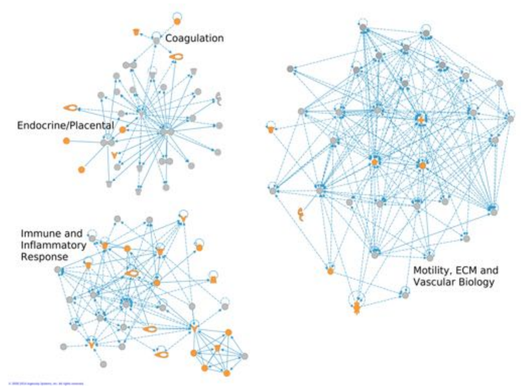
- Ingenuity Pathway Analysis illustrates the density of significant analytes in select functional categories of relevance to PTB.



- LASSO classification results.

	Mean Bootstrap AUC	pvalue
Lasso	0.83	<0.01

- Results are reported as the mean area under the receiver operating characteristic (AUC) curve for 1000 bootstraps using a LASSO classifier composed of the top univariate analytes.

Conclusions

- Our 2-phase strategy lead to the identification of biomarkers across multiple pathways of relevance to PTB.
- Good performance in training sets using multivariate models constructed from these analytes suggests that together they are promising candidates for prediction of PTB.
- Once classifiers have been finalized, their performance will be assessed in follow-up validation studies.

References

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