Novel Biomarkers of Acquired TTP Disease Activity Identified by Metabolomic Profiling

Jay S. Raval\textsuperscript{1}, Raj S. Kasthuri\textsuperscript{2}, Wanda Bodnar\textsuperscript{3}, Yara A. Park\textsuperscript{1}, Marshall A. Mazepa\textsuperscript{1}

\textsuperscript{1}Pathology & Laboratory Medicine
\textsuperscript{2}Internal Medicine
\textsuperscript{3}Environmental Sciences and Engineering
Disclosures

- No relevant conflicts of interest, financial relationships, or commercial interests
Background

- Thrombotic thrombocytopenic purpura (TTP)
  - Thrombocytopenia
  - Microangiopathic hemolytic anemia
  - Presumptive diagnosis is made if no other clear etiology explains these findings

- Incidence = ~5 per 1,000,000 per yr

- Without treatment, >90% patients die
Pathophysiology

- Autoantibodies directed against enzyme ADAMTS13 (the sole human vWF-cleaving protease)
- Depressed enzyme function decreases conversion of ULvWF into smaller vWF multimers
- Disrupts normal endothelial-VWF-platelet homeostasis
- Hyperactive interactions between ULvWF and platelets
- Results in platelet-vWF thrombi within microvasculature
Why Therapeutic Plasma Exchange (TPE) Works?

• First-line therapy consists of daily TPE until treatment response achieved

• By removing patient plasma
  » ADAMTS13 autoantibodies

• By giving donor plasma
  » Provide functional protease

• Decreases mortality to <10%
The Problem

• Despite rapid treatments and achieving treatment responses, **up to 50% of patients have exacerbations**

• Mortality risk is ~90% with each episode of recurrence

• **Currently there are no reliable early clinical or laboratory predictors of disease activity**

• TTP biomarkers exist, but these are **end-stage metrics of disease activity that herald the onset of full-blown TTP**
  » Thrombocytopenia
  » Biochemical evidence of hemolysis
What is Needed

• Identification of alternative biomarkers of TTP disease activity

• Ideal if these biomarkers precede the development of full-blown clinical TTP

• Analysis performable on a diagnostic platform which is available to laboratories located within hospitals treating TTP patients
What is Metabolomics?

- Field of "omics" research focusing on comprehensive characterization of the small molecule metabolites in biological systems

- Provides a view of the metabolic status and global biochemical events associated with a cellular or biological system
  
  » Systematic study of “unique chemical fingerprints” that specific cellular processes leave behind

- Depicts both the steady-state physiological condition and dynamic responses to environmental modulation
Goals of Our Study

• Primary
  » Identify novel biomarkers of TTP disease activity utilizing commercially-available metabolomic profiling technologies

• Secondary
  » Describe a modern metabolomic analytical technique and illustrate how analytical chemistry might further assist in TTP research
Methods

- **TTP clinical database**
  - Demographics, plasma exchange parameters, laboratory data, immunosuppressive medications, clinical responses and outcomes

- **TTP biosample repository**
  - First 50 mL of waste plasma during performance of daily TPE collected, aliquotted, and frozen ≤4 hours at -80°C
Methods

- IRB-approved retrospective pilot, exploratory study
- Samples from 10 randomly selected *de novo* TTP patients with severe deficiency of ADAMTS13 activity (<10%) were chosen for metabolomic analysis
  > “Initial sample” = Immediately prior to initial TPE treatment
  > “Final sample” = From the final day of daily TPE after achieving treatment response (PLT >150,000/μL x 2 days)
- Frozen plasma samples from 8 healthy blood donors obtained
Methods

• Paired samples (for each patient) and single samples (for healthy donors) were interrogated in triplicate by
  » Ultra-high-performance liquid chromatography OR Flow injection analysis
  » Triple quadrupole tandem mass spectrometry
    • Linear ion trap capabilities
    • Multiple reaction monitoring mode
    • Positive and negative electrospray ionization
Methods

- Ultra high performance liquid chromatography (UHPLC) or Flow Injection Analysis (FIA) separates analytes in a complex mixture.
- Mass spectrometry (MS) method ionizes molecules and then sorts them based on mass-to-charge ratio (m:z).
- Tandem mass spectrometry (MS/MS) uses three “quadrupoles” to select and/or fragment different ions with specific m:z (we care about all ions, so + and – selected).
- Combination of UHPLC and MS/MS highly sensitive and specific (pg/ml range, approaching 100% accuracy, CV<10%).
- Create mass spectra that plots ion signals vs. m:z.
- Used to identify compounds in complex mixtures.
How FIA/UHPLC + MS/MS Works

- Detection
- Quadrupole mass filter “Q1”
- Quadrupole Collision Cell “q2”
- Quadrupole mass filter “Q3”
- Fragmentation
- m/z selection
- m/z selection of fragments
- Sample
- HPLC Column Packing Material
- Pump Solvent Manager Solvent Delivery System
- Injector AutoSampler Sample Manager
- Particle Multiplier
- Detection
- Sample stream
- Acceptor stream
- Semipermeable membrane
- Detector
- OR
- OR
Methods

• Mass spectra obtained are compared against existing mass spectra from a library
  » Compounds preliminarily identified

• In order to feel confident about this process, a commercially-available research assay for quantifying up to 188 endogenous metabolites from 5 different compound classes was used
  » Calibrators
  » Standards (high, intermediate, and low)
  » Integrated software with mass spectra library

• For each metabolite, results from the 8 healthy donors were averaged to define a normal value
Methods

• For each patient, initial and final sample ratios were calculated by expressing concentrations of each metabolite as multiples of the normal values.

• We assumed that a valuable biomarker concentration in TTP patients must have
  » Initial ratio of <0.5X or >1.5X the normal value
  » Final ratio approach 1 after achieving treatment response

• Identified candidate biomarkers further interrogated through use of specialized functional metabolic and biochemical pathway mapping software
  » More confidently associate these compounds with mechanistic processes/interactions known to occur in TTP.
What This Means (a metaphor)

• Say there were 188 people with at least one unique identifier
  » DNA, dental records, art they had created, et cetera
• All these items were mixed together in a big bag
• Metaphorically similar technology could
  » Separate the items in this grab-bag
  » Identify each of the owners
  » Indicate where they lived, what was their occupation
  » Hypothesize how they commuted to work and the route they took
## Results

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Acylcarnitines</th>
<th>Glycerophospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>C12</td>
<td>LysoPC a C17:0</td>
</tr>
<tr>
<td>Ile</td>
<td>C14:1</td>
<td>LysoPC a C18:0</td>
</tr>
<tr>
<td>Met</td>
<td>C14:2</td>
<td>LysoPC a C18:2</td>
</tr>
<tr>
<td>Orn</td>
<td>C16</td>
<td>PC ae C34:3</td>
</tr>
<tr>
<td>Pro</td>
<td>C16:2</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>C18:1</td>
<td>C18:2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C5:1-DC</td>
</tr>
</tbody>
</table>
Results

• Pathway mapping software analyses demonstrated these biomarkers were associated with platelets, endothelial cells, vWF, or red cells
  » Linked to TTP pathophysiology
  » Hypothesized to be involved directly in TTP pathophysiologic pathways or sites of injury

• Follow-up analyses of plasma samples from patients with myasthenia gravis (another autoimmune disease)
  » No overlapping metabolites detected after functional pathway analysis
  » Not just an autoimmune disease profile
Conclusions

• Identified a preliminary panel of novel candidate biomarkers associated with TTP disease activity through metabolomic profiling

• Ability of these metabolites to identify active TTP disease will need to be validated in additional samples
  » Will be working in cooperation with RTI International Eastern Regional Comprehensive Metabolomics Resource Core

• Future directions include analysis of the predictive value of these biomarkers to identify patients at risk for disease recurrence prior to developing full-blown TTP
  » Hope of personalizing/tailoring TPE and immunosuppression