Platelet-Dependent Thrombin Generation in Children with Sickle Cell Anemia

Neethu M Menon, MD
Clinical Fellow
Pediatric Hematology/Oncology
9/8/18
Overview

- Background on sickle cell anemia (SCA) and thrombin generation testing
- Aims and Methodology
- Results
- Conclusions
Background: Hypercoagulability plays a role in SCA

- Intracellular polymerization of HbS
- Distorted red cell morphology
  - Altered blood viscosity
  - Circulatory sludging
- Hemolytic anemia
- Vaso-occlusion
- Pain and organ damage

Hypercoagulability

- Thrombotic complications
  - Ischemic stroke
  - Venous thromboembolism
- Acute chest syndrome
- Pulmonary hypertension
- Avascular necrosis
- Nephropathy

Background: Markers of in-vivo coagulation are elevated in SCA at steady state

Platelets meet subendothelium

Endothelial injury

Factor VII meets Tissue factor

Pro-coagulant and Anti-coagulant proteins

Activation of clotting cascade

Products of thrombin generation

Pro-coagulant proteins↑

Factor VIII
VWF

Anti-coagulant proteins↓

Protein C
Protein S

GPIIbIIIa binds to fibrinogen

Platelet aggregation

Pro-coagulant and Anti-coagulant proteins↑

Thrombin-antithrombin complexes (TAT-1)
Prothrombin Fragment 1.2 (PF 1.2)
Fibrinopeptide A
D-dimer
In vivo TG is a smoke detector signaling ongoing evil; ex vivo TG is like the smell of gasoline indicating an increased risk
Dr. Hemker
Background: The best way to study coagulation is an ex vivo global coagulation test.
Background: Thrombin Generation (TG) Assay by Calibrated Automated Thrombography (CAT)

Coagulation begins!

Tissue Factor (TF)

Phospholipids (PL)

Plasma sample

Fluorogenic substrate +Calcium

Coagulation begins!

ETP: Endogenous Thrombin Potential
### Review of Literature: TG studies in SCA so far show variable results

<table>
<thead>
<tr>
<th>TGA Study</th>
<th>SCA subjects (age range in yrs)</th>
<th>Sample/Analytic conditions</th>
<th>Steady state versus healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wijnberge et al, 2018</td>
<td>77 (18-66)</td>
<td>PPP/1pM TF</td>
<td>No difference in peak, ↓ETP</td>
</tr>
<tr>
<td>Gupta et al, 2017</td>
<td>13 (2-14)</td>
<td>PPP/1pM TF</td>
<td>No difference in peak or ETP</td>
</tr>
<tr>
<td>Whelihan et al, 2016</td>
<td>25 (18 and above)</td>
<td>PPP+CTI/5pM TF</td>
<td>↓peak, ↓ETP</td>
</tr>
<tr>
<td>Amin et al, 2015</td>
<td>17 (20-50)</td>
<td>PPP+CTI/1pM TF</td>
<td>↓ETP, ↓TTP</td>
</tr>
<tr>
<td>Noubouossie et al, 2012 and 2013</td>
<td>97 (2-20)</td>
<td>PPP/1pM TF with and without TM</td>
<td>↑peak, ↑ETP, ↓lag time</td>
</tr>
<tr>
<td>Gerotziafas et al, 2012</td>
<td>92 (16-35)</td>
<td>PPP/5pM TF</td>
<td>↑peak, ↓TTP, No diff in ETP</td>
</tr>
<tr>
<td>Chaari et al, 2009</td>
<td>78</td>
<td>PPP/5pM TF</td>
<td>No difference</td>
</tr>
<tr>
<td>Betal et al, 2009</td>
<td>23</td>
<td>PPP+CTI/1pM TF and 5pM TF</td>
<td>↓ETP, ↓slope for both TF concentrations</td>
</tr>
</tbody>
</table>
Review of literature: TG interpretation depends on underlying methodology

- Method of blood draw
- Corn trypsin inhibitor (CTI) or not
- TF concentration: 1pM vs 5pM
- Thrombomodulin (TM) or not
- Platelets or not
  - Platelet-poor plasma (PPP) or platelet-rich plasma (PRP)
Review of Literature: TG in the presence of platelets

- ETP is comparable in healthy adults with and without platelets
- ETP is higher in young adults with stroke (without SCA) in presence of platelets
- TG in the presence of platelets (PRP) closely resembles the physiological milieu
  - This is especially important when multiple cellular interactions are involved
- TG in PRP is a better predictor of hypercoagulability in SCA where platelets are chronically activated
To summarize so far...

- Hypercoagulability contributes toward the long-term complications of SCA
- There is a paucity of biomarkers to predict these complications
- TGA is a global coagulation test used to predict the risk of thromboembolism
- TGA using platelet-rich plasma accounts for the role of platelets in clotting
- There are no previous studies of TG using PRP in SCA
Hypotheses and Aims

- **Hypothesis:** TG will be higher in PRP due to the presence of chronically activated platelets in SCA

- **Primary Aim**
  - To determine TG in PRP compared to PPP in children with SCA at steady state
  - To determine clinical variables predictive of the difference between the two sample types
Hypotheses and Aims

- **Secondary Aims**
  - To study the relationship of platelet-dependent TG with cerebral velocities, hemolysis and occurrence of clinical complications
  - To assess TG with and without addition of TM

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis of HbSS or HbSβ° thalassemia</td>
<td>Use of aspirin containing medications in last 7 days</td>
</tr>
<tr>
<td>Age 2-15 years</td>
<td>Use of other anti-platelet agents like NSAIDs in last 2 days</td>
</tr>
<tr>
<td>In the baseline or steady-state: no SCA related acute</td>
<td>Subject, guardian or parent not willing to participate</td>
</tr>
<tr>
<td>clinical events in the preceding 4 weeks</td>
<td></td>
</tr>
</tbody>
</table>
Methodology

1. Identify potential subjects in clinic
2. Obtain consent prior to routine phlebotomy
3. Collect 6mls of blood in sodium citrate tubes
4. Centrifuge in translational research lab
5. Obtain 1 PRP and 2 PPP samples
6. Run platelet count on PRP sample
7. Use 1st PPP sample to calibrate PRP to 150K
8. Store 2nd PPP sample at -70C in designated freezer
9. Run PRP TGA fresh within 2 hours
10. Run PPP TGA within 4 months
Study Schema

Enrolled subjects based on inclusion and exclusion criteria (65)

Processed samples (59)

- Excluded prior to TG testing
  Failed blood draw (3)
  Elevated HbF (1)
  Processing error (2)

- Excluded for PPP and PRP comparison
  Ibuprofen intake (2)
  Calibration error in PRP (1)
  Platelet count <100K (5)
  Unable to run PRP within 2 hours (2)
  Reagent mixing error for PRP (1)
  Curve not reliable for PRP (1)
  Machine error for PPP (4)

- Excluded for PPP and PPP with TM comparison
  Machine error for PPP (4)
  Insufficient sample for TM addition (2)

PRP (59)
PPP (59)
PPP with TM (59)

- PRP (47)
- PPP (55)
- PPP with TM (53)

Analyzable for comparison between PPP and PRP (43)
Analyzable for comparison between PPP and PPP without TM (53)
## Demographic data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number (percentage)* or range (mean)^</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All patients with analyzable data (55)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender*</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>34 (62)</td>
</tr>
<tr>
<td>Male</td>
<td>21 (38)</td>
</tr>
<tr>
<td>Age range in years*</td>
<td></td>
</tr>
<tr>
<td>2-5</td>
<td>18 (33)</td>
</tr>
<tr>
<td>6-10</td>
<td>27 (49)</td>
</tr>
<tr>
<td>11-15</td>
<td>10 (18)</td>
</tr>
<tr>
<td>On Hydroxyurea*</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin on day of blood draw in g/dl^</td>
<td></td>
</tr>
<tr>
<td>Reticulocyte percentage on day of blood draw^</td>
<td></td>
</tr>
<tr>
<td>TAMMV on TCD in cm/sec*</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>29 (81)</td>
</tr>
<tr>
<td>Conditional</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>3 (8)</td>
</tr>
<tr>
<td>Number of patients with pain crises in the last year needing admission*</td>
<td></td>
</tr>
<tr>
<td>Number of patients with acute chest syndrome in lifetime*</td>
<td></td>
</tr>
<tr>
<td>Number of patients with acute splenic sequestration in lifetime*</td>
<td></td>
</tr>
</tbody>
</table>
## Primary Aim Results

<table>
<thead>
<tr>
<th>ETP in PRP</th>
<th>ETP in PPP</th>
<th>Absolute difference</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1151.6 +/- 223.3</td>
<td>1239.9 +/- 224.1</td>
<td>88.4 +/- 219.2 (p=0.01)</td>
<td>5.9 +/- 16.7 (p=0.026)</td>
</tr>
</tbody>
</table>
### Primary Aim Results: Age based

<table>
<thead>
<tr>
<th>Age Group</th>
<th>PPP Mean</th>
<th>PPP SD</th>
<th>PRP Mean</th>
<th>PRP SD</th>
<th>Percentage Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-5 yrs</td>
<td>1163.7</td>
<td>218.5</td>
<td>1268.02</td>
<td>291.9</td>
<td>5.4 +/- 21.4</td>
</tr>
<tr>
<td>6-10 yrs</td>
<td>1078.9</td>
<td>154.6</td>
<td>1204.6</td>
<td>168.01</td>
<td>9.5 +/- 14.1</td>
</tr>
<tr>
<td>11-15 yrs</td>
<td>1352.8</td>
<td>296.5</td>
<td>1307.3</td>
<td>276.01</td>
<td>(-4.2) +/- 14.1</td>
</tr>
</tbody>
</table>

**Graph:**

- **ETP in children based on TGA method**
- **X-axis:** Age range in years
- **Y-axis:** ETP in nmol/min
- **Legend:**
  - PRP
  - PPP

- The graph shows a comparison of ETP levels between PRP and PPP across different age ranges.
- The graph indicates a trend where ETP levels in PPP are generally lower than in PRP, with the difference being most pronounced in the 11-15 years age group.
## Primary Aim Results: Clinical Predictors of the difference between PPP and PRP

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.34</td>
<td>0.62</td>
</tr>
<tr>
<td>Female gender</td>
<td>-3.88</td>
<td>0.39</td>
</tr>
<tr>
<td>Use of hydroxyurea</td>
<td>1.5</td>
<td>0.74</td>
</tr>
<tr>
<td>Occurrence of pain in the last year needing admission</td>
<td>0.98</td>
<td>0.86</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-1.24</td>
<td>0.53</td>
</tr>
<tr>
<td>Reticulocyte count</td>
<td>-0.058</td>
<td>0.35</td>
</tr>
</tbody>
</table>
### Secondary Aim Results

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Pearson’s r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocyte count (%)</td>
<td>0.03</td>
<td>0.84</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max velocity on TCD (cm/sec)</td>
<td>0.06</td>
<td>0.74</td>
</tr>
<tr>
<td>Sequestration over lifetime</td>
<td>0.3</td>
<td>0.055</td>
</tr>
<tr>
<td>ACS over lifetime</td>
<td>-0.07</td>
<td>0.66</td>
</tr>
</tbody>
</table>

#### ETP in children based on TGA method

![ETP graph](image)

#### Percentage difference between PPP without TM and with TM

<table>
<thead>
<tr>
<th>Age group</th>
<th>Mean</th>
<th>SD</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-5 yrs</td>
<td>927.8</td>
<td>185.09</td>
<td>16.3 +/-12.1</td>
</tr>
<tr>
<td>6-10 yrs</td>
<td>1041.9</td>
<td>204.6</td>
<td>-0.7 +/-16.8</td>
</tr>
<tr>
<td>11-15 yrs</td>
<td>1175.9</td>
<td>178.8</td>
<td>11.6 +/-15.8</td>
</tr>
</tbody>
</table>
Conclusions

- Significantly higher thrombin generation in the presence of platelets supports the use of platelet-rich samples to study clotting in children with SCA.

- Impairment of the protein C/S pathway in SCA is evident based on minimally reduced thrombin generation after addition of thrombomodulin.

- This pilot study also demonstrates the feasibility of using platelet-rich plasma to study thrombin generation.

- TG using PRP in the presence of TM has the potential to study the combined role of platelets and endothelial interactions; this should be explored in future studies.
Acknowledgements

Primary Mentors
Janna Journeycake, MD
Ayesha Zia, MD

Faculty Sponsor
Stephen Skapek, MD

SOC Members
George Buchanan, MD
Zora Rogers, MD
Michael Dowling, MD
Tanya Watt, MD
Jim Amatruda, MD

Research Coordinators
Leah Adix
Amanda Richards
Zain Rahimi

Grants and Contracts
Specialist
Lene Abraham
Thomson Thomas

Grant Support
CCRAC Fellow Award

Gill Center for Cancer and Blood Disorders
Jennifer Marshall
Sharon Stewart
Fatima Gonzalez
Christina Garcia