Two Serum Biomarkers Identify Sustained Injury in mild TBI cohorts and American Football Players.

Timothy Van Meter1, Nazanin Mirshahi2, Hayley Falk1, Vani Rao3, Haris Sair4, W. Frank Peacock5, Alon Friedman6,7

1 Program for Neurological Diseases, Immunocore, Inc., Richmond, VA; 2 Departments of Emergency Medicine, Psychiatry, and Radiology, Johns Hopkins University School of Medicine, Baltimore, MD; 3 Department of Emergency Medicine, Baylor College of Medicine, Houston, TX; 4 Center for Neuroscience and Regenerative Medicine, ULSMS, Rockville, MD; 5 Departments of Emergency Medicine, University of Michigan, Ann Arbor, MI; 6 Department of Physiology and Cell Biology, Brain and Cognitive Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel; 7 Departments of Medical Neuroscience and Pediatrics, Dalhousie University, Nova Scotia, Canada.

Introduction
Head injury brings nearly 5 million patients into emergency departments per year in the US1. The majority of patients receiving a CT (90%) have a negative CT result. Structural MRI scanning reveals structural abnormalities in up to one-third of those patients, and advanced neuroimaging methods, such as diffusion tensor imaging (DTI), detects abnormalities in an even larger fraction. There is a great need to identify TBI in patients using objective laboratory tests, as these patients are at risk for persistent post-concussive symptoms that may affect their overall quality of life. These patients could be evaluated with new therapies. Recent basic science and diagnostic tests measuring changes in physiological levels of circulating biomarkers may aid in identifying patients at risk for long-term symptoms of TBI, and allow stratification of patients for more effective treatment planning. In this study, we contrast mild traumatic brain injury patients with football players sustaining repetitive head injuries.

Several protein biomarkers discovered in serum and CSF have been detected in TBI, including Brain Derived Neurotrophic Factor (BDNF), Neurogranin (NRGN) and Glial Fibrillary Acidic Protein (GFAP)4,5. However, none of the antigens tested to date have been useful as single biomarkers to help confirm a diagnosis of brain injury. The current study evaluated 5 brain-specific serum protein biomarkers, tested individually and in combination, to diagnose brain injury. This study resulted in the development and validation of a new diagnostic tool for the early diagnosis of TBI.

Serum Biomarker Trial Design: HeadSMART Mild TBI Cohort

6 Month Study Time Course

First 30 Days (approx.)
Neurological dysfunction occurs with initial insult and as the injured brain rebuilds itself.

1. Day 1 Child TBI diagnosis and acute cell injury and death
2. Day 7 Injury biomarker peak
3. Day 30 Autoantibody attachment

Beyond 30 Days (approx.)
Neurological dysfunction may re-emerge with the potential induction of autoantibodies against circulating signaling molecules and brain structures.

Methods
Samples: Serum biomarker assays were performed on three separate cohorts. Football Players: Athletes playing American football at Ben Gurion University were enrolled in the study and blood samples were taken on and off season. All players were aged 18-39 years (median 26.9 years). MRI studies including blood brain barrier permeability (BBB) and diffusion weighted imaging (DTI) were conducted as well as collection of clinical information and administration of the NFL Assessment Questionnaire. Mild Traumatic Brain Injury: Mild traumatic brain injury (mTBI) patients were enrolled in the HeadSMART trial at Johns Hopkins University (2 sites). The HeadSMART trial is an IRB-approved prospective clinical trial that was initiated in 2014 and is still accruing. From the current biomarker studies, 254 mTBI patients were enrolled; all patients met the ACEP guidelines for head CT due to head injury. Clinical data, outcome measures (GSQSC, PCS), neuropsychiatric testing and neuroimaging findings were collected on all patients. Median baseline blood draw was taken 4.2 hours from injury. Subsequently, patients were evaluated at 2 additional time points out to 6 months post injury. Health-related quality of life (HRQoL) and healthy controls were enrolled using IRB-approved screening questionnaires as siblings and family members of ED patients. Major diseases and health concerns were excluded. Male age matched controls from this cohort (Median age 28, range 18-39; n=52) were used to compare with athletes from the football cohort.

Biomarker Assays: Serum biomarker concentrations for Brain-Derived Neurotrophic Factor (BDNF), Glial Fibrillary Acidic Protein (GFAP), Neurogranin (NRGN), Neuron Specific Enolase (NSE), and Beta-Synuclein (SNCB) were assessed in duplicate tests using high sensitivity sandwich ELISA tests across replicate assays. Detection technologies were either Mass Spectroscopy Discovery (MSD) electro-chemiluminescence or peroxidase-mediated chemiluminescent detection with 3,3′,5,5′-tetramethylbenzidine (TMB). Model building was performed using logistic regression with 30 bootstrap replicates and the models were tested on the out-of-bag sample set. Model performance was estimated in RStudio version 0.99.896 using the “pROC” package.

Results
Wilcoxon Rank Sum tests were used to compare median biomarker levels in healthy controls, athletes, and mTBI patients (Figure 3). Significant differences in biomarker levels for SNRB were found in acute (p<0.0001) and 1 month time points (p<0.0004) in mild TBI patients; whereas SNRB was significant at the 3 month blood draw (p<0.0001), but not during acute testing (p=0.3024). In contrast, athletes sustaining repetitive injury showed significantly elevated NRGN (p<0.0001), and no difference in SNRB (p=0.621). All median comparisons were performed in RStudio version 0.99.896.

Results from ROC analysis indicate area under the curve (AUC) values for individual biomarkers were below clinically relevant levels for sensitivity and specificity (i.e., where both >0.7) in distinguishing healthy individuals from mTBI and athletes. Combining SNRB and SNBC biomarkers in a panel gave AUC values greater than 0.95 (AUC = 0.969; sensitivity=0.997, specificity=0.922).

Significant improvements in the combined sensitivity and specificity for the classification of athletes as brain-injured are observed with three- and four-analyte panels (see Future Directions). These results were used using logistic regression analysis (model building with 30 bootstrap replicates, in RStudio, “pROC” package. Table 1, below).

Biomarker Panel Performance
• Testing of three additional biomarkers, BDNF, GFAP, and NSE, in age-matched controls and football players showed significant median differences in NSE (p<0.001), but not GFAP (p=0.533) or BDNF (p=0.059). Wilcoxon Rank Sum test.
• ROC analyses performed with two-, three-, and four-analyte biomarker panels was used to optimize the balance of sensitivity and specificity in detecting injury in athletes during the playing season.
• Addition of additional markers to NRGN and SNBC enhances sensitivity and specificity by more broadly covering the spectrum of injury in athletes suffering repetitive injury.

Future Directions: Additional Biomarkers and Panels Enhance Sensitivity and Specificity.

Figure 4. Comparison of Biomarker Levels in Football Athletes and Age-Matched Healthy Controls. BDNF, GFAP, and NSE were tested in immunoassays.

Table 1: Performance of Biomarker Panels in Athletes

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Area under the curve (AUC)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>p-Value</th>
<th>Number of Athletes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRGN, SNBC</td>
<td>0.907</td>
<td>0.958</td>
<td>0.912</td>
<td>0.592</td>
<td>12</td>
</tr>
<tr>
<td>NRGN, GFAP</td>
<td>0.956</td>
<td>0.989</td>
<td>0.008</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>NRGN, BDNF</td>
<td>0.667</td>
<td>0.789</td>
<td>0.135</td>
<td>52</td>
<td>5</td>
</tr>
<tr>
<td>NRGN, SNBC, NSE</td>
<td>0.957</td>
<td>0.993</td>
<td>0.015</td>
<td>52</td>
<td>17</td>
</tr>
<tr>
<td>NRGN, SNBC, GFAP</td>
<td>0.953</td>
<td>0.984</td>
<td>0.011</td>
<td>41</td>
<td>23</td>
</tr>
<tr>
<td>NRGN, SNBC, BDNF</td>
<td>0.975</td>
<td>0.990</td>
<td>0.028</td>
<td>52</td>
<td>17</td>
</tr>
<tr>
<td>NRGN, SNBC, GFAP, BDNF</td>
<td>0.960</td>
<td>0.980</td>
<td>0.017</td>
<td>41</td>
<td>17</td>
</tr>
</tbody>
</table>

1. Members of the HeadSMART consortium