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Diagnostics of Brain Rehabilitation

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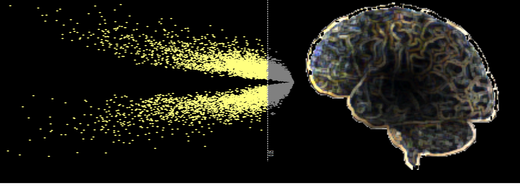
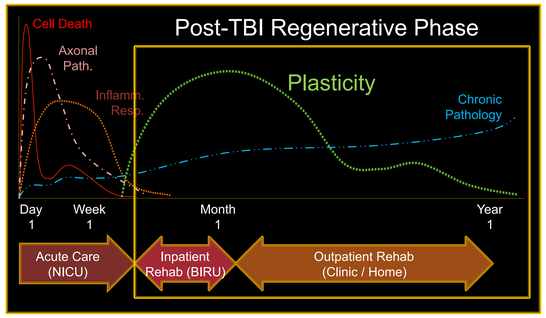


Abstract

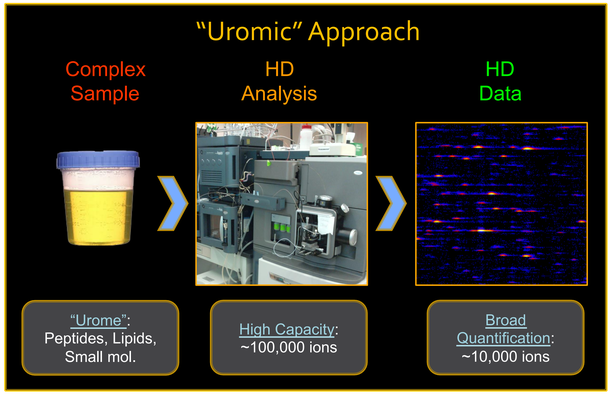
Traumatic brain injury (TBI) is a leading cause of long-term morbidity among the young resulting in significant societal impacts. Yet advances in TBI therapeutic care have been largely limited by the complexity of the pathobiology, heterogeneity among patients, and imprecise endpoint assessments with which to evaluate efficacy. Thus, there remains a significant need for improved diagnostics, particularly for guiding novel therapeutic use and outcomes. So-called theragnostic assays are of particular interest in the new area of TBI rehabilitation, which ideally would target a window of heightened brain plasticity during which circuit remodeling would support recapitulation of lost function. The biochemical processes associated with brain plasticity following TBI produce metabolized components that are small enough in size to passively diffuse into peripheral fluid and by natural means are excreted into urine. We employ high performance mass spectrometry to quantify these byproducts, comprising a "TBI urinary signature" of some 2,500 TBI selective molecules. In this study we hypothesized that the urinary signature would evolve with the advent of a plasticity window during the course of inpatient rehabilitation. Urine samples from eight TBI patients were collected at admission and discharge from the VCU Health Science Center Brain Injury Rehabilitation Unit. Application of non-supervised dimensional reduction analysis demonstrates that the TBI urinary signature is highly effective at classifying TBI patients from non-traumatized age / sex matched individuals. Further, our data demonstrate that the TBI urinary signature evolves distinctively between admission to rehabilitation (mean of 22 days post-TBI) and discharge from the unit (mean of 32 days post-TBI), clearly differentiating the point in recovery. Results further suggest individualized features grouping subjects into recovery classes that are being evaluated for functional correlates. Future research with these results will further evaluate the prognostic capacity of the TBI urinary signature as subjects are followed out one year from their injury.



Design and Methods

Study Design &Sample Collection
A controlled demographic of young adult Caucasian male subjects was recruited with informed consent and approval by the Virginia Commonwealth University Institutional Review Board (Richmond, VA). TBI subjects were enrolled upon admission to inpatient rehabilitation at a mean 22 days post injury (n=8; 26±8 years old; 5 ± 3 Glasgow Coma Scale [GCS] score assessed acutely after injury). Non-traumatized matched control subjects were then recruited (n=8; 26±8 years old). Criteria excluded subjects with non-cranial bone fractures, renal dysfunction at time of rehabilitation admission, and a positive history for past brain injury or neurological disease. Admission to the Brain Injury Rehabilitation Unit was based on standards of care for demonstrating readiness, with required medical stability and capacity to progress in an acute rehabilitation program. Beginning at the 72 h on unit, three mid-stream urine specimens were acquired within a 48-h window. Subsequently an additional three mid-stream urine specimens were collected within a 48-h window approaching discharge of the subject from the Brain Injury Rehabilitation Unit. Urine specimens were placed at 4℃ after collection and centrifuged at 1500xg and 4℃ for 15 min. Aliquots were then stored at -80℃.

Sample Processing
Specimens (six per TBI subject and three per control) were load-normalized to an osmolarity measure of 130 mOsm/kg with Nanopure water. Balanced specimens (100 μL) were filtered with 0.1-μm pore Ultrafree-MC units (Millipore, Billerica, MA), with the supernatants transferred to vials for direct injection (8 μL on column) in a group-interspersed order. Reversed-phase separation was performed with a nano-Acquity chromatography system, using a Symmetry C18 trapping column (2 cm x 180 μm i.d.) and an HSS T3 nanoAcquity (15 cm x 75 μm i.d.) capillary column (Waters, Milford, MA). Components were gradient separated using 0.1% formic acid modified acetonitrile and water. Eluting analytes were electrosprayed into a Synapt G2 hybrid ion mobility/mass spectrometer (Waters), operated in a data-independent analysis mode. All analytical work was performed within a climate-controlled clean room.

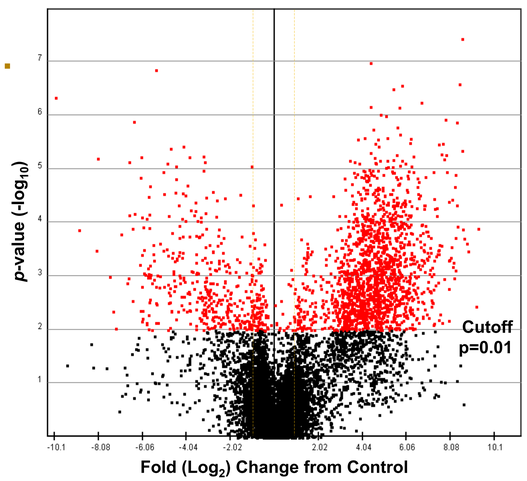


Data Processing
Data were processed using PLGS software v.2.5.2 (Waters). Accurate mass and retention time (AMRT) tables for triplicate specimens were merged to generate a single composite molecular profile per subject that accounted for intraday variance. All subject profiles were aligned by AMRT values (±7 ppm mass accuracy; ± 0.5 min retention time) using Expressions software (v.2.5). Non-reproducing AMRT measures (<3/group) were removed. Values from a simulated Gaussian distribution randomized about the limit of quantification were imputed for left censored data denoting a non-random, group-specific level below the detection limit. Inter-subject normalization (median intensity, 1000 most intense ions) and log(2) transformation procedures were performed.

Statistical Analysis
Aligned composite molecular profiles (separate admission and discharge profiles per TBI subject) were statistically tested using the MultiExperimentViewer (v. 4.8.1) informatics package for array data. Volcano plot presentations were generated following a Welch's t-test methods with alpha adjusted to 0.01. The TBI-responsive "Urome" was evaluated across all 24 specimens using a non—parametric Kruskal-Wallis method with alpha adjusted to 0.01, with results presented in a heatmap format. Principle component analysis was performed for data-reduction, with the first two components plotted.

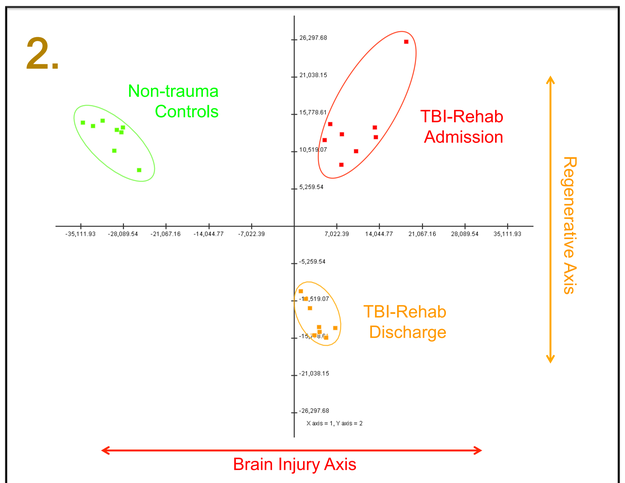
Results

1.



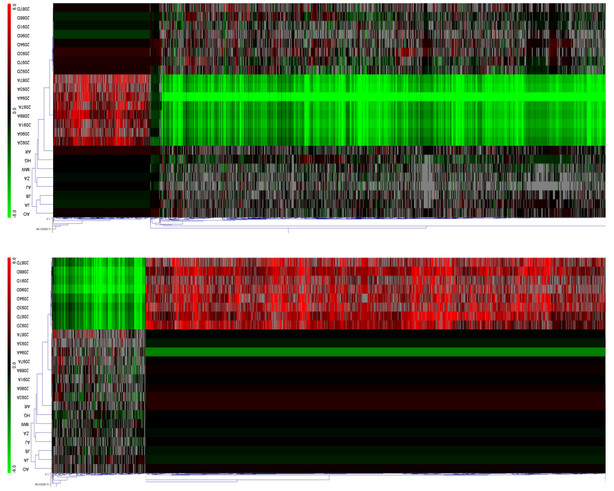
Volcano plot resolving molecular factors that differentiate the discharge urinary signature ('urome") from the admission urinary signature of eight TBI patients. *Paired t-test, p<0.01.* There **11,763** biomolecules that were identified and **2,139** (18%) met significance criteria differentiating TBI admission from TBI discharge.

2.



Principle component analysis presents a multivariate reduction of TBI "urome" signatures for **admission** (red) separated from **discharge** (orange) as well as matched non-traumatized **controls** (green). The x-axis depicts the distinction of TBI subjects regardless of the collection time post-TBI. The y-axis is discriminating based on time after injury suggesting separate factors may be by-products of regenerative **neuropathobiology** evolving during the post acute period.

3.

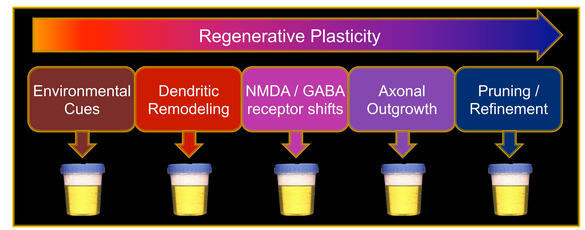


Heatmap of differential molecular factors within the TBI urinary signature ("urome"). **Top:** heatmap comprised of molecular factors selectively present during admission to brain injury rehabilitation. **Bottom:** heatmap comprised of molecular factors selectively present during discharge from brain injury rehabilitation. These results underlie the "regeneration axis" in Figure 2, classifying specimens based on time post-TBI / within rehabilitation. Red = increased, green = decreased, relative to non-traumatized control subjects. *Kruskal-Wallis, p<0.01.*

Conclusions

The TBI urinary signature is distinct from matched controls. Further, the TBI urinary signature evolves with time in recovery. Work is ongoing to evaluate the clinical correlation of the temporally distinct signatures and the precise nature of the underlying constituents. To date we have determined that the TBI urinary signature includes a compilation of peptides. These peptides are by-products from proteins with an enriched relevance to neuroplasticity as illustrated in the table below. Many peptides are derivatives from the terminal signaling motif of the parent protein, denoting functional significance to their release during a timeframe critical to rehabilitative intervention and rewiring of circuits associated with lost function.

Name	Class	ID	Qty	P-value
DLG4 (PSD95) binding/activity partners	Interactions	Int:DLG4	8	$2.47e^{-3}$
Ncam signaling for neurite out-growth	Reactome	MSigDB:375165	7	$5.65e^{-5}$
Involved in axon guidance	Reactome	MSigDB:422475	8	$1.75e^{-3}$
Proteinaceous extracellular matrix	Cell Comp.	GO:0005578	18	$5.86e^{-9}$
Tissue development	Bio. Process	GO:0009888	24	$1.18e^{-3}$
Cell projection morphogenesis	Bio. Process	GO:0048858	15	$2.78e^{-2}$
Extracellular matrix structural constituents	Mol. Function	GO:0005201	10	$1.63e^{-8}$
Growth factor binding	Mol. Function	GO:0019838	6	$3.87e^{-2}$



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