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Principal Components Derived from CSF Inflammatory Profiles Predict Outcome in Survivors after Severe Traumatic Brain Injury

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Abstract

Studies have characterized absolute levels of multiple inflammatory agents as significant risk factors for poor outcomes after traumatic brain injury (TBI). However, inflammatory marker concentrations are highly inter-related, and production of one may result in the production or regulation of another. Therefore, a more comprehensive characterization of the inflammatory response post-TBI should consider relative levels of markers in the inflammatory pathway. We used principal component analysis (PCA) as a dimension-reduction technique to characterize the sets of markers that contribute independently to variability in cerebrospinal (CSF) inflammatory profiles after TBI. Using PCA results, we defined groups (or clusters) of individuals (n=111) with similar patterns of acute CSF inflammation that were then evaluated in the context of outcome and other relevant CSF and serum biomarkers collected days 0-3 and 4-5 post-injury. We identified four significant principal components (PC1-PC4) for CSF inflammation from days 0-3, and PC1 accounted for the greatest (31%) percentage of variance. PC1 was characterized by relatively higher CSF sICAM-1, sFAS, IL-10, IL-6, sVCAM-1, IL-5, and IL-8 levels. Cluster analysis then defined two distinct clusters, such that individuals in cluster 1 had highly positive PC1 scores and relatively higher levels of CSF cortisol, progesterone, estradiol, testosterone, brain derived neurotrophic factor (BDNF), and S100b; this group also had higher serum cortisol and lower serum BDNF. Multinomial logistic regression analyses showed that individuals in cluster 1 had a 10.9 times increased likelihood of GOS scores of 2/3 versus 4/5 at 6 months compared to cluster 2, after controlling for covariates. Cluster group did not discriminate between mortality compared to

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GOS scores of 4/5 after controlling for age and other covariates. Cluster groupings also did not discriminate mortality or 12 month outcomes in multivariate models. PCA and cluster analysis establish that a subset of CSF inflammatory markers measured in days 0-3 post-TBI may distinguish individuals with poor 6-month outcome, and future studies should prospectively validate these findings. PCA of inflammatory mediators after TBI could aid in prognostication and in identifying patient subgroups for therapeutic interventions.

Keywords

TBI; inflammation; principal component analysis; cluster analysis; interleukins; cytokines; soluble cell surface markers; outcome; prognosis; Rehabilomics

1. Introduction

Traumatic Brain Injury (TBI) occurs in 2.5 million Americans yearly, resulting in 50,000 deaths annually as a direct result of injury (CDC, 2010). There have been numerous experimental and clinical studies of secondary injury cascades. Further, TBI has been characterized by: direct disruption of brain tissue, excitotoxicity, hormone pathophysiology, oxidative stress, as well as an aseptic central and peripheral inflammatory response. TBI is heterogeneous with respect to age, sex, initial severity, imaging findings, mechanism of injury, and development of infections and other complications. Clinical trials have not been successful to date in identifying any definitive neuroprotective treatment (Maas *et al.*, 2010). This failure could be due, in part, to a lack of reconciliation between the nuances associated with human patient heterogeneity that occurs with TBI and the clean experimental modeling conditions of preclinical research. The ability to utilize an adaptive trial design to triage and stratify subgroups based on this heterogeneity prior to enrollment and randomization could enhance the identification of clinical intervention targets for future therapies that are efficacious for relevant subsets of the population. However, it is possible that the search for biomarkers in the field of TBI has largely failed because a majority of efforts have focused on identifying a single “magic bullet” that hits a singular therapeutic target in a relatively homogeneous population, which likely oversimplifies the pathophysiology and the approach to clinical trial investigations for individuals with TBI.

Post-traumatic inflammation is a complex component of the secondary injury cascade that has been well-documented in both humans and experimental models (Jeong *et al.*, 2013; Lucas *et al.*, 2006; Woodcock and Morganti-Kossmann, 2013). Studies have characterized certain candidate cytokines, chemokines, cell-surface markers, and microglia as elevated early after injury compared to uninjured controls (Woodcock and Morganti-Kossmann, 2013). Contemporary concepts contend that controlled inflammation is necessary to clear debris and damaged cells early following TBI, while sustained elevations of inflammatory markers, such as IL-1β, TNFα, and IL-6, are deleterious if not physiologically regulated and can lead to an increased risk of depression (Juengst *et al.*, 2014), epilepsy (Diamond *et al.*, 2014), cognitive deficits (Clausen *et al.*, 2009, 2011) and poor global outcomes (Kumar *et al.*, 2014).

Work using lipopolysaccharide (LPS)-challenge as an experimental model of inflammation suggests that cytokines are highly correlated with one another, and the production of one biomarker directly or indirectly impacts production and release of others (Hang *et al.*, 2004). Despite this consideration, human studies to date have strongly focused on absolute, not relative, levels of CSF and serum inflammatory biomarkers produced after TBI. That is, there exists little knowledge of which markers account for similar patterns of variance among patients or which inflammatory agents may "track together" after TBI. It could be of considerable clinical significance to not only know which markers are elevated relative to controls, but also which sets of markers share some discriminatory capacity among patient outcomes early after injury. Such information may be useful to inform prognosis and guide therapy. For example, a given biomarker may be elevated 10-fold in patients vs. controls; however, it may have little variability among patients, making it less useful as a prognostic marker compared to other markers that may have a wide range of concentrations in the patient population.

Taking a data-driven approach to discriminating patient subgroups, we evaluated relative CSF inflammatory levels in the first week after injury to identify which sets of markers account for the greatest variability among patients. To this end, we used dimension reduction methods, including principal component analysis (PCA) and cluster analysis, to identify independent subgroups of patients with similar inflammatory responses following TBI, without incorporating any prior knowledge of post-TBI immunity into the modeling strategy and independent of any known relationships to outcome or recovery after injury. PCA is a statistical technique that has been applied into a number of disciplines, including biology, medicine, and the social sciences. In the healthcare field, PCA has been applied to a variety of diseases including cardiovascular disease (Nettleton *et al.*, 2007), autism (Tadevosyan-Leyfer *et al.*, 2003), depression (Hamilton, 1967), and cancer (Machado *et al.*, 2005). In TBI, the data are limited; one small study of 12 individuals used microdialysis to examine the inflammatory profiles using PCA methodology (Helmy *et al.*, 2012).

In this study, 1) we applied PCA to CSF inflammatory marker data derived from our large cohort with severe TBI to identify parameter combinations (known as principal components) that account for the variability across individuals, 2) we used these principal components to identify meaningful clusters of individuals in our study population, and 3) we assessed the association between cluster group membership and relevant demographic and clinical variables, previously measured biomarkers, and outcomes in the first year after TBI.

Using relative levels of inflammatory agents to characterize sets of markers that account for the greatest variation among individuals with TBI could have significant implications for 1) prognostication, 2) identifying individuals who may be good candidates for therapeutic intervention, 3) detecting which *sets of markers* have strong discriminatory potential and could represent targets for interventions, and 4) delineating potential treatment windows for inflammation-related interventions in a clinical trial. Overall, this data-driven approach provides a novel assessment of the potential of patterns among TBI-associated inflammatory biomarkers to predict long-term outcomes after TBI.

2. Materials and Methods

2.1. Study Protocol

This prospective, observational cohort study was approved by the University of Pittsburgh Institutional Review Board. We enrolled 114 adults with severe closed-head TBI at our level 1 trauma center. Patients were eligible if they were between ages 16-75 years, had a severe TBI based on an admission Glasgow coma scale (GCS) score ≤8 with positive findings on head CT, required an extraventricular drainage catheter (EVD) for intracranial pressure (ICP) monitoring and management, and had at least two CSF and/or serum samples collected during the first week post-injury available for analysis.

Individuals were excluded from our analysis if they exhibited any of the following: a penetrating head injury, documented prolonged cardiac or respiratory arrest at injury (>30 minutes occurring prior to admission), or evidence of brain death within the first three days after injury; an Abbreviated Injury Scale (AIS) score of 5 in any region other than the head/neck; a previous history of pituitary or hypothalamic tumor, history of breast cancer requiring chemotherapy treatment/tamoxifen, history of prostate cancer requiring orchiectomy or LH suppression agents, or untreated thyroid disease.

Individuals with TBI received care consistentwith *The Guidelines for the Management of Severe Head Injury* (Brain Trauma Foundation *et al.*, 2007). This care included initial EVD placement, central venous catheter and arterial catheter placement, and surgical intervention for decompression of mass lesions when clinically indicated. Intracranial pressure was treated in a stepwise fashion to maintain pressure within normal parameters (<20 mmHg), and cerebral perfusion pressure (CPP) was maintained at >60mmHg. Also, there were a total of n=6 participants enrolled in a randomized controlled trial evaluating maintenance of moderate hypothermia (temperature 32.5-33.5°C) after severe TBI. All the patients not enrolled in the trial were treated to maintain a normothermic state.

2.2. Sample Collection and Processing

CSF samples (n=567) were collected passively via EVD placed for clinical care, and samples were collected up to twice daily for up to 5 days after injury. The samples were collected at 7 AM or 7 PM, whenever possible, and were stored at 4°C until processing. For some individuals, clinical care, medical stability, minimal CSF output, or removal from the intensive care unit (ICU) precluded the acquisition of CSF samples at certain time points. Serum samples (n=610) were also collected on a subset of individuals (n=84). All CSF and serum samples were centrifuged, aliquoted, and stored at -80°C until batch analysis.

CSF inflammatory markers were measured using a Luminex™ bead array assay (Millipore, Billerica, Massachusetts). Multiplex bead array assays use a microsphere tagged with multiple fluorescent-labelled markers. A fluorescence detection laser optic system was used to analyze simultaneous individual protein binding. Single samples were used for analysis for each Luminex assay. The markers measured in CSF included the following cytokines and cell-surface markers: interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, and tumor necrosis factor alpha (TNF-α), soluble vascular adhesion molecule-1 (sVCAM-1), soluble intracellular adhesion molecule-1 (sICAM-1), and soluble Fas (sFAS). The

minimum detectable limit and coefficient of variance for each marker have been previously reported in detail by our group (Santarsieri *et al.*, 2015).

In addition to inflammatory markers, a battery of steroid hormones, brain-derived neurotrophic factor (BDNF), and S100b were also assessed as TBI-relevant biomarkers in CSF and serum. These markers have been previously measured and reported on independently in prior studies (Failla *et al.*, 2015; Goyal *et al.*, 2013; Santarsieri *et al.*, 2015; Wagner *et al.*, 2011). CSF and serum cortisol, as well as serum testosterone (T), estradiol (E2), and progesterone, were measured using radioimmunoassay with the Coat-A-Count ® In-vitro Diagnostic Kit (Siemens Healthcare Diagnostics Inc., Los Angeles, CA). Estradiol and testosterone were measured using high sensitivity enzyme immunoassay (EIA) kits (Salimetrics, LLC. State College, PA). A ratio was also created with E2 over T, as previously reported, to represent a measure of aromatization (Garringer *et al.*, 2013). CSF and serum BDNF levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech, Norcross, GA). Similarly, CSF and Serum S100b levels were also measured using ELISA kits (International Point of Care Inc., Toronto, Ontario, Canada).

2.3. Demographic and Clinical Variables

Relevant demographic and clinical variables were gathered through a combination of personal interview and medical record abstraction. The variables collected for this study include: age, sex, body mass index (BMI), GCS score, Injury Severity Scale (ISS) score, mechanism of injury, initial computed tomography (CT) imaging findings, hospital length of stay, and the development of sepsis and pneumonia during hospital stay. The GCS, a ubiquitously used measure of neurological injury severity based on verbal, motor, and eye responses, was assessed serially by trained clinical ICU staff. The best GCS score in the first 24 hours was used in this study. Trauma research staff abstracted ISS scores from the top three most injured body regions detailed from regional AIS score assignments, which is a commonly used anatomical trauma scoring scale that quantifies overall severity of injury across multiple anatomical regions (Baker *et al.*, 1974). Pneumonia and sepsis status was abstracted from our local hospital trauma registry.

2.4. Six and 12 Month Outcome Assessment

Individuals with acute biomarker samples were followed-up at six (85.6% follow-up rate) and 12 months (80.2% follow-up rate) for the assessment of global recovery using the Glasgow Outcome Scale (GOS). The GOS is a global measure of neurological recovery ranging from 1-5, with scores corresponding to: 1) dead, 2) vegetative state, 3) severe disability, 4) moderate disability, and 5) good recovery (Jennett and Bond, 1975). Participant's GOS scores were divided into three categories: 1) dead (GOS=1); 2) severe disability (GOS=2/3); or 3) favorable outcomes (GOS=4/5).

In addition to GOS, a post-hoc analysis was conducted using Disability Rating Scale (DRS) scores at 6 and 12 months. The DRS is a scale developed to assess individuals in the rehabilitation phase of recovery, and includes eight items divided into four subscales: 1) arousal and awareness, 2) cognitive ability to handle self-care functions, 3) physical dependence upon others, and 4) psychosocial adaptability for work, housework, or

school(Rappaport *et al.*, 1982). The scores range from 0 to 30, with lower scores indicating no disability and 30 indicating death. For the purposes of the post-hoc analysis among only survivors, DRS scores were utilized for the purpose of having an outcome scale with slightly more granularity among survivors than the GOS. Scores were divided into three categories, 1) partial to no disability (DRS=0-3); 2) moderate or severe disability (DRS=4-14); 3) extreme severe disability, vegetative state or dead (DRS=15-29).

2.5. Statistical Analysis

All statistical analyses were performed using SAS version 9.4. Descriptive statistics, including medians, means, and standard error of the mean (SEM), were computed for continuous variables. Frequencies and percentages were determined for categorical variables. Non-parametric Mann Whitney U tests were conducted for continuous variables, and chi-square tests, or Fisher's Exact Test when appropriate, were used for categorical variables. All tests were two-tailed, with a significance level set at α=0.05.

For the purposes of this study, samples were divided into two time epochs after injury, days 0-3 and days 4-5, which represent an early and late measure for biomarker levels in the first week following TBI. These time frames were chosen based on prior biomarker work from our lab showing these time points as sensitive to mortality and global outcomes (Wagner *et al.*, 2015).

Average values for each biomarker were calculated within days 0-3 and days 4-5. The data were analyzed in three stages outlined below, which include: 1) PCA, 2) cluster analysis, and 3) bivariate and multivariate analyses stratified by cluster groups.

In addition, a post-hoc analysis was conducted exploring differences in levels of CSF inflammatory markers among individuals in the 75th percentile or above for age, compared to those below the 75th percentile for age.

2.5.1. Principal Component Analysis—PCA is a statistical method that identifies sources of variation within data and serves as a dimension-reducing procedure for a set of correlated continuous variables (Bryant and Yarnold, 1995; Grimm and Yarnold, 1995). PCA is an ideal analytical approach to use with hypothesis-generation studies, as it does not rely on any *a priori* knowledge of the relationship amongst the variables of interest. This data-driven technique identifies uncorrelated linear combinations of observed variables, called principal components, which explain the greatest degree of variance among a set of observed variables, within a specified study population (Suhr, 2005). The principal components are organized in order of descending independent contribution to discrimination of variance in the data (i.e. PC1>PC2, PC2>PC3, etc.). Mathematically, the principal components correspond to eigenvectors of a covariance matrix formed from the data, while the associated eigenvalues quantify components' contributions to discrimination of variance in the data.

The principal components can be thought of as providing a new coordinate system for the data, with the greatest spread of data along the first coordinate, as illustrated in the schematic diagram in Figure 1. For example, in a hypothetical real-world application of

PCA, measurements could be gathered from a cohort for Hemoglobin-A1C and capillary blood glucose. PCA could then yield principal components PC1 and PC2, each corresponding to a different linear combination of the measured quantities. Although PCA does not provide biological interpretations for the principal components, in this simple example, we can hypothesize that PC1 might represent variance associated with *severity of diabetes*, while PC2 would account for the remaining variance in the population not captured by PC1. With data sets of dimensions greater than two, such as our CSF inflammatory biomarker data, dimension reduction is obtained by identifying a subset of dominant principal components that account for a sufficiently large proportion of the variance in the data and projecting data onto this lower-dimensional subset. In such settings, there is rarely as clear a biological interpretation of the dominant principal components as there is in our hypothetical example yet, crucially, the link with variance remains.

Prior to performing PCA, days 0-3 and 4-5 averages for each CSF inflammatory marker were z-score standardized to account for inherent differences in absolute concentrations of certain markers relative to one another. The formula utilized for z-score standardization was: $z\text{-score} = (x-\mu)/\sigma$, where x corresponds to subject mean, μ corresponds to study population mean, and σ corresponds to study population standard deviation.

After z-score standardization, data in days 0-3 and 4-5 were assessed independently by PCA using the SAS procedure PROC FACTOR, specifying “prin” as the analytic method. We utilized the Kaiser criterion for significant principal component inclusion, which specifies that only principal components that have eigenvalues greater than 1 will be retained (Kaiser, 1960). No rotations were made on the data.

For each inflammatory marker, there was a “loading” associated with each principal component, which ranged from -1 to 1. The size of this loading measures how significant a contribution a marker makes to a principal component, while its sign determines whether a larger-than-average (positive) or smaller-than-average (negative) level of that marker is associated with positive variation in that principal component. Given these loadings, each subject received a “score” for each significant principal component for days 0-3 and 4-5, respectively (e.g. PC1 score, PC2 score, etc. for each time range). A subject's score for a principal component was based on that individual's particular data for the variables that load with that component and was computed by taking the dot product of the patient's z-score standardized data with the corresponding principal component. That is, if a subject had high levels of markers with large positive loadings for a principal component, then that individual would be assigned a large positive score for that component, whereas high levels of markers with large negative loadings would lead to a negative score, and levels of markers with small loadings would have little influence on the subject's score.

2.5.2. Cluster Analysis—After PCA, a non-hierarchical, k-means cluster analysis was run using all of the scores for significant principal components generated from PCA for days 0-3 and 4-5, respectively. The purpose of clustering was to derive meaningful subpopulations of patients with TBI that had similar relationships among inflammatory markers that loaded(i.e. had large positive or negative loadings) for each significant principal component (e.g. PC1, PC2). K-means cluster analysis involves the following steps:

1) arbitrarily choosing k observations as *seeds*, 2) assigning each remaining observation to the *seed* closest to it in Euclidean distance, in order to form temporary clusters, and 3) repeating this process until convergence is reached and final clusters are formed (MacQueen et al., 1967). All clustering was performed using the SAS procedure PROC FASTCLUS. The cubic clustering criterion (CCC) was noted for days 0-3 and 4-5 clusters; values greater than 2 were considered a benchmark for good cluster grouping, while values less than 2 were considered to be a poor cluster grouping (Sarle, 1983). If a cluster group contained only 1 individual, it was removed from analyses. Author expert judgment was used to combine certain nearby cluster groups, as appropriate.

2.5.3. Bivariate and Multivariate Analyses—The primary outcome of interest was GOS category (dead, GOS=1; vegetative state/severe disability, GOS=2/3; or moderate disability-good recovery, GOS=4/5). Relevant demographic and clinical variables were compared by cluster group and GOS in bivariate analyses. To control for the potential effects of confounders, variables significantly associated with both cluster group and GOS were controlled for in multivariate analyses. First, the proportional odds assumption was tested for the multivariate ordinal logistic regression model. Since the proportionality assumption was violated, a multinomial logistic regression was performed with the data. In addition to GOS, DRS scores were compared among survivors in a bivariate relationship to cluster membership. Finally, additional TBI-related serum and CSF biomarker levels (steroid hormones, BDNF, and S100b) were compared by cluster group in bivariate analyses.

3. Results

3.1. Principal Component Analysis: Days 0-3 and Days 4-5

PCA produced four significant principal components (eigenvalue>1) for days 0-3 and three significant principal components for days 4-5. Each principal component consists of a vector of coefficients weighting the contribution of each measured biomarker to that component. The coefficients of each biomarker in the two dominant principal components for days 0-3 and 4-5 (specifically PC1 and PC2 for each time interval) are graphed in Figure 2a/b. The principal component loadings for days 0-3 and 4-5 are provided in Table 1, with bolded values representing inflammatory markers that loaded greater than or equal to |0.4| for a given principal component.

3.2. Cluster Analysis: Days 0-3 and Days 4-5

For each individual, we derived a principal component score for each significant principal component (i.e. PC1 score, PC2 score, etc. for days 0-3; PC1 score, PC2 score, etc. for days 4-5). These scores were tested for significant cluster groupings among the study population using k-means cluster analysis. The k-means clustering algorithm works by partitioning n observations into k clusters, where each observation (subject) is assigned to the cluster with the closest mean. For days 0-3, five clusters were obtained; two of them contained only one individual each and were removed from the analysis. One cluster contained only four individuals and was therefore combined with another larger cluster that had similar (non-significantly different) mean PC1 scores (p=0.243). These steps yielded two cluster groups

that were extracted and utilized for subsequent analysis: cluster 1 (n=32) and cluster 2 (n=79). The CCC for this cluster grouping was 3.674, which values greater than two are indicative of good cluster groupings(Sarle, 1983). For the day 0-3 data for each individual, we plotted the score for PC1 against the score for PC2; Figure 3 shows these data, stratified by cluster group. Importantly, the additional significant principal components PC3 and PC4 for days 0-3 were utilized in the k-means clustering, although they are not portrayed in the figure to simplify the visual interpretation to two dimensions. The two cluster groups primarily differ based on scores for PC1, wherein 100% of individuals in cluster 1 have a positive score for PC1, and a majority of individuals (61%) in cluster 2 have negative values for PC1.

The k-means clustering algorithm produced poorly separated clusters for days 4-5, where a predominant proportion (91%) of the study population was assigned to one nondescript cluster, with a small minority divided between two other clusters. As a result, further analyses were not performed with cluster groups for days 4-5.

3.3. Demographic and Clinical Variable Relationships to Day 0-3 Cluster Groups

The relationship between relevant demographic and clinical variables and cluster group are reported in Table 2. The average age of subjects in cluster 1 was significantly higher than in cluster 2 (46.09 vs. 31.75, p=<0.001). There was a lower proportion of men in cluster 1 compared to cluster 2 (71.88% vs. 87.34%, p=0.05). The GCS, ISS, BMI, sepsis and pneumonia status for both cluster groups were not significantly different. Cluster 1 had a greater proportion of subdural hematomas (SDH) (87.50% vs.59.49%, p=0.004); however, cluster 1 had a lower proportion of diffuse axonal injuries (DAI) (9.38% vs. 43.04%, p=<0.001). The mechanism of injury significantly differed between the two clusters (p=<0.001), with a cluster 2 having greater frequencies of MVA or motorcycle accidents, and cluster 1 having more falls. The average length of acute hospital stay was significantly less for individuals in cluster 1 compared to cluster 2 (17.49 vs. 23.19 days, p=0.013).

A post-hoc analysis was conducted to examine differences in levels of CSF inflammatory markers, stratified above and below the 75th percentile of age, which was age 48 in our population. Further, as shown in Table 3, individuals above age 48 had significantly higher levels of IL-5, IL-6, IL-8, IL-10, sICAM-1, sVCAM-1, and sFAS (p<0.05 for all comparisons).

3.4. Demographic and Clinical Variable Relationships to GOS scores at 6 months

The relationship between relevant demographic and clinical variables and 6 month GOS are reported in Table 4. Age was significantly different by GOS categories at 6 months, with deceased individuals having a significantly higher average age at injury, while those with GOS scores of 2/3 and 4/5 had similar average ages (47.03 vs. 32.44 and 30.47). Presence of DAI was significantly different by outcome group (p=0.030), with the greatest proportion of DAI injuries among individuals with GOS scores of 4/5. The average acute length of stay among those that die was 15.70 days, while it was 25.56 days among those with GOS scores of 2/3 and 21.56 days for those with GOS scores of 4/5(p=0.003). No other variables tested were significantly different by GOS group. Demographic and clinical variables were also

examined for 12 month outcomes, and similar findings were seen to those reported for 6 month GOS (data not shown). Therefore, based on the bivariate comparisons by cluster group and outcome, age, DAI, and length of acute hospital stay were controlled for in the multivariate model. Also, we added the best GCS score in the first 24 hours as a covariate in the model: 1) because of its marginal significance in bivariate analyses to cluster group and outcome; and 2) to control for a measure of injury severity in the multivariate model.

3.5. Relationship Between Cluster Group and 6 and 12 Month Outcomes

In bivariate analyses at 6 months, there was a significant association between cluster membership and GOS scores (p=<0.001). In cluster 1, 14 individuals (48.3%) were deceased at 6 months (GOS=1), 13 had GOS scores of 2/3 (38.2%), and 2 had GOS scores of 4/5 (6.3%). In cluster 2, 15 individuals (22.7%) were deceased, and 21 (61.8%) and 30 (45.5%) of individuals had GOS scores of 2/3 and 4/5, respectively. There were also a significant association between cluster group and 12 month GOS scores (p=0.025) (data not shown).

All multivariate models examining the association between cluster group and outcome were adjusted for age, GCS, DAI, and acute hospital length of stay (see Table 5). The proportional odds assumption for the GOS score was checked before performing a multivariate ordinal logistic regression model. This test was significant ($\chi 2$=15.908, p=0.007), which indicates that proportionality was violated across levels of GOS; therefore, a multinomial logistic regression was conducted. This type of regression model does not assume proportionality among levels of the outcome (e.g. GOS=1 to GOS=2/3 to GOS=4/5). Therefore, each independent variable was modeled for its effects on GOS 1 vs. GOS 4/5, and GOS 2/3 vs. GOS 4/5.

Further, for 6 month GOS outcome among the study population, individuals in cluster 1 compared to cluster 2, had a 10.9 times increased odds of GOS scores of 2/3 vs. 4/5 (adjusted odds ratio (OR)=10.941, 95% CI (1.963, 60.978), p=0.006), after controlling for covariates. Individuals in cluster 1 vs. 2 did not differ in odds of GOS scores of 1 vs. 4/5 (adjusted OR=4.142, 95% CI (0.663, 25.891), p=0.129), after controlling for covariates. Due to wide confidence intervals observed, post-hoc power analyses were conducted for each outcome comparison. Among GOS scores of 2/3 vs. 4/5, we calculated a power of 0.980 using a Pearson chi-square test for two proportions, for an effect size of 10.94 between cluster groups, with a sample size of 34 and 32 for GOS 2/3 and GOS 4/5, respectively. Among GOS scores of 1 vs. 4/5, with a sample size of 29 for GOS 1 and 32 for GOS 4/5, and an effect size of 4.142, we calculated a modest power of 0.698.

Importantly, age was a significant predictor of GOS 1 vs. 4/5 (adjusted OR=1.061, 95% CI (1.011, 1.112), p=0.015). At 12 months, there were no significant multivariate relationships between cluster group and outcome (data not shown).

A post-hoc analysis was conducted examining the association between cluster group membership and DRS scores among survivors. As shown in Figure 4, there was a strong association between cluster group and DRS category (p=0.008), such that over half (56.9%) of subjects in cluster 2 had partial to no disability, while a majority of subjects (66.7%) in cluster 1 had moderate to severe disability. Also, the association between cluster group and

DRS at 12 months was not significant (data not shown).Of note, to protect against potential confounding, demographic and clinical covariates were checked for associations with DRS scores among survivors. We found that only DAI injury type was significantly different by DRS category, though its effect did not meaningfully change(i.e., >10% change in effect size) the association between cluster group and DRS at 6 months. Therefore, for simplicity of reporting this post-hoc analysis, the bivariate association is reported between cluster and DRS.

3.6. Relationship Between Cluster Group and TBI-relevant CSF and Serum Biomarkers

Average serum and CSF steroid hormone, BDNF, and S100b levels, stratified by cluster group are provided in Table 6. In CSF, day 0-3 average levels of cortisol, progesterone, E2, testosterone, BDNF, and S100b are higher in cluster 1 compared to cluster 2 ($p<0.05$ for all comparisons). In serum, average cortisol levels were higher in cluster 1 vs. cluster 2 ($p=<0.001$). Average BDNF levels were lower in cluster 1 vs. cluster 2 ($p=0.042$).

Discussion

This study employs PCA and clustering methodology to characterize the neuro-inflammatory response following TBI. The results offer a valuable addition to the field by providing a novel shift in approach in describing inflammation, from an absolute to a *relative* perspective. Immunology is a cybernetic physiological process where compensatory mechanisms (i.e. anti-inflammatory and pro-inflammatory markers) influence production and regulation of inflammatory agents (Hallenbeck, 1977). To date, the field of TBI has largely characterized inflammation using descriptive values of peaks or weekly means for a single marker at a time. Thus, there has been limited study into relative biomarker interrelationships in the clinical TBI setting, which has constrained the neurotrauma field to date in fully understanding the complexity of the inflammatory system and its role in injury and recovery. We contend that the overall approach to analyzing TBI biomarkers requires a holistic approach that considers multiple markers taken together. Specifically, our analyses highlight the importance of examining variations in an ensemble of inflammatory markers and considering inflammation data in the larger context of multiple markers representing other secondary injury pathways.

Age and Inflammation in the Context of TBI Recovery

The primary outcome of interest in this study was global outcome, which was assessed at 6 and 12 months. Our results elucidate interesting and novel relationships between age, post-traumatic inflammation, and recovery that are worthy of discussion. Namely, our data show that after multivariate adjustment in a multinomial model, age was the strongest predictor of risk for mortality compared to favorable outcomes at 6 months (GOS=4/5). However, inflammatory cluster membership was the most significant predictor of severe disability (GOS=2/3) compared to favorable outcomes (GOS=4/5).Similar results were seen using another disability scale, the DRS, where the majority of participants in cluster 1 had moderate or severe disability, and a majority of participants in cluster 2 had partial to no disability.

From these findings, it can be postulated that age and inflammation are closely related, though it appears their association to TBI recovery differs in keys aspects. With this in mind, we postulate three key mechanisms, conceptually outlined in Figure 5, that underlie an "*age-inflammation hypothesis"* of TBI recovery. These include: 1) age effects independent of inflammation, 2) age-related inflammatory response, and 3) inflammation-related effects independent of age.

The first mechanism, age effects independent of inflammation, is well documented in TBI (Crownover *et al.*, 2012; De Guise *et al.*, 2015; Røe *et al.*, 2013). The single greatest predictor of TBI-related deaths is older age, with nearly a 6% increase in risk of death for each year increase in age after injury (Harrison-Felix *et al.*, 2004). The CDC estimates that individuals aged 65 and older at injury have over a 2.5 times increased risk of death compared to the next oldest age group, 45-64 year olds (CDC, 2001-2010). Importantly, individuals with TBI are twice as likely to die compared to individuals in the general population of similar age (Harrison-Felix *et al.*, 2004). It is possible that some of this increased risk for mortality can be attributed to inflammatory-related pathways; however, it is also likely that other factors, unmeasured in this study, influence increased mortality burden, such as chronic comorbidities like hypertension, diabetes, and coronary artery disease(Centers for Disease Control and Prevention, 2010). Also, in our cohort older age was associated with specific mechanisms of injury (e.g. motor vehicle collisions and falls) that lead to particular brain injury pathology (e.g. DAI and SDH) that could have also influenced recovery course.

With respect to the second mechanism, the age-related inflammatory response, the literature suggests that age is associated with changes in microglial reactivity/functionality and a greater pro-inflammatory load (Lourbopoulos *et al.*, 2015; Norden *et al.*, 2014). Further, increases in pro-inflammatory load associated with aging may have an adverse impact on the secondary injury cascade, and the result could reduce antioxidant reserve and lead to mitochondrial dysfunction, which could accelerate neurodegeneration (Friedland-Leuner *et al.*, 2013; Mocchegiani *et al.*, 2014; Salminen and Paul, 2014; Xu *et al.*, 2008). In our cohort, the PCA and cluster analysis were conducted with no *a priori* goal of observing associations between CSF inflammatory load and age; however, our data rendered a strong relationship between inflammatory cluster and older age. Specifically, in a post-hoc analysis conducted to examine differences in specific inflammatory markers associated with aging, we found that individuals above the 75th percentile for age in our cohort (age ≥48) had significantly higher levels of *the exact set of seven inflammatory markers* included in our PC1 that surpassed the |0.4| threshold using day 0-3 data. This suggests a unique inflammatory pattern that is characteristic among older adults with TBI that is distinct from the inflammatory response mounted by their younger counterparts with TBI.

The third mechanism refers to inflammation effects independent of age. This mechanism involves the role of inflammation in propagating the secondary injury cascade that is characteristic of TBI. This mechanism stresses the fundamental importance of how immunology interacts with and influences many other pathophysiological cascades and components characteristic of TBI. Our lab recently has explored how CNS inflammation interfaces with other pathways, specifically the neuroendocrine system. The work

demonstrated cortisol as a key mediator of inflammatory effects on outcome in TBI (Santarsieri *et al.*, 2015). The data reduction capabilities of PCA provided an opportunity to characterize the complexities of the inflammatory response as an overall entity, explained by key principal components. Using data generated previously from our group, we identified key relationships that highlight the central role of acute post-traumatic inflammation in driving neurological and peripheral secondary injury responses across multiple pathways. Though only correlational, our results offer insights that may guide future research that explores in more depth how inflammation influences and interacts with neurotrophins and sex hormone physiology as well as, the relationships between inflammation and damage-associated molecular pattern molecules, like S100b. We postulate that through its influence on the secondary injury cascade, increased inflammatory burden is associated with greater overall disability that is age-independent.

PCA as a Novel Approach to Characterize TBI Inflammation

In addition to unique age and inflammation related mechanisms on TBI recovery, the PCA approach to classification based on the inflammatory response provided several key novel insights, by identifying the relative importance of certain markers in explaining variability in post-traumatic inflammation. For example, IL-1β and TNFα are two of the most characterized and studied markers in the field of TBI. It is well-documented that these two markers are dramatically increased and are important mediators of the inflammatory response following TBI (Hayakata *et al.*, 2004; Morganti-Kossman *et al.*, 1997; Woodcock and Morganti-Kossmann, 2013). However, somewhat surprisingly, our data showed that IL-1β and TNFα provide a rather limited and similar contribution to variance in day 0-3 inflammatory profiles compared to other PC1 and PC2 markers. This finding seems in contrast to the work by Helmy and colleagues that found that TNF and IL-1β were important contributors to their PC2 in the first 48 hours after injury among a small case series of individuals with TBI (Helmy *et al.*, 2012). It could be interpreted that these markers are *similarly* elevated across the population in our study, and, although they are elevated considerably versus controls and related to recovery after severe TBI (Santarsieri *et al.*, 2015), they may provide little discriminative capacity in gauging degree of early inter-individual variability after TBI.

From days 0-3 post-TBI, we derived four principal components that we used to form two independent clusters of CSF inflammatory profiles. The most dominant principal component, PC1, accounts for >31% of the variance in day 0-3 inflammatory profiles after TBI. The inflammatory markers with the strongest PC1 loading (>0.7) include the soluble cell-surface markers sICAM-1 and sFAS and the inflammatory cytokines IL-6 and IL-10. The absolute production of each of these markers in the context of TBI has been described previously (Ertel *et al.*, 1997; Kirchhoff *et al.*, 2006; Pleines *et al.*, 1998; Santarsieri *et al.*, 2015; Shiozaki *et al.*, 2005), though prior studies have not demonstrated the relatively similar importance of these markers in explaining variability of the inflammatory response after TBI. Because these markers collectively make similar contributions to variance, individuals with high PC1 scores are likely to have relatively high levels of sICAM-1, IL-10, IL-6 and sFAS. For PC2, for days 0-3, the markers with the greatest loading include: IL-7, IL-12, IL-4, and IL-6. Unlike PC1, however, not all of the loadings were positive.

Specifically, IL-6 had a loading of -0.4, which was approximately equal in magnitude and opposite in direction to IL-4 (loading=0.42), suggesting that higher PC2 scores are indicative of relatively *higher* IL-4 and *lower* IL-6. This could imply that compensatory mechanisms occur that involve IL-4, and perhaps also IL-7 or IL-12, which regulate the production of IL-6.

From days 4-5 after injury, PCA reveals that markers explaining the greatest variability are unique and largely different from those observed with the principal components generated for days 0-3. IL-1β and TNFα, which show relatively little variability early after injury, appear to have greater discriminative capacity days 4-5 after injury. We believe that this finding could indicate a transition for these markers later in the first week post-injury, from a more uniform role in initiating the inflammatory response early on, to markers that may be more dynamic and heterogeneous in their roles with perpetuating and regulating inflammation during the acute care phase post-TBI. A dynamic role for TNFα is well known from pre-clinical research where classic studies of TNFα knockout mice revealed a transition from early detrimental effects to delayed beneficial effects on behavioral outcomes over the initial 5 days post TBI (Scherbel *et al.*, 1999). Conversely, IL-4 and IL-12 were two markers showing strong loading to day 0-3 PC2. Biologically, these markers have been implicated in the activation of microglia through T-cell mediation (Germann *et al.*, 1993). However, by days 4-5, these markers appear to make little contribution to variation in inflammatory profiles. Thus, IL-4 and IL-12 cytokine activity may be critical for distinguishing patient status early on, whereas activity is likely similar across the population at later time points post-injury, suggesting either saturation or return to baseline state for these markers. Overall, day 4-5 data did not discriminate subjects into unique cluster groupings. Early microglial activation could vary and be critical to affecting outcome. Alternatively, it is possible that over time, considerable heterogeneity with treatment course, hospital complications, and surgical interventions confounds the clear discrimination of distinct groups.

Limitations

Despite the novelty and implications of this work, this study was not without limitations. First, the current study averaged cytokine levels across days 0-3, and there is without doubt some degree of inflammatory marker variability that occurs within this time frame. Explaining the most variance in the data does not automatically equate to providing the best prediction of outcome, and that in theory, many possible weighted combinations of subsets or markers could be tested for predictive power on a trial-and-error basis. However, the point of this study was to specifically evaluate the utility of the PC-based approach for clustering patients and predicting their outcomes, and thus the trial-and-error exploration of subsets of markers is outside the scope of this work. With that said, we have explored various temporal groupings for deriving meaningful clusters that discriminate outcome (data not shown). The day0-3 grouping performed best, and this analysis is what is reported for the manuscript. Future studies should be designed with a greater time resolution of blood draws (e.g., every six hours) to provide enough data to run separate daily PCA analyses over the first week post-injury. Importantly, the confidence intervals observed in this study were considerably wide. Nonetheless, post-hoc power analysis show sufficient power (∼98%) to

detect a difference in effect by cluster group in terms of capacity to discriminate between severe disability (GOS=2/3) versus moderate disability/good recovery (GOS=4/5), due to the magnitude of the effect size (OR=10.94). It is likely that this OR estimate is inflated, and we hypothesize that with a greater sample size, the effect size will still show a significant effect, but its magnitude will attenuate towards the null to some degree and the confidence interval will become narrower.

Also of note, the findings observed in this study were limited to 6 months post-injury and not apparent at 12 months. This apparent difference in significance is not entirely surprising, because as individuals with TBI become more removed from their point of injury, there is an increasing impact of psychosocial factors (e.g. environmental exposures, rehabilitative treatments, and social supports) on outcomes reported, particularly for global outcomes like GOS and DRS. This increasing contribution of later exposures, treatments and social support then naturally renders early biomarker (biological) characterizations associated with the initial phases of injury less informative. It is more likely that biological markers collected during later time points after the acute time frame will be better prognostic markers of long-term outcomes. In fact, based on our previous work, we know that inflammatory cytokine levels during the subacute period (2wk-3mo) after injury are predictive of global outcomes at both six and 12 months (Kumar *et al.*, 2014).

Further, due to the strong association between aging and inflammation observed in this study, future work may benefit from prospectively collecting data on chronic diseases related to aging, such as hypertension, diabetes mellitus, and cardiovascular disease. Another limitation was the observational nature of this study makes it difficult to know whether biological relationships among cytokines, as well as between cytokines and other biomarkers, are due to direct effects or simply epiphenomena derived from other causal components of the secondary injury cascade. This clinical data can suggest productive directions for future experimental studies designed to elucidate biochemical interactions between markers.

Conclusions

Results from this study may have considerable implications to the field of TBI. Importantly, individuals with TBI have *distinct patterns involving multiple CSF inflammatory markers* that emerge and are detectable soon after injury. The data also show that unique groups of individuals can be distinguished acutely based on their CSF inflammatory profiles. This information provides some insight into which individuals are at an early risk for a prolonged, deleterious inflammatory response and, thus, may be good candidates for anti-inflammatory treatment interventions. Markers identified as loading to PC1 appear to have the greatest *relative* importance in explaining variability observed with inflammatory cascades acutely after TBI. These candidate markers may be leveraged to generate reliable and informative screening tools for prognosis in TBI clinical care, particularly among survivors of TBI for discrimination of severity of disability. It is important to note that not all biomarkers with discriminative capacity for TBI outcomes may be effective discriminators of mortality. In contrast, other markers we have evaluated (e.g. serum

estradiol) are potent mortality predictors (Wagner *et al.*, 2011) but have less predictive capacity among survivors (unpublished data).

As subsequent steps, we believe that PCA and clustering should be leveraged as data reduction methods that can be utilized in future adaptive clinical trials to identify patients at pre-specified time points who *are most likely to benefit* from an anti-inflammatory treatment. Additionally, older age and sex effects on variance in inflammatory profiles are substantial and require closer examination. Also, the association between cluster group and other biomarkers associated with the secondary injury response warrants further investigation in future studies. For example, experimental studies may benefit from examining how manipulation of one or more inflammatory markers affects other secondary injury responses, such as sex hormone physiology. Finally, future studies may build upon our work by using *in silico* mathematical modeling tools like differential equation models (Daun *et al.*, 2008) to examine how manipulating one or more markers affects the intricacies of the inflammatory response and to suggest potential therapeutic targets to test in clinical trials.

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Highlights

- We report principal components of acute CSF inflammation in severe TBI
- Using principal components, two unique clusters of TBI patients are identified
- Day 0-3 cluster group is a significant predictor of poor outcomes at 6 months
- Cluster groups discriminateCSF and serum hormone, BDNF, and s100b pathophysiology

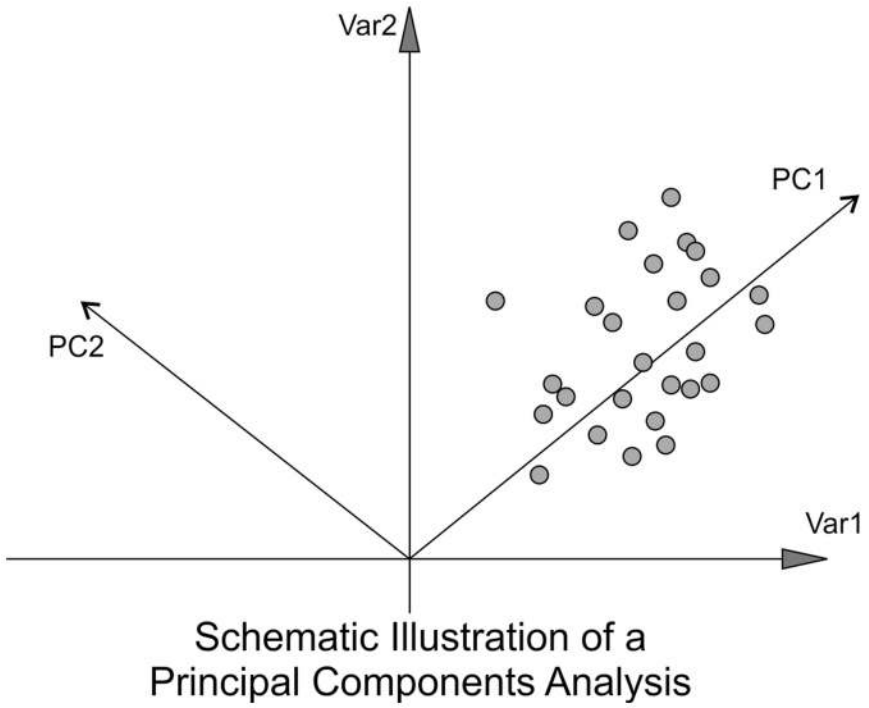

Figure 1.

In this schematic, Var 1 corresponds to Hemoglobin-A1C and Var 2 corresponds to capillary blood glucose. PC1 is the first principal component derived from PCA that accounts for the greatest percentage of variance in the data. PC2 accounts for the remaining variance, not accounted by PC2. Abbreviations: Var=variable; PC=principal component

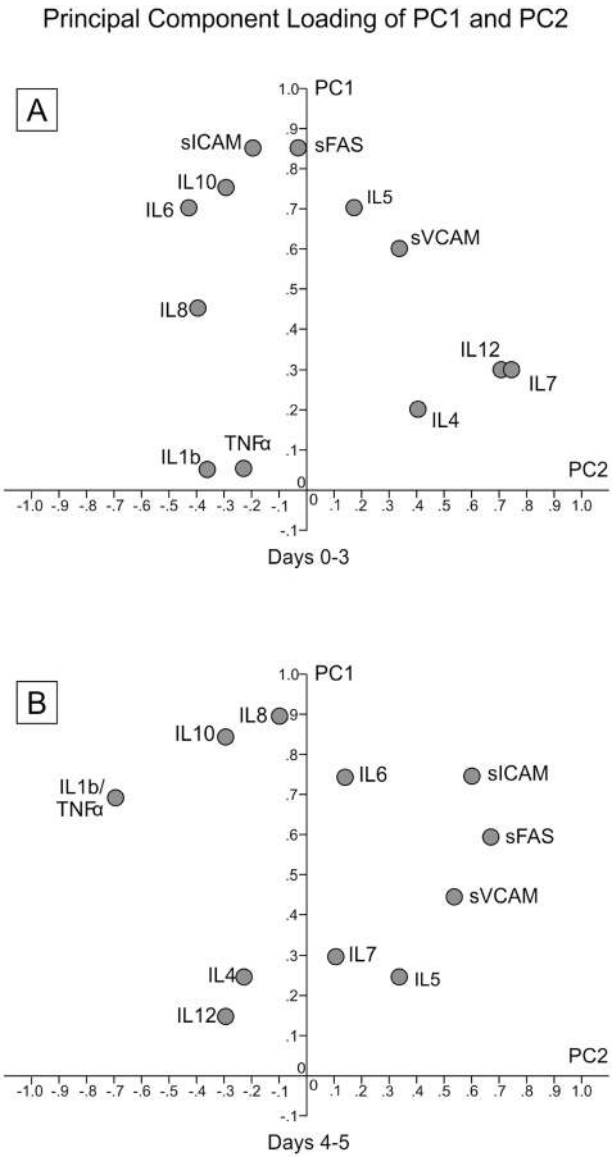

Figure 2.

Panel A: The loading of each CSF inflammatory biomarker to PC1 and PC2 for days 0-3 post-injury. The markers significantly loading (≥0.4) to PC1 were: IL-5, IL-6, IL-8, and IL-10. The markers significantly loading to PC2 were: IL-4, IL-6, IL-7, IL-12.

Panel B: The loading of each CSF inflammatory biomarker to PC1 and PC2 for days 4-5 post-injury. The markers significantly loading (≥0.4) to PC1 were: IL-1β, IL-6, IL-8, IL-10, TNFα, sVCAM-1, sICAM-1, sFAS. The markers significantly loading to PC2 were: IL-1β, TNFα, sVCAM-1, sICAM-1, and sFAS.

Abbreviations: CSF=cerebrospinal fluid; PC=principal component; IL=interleukin

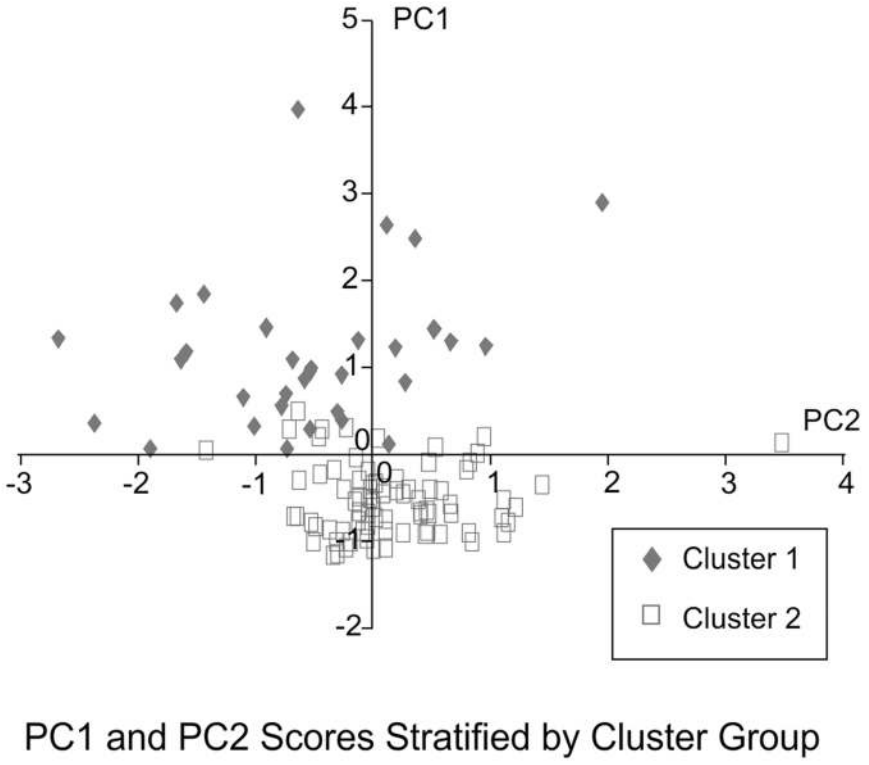

Figure 3.

PC1 and PC2 scores are plotted for each individual for days 0-3 post-injury, stratified by cluster group 1 and 2. PC3 and PC4 scores were utilized in the assignment of cluster groups, though they are not portrayed in this graphic; Abbreviations: PC=principal component

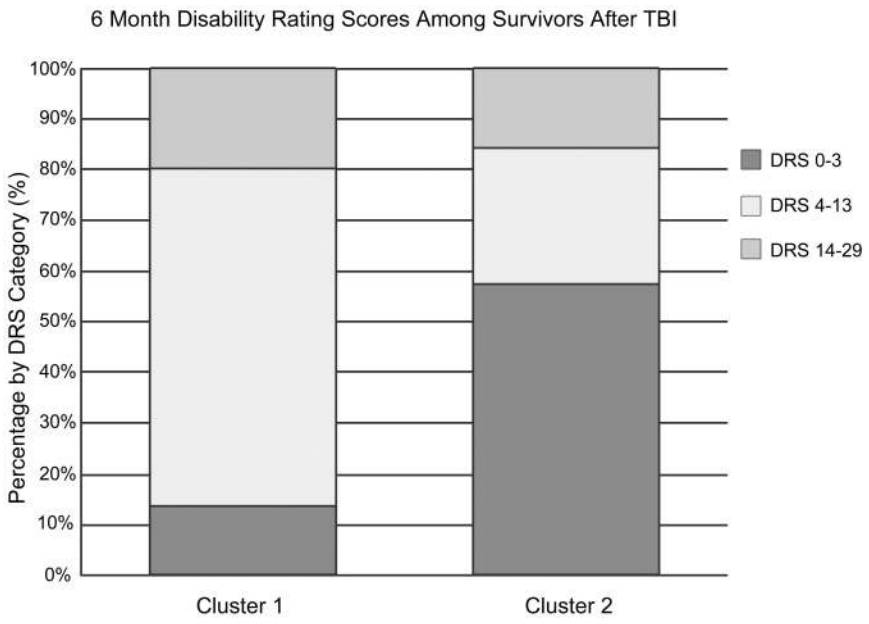

Figure 4.

The Disability Rating Scale is divided into three categories: partial to no disability (DRS=0-3); 2) moderate or severe disability (DRS=4-14); 3) extreme severe disability, vegetative state or dead (DRS=15-29). Our data indicate a significant association between cluster group membership and DRS scores at 6 months (p=0.008). A majority of individuals in Cluster 1 had moderate or severe disability; however, a majority of individuals in Cluster 2 had partial to no disability.

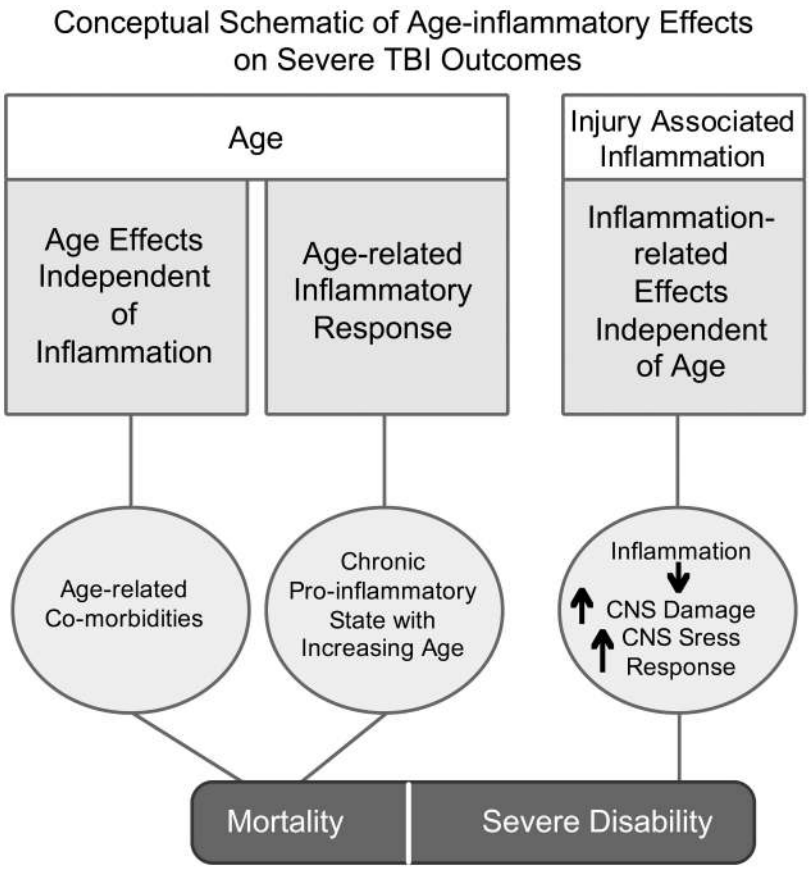

Figure 5.

We hypothesize that older age has the strongest influence on mortality after TBI through both inflammation independent and dependent effects; however, among survivors, an individual's CNS inflammatory load is a strong prognostic indicator of severity of disability post-injury

Table 1

Principal Components Analysis Loadings of CSF Inflammatory Markers

Days Post-Injury:	Days 0-3				Days 4-5		
Components (% variance)	**PC1 (31.4%)**	**PC2 (16.9%)**	**PC3 (10.8%)**	**PC4 (9.1%)**	**PC1 (36.8%)**	**PC2 (19.9%)**	**PC3 (11.3%)**
IL-1β			**0.45**	**0.45**	**0.69**	**-0.67**	
IL-4		**0.42**		**0.50**			**0.72**
IL-5	**0.69**						
IL-6	**0.72**	**-0.40**			**0.76**		
IL-7		**0.75**					**0.82**
IL-8	**0.45**			**0.53**	**0.91**		
IL-10	**0.77**				**0.86**		
IL-12		**0.73**					
TNFα			**0.79**		**0.70**	**-0.67**	
sVCAM-1	**0.62**				**0.46**	**0.55**	
sICAM-1	**0.83**				**0.73**	**0.62**	
sFAS	**0.83**				**0.58**	**0.68**	

Note: Bolded values were principal components greater than or equal to |0.4|, our minimum threshold for significant loading

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Table 2
Clinical and Demographic Associations with Cluster Group

	Cluster 1 (n=32)	Cluster 2 (n=79)	p-value
Age, mean (SE)	**46.09 (3.27)**	**31.75 (1.55)**	**<0.001***
Sex, Men (%)	**23 (71.88)**	**69 (87.34)**	***0.050***
GCS, Median (IQR)	6 (5-7)	7 (6-7.5)	0.166
ISS, Mean (SE)	32.81 (1.72)	34.38 (0.87)	0.235
BMI, Mean (SE)	26.14 (0.90)	26.92 (0.67)	0.815
Injury type from CT			
SDH	**28 (87.50)**	**47 (59.49)**	**0.004***
SAH	28 (87.50)	57 (72.15)	0.084
DAI	**3 (9.38)**	**34 (43.04)**	**<0.001***
EDH	2 (6.25)	14 (17.72)	0.119
Contusion	16 (50.00)	35 (44.30)	0.585
IVH	11 (34.38)	21 (26.58)	0.411
ICH	15 (46.88)	27 (34.18)	0.212
Mechanism of Injury, n (%)			
MVA	13 (40.63)	49 (62.82)	
Motorcycle	4 (12.50)	18 (23.08)	**0.005***
Fall	11 (34.38)	6 (7.69)	
Assault/fight	2 (6.25)	3 (3.85)	
Other	2 (6.25)	2 (2.56)	
Length of Stay in Acute Care (days), Mean (SE)	**17.49 (1.77)**	**23.19 (1.34)**	**0.013***
Length of Stay in Rehab (days), Mean (SE)	22.11 (5.55)	23.63 (3.73)	0.982
Sepsis, n (%)	3 (9.38)	2 (2.78)	0.712
Pneumonia, n (%)	20 (62.50)	45 (56.96)	0.592

Table 3

CSF Inflammatory Cytokine Levels (pg/mL) in Age Above and the Below 75th Percentile Age

	Age Quartile 1-3	Age Quartile 4†	p-value
IL-1β (Mean, SE)	0.14 (0.04)	0.28 (0.12)	0.975
IL-4 (Mean, SE)	0.48 (0.06)	0.64 (0.12)	0.287
IL-5 (Mean, SE)	**0.14 (0.02)**	**0.25 (0.05)**	**0.007***
IL-6 (Mean, SE)	**732.33 (86.72)**	**1433.12 (180.73)**	**<0.001**
IL-7 (Mean, SE)	0.69 (0.04)	0.57 (0.08)	0.072
IL-8 (Mean, SE)	**600.01 (94.08)**	**811.68 (170.95)**	**0.025***
IL-10 (Mean, SE)	**22.72 (4.04)**	**47.98 (10.54)**	**<0.001**
IL-12 (Mean, SE)	0.10 (0.01)	0.10 (0.01)	0.922
TNFα	1.03 (0.14)	1.32 (0.45)	0.321
sICAM-1	**36428.79 (12065.77)**	**86993.57 (32294.69)**	**<0.001**
sVCAM-1	**4120.93 (376.01)**	**9975.85 (1457.28)**	**<0.001**
sFAS	**203.20 (13.91)**	**432.42 (54.90)**	**<0.001**

† Quartile 4 corresponds to age 48 or older

Table 4
Clinical and Demographic Associations with 6 Month GOS Group

	GOS=1 (n=29)	GOS=2/3 (n=34)	GOS=4/5 (n=32)	p-value
Age, mean (SE)	**47.03 (3.20)**	**32.44 (2.56)**	**30.47 (2.40)**	**<0.001***
Sex, Men (%)	23 (79.31)	27 (79.41)	28 (87.50)	0.620
GCS, Median (IQR)	48 (36-60)	26 (20-44)	28 (21-35)	0.120
ISS, Mean (SE)	34.97 (1.64)	33.24 (1.52)	32.94 (1.35)	0.717
BMI, Mean (SE)	26.55 (0.97)	25.59 (1.01)	27.3 (1.03)	0.342
Injury type from CT, n (%)				
SDH	21 (72.41)	25 (78.13)	17 (54.84)	0.119
SAH	25 (86.21)	23 (71.88)	22 (70.97)	0.303
DAI	**5 (17.24)**	**9 (28.13)**	**15 (48.39)**	**0.030***
EDH	5 (17.24)	3 (9.38)	4 (12.90)	0.660
Contusion	18 (62.07)	14 (43.75)	13 (41.94)	0.228
IVH	7 (24.14)	9 (28.13)	11 (35.48)	0.617
ICH	10 (34.48)	11 (34.38)		0.873
Mechanism of Injury, n (%)				
MVA	10 (34.48)	17 (51.52)	20 (62.50)	
Motorcycle/Bicycle	6 (20.69)	7 (21.21)	7 (21.88)	
Fall	10 (34.48)	3 (9.09)	3 (9.38)	0.159
Assault/fight	1 (3.45)	2 (6.06)	1 (3.13)	
Other	2 (6.90)	4 (12.12)	1 (14.29)	
Length of Stay in Acute Care (days), Mean (SE)	**15.70 (2.02)**	**25.56 (1.94)**	**21.56 (1.51)**	**0.003***
Length of Stay in Rehab (days), Mean (SE)	n/a	25.14 (5.13)	21.14 (3.38)	0.982
Sepsis, n (%)	2 (7.69)	1 (3.03)	2 (6.90)	0.702
Pneumonia, n (%)	15 (51.72)	23 (67.65)	18 (56.25)	0.410

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Table 5

Multinomial Logistic Regression

		Glasgow Outcome Scale Score			
		6 month		12 month	
		Odds Ratio (95% CI)	p-value	Odds Ratio (95% CI)	p-value
Age	**GOS 1 vs. 4/5**	**1.061 (1.011, 1.112)**	**0.015***	**1.058 (1.015, 1.103)**	**0.007***
Age	GOS 2/3 vs. 4/5	0.995 (0.949, 1.044)	0.838	0.948 (0.886, 1.013)	0.114
GCS	GOS 1 vs. 4/5	0.713 (0.511, 0.944)	**0.046***	0.710 (0.514, 0.981)	**0.038***
GCS	GOS 2/3 vs. 4/5	0.914 (0.718, 1.163)	0.463	1.249 (0.921, 1.693)	0.153
DAI (Yes vs. No)	GOS 1 vs. 4/5	0.525 (0.115, 2.392)	0.405	0.568 (0.145, 2.224)	0.417
DAI (Yes vs. No)	GOS 2/3 vs. 4/5	0.593 (0.174, 2.020)	0.403	0.299 (0.063, 1.418)	0.129
Acute Care Length of Stay	GOS 1 vs. 4/5	0.954 (0.888, 1.024)	0.192	0.968 (0.910, 1.029)	0.291
Acute Care Length of Stay	GOS 2/3 vs. 4/5	1.048 (0.995, 1.105)	0.075	**1.049 (1.029, 1.163)**	**0.004***
Cluster (1 vs. 2)	GOS 1 vs. 4/5	4.142 (0.663, 25.891)	0.129	1.123 (0.262, 4.814)	0.876
Cluster (1 vs. 2)	**GOS 2/3 vs. 4/5**	**10.941 (1.963, 60.978)**	**0.006***	4.840 (0.858, 27.294)	0.074

Table 6

Day 0-3 CSF and Serum TBI-relevant Biomarkers by Cluster grou

	Cluster 1 (n=32)	Cluster 2 (n=79)	p-value
CSF			
Cortisol (Mean, SE)	**40.16 (4.04)**	**22.62 (2.19)**	**<0.001***
Progesterone (Mean, SE)	**137.30 (25.24)**	**52.72 (4.83)**	**<0.001***
E2 (Mean, SE)	**6.30 (0.98)**	**4.14 (0.41)**	**0.010***
Testosterone (Mean, SE)	**410.50 (103.28)**	**190.49 (31.20)**	**0.017***
E2:Testosterone Ratio (Mean, SE)	0.07 (0.03)	0.06 (0.01)	0.391
BDNF (Mean, SE)	**0.246 (0.044)**	**0.209 (0.047)**	**0.050***
S100b (Mean, SE)	**4.83 (0.78)**	**2.73 (0.49)**	**0.001***
Serum			
Cortisol (Mean, SE)	**290.43 (20.03)**	**196.31 (11.73)**	**<0.001***
Progesterone (Mean, SE)	3.23 (0.74)	2.44 (0.43)	*0.074*
E2 (Mean, SE)	80.12 (12.87)	62.61 (4.27)	0.570
Testosterone (Mean, SE)	3.83 (0.54)	4.00 (0.45)	0.920
E2:Testosterone Ratio (Mean, SE)	31.94 (5.04)	31.77 (3.42)	0.969
BDNF (Mean, SE)	**131.59 (14.78)**	**174.52 (11.25)**	**0.042***
S100b (Mean, SE)	12.16 (3.05)	16.15 (2.81)	0.347