CHRONIC SERUM CORTISOL MEDIATES THE RELATIONSHIP BETWEEN IL-6/sIL-6R COMPLEX AND LONG-TERM GLOBAL OUTCOME AFTER MODERATE TO SEVERE TRAUMATIC BRAIN INJURY

by

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ABSTRACT

Stress-induced hypothalamic-pituitary-adrenocortical (HPA) axis is a dominant mechanism for maladaptive outcomes after severe traumatic brain injury (TBI). Cortisol, a stress hormone released from the adrenal gland, in an acute setting can act as an anti-inflammatory agent. However, chronic sustained elevations of cortisol are an exacerbating factor for pathogenic responses in a number CNS disorders. Specifically, interleukin 6 (IL-6), a potent pro-inflammatory mediator associated with 6 and 12-month global outcome, is a stress-responsive cytokine that has detrimental effects in the brain. IL-6 and its affinity to its soluble receptor, sIL-6R, is shown to stimulate the HPA axis into a state of sustained chronic stress. Chronic serum cortisol paired with inflammatory cytokines has not been extensively studied in TBI. Thus, the purpose of this study was to investigate the role of IL-6 signaling and the mediating (regulating) effects of cortisol in differentiating global 12-month outcome for N=103 individuals with moderate to severe TBI. Exposure was measured as monthly levels of serum cortisol, IL-6, and sIL-6R. Biomarkers were collected from 2 weeks – 6 months post-injury. Monthly IL-6:sIL-6R ratios were averaged to produce a 6-month mean, and deciled. Outcome was assessed as twelve month GOS scores dichotomized as poor (GOS of 2/3) and good (GOS of 4/5). Group based trajectory analysis on 6-month mean cortisol identified three subgroups – a *high*, *decliner*, and

riser group. Mediation analysis, adjusting for cortisol trajectory, attenuated the relationship of IL-6:sIL-6R to GOS membership (OR=1.2, p=0.332). These results indicate that IL-6 signaling through the sIL-6R is influenced by cortisol levels. Further, this study begins to characterize propagation of chronic inflammation and the long-term stress response post-TBI as it relates to HPA axis activation and the IL-6 family of cytokines.

Public Health Significance: TBI contributes to a significant portion of disability and death in the world. TBI currently lacks effective neuroprotective treatment. However, the bi-directional communication between the HPA axis and neuroinflammatory markers provides novel insight onto the resolution of chronic inflammation, sustained stress, and poor recovery after injury. Further, IL-6 signaling may be a potential target for treatment intervention, which could alleviate the burden survivors of a brain injury experience.

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1.0 INTRODUCTION

Approximately 1.7 million people in the United States sustain a traumatic brain injury (TBI) each year.[1] TBI is considered a serious public health concern due to its high prevalence. In 2010, it was reported that 275,000 hospitalizations and 52,000 fatalities were due to TBI.[2] The secondary injury response, after TBI is characterized by an acute innate immune response, which consists of the initial debris clean-up mechanisms, such as clearing up damaged tissue as a result from the injury. Prolonged inflammation in the first few months after TBI is a large contributor to the persistent complications that occur post-injury. While much of the research to date has focused on acute neuro-inflammatory markers, our prior work begins to identify potential sub-acute and chronic peripheral inflammatory markers that influence long-term functional outcome.[3] Specifically, we have shown that pro-inflammatory mediators, including interleukin (IL) 6 in the sub-acute phase, are associated with worse global outcomes at 6 and 12 months after moderate to severe TBI.[3]

Cortisol, a stress hormone primarily released from the adrenal glands in response to adrenocorticotropic hormone (ACTH) through the hypothalamic-pituitary-adrenal (HPA) axis,[4] is elevated after a TBI. Chronically elevated cortisol levels can perpetuate inflammatory effects post-injury. In some cases, cortisol can have anti-inflammatory[5,6] effects; however sustained cortisol elevations increase pro-inflammatory effects, both centrally and systemically. Both serum and cerebrospinal fluid (CSF) cortisol levels are elevated early after TBI. Our previous

work identified acute CSF profiles as predictors of long-term outcome.[7] Acute cortisol trajectories (low vs. high groups) in CSF had variable inflammatory patterns which had subsequent relationships to outcome.[7] Specifically, the cortisol trajectories influenced both sub-physiological and pathological relationships to inflammatory cytokine patterns, where only a subgroup of individuals experienced a physiological relationship to inflammatory cytokines, and favorable outcome, suggesting cortisol might serve as a useful diagnostic measure through which to discern whether inflammation leads to favorable or unfavorable outcome.[7] Additionally, elevated chronic cortisol is shown to exacerbate inflammation in the central nervous system (CNS), measured by pro-inflammatory cytokines as an index for lipopolysaccharide (LPS) induced inflammation in the rat brain.[8] Thus, cortisol measurements in the post-acute phase after an injury may provide unique insights into effects on the inflammatory cascades after TBI. There are distinct signaling mechanisms that exist between inflammatory pathways that also serve as potent activators of the HPA axis. Specifically, IL-6 can stimulate the HPA axis into a state of sustained chronic stress, measured by elevated glucocorticoid levels,[9] which contributes to poor recovery after an injury.

IL-6 signaling and its relationship to HPA-axis dysfunction has not been studied in the context of TBI. IL-6 cytokine receptors exist both in membrane-bound form and in soluble form. The soluble receptors of the IL-6 family include soluble IL-6R (sIL-6R) and soluble IL-11R. IL-6 binding to its membrane bound receptor is termed *classical signaling*.[10,11] Cells expressing the membrane bound receptor, once bound by IL-6, form a signaling complex with membrane-bound glycoprotein (gp130)[11], which can have anti-inflammatory effects, by controlling the innate immune response.[12] Interestingly, other studies suggest IL-6 bound to sIL-6R potentially perpetuates individuals into a chronic inflammatory state[10] through an alternative

pro-inflammatory *trans-signaling* pathway. The divergent outcomes of the soluble receptor versus the membrane bound receptors is a function of the cell types the soluble receptors can attach to. The membrane bound, classical signaling, is active primarily in hepatocytes and lymphocytes; however, the soluble receptor used in the trans-signaling pathway has ubiquitous activity, as it can bind to any cell type.[11] Importantly, soluble gp130 (sgp130) has inhibitory effects on the IL-6/sIL-6R complex, such that sgp130 can attenuate these trans-signaling induced IL-6 cascades. Previous studies report a molar excess of sgp130 restricts the expression of the IL-6/sIL-6R complex[10,13,14], identifying the need to elucidate the regulatory mechanisms of the IL-6 cytokine family underlying its influence on outcome. The bi-directional communication between the HPA axis and neuro-inflammatory markers may be an important process that informs the relationship between the stress response and secondary inflammatory response post-TBI.

While most current biomarker discovery and clinical development efforts have focused on CNS specific markers, biomarkers reflective of a systemic response to TBI may provide unique prognostic information. Following secondary injury, centrally derived cytokines included IL-1β, IL-6 and TNFα, contribute to increased blood-brain barrier (BBB) permeability[15], and large IL-6 levels have been observed after TBI.[16] Increased permeability contributes to the potential for peripheral IL-6 relevant cytokine levels to serve as biomarkers for both outcome prediction and treatment approaches for intervention techniques post-injury. As such, this study begins to characterize chronic serum IL-6 and sIL-6R and its relationship to chronic serum cortisol, as informative markers for long-term global outcome. The role of IL-6 signaling and its regulatory affects on the adrenal gland may be useful markers in differentiating global 12-month

outcome for individuals with moderate to severe TBI. Thus, we hypothesize that chronic cortisol levels would mediate (regulate) IL-6 signaling and act as a biological predictors of outcome.

2.0 MATERIALS AND METHODS

2.1 STUDY DESIGN AND PARTICIPANTS

This study was approved by the Institutional Review Board at the University of Pittsburgh. We assessed 103 men and women (n=80 men and n=23 women) between 17 to 78 years of age, with moderate to severe TBI characterized by the Glasgow Coma Scale (GCS) ≤12. All participants were recruited through our level 1 trauma center and received the standard of care outlined in *The Guidelines for the Management of Severe Brain Injury*.[17] Study participants met additional inclusion criteria for positive findings on head computed tomography and extraventricular drainage catheter (EVD) placement for intracranial pressure (ICP) monitoring. Exclusion criteria included penetrating brain injury, history of hypothalamic or pituitary tumors, history of breast cancer requiring chemotherapy treatment, history of prostate cancer requiring orchiectomy or untreated thyroid disease.

Various demographic and clinical variables were collected and included in this study. These include age, sex, race, mechanism of injury, GCS (best in 24 hours) [18], Non-head injury severity score[19], length of hospital stay, hospital complications, and admission CT findings assessing brain injury type. Hospital complications are shown in **Table 1**.

Table 1. Description of Hospital Complications

Pulmonary	Acute respiratory distress syndrome Acute respiratory failure Aspiration/Pneuomonia atelectasis Pleural effusion Pneumonia Pulmonary embolus Bronchial mainstem intubation Acute sinusitis Empyema
Infection	CNS infection Sepsis/septicemia
Cardiovascular	Acute arterial occlusion Cardiopulmonary arrest (not cause of death) Major arrhythmia Myocardial infarction
Musculoskeletal	Extremity compartment syndrome
Hematological	Coagulopathy Postoperative hemorrhage
Renal	Acute renal failure Renal failure Urinary tract infection
Wounds	Wound infection Decubitis ulcer Wound dehiscence
Gastrointestinal	*Clostridium difficile* colitis Esophageal intubation GI bleed Bowel Obstruction Pancreatitis Small bowel obstruction
Neurological	CNS infection Diabetes insipidus Neuro-sequelae Progression of neurologic insult Seizures

2.2 SAMPLE COLLECTION AND BIOMARKER MEASUREMENTS

Blood samples for participants with TBI were collected in up to 2 week intervals for up to 6 months post-injury. Each sample collected was centrifuged, aliquoted, and stored at -80°C until the time of assay. Serum hormone assessments were initially measured for cortisol with radioimmunoassay (RIA) with the Coat-A-Count ® In-Vitro Diagnostic Test Kit (Siemens Healthcare Diagnostic). A second batch of serum Cortisol measurements were completed using competitive enzyme-linked immunosorbent assay (ELISA) (1-3002, Salimetrics, PA, USA). Although the Salimetrics kit is developed for saliva samples, a pilot experiment on serum samples resulted in strong linearity with a serial dilution,[20] showing no signs of matrix effects with the use of serum versus saliva. Several samples (n=38) from the original batch of experiments were re-run using the ELISA kit, allowing all of the new measurement values to be linearly transformed back to the original cortisol measurements. The linear transformation equation is as follows:

$$\text{Cortisol}_{\text{RIA}} = 41.74 + 0.96*\text{Cortisol}_{\text{ELISA}}$$

Serum IL-6 and sIL-6R were measured using a high-sensitivity Luminex bead assay (Millipore, Billerica, Massachusetts; Milliplex High Sensitivity 12-plex and 9-plex). The assays use a microsphere process as an immunoassay tagged with multiple fluorescent-labeled markers. The binding for each individual protein onto the multiplex bead was analyzed with a fluorescence detection laser optic system.

2.3 OUTCOME VARIABLE

The outcome variable of interest for this study was 12-month Glasgow Outcome Scale (GOS). This outcome variable was assessed at 12-months post-injury, and each individual received a score ranging from 1-5. The GOS is a widely utilized global scale that quantifies functional outcome.[21] The description of each score is as follows: 5 = good recovery, 4 = moderate disability, 3 = severe disability, 2 = persistent vegetative state, and 1 = death.[21] A GOS of 1 was excluded, as only survivors were included in this analysis. GOS was dichotomized to reflect a poor (GOS = 2/3) or good (GOS = 4/5) outcome group for GOS scores obtained at 6 and 12-months.

2.4 STATISTICAL ANALYSES

Statistical analyses were performed using SAS software (version 9.4 SAS Institute, INC., Cary NC) and STATA software (version 15.0 SE StataCorp, College Station, TX). Descriptive statistics including means and standard errors for all continuous variables were computed. Categorical variables were summarized using frequencies and percentages. The distribution of all continuous serum biomarkers was assessed for normality using the Shapiro-Wilk test. Mean comparisons for serum biomarkers between categories were performed using the Mann-Whitney test or Kruskal Wallis test where appropriate. Associations between categorical variables were assessed using the Chi-Square analysis or Fisher's Exact test where appropriate. All p-values that were considered statistically significant were at an alpha level of 0.05. All p-values that fell

above alpha, but below 0.10 were considered a trend, and indicated by italicization. Each independent variable was assessed for outliers.

2.4.1 Inflammatory Biomarker Analysis

A ratio of serum IL-6 to sIL-6R was produced to create a more biologically relevant marker of the IL-6/sIL-6R complex. The complex formed from IL-6 binding to its soluble receptor, in some part, is mechanistically driven by the bioavailability of each of the molecules. Thus, the ratio of IL-6 to sIL-6R quantifies the relative relationship of the two markers, and the ratio may be an informative overall marker indicative of the trans-signaling IL-6/sIL-6R complex. The ratio of IL-6 to sIL-6R was produced at each monthly bin from 2 weeks to 6-months post-injury. The monthly ratios across the 6-month time course were averaged to create a marker. The distribution of the new averaged value was not normal (**Figure 1**), and was therefore deciled for further analyses.

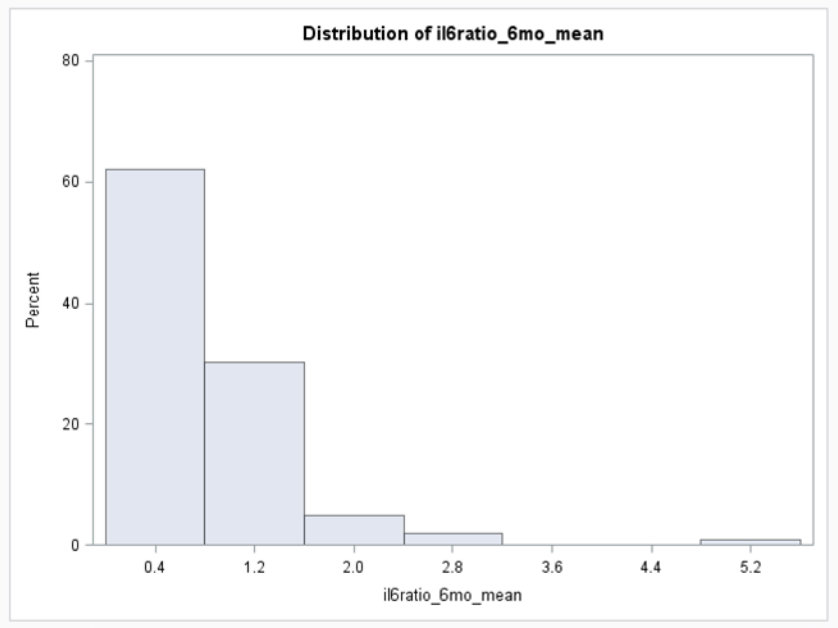

Figure 1. Distribution of IL-6:sIL-6R 6-month Average

2.4.2 Cortisol Trajectory Analysis

The temporal profiles for serum cortisol for the first 6-months were assessed using group-based trajectory analyses (GBTA) using the PROC TRAJ Macro from the SAS software. Individuals who had a minimum of two time points in the time course were included in this analysis. The trajectory (TRAJ) analysis identifies subgroups of individuals whose biomarker profiles have similar patterns longitudinally. The TRAJ procedure uses maximum likelihood estimation to handle missing values[22–24] and identifies trajectory groups also using likelihood. The TRAJ procedure also assumes normal data; therefore, data transformations to achieve normality were assessed. Log transformation of the cortisol data did not follow a normal distribution; thus, cortisol values for the 6-month time course were deciled for each month prior to running the TRAJ analysis. The initial step for a GBTA includes finding the number of groups that best describes the data. The groups identified by the statistical procedure are subgroups of individuals whose cortisol levels have similar profiles longitudinally over the 6-month time course. The number of groups in the final model were determined by first using a baseline of a fourth order polynomial model with one group as a comparison.[25]

The polynomial order refers to the exponent value of a particular variable. The order of the polynomial model was kept constant, while the number of groups was increased by one integer value. We evaluated one to four groups, as analyzing a high number of groups contributes to lower percentage of individuals within each of the groups. The Bayesian information criterion (BIC) was used as an indicator for determining the optimal number of groups in the final model. A lower BIC value indicated a better model fit. Each subject was assigned to a trajectory group based on posterior probability. The posterior probability is a function of the BIC, and it is the probability assigned for each individual falling within each

trajectory group. The average posterior probability in each group ranged from 0.71 to 0.81 in the final model. The recommended posterior probability is 0.7[26], indicating that our final model was above the criteria. The final cortisol TRAJ model resulted in a linear order model for two of the groups and an intercept model for one of the groups.

A linear model includes first order independent variables where each of the independent variables have a specific intercept and a constant rate of change.

- $Y = \beta_0 + \beta_1 * X$

An intercept model is often termed a constant model, as it is the model where all the independent variables are equal to zero

- $Y = \beta_0$

The groups from the GBTA were labeled based on their patterns with respect to the time course. Based on their visual patterns, the three different TRAJ group profiles included a *decliner*, *riser*, and *high* group, shown in **Figure 2.**

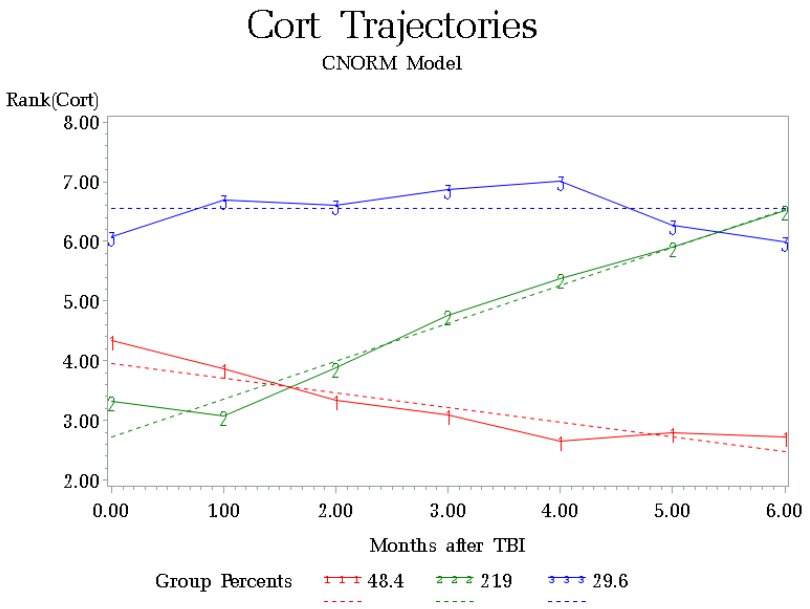

Figure 2. Ranked Cortisol Trajectories for 2 weeks to 6 months post-injury

2.4.3 Mediation and Regression Analyses

A logistic regression is a regression method for which the association of independent variable to a dichotomous dependent variable is tested. The logistic regression model estimates the adjusted probabilities from a fitted model which follows the form:

$$\text{Logit}(p_i) = \beta_0 + \beta_1 * X_i \text{ where i = number of predictors}$$

A logistic regression model assumes both that the dependent variable be binary and that the error terms are independent and identically distributed.

An ordinal logistic regression is a regression method used to determine the association of independent variables to a dependent variable that is grouped into ordinal categories that are two or more levels (J = number of levels). The model estimates cumulative probabilities for each of the outcome levels. The ordinal logistic regression model has J-1 logit functions:

$$\text{Ln}(\text{odds}_j) = \alpha_i + \beta^T * X, \text{ for all } j = 1, \ldots., J-1$$

The ordinal logistic regression assumes that the covariate effects, β, are the same for different logit functions. This is also termed the proportional odds assumption. In other words, the underlying relationship between each ordered pair of outcome groups is the same, and has parallel-lines. The ordinal logistic regression model also assumes independent and identically distributed error terms.

Lastly, a mediation analysis seeks to identify whether the direct causal relationship between an independent and dependent variable is influenced by another variable called a mediator. The mediator variable clarifies the true causal pathway between the independent and dependent variable. A variable functions as a mediation variable if: 1) the change in the level of the independent variable significantly accounts for the variable that is presumed the mediator variable 2) the change in the level of the presumed mediator significantly accounts for variations

in the dependent variable 3) If the presumed mediator variable strongly dominates the significant relationship between the dependent and the independent variable, then it is termed the mediator variable. In other words, if the relationship between the dependent and the independent variable no longer exists and their variations are controlled by some other variable, then that variable is termed as the mediator variable.

Thus for the mediation, when the outcome was binary (i.e. GOS 12-Month scores of 2,3 vs. 4,5), we performed a logistic regression model to test associations between the independent and dependent variables. When the outcome was greater than two groups (i.e. cortisol TRAJ), we performed an ordinal logistic regression model to test associations between the independent and dependent variables. We hypothesize that the different cortisol TRAJ groups have an ordering, as declining levels may lead to better outcome, compared to sustained elevations in cortisol levels, which may lead to worse outcome. Lastly, the proportionality assumption was tested before use of the ordinal logistic regression model.

Baron and Kenny were the first to delineate the conceptual and methodological implications of mediators.[27] They also provided the first specific analytic procedures that elucidated the causal system framework appropriate for making inferences on mediators.[27] The Baron and Kenny method outlines several steps for statistically testing certain relationships, which include testing each leg of the mediation to determine if:

1) The exposure is associated with the outcome.

   Independent variable → dependent variable

   Outcome = $\beta_{10} + \beta_{11}$*Exposure + ε_1 where β_{11} is significant

2) The exposure is associated with the mediator

   Independent variable → mediator

   Mediator = $\beta_{20} + \beta_{21}$*Exposure + ε_2 where β_{21} is significant

3) The mediator is associated with the outcome

   Mediator → dependent variable

   Outcome = $\beta_{30} + \beta_{31}$*Mediator + ε_3 where β_{31} is significant

4) The mediator attenuates the association between the exposure and outcome (indirect effect).

   Outcome = $\beta_{40} + \beta_{41}$*Exposure + β_{42}*Mediator + ε_4 where β_{42} is significant and β_{41} is smaller in absolute value than the original mediation effect (β_{11})

In this analysis, the legs of the mediation assessed for mediation effects were: 1) IL-6/sIL-6R complex (exposure) association with 12-month GOS (outcome), 2) IL-6/sIL-6R complex (exposure) association with cortisol TRAJ group (mediator), 3) cortisol TRAJ group (mediator) association with 12-month GOS (outcome), and 4) whether the relationship between IL-6/sIL-6R complex (exposure) and 12-month GOS (outcome) was attenuated when adjusting for cortisol TRAJ group (mediator). Here, we define the final model as the combined legs of the mediation analysis, or in other words, the assessment of the fourth mediation criteria. The mediation percentage was calculated by considering the natural logarithm (ln) of the odds ratios (OR) with the following equation:

$$\text{Mediation percentage} = \{[\ln(OR_{\text{Total Effect}}) - \ln(OR_{\text{Direct Effect}})]/\ln(OR_{\text{Total Effect}})\}*100\%$$

The mediation percentage illustrates the amount the mediator variable accounts for the true causal pathway between an independent and dependent variable.

2.5 MODEL SELECTION

The variables in the mediation were included based on investigating the bivariate relationships between the various clinical and demographic variables to global outcome. Injury severity is an important covariate for TBI studies, and therefore, GCS was included in the final model, regardless of statistical significance in the bivariate models. Similarly, since age is an important demographic variable to consider, it was kept in the model regardless of statistical significance bivariately. Given *a priori* knowledge that the cohort was primarily Caucasian men, race and sex were not included in the model regardless of statistical significance bivariately. The variables included in the final model were cortisol TRAJ, IL-6:sIL-6R ratio, age, and GCS. These variables consist of the biomarker variables of interest, as well as basic demographic and injury severity variables that are typical confounders in a TBI population.

2.6 MODEL DIAGNOSTICS

2.6.1 Individual Level Diagnostics

The detection of outlier points and influential points were assessed. The detection of outliers was characterized by calculating the Pearson residuals of the final model. This

calculation was done by taking the difference between the observed and predicted outcome, divided by the standard error. Large residuals indicate deviation from the observed data. A value greater than the absolute value of 2 indicates a large residual. Similarly, the presence of influential points was characterized by the delta beta influential statistic. A value of dbeta>1 is considered large, and indicates an influential point.

2.6.2 Overall Goodness-of-Fit

The Hosmer-Lemeshow test was used to determine the overall goodness-of-fit for the model. This test compares the predicted probabilities and the observed probabilities of the model that was fit. The predicted probability and the observed probability should match closely, and the more closely they match, the better the fit. The null and alternative hypothesis of the test are as follows:

- H_0 : E (Y|X) = Pr (Y|X) = exp(Xβ)/(1+exp(Xβ))

  Correct model (goodness-of-fit)

- H_1 : E (Y|X) = Pr (Y|X) ≠ exp(Xβ)/(1+exp(Xβ))

  Miss-specified model (lack of fit)

3.0 RESULTS

3.1 DISTRIBUTION OF CONTINUOUS EXPOSURE VARIABLE

The untransformed distribution of IL-6/sIL-6R 6-month ratio (exposure) was tested for normality using the Shapiro-Wilk test. This test resulted in a $p<0.0001$, indicating that this variable failed to meet the normality assumption. The ratio was deciled to produce a more even distribution of the predictor variable, shown in **Figure 3**.

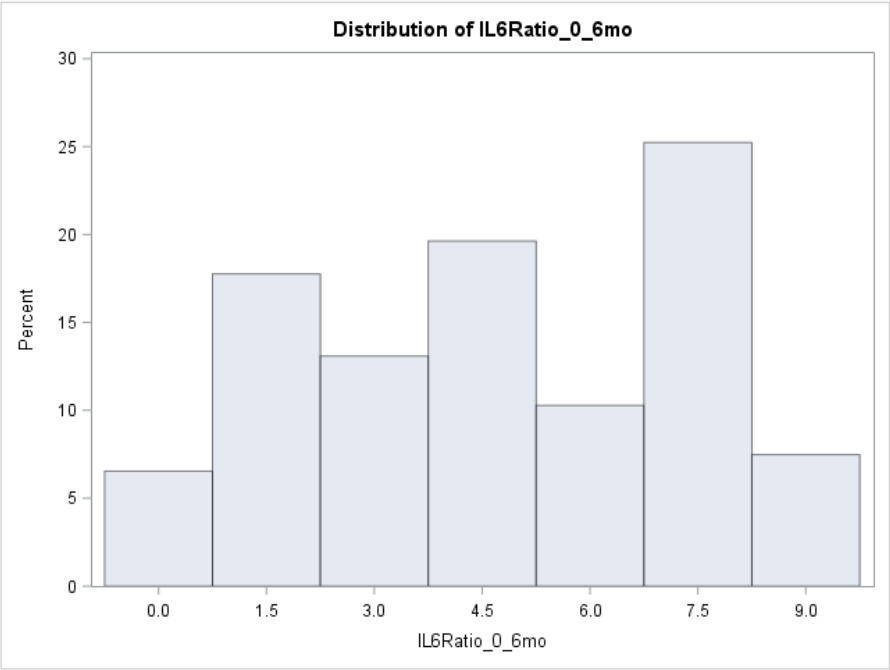

Figure 3. Distribution of IL-6/sIL-6R after Deciling

As a method for binning the individuals in the cohort, each individual received a value ranging from 0 to 9 after deciling the ratio. The lowest number of individuals within a given bin was N=7 for bin 0. Similarly, the highest number of of individuals within a given bin was N=16 for bin 8. The median number of individuals for all of the bins was 11.

3.2 DEMOGRAPHIC AND CLINCAL INFORMATION FOR COHORT

A detailed description of the cohort by cortisol TRAJ group (mediator) assignment is presented in **Table 2.**

Table 2. Demographic and Clinical Information for Cohort by Cortisol TRAJ

Variable	Cort Decliner TRAJ (N=56)	Cort Riser TRAJ (N=17)	Cort High TRAJ (N=34)	p-value
Age at injury, Mean (SE)	36.92 (2.31)	35.59 (3.74)	35.88 (3.05)	p=0.907
Sex Men n (%)	46 (82.14)	15 (88.24)	23 (67.65)	p=0.152
Race n (%)				
Caucasian	50 (90.91)	16 (94.12)	31 (93.94)	p=0.951
African American	4 (7.27)	1 (5.88)	1 (3.03)	
Best in 24 GCS, Median (IQR)	7 (2)	8.5 (5)	7 (3)	p=0.174
Non-head ISS, Mean (SE)	13.21 (1.86)	5.83 (1.80)	13.08 (2.18)	*p=0.068*
Length of stay (days), Mean (SE)	22.84 (1.94)	17.00 (2.69)	23.96 (2.81)	p=0.351
MOI, n (%)				
MVA	17 (43.59)	5 (41.67)	13 (52.00)	
Motorcycle	10 (25.64)	4 (33.33)	6 (24.00)	
Truck	1 (2.56)	0 (0)	0 (0)	
Off-Road Vehicle	5 (12.82	0 (0)	1 (4.00)	
Bicycle	1 (2.56)	1 (8.33)	1 (4.00)	
Fall/jump	3 (7.69)	2 (16.67)	3 (12.00)	
Assault/Fight	0 (0)	0 (0)	1 (4.00)	
Other	2 (5.13)	0 (0)	0 (0)	
Complications, n (%)				
Pulmonary	26 (72.22)	6 (50.00)	16 (69.57)	p=0.352
Infectious Disease	4 (11.11)	0 (0)	6 (26.09)	*p=0.089*
Cardio	3 (8.33)	0 (0)	0 (0)	p=0.281
MSK	0 (0)	0 (0)	0 (0)	
HEME	3 (8.33)	1 (8.33)	2 (8.70)	p=1.000
Renal	4 (11.11)	0 (0)	4 (17.39)	p=0.366
Wounds	3 (8.33)	0 (0)	2 (8.70)	p=0.701
GI	6 (16.67)	0 (0)	2 (8.70)	p=0.316
Neurological	7 (19.44)	3 (25.00)	4 (17.39)	p=0.850
Other	3 (8.33)	0 (0)	0 (0)	p=0.281
Injury type from CT, n (%)				
SDH	24 (63.16)	7 (58.33)	18 (72.00)	p=0.661
SAH	22 (56.41)	9 (75.00)	17 (68.00)	p=0.419
DAI	14 (36.84)	4 (33.33)	7 (28.00)	p=0.767
EDH	5 (12.82)	2 (16.67)	5 (20.00)	p=0.695
Contusion	13 (34.21)	5 (41.67)	10 (40.00)	p=0.848
IVH	9 (23.68)	3 (25.00)	4 (16.00)	p=0.724
ICH	12 (31.58)	4 (33.33)	10 (40.00)	p=0.785
Other	0 (0)	1 (8.33)	1 (4.00)	p=0.240

There were no statistically significant differences for the demographic and clinical variables by cortisol TRAJ group. All groups included more men than women, with most participants being Caucasian. Motor vehicle and motorcycle collisions were the most common mechanism of injury across all three TRAJ groups. The *decliner* and *high* groups had a higher Non-head ISS score than the *riser* group (p=0.068).

Similarly, a description of the cohort stratified by 12-month GOS (outcome) group category is presented in **Table 3**.

Table 3. Demographic and Clinical Information by Good vs. Poor 12-Month GOS

Variable	Good GOS Outcome Group (N=77)	Poor GOS Outcome Group (N=26)	P value
Age, Mean (SE)	37.29 (1.98)	29.92 (2.38)	*p = 0.056*
Sex, Men (%)	67 (82.72)	17 (65.38)	*p = 0.061*
Race, n (%)			**p = 0.038**
Caucasian	72 (94.74)	21 (84.00)	
African-American	4 (5.26)	2 (8.00)	
GCS best in 24, Median (IQR)	7 (4)	7 (1)	**p = 0.041**
Non-head ISS, Mean (SE)	10.58 (2.38)	15.79 (2.82)	*p=0.089*
Length of Stay, Mean (SE)	19.98 (1.52)	28.75 (3.01)	**p = 0.002**
MOI, n (%)			
MVA	26 (47.27)	9 (42.86)	
Motorcycle	14 (25.45)	6 (28.57)	
Truck	1 (1.82)	0 (0.00)	
Off-Road Vehicle	4 (7.27)	2 (9.52)	
Bicycle	2 (3.64)	1 (4.76)	
Fall/jump	7 (12.73)	1 (4.76)	
Assault/Fight	0 (0.00)	1 (4.76)	
Other	1 (1.82)	1 (4.76)	
Complications, n (%)			
Pulmonary	33 (63.46)	15 (78.95)	p = 0.217
Infectious Disease	5 (9.62)	5 (26.32)	*p = 0.065*
Cardio	3 (5.77)	0 (0.00)	p = 0.387
MSK	0 (0.00)	0 (0.00)	
HEME	5 (9.62)	1 (5.26)	p = 0.345
Renal	6 (11.54)	2 (10.53)	p = 0.327
Wounds	3 (5.77)	2 (10.53)	p = 0.290
GI	5 (9.62)	3 (15.79)	p = 0.237
Neurological	7 (13.46)	7 (36.84)	**p = 0.043**
Other	3 (5.77)	0 (0.00)	p = 0.387
Injury Type, n (%)			
SDH	33 (61.11)	16 (76.19)	p = 0.272
SAH	33 (60.00)	15 (71.43)	p = 0.356
DAI	19 (35.19)	6 (28.57)	p = 0.585
EDH	9 (16.36)	3 (14.29)	p = 0.273
Contusion	20 (37.04)	8 (38.10)	p = 0.932
IVH	12 (22.22)	4 (19.05)	p = 0.240
ICH	17 (31.48)	9 (42.86)	p = 0.353
Other	1 (1.85)	1 (4.76)	p = 0.409

The poor outcome group had a lower mean age (29.92 (SE, 2.38)) compared to the good outcome group (37.29 (SE, 1.99)) with a p-value of 0.057. Both groups had more men than women, with a greater percentage of participants self-reporting being Caucasian. GCS reached statistical significance bivariately to 12-month GOS (p=0.041). The median GCS was 7 among those with good outcome and 6 among those with poor outcome. Additionally, the poor outcome group tended to have a higher Non-head ISS score (p=0.090). The poor outcome group also had a statistically significant longer hospital length of stay compared to the good outcome group (p=0.002). Motor vehicle and motorcycle accidents were the most common mechanisms of injury for both outcome groups. There were no complications or CT findings that significantly differed between the groups.

3.3 SERUM CORTISOL TRAJECTORY PROFILES

The trajectory analysis resulted in three different TRAJ group profiles which included a *decliner, riser,* and *high* group. Less than 20% of individuals contributed a minimum of two time points. Further, approximately 50% of individuals fell in the *decliner* group, and the other half of the cohort fell evenly between the *riser* and *high* groups. The final model has a BIC of -1389. The *high* group was an intercept model (p<0.0001), where the *decliner* (p=0.023) and *riser* (p=0.005) groups were both linear order models. The *high* group stayed consistently high for the first five months post-injury. After month 5, the stable high group started to decline by month six. The *riser* group consistently increased for the first six months, surpassing the *stable high* group after month five. While the *decliner* group consistently decreased for the first six months,

plateauing after month five. Also a Chi-Square test identified that the *high* cortisol TRAJ group was associated with increased poor long-term outcome (p=0.005).

The posterior probabilities of the model were assessed, and the average posterior probability in each group ranged from 0.71 to 0.81. These values were above the recommended statistical cut-off of 0.70. Further, the cortisol levels were graphed by the trajectory group assignment, shown in **Figure 4**.

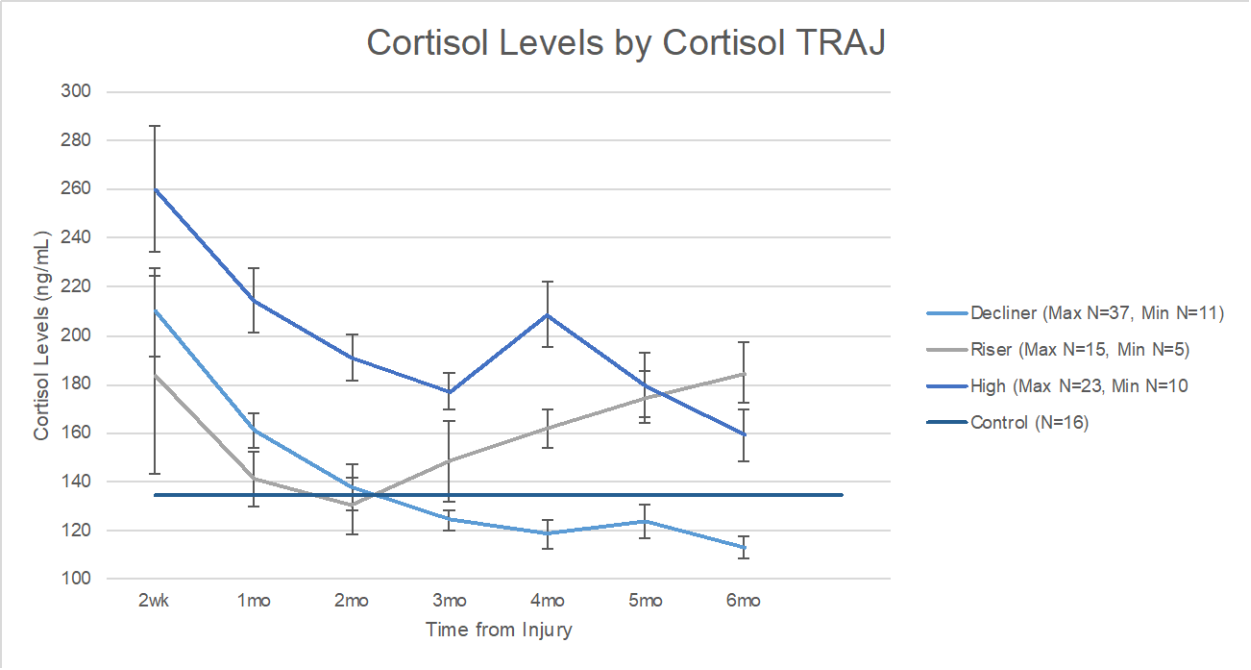

Figure 4. Cortisol Levels by Trajectory Group Assignment

The graphical representation of the levels produced similar results to the GBTA. Visually, a *high*, *decliner*, and *rise* group is still depicted by the graph. The minimum and maximum number of individuals comprised in the averages for each TRAJ group for each time point are provided in the legend. The *high* group was not as stable as represented by the model output; however, the levels are higher than the other two groups, and the confidence intervals do

not overlap with other groups at most time points. The *decliner* group, based on the levels, decreases over the 6-month time course. The *riser* group has a more pronounced dip in the early part of the time course, but continues to gradually increase for the remainder of the time points. Notably, only the *decliner* group has resumption of cortisol levels to below control values.

3.4 IL-6:SIL-6R COMPLEX BIVARIATE RELATIONSHIPS TO CORTISOL TRAJ AND GLOBAL OUTCOME

IL-6:sIL-6R ratios discriminated between cortisol trajectories and also between good and poor global outcome. Average levels for the 6-month IL-6:sIL-6R ratios by 12-Month GOS and cortisol TRAJ are shown in **Figure 5** and **Figure 6** respectively.

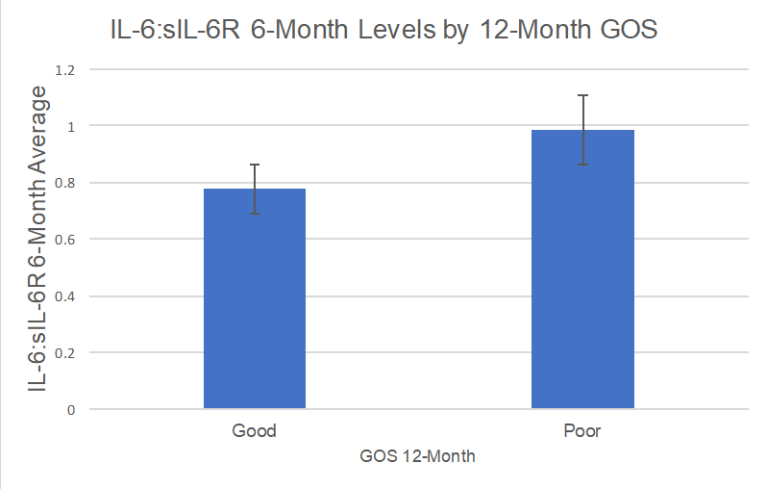

Figure 5. IL-6:sIL-6R by 12-Month GOS

A Mann-Whitney test indicated higher IL-6:sIL-6R levels were significantly associated to poor global outcome (p=0.036).

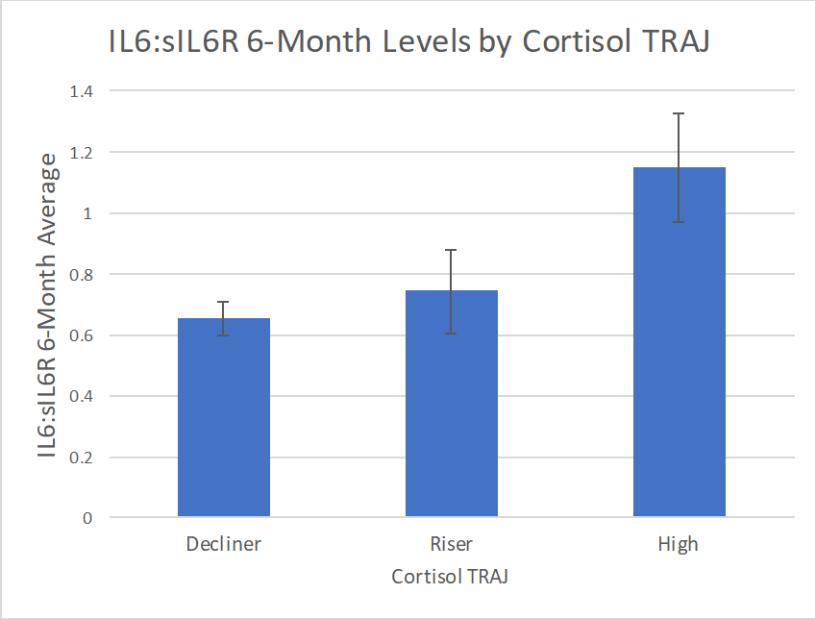

Figure 6. IL-6:sIL-6R by Cortisol TRAJ

A Kruskal Wallis test indicated IL-6:sIL-6R levels were significantly different among the different cortisol TRAJ groups (p=0.014). A pairwise comparison showed a significant difference between the *decliner* and *high* group (p=0.012), while the other pairwise comparisons were not statistically significant.

3.5 CORTISOL TRAJ MEDIATES IL-6/SIL-6R ASSOCIATIONS TO OUTOCME

The first leg of the mediation tested the association between IL-6:sIL-6R and 12-month GOS while adjusting for age and GCS score. The logistic regression is modeled with poor outcome. Each increase in IL-6:sIL-6R decile rank has 1.2 times the odds of experiencing poor outcome (p=0.061). These results are shown in **Table 4.**

Table 4. IL-6:sIL-6R to 12-Month GOS

Logistic Model	**Point Estimate (95%CI)**	P value
Age	0.977 (0.944, 1.012)	0.204
GCS	0.889 (0.733, 1.077)	0.230
IL6:sIL6R	1.187 (0.992, 1.420)	*0.061*

The second leg of the mediation tested the association between cortisol TRAJ and 12-month GOS while adjusting for age and GCS score. The logistic regression is modeled with poor outcome. The *high* TRAJ group had 5.8 times the odds of experiencing poor outcome compared to the *decliner* group (p=0.002), shown in **Table 5.**

Table 5. Cortisol TRAJ to 12-Month GOS

Logistic Model	**Point Estimate (95%CI)**	P value
Age	0.980 (0.944, 1.017)	0.275
GCS	0.813 (0.660, 1.001)	*0.052*
Cort TRAJ *Riser* vs *Decliner*	0.811 (0.149, 4,431)	0.190
Cort TRAJ *High* vs *Decliner*	5.787 (1.951, 17.170)	0.002

The last leg of the mediation tested the association between IL-6:sIL-6R to cortisol TRAJ. The ordinal logistic regression is modeled in reference to the *high* TRAJ group. The proportionality assumption was met (p=0.818), as the not statistically significant p-value fails to

reject the null hypothesis of proportional odds. An increase in IL-6:sIL-6R decile rank has 1.2 times the odds of being in the *high* TRAJ group (p=0.004) when adjusting for age and GCS score. The results are shown in **Table 6.**

Table 6. IL-6:sIL-6R to Cortisol TRAJ

Ordinal Logistic Model	**Point Estimate (95%CI)**	P value
Age	0.989 (0.964, 1.016)	0.431
GCS	1.222 (1.047, 1.426)	0.011
IL6:sIL6R	1.243 (1.071, 1.443)	0.004

The mediation analysis adjusting for age, and GCS was a logistic regression modeled with GOS group. The results showed that cortisol TRAJ was associated with 12-month GOS (p=0.010), shown in **Table 7**.

Table 7. Multivariable Model to 12-Month GOS

Logistic Model	**Point Estimate (95%CI)**	P value
Age	0.980 (0.944, 1.017)	0.285
GCS	0.823 (0.666, 1.017)	*0.071*
IL6:sIL6R	1.099 (0.909, 1.328)	0.332
Cort TRAJ *Riser* vs *Decliner*	0.765 (0.139, 4.218)	0.200
Cort TRAJ *High* vs *Decliner*	4.934 (1.594, 145.274)	0.004

The *high* TRAJ group had 4.9 times the odds of experiencing poor outcome compared to the *decliner* TRAJ group. Notably, the relationship between IL-6:sIL-6R and GOS was attenuated with the addition of cortisol TRAJ. The effect of IL-6:sIL-6R was slightly also dampened compared to the univariable regression (1.1 vs. 1.2). Cortisol accounts for 52% of the true causal pathway between IL-6:sIL-6R and global outcome. This number can be formally calculated by referring to the odds ratios (point estimates) for the total effect of IL-6/sIL-6R (Table 5) and for the direct effect of IL-6/sIL-6R (Table 7). The calculation is as follows:

$$\{[\ln(1.22) - \ln(1.10)]/\ln(1.22)\}*100 = 52\%$$

3.6 MODEL FITS

The individual level diagnostics for potential outlier points and potential influential points are displayed in **Figures 7 & 8**, respectively.

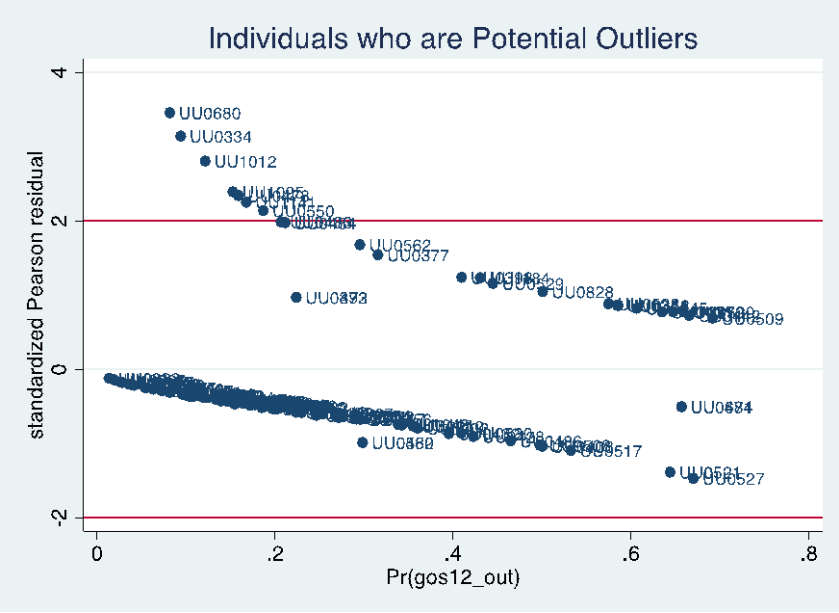

Figure 7. Potential Outlier Points

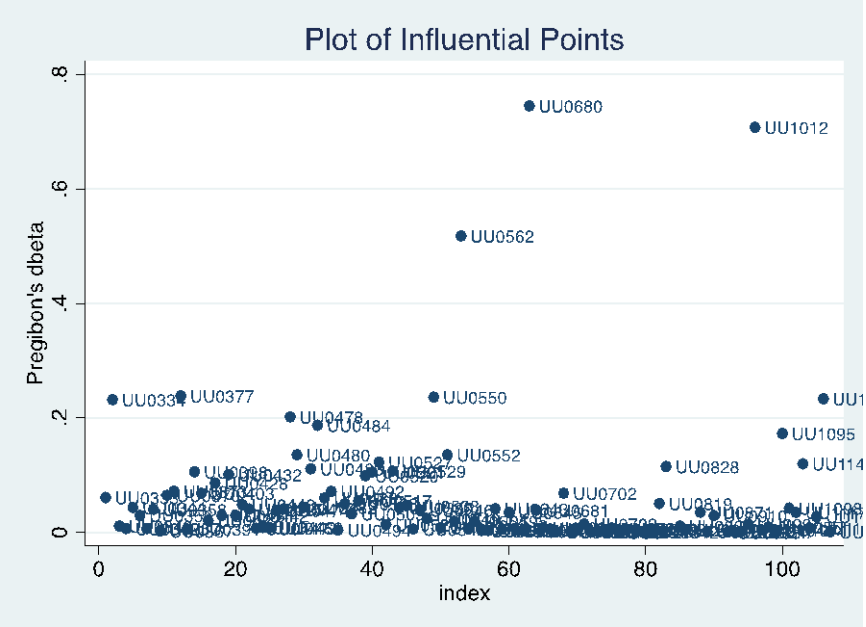

Figure 8. Potential Influential Points

There were several outlier points that were detected by investigated the Pearson residuals. However, there were no influential points detected in the final model. Despite the presence of outlier points, the Hosmer-Lemeshow test for goodness-of-fit resulted in a not statistically significant p-value (p=0.456). This result indicates that the final model did not have a lack of fit.

4.0 DISCUSSION

Effective neuroprotective treatments for individuals who have sustained a TBI are lacking, and previous research has examined inflammatory markers, sex hormones, and neuronal damage markers as potential biomarkers for studying global disability and mortality after TBI.[28,29,30,31] This study begins to characterize the stress-induced IL-6 signaling response and its relationship to 12-month global outcome after moderate to severe TBI. This is the first study to describe stress-induced pro-inflammatory trans-signaling mechanisms in long-term outcome prediction in the context of TBI. This study only includes individuals who survive to acute care discharge. The lower age for the poor outcome group is most likely due to higher rates of mortality for older individuals in the acute phase post-injury. Our data show that higher and more prolonged cortisol exposure is associated with higher levels of IL-6 trans-signaling, shown by elevated IL-6:sIL-6R ratios in the *high* cortisol TRAJ, which are also associated with poor outcome. These results indicate that IL-6 signaling may hyper-stimulate and sustain activation of the HPA axis, and these relationships together influence global outcome. Our data show that the serum IL-6 to sIL-6R ratio may be a relevant systemic marker for the trans-signaling complex in the brain, which has been shown in a number of studies to perpetuate inflammation[10,11,13,14]. Further, the HPA axis coordinates the primary physiological stress response after injury, and our data show that higher levels of IL-6:sIL-6R was more likely to be associated with the *high* cortisol group and poor 12-month outcome. Therefore, IL-6 and cortisol together provide unique long-term outcome discrimination in TBI.

There is growing importance placed in the relationship between neuroinflammation and HPA axis dysfunction. HPA axis dysregulation is demonstrated in a number of maladaptive CNS outcomes, such anxiety, panic, and bipolar disorders.[32–35] IL-6 signaling can stimulate the HPA axis at the level of the hypothalamus, pituitary, and adrenal gland.[9] Elevated IL-6 and sIL6R has been studied in relation to cortisol-linked disorders like depression and PTSD,[9,36]where patients who experienced PTSD had higher levels of these inflammatory markers. Further, sustained elevated cortisol levels and IL-6 signaling can lead to a disruption of gap junction proteins in the BBB and subsequent neuron dysfunction, which may allow for brain-to-blood or blood-to-brain cytokines or hormone exchange that does not usually occur under normal conditions.[37] Specifically, chronic serum IL-6 signaling is a dominant mechanism for pathogenic roles on BBB disruption after injury[15], and may potentially be an indicator for IL-6 trans-signaling occurring within the brain. However, IL-6 signaling has pleiotropic inflammatory functions, relaying the importance to elucidate its dichotomous roles on outcome. IL-6 protects against septic shock and directs resolution of acute inflammation.[38] However, IL-6 family cytokines elicit a detrimental response in the context of trans-signaling and the perpetuation of chronic inflammation.[10] Peripheral pro-inflammatory signals are shown to mimic microglia in the brain, and the presence of sIL-6R elicits an exaggerated response by microglia and neurons in the brain to secrete IL-6.[15]

Neuroendocrine hormones profiles, which are altered after injury, may act as important predictors of outcome post-TBI.[7,28,31,39] The sympathetic nervous system (SNS) plays a primary role in the activation of the HPA axis and can lead to a sustained elevation of cortisol. Studies have identified anti-inflammatory effects of cortisol as it can aid in the removal of immune cells from the periphery and CNS[5,6,40]. However, chronically elevated cortisol levels can have

perpetuating inflammatory effects post-injury. The disruptions in the HPA feedback loop are likely due to a decrease in the sensitivity of the receptors to cortisol and other signaling mechanisms of the axis.[41] Our previous work identified a relationship between CSF cortisol and BDNF, where cortisol predicted mortality and mediated the relationship between BDNF and mortality.[20] Thus, cortisol may provide unique insights the secondary injury cascades.

Rodent models have shown evidence that both acute and chronic stress can increase levels of IL-6 mRNA in the rat hypothalamus[42–44]. Interestingly, neutralization of IL-6 signaling in mice undergoing stress prevented cortisol elevations in these studies. Stress also alters IL-6 levels in the rat brain; however, the underlying mechanism for this phenomenon remains unclear[41,44]. Our work shows cortisol mediation of IL-6:sIL-6R as evidence that there may be a mechanism connecting these two signals in the context of TBI and global outcome. The direct relationship between IL-6:sIL-6R complex is mediated through cortisol, which identifies the true causal pathway. Cortisol accounted for 52% of the relationship between IL-6:sIL-6R and global outcome. In most clinical settings, this is a large amount of the total effect of IL-6:sIL-6R complex that is mediated by cortisol. This partial mediation explains a great portion of the trans-signaling causal pathway.

While one of our legs did not reach statistical significance, the sample size likely contributed to this attenuated relationship, as the unadjusted univariable association (N=107) was statistically significant at an α=0.05. After adjustment for covariates, this relationship was likely attenuated due to the decrease in sample size. There were missing GCS values, which contributed to the decreased sample size reported in this analysis. Additionally, our final model did not show a lack of fit. There were no influential points in the final model. There were several outlier points identified when comparing the Pearson residuals to the fitted values. Several

outlier checks were assessed prior to analyzing the data, including assessing the interquartile range of the data, and investigating unusually large or unusually small data points. There were only a handful of individuals identified who may be potential outliers, and given that the sample size is over a hundred individuals, they are likely not contributing to biased estimates of the final model.

Despite these preliminary results, there is still need to clarify whether trans-signaling may be a target for reducing long-term functional burden. Targeting trans-signaling may direct resolution of chronic inflammation as both *in vitro* and *in vivo* studies using various inflammatory models report specific inhibition of trans-signaling with an fc-dimerized version of sgp130 (sgp130fc)[45–51]. One particular study conducted in mice with hepatocellular carcinoma examined how IL-6 trans-signaling affects STAT3 phosphorylation, a major signal transducer downstream of the initial IL-6/sIL-6R complex formation, after recombinant sgp130 administration[46]. In this study, recombinant sgp130 significantly decreased IL-6 induced STAT3 phosphorylation[46]. Thus, sgp130 may be a potential treatment measure for trans-signaling; however, it does not provide a direct measure to resume HPA axis dysfunction. While biologically induced in the context of TBI, elevated CNS cortisol levels can negatively impact mood, cognition, and neuronal damage.[52–54] Previously, our group reported that in CSF the association between inflammatory markers and outcome is mediated by cortisol TRAJ group membership[28], indicating cortisol may be an important mechanism to target. The influence of exercise on stress reduction has been reported in a number of studies[55–57], and in particular, one specific study reported exercise facilitated neuronal repair after CNS injury.[58] Further, cognitive behavioral therapy (CBT) is shown to help with reducing elevated cortisol levels in women with hypercortisolemia.[59] Future work would include understanding how sgp130 potentially serves as

a modifiable target for prevention and/or resolution of chronic inflammation, as well as understanding the role of stress-reduction therapies in resolution of poor outcome post-TBI.

This study is not without limitations. An important assumption of the PROC TRAJ procedure is the use of normally distributed data when approximating the group-based trajectory model. The distribution used in our analysis was a uniform distribution of the cortisol levels, as the levels were deciled, but were not normally distributed. However, the goal of the GBTA specific to this work was to identify subgroups of individuals based on longitudinal cortisol profiles. As such, both statistical and clinical diagnostic measures were performed to assess the model output. Statistically, the posterior probabilities that were assessed were on average above the recommended 0.70 cut-off. This indicates that generally, most individuals had a high probability of falling in just one unique trajectory group. While statistical cut-offs may be somewhat arbitrary for clinical applications, the graphical representation of the actual cortisol levels by trajectory group indicate similar results. Thus, our work supports that the GBTA was able to discriminate the true levels, despite using uniform distribution to approximate the groups. Another limitation to this study is that full sample collection was not completed for all patients. Therefore, biomarker levels were not generated for these time points. As a result, this reduces statistical power for the analysis and may have affected trajectories or group assignments in patients without full sample collection.

This work needs to be replicated in larger cohorts and further consideration of the outlier points may provide useful information into more accurate model prediction. However, these data discriminate outcome through a mediation of HPA axis activation. This study indicates that at least part of the role cortisol plays in mediating 12-month outcome is influenced by activating IL-6 trans-signaling. Our results demonstrate that IL-6 signaling can independently influence

outcome, but cortisol contributed to an important shared and independent role in outcome prediction. Particularly, these data begin to clarify the pathogenic roles of HPA axis disruption and IL-6 signaling, which could be a potential target for therapeutic intervention.

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