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Full Length Article

# Metabolomic association between venous thromboembolism in critically ill trauma patients and kynurenine pathway of tryptophan metabolism

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ABSTRACT

*Objective:* Incidence of venous thromboembolism (VTE) in critically ill patients remains unacceptably high despite widespread use of thromboprophylaxis. A systems biology approach may be useful in understanding disease pathology and predicting response to treatment. Metabolite profile under specific environmental conditions provides the closest link to phenotype, but the relationship between metabolomics and risk of VTE in critically ill patients is unknown. In this study, metabolomics signatures are compared in patients with and without VTE.
*Design:* Multicenter case-control study using prospectively collected data from the Inflammation and Host Response to Injury program, with pathway and in silico gene expression analyses.
*Setting:* Eight level 1 US trauma centers.
*Patients:* Critically ill adults with blunt trauma who developed VTE within the first 28 days of hospitalization compared to patients without VTE (N-VTE).
*Interventions:* None.
*Measurements and main results:* Patients included in the study (n = 20 VTE, n = 20 N-VTE) were mean age of 34 years, injury severity score of 35, and VTE diagnosed a median of 10.5 days after admission. Global metabolomics revealed two kynurenine metabolites, *N*-formylkynurenine (AUC = 0.77; 95% CI: 0.59–0.89) and 5-hydroxy-*N*-formylkynurenine (AUC = 0.80; 95% CI:0.63–0.90) significantly discriminated VTE and N-VTE; ratio between N-formylkynurenine/5-hydroxy-*N*-formylkynurenine improved predictive power (AUC = 0.87; 95% CI: 0.74–0.95). In the pathway analysis, tryptophan was the only significant metabolic pathway including *N*-formylkynurenine and 5-hydroxy-*N*-formylkynurenine ($p < 0.001$), and 8 proteins directly or indirectly interacted with these metabolites in the interaction network analysis. Of the 8 genes tested in the in silico gene expression analyses, KYNU ($p < 0.001$), CCBL1 ($p < 0.001$), and CCBL2 ($p = 0.001$) were significantly different between VTE and N-VTE, controlling for age and sex.
*Conclusions:* Two novel kynurenine metabolites in the tryptophan pathway associated with hospital-acquired VTE, and 3 candidate genes were identified via pathway and interaction network analyses. Future studies are warranted to validate these findings in diverse populations using a multi-omics approach.

## 1. Introduction

In 2015 the Precision Medicine Initiative (PMI) was launched with a goal of accelerating research to assist clinicians with innovative tools and treatments to provide optimal care to individual patients, as opposed to a 'one size fits all' strategy. Precision medicine is an innovative approach to disease prevention and treatment that takes into account individual differences in genes, environments, and lifestyles to determine mechanisms of disease and explain why treatments are effective in some patients but not others.

An emerging '-omics' field vital to the PMI, metabolomics is the study of small breakdown products of metabolism (amino acids, lipids, etc.), to provide a cross-sectional link between an organism's genome and downstream interaction with the environment. Numerous body fluids and tissues are suitable for analysis and mass spectrometry or nuclear magnetic resonance methods are used to compare metabolomic profiles by disease or responsiveness to treatment (e.g. pharmacometabolomics). Unfortunately, there are few metabolomics studies that have been performed in critically ill and injured patients [1–4].

Because the incidence of venous thromboembolism (VTE) in critically ill patients remains unacceptably high despite widespread use of thromboprophylaxis [5,6] metabolomics may be particularly useful for

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further understanding of disease pathology and predicting response to treatment. Previous studies have assessed specific biomarkers in patients with confirmed VTE but none to date have assessed the relationship between metabolomics and risk of VTE in acutely ill, hospitalized patients (i.e. in those without disease). Hong et al. reported perfect discrimination with a single protein biomarker in a group of patients without documented risk factors for VTE. However, patients with trauma, surgery, and those who were immobile were excluded from the study [7]. Analysis of the soluble glycoprotein V (sGPV) in patients admitted to the emergency department with suspicion of VTE revealed an increase in sGPV in those with confirmed VTE but was insufficient as a single protein biomarker since the positive and negative predictive values were low [8].

Using an untargeted metabolomics approach, the purpose of this study was to determine the metabolomic signatures for patients with VTE and a group of controls with similar characteristics. We hypothesized that the metabolic profile in patients diagnosed with VTE is statistically different compared to critically ill and injured patients without VTE.

## 2. Materials and methods

The 'Inflammation and the Host Response to Injury' research study, funded by the National Institute of General Medical Sciences (NIGMS), was a multicenter, prospective study that enrolled patients with severe blunt trauma in eight Level I trauma centers, including the University of Florida (http://www.gluegrant.org). The study is registered at clinicaltrials.gov, NCT00257231. The purpose of the study was to improve understanding of the biology involved in the body's response to serious trauma or burn injury.

Outcome data, including VTE were collected from the time of hospitalization through hospital day 28. Deep vein thrombosis (DVT) was confirmed by autopsy, venogram, duplex ultrasound or other non-invasive vascular evaluation. Pulmonary embolism (PE) was diagnosed by at least one of the following: confirmation of pulmonary embolus via diagnostic angiography, computed tomography, or moderate to high probability ventilation/perfusion radionucleotide scan. The utilization of DVT screening practices, as well as clinical criteria used to initiate workup of suspected DVT and PE were institution and provider specific and not uniformly protocolized across centers [9]. The current study is a secondary pilot analysis of this study in that we assessed global metabolomics using frozen plasma samples from patients who experienced in-hospital VTE and compared these signatures to a matched sample of patients who did not develop VTE (N-VTE). Expedited approval was granted by the Institutional Review Board at the University of Florida.

### 2.1. Study criteria

Inclusion criteria for enrollment in the trauma study were: blunt trauma without isolated head injury, defined as either abbreviated injury score (AIS) head < 4 OR Glasgow Coma Scale (GCS) motor > 3 within 24 h of injury, emergency department arrival within 6 h from time of injury, blood transfusion within 12 h of injury, base deficit of at least 6 or systolic blood pressure < 90 mm Hg within 60 min of emergency department arrival, and fully or partially intact cervical spinal cord. Exclusion criteria were: age < 16 years, anticipated survival of < 24 h from injury, anticipated survival < 28 days due to pre-existing medical condition, inability to obtain first blood draw within first 12 h after injury, severe traumatic brain injury (i.e., GCS ≤8 after ICU admission AND brain computerized tomography scan abnormality within 12 h after injury), immunosuppression, significant pre-existing organ dysfunction, and receiving home oxygen therapy.

To generate a homogenous sample, additional inclusion criteria for the current study were white race, male gender, and age 18 to 55 years. A random sample of patients with VTE was collected from the 'Inflammation and the Host Response to Injury' data bank and a control group (N-VTE) was matched (nearest neighbor) 1:1 using age and injury severity score (ISS).

Variability in clinical care would be expected to impact proteomic and genomic expression patterns. To obviate these concerns, the Patient-Oriented Research Core Trauma Research Group developed standardized protocols for patient care. These SOPs, developed through consensus among the investigators at the participating centers, are grounded in evidence-based medicine. As implemented, they are considered the standard of care for patient management and are mandated for all enrolled patients as well as for uniform routine care at each of the participating trauma centers. SOPs for the following have been published elsewhere: acute ventilatory management, treatment of ventilator associated pneumonia, shock resuscitation, transfusion, sedation and analgesia, venous thromboembolism, nutrition, insulin, antibiosis, and cardiac support [9–16].

### 2.2. Sample collection and processing

Whole blood samples were collected within 12 h of hospital admission into EDTA-containing vacutainers (purple top). Samples were centrifuged at 2500*g* for 10 min to generate plasma which was then frozen continuously at −80 °C. Global metabolomics were performed in the high-throughput core of the Southeast Center for Integrated Metabolomics (SECIM) following established protocol. Plasma samples were thawed and 100 μL aliquoted to a new microcentrifuge tube. Next, 20 μL of internal standard solution containining myo-inositol D6 at 8 μg/mL, leucine-13C6 at 4 μg/mL, leucine-D10 at 4 μg/mL, creatine-D3 at 4 μg/mL, citric acid-13C6 at 8 μg/mL, tyrosine-13C6 at 4 μg/mL, phenylalanine-13C6 at 4 μg/mL, trpytophan-13C11 at 40 μg/mL, propionic acid-13C3 at 8 μg/mL, succinic acid-D4 at 4 μg/mL, salicylic acid-D6 at 4 μg/mL and caffeine-D3 at 4 μg/mL (Cambridge Isotopes Tewksbury, MA) and mixed. Then, 800 μL of precipitation solution was added (Acetonitirile:Methanol:Acetone 8:1:1, 800 μL). The samples were cooled and then spun at 20,000 rcf, 10C for 10 min. The supernatant was transferred to a new tube and dried down with clean nitrogen. The dried pellet was reconstituted in 100% water containing 0.1% formic acid (100 μL). Analysis was performed on a ThermoScientific QExactive high-resolution mass spectrometer with ultra-high performance liquid chromatography (UHPLC) in both positive and negative electrospray ionization modes with a mass resolution of 35,000 at $m/z$ 200 and mass range collected from 70 to 1000. Separation under gradient elution was achieved with an ACE C18-PFP column with 0.1% formic acid in water and acetonitrile. Data provided for statistical analysis was deisotoped, adduct matched and retention time aligned, and searched against an internal metabolite library generated from authentic standards using MZmine [17].

### 2.3. Experimental approach

The overall experimental framework used in this study consists of five steps, as depicted in Fig. 1. The first step includes the removal of metabolites with > 20% missing values followed by imputing the remaining missing values using K nearest-neighbor (KNN) method. Values for peak area of metabolites corresponding to concentration were then set to a log2 scale.

As a second step, an un-paired *t*-test was used to identify metabolites that were significantly different between the VTE vs. the N-VTE group. A false discovery rate (FDR) was used to adjust for multiple metabolites testing. Metabolites with a FDR < 0.05 were considered significant.

The third step of our analyses included the evaluation of the significant metabolites identified in step 2 as potential biomarkers that can differentiate between VTE and the control group. Receiver operating characteristic (ROC) curve analyses were used to determine the diagnostic power of the significant metabolites. Since ratios between metabolites may carry more information than absolute levels of metabolites alone, we calculated ratios of possible metabolite pairs based on