NANOTECHNOLOGY-ENHANCED BLOOD AND URINE ANALYSIS FOR THE IDENTIFICATION OF BIOMARKERS RELATED TO SEVERE TRAUMATIC BRAIN INJURY AND ACUTE RESPIRATORY DISTRESS SYNDROME

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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Date: _____ Spring Semester 2018
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Nanotechnology-enhanced Blood and Urine analysis for the identification of biomarkers related to Severe Traumatic Brain Injury and Acute Respiratory Distress Syndrome

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

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Bachelor of Science
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Spring Semester 2018
George Mason University
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DEDICATION

This is dedicated to my family, my Belgian “grandma cat”, and my lovely boyfriend Animesh, who have given me their great support and a huge inspiration. I also want to dedicate this to Kelsey, my fierce queen of the lab, for her sense of humor, her hilarious stories, and her funny, cute pets.

ACKNOWLEDGMENTS

First, I would like to express my appreciation to Dr. Alessandra Luchini for her guidance during my thesis work. I would also like to thank Dr. Lance Liotta, Dr. Emanuel Petricoin for allowing me to conduct research on this project.

Second, I would like to thank Kelsey Atkin Mitchell for introducing me to the topic and clarifying any concerns that come up. I am also indebted to Dr. Ancha Baranova and Dr. Mariaelena Pierobon for their support and cooperation.

Finally, special thanks to the student members of the lab, the School of Systems Biology at George Mason University, and the INOVA Health Systems for their resources.

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LIST OF ABBREVIATIONS AND SYMBOLS

Acute Respiratory Distress Syndrome.....ARDS
Acrylic Acid .....AAc
Aldehyde dehydrogenase 1 family member L1 .....ALDH1L1
Allylamine .....AA
Angiotensinogen .....AGT
Angiotensin I converting enzyme .....ACE
Annexin A4 .....ANXA4
Apolipoprotein E .....APOE
Area under curve .....AUC
ATPase H+ transporting V1 subunit H ATP6V1H
Bismarck Brown Y.....BM
Blood-brain barrier.....BBB
Cathepsin D .....CTSD
Carbonyl reductase 1 .....CBR1
Cell division cycle 42.....CDC42
CD 14 molecule.....CD14
CD 59 molecule.....CD59
Cibacron Blue F3G-A.....CB
Clusterin .....CLU
Collagen type XVIII alpha 1 chain COL18A1
Collision-induced dissociation.....CID
Controlled cortical impact CCI
Creatine kinase B .....CKB
Crystallin alpha B .....CRYAB
Degrees Celsius ..... ºC
Dimethylarginine dimethylaminohydrolase 2.....DDAH2
Enolase 1 .....ENO1
Eukaryotic translation elongation factor 1 alpha 1 .....EEF1A1
False discovery rate .....FDR

Galectin 3 ..... LGALS3
Glyceraldehyde-3-phosphate dehydrogenase..... GAPDH
Granulin precursor ..... GRN
Greater than or equal to..... ≥
Greater than ..... >
Growth differentiation factor 15..... GDH15
Heat shock protein family A member 8 ..... HSPA8
Heat shock protein family B member 1 ..... HSPB1
High-performance liquid chromatography..... HPLC
Iodoacetamide ..... IAA
Lactate dehydrogenase A ..... LDHA
Less than or equal to ..... ≤
Less than ..... <
Lipopolysaccharide binding protein ..... LBP
Liquid chromatography-tandem mass spectrometry..... LC-MS/MS
Macrophage migration inhibitory factor ..... MIF
Malate dehydrogenase 1 ..... MDH1
Meter ..... m
Membrane metalloendopeptidase ..... MME
Microliter ..... µL
Milligram ..... mg
Milliliter ..... mL
Millimole ..... mmoL
Minute ..... min
Molarity ..... M
N-(3 dimethylaminopropyl)-N′-ethyl carbodimide hydrochloride..... EDC
N-N'-methylenebis(acrylamide)..... BIS
N-isopropylacrylamide..... NIPAm
Parkinsonism associated deglycase PARK7
Peptide-spectrum match..... PSM
Peroxiredoxin 6 ..... PRDX6
Phosphoenolpyruvate carboxykinase 1 ..... PCK1
Plus or minus..... ±
Potassium persulfate..... KPS
Prosaposin ..... PSAP
Pyruvate kinase, muscle ..... PKM
Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial isoform X2 ..... PDHA1
Ras-related C3 botulinum toxin substrate 1 ..... RAC1
Receiver Operating Characteristic ..... ROC
Rotations per minute ..... RPM
S100 calcium binding protein A8 ..... S100A8
Second ..... s
Serpin family A member 1 ..... SERPINA1

Serpin family A member 3 ..... SERPINA3
Solute carrier family 3 member 2 ..... SLC3A2
Standard deviation ..... SD
Transthyretin .....TTR
Traumatic Brain Injury..... TBI
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation zeta ..... YWHAZ
Ubiquitin C-terminal hydrolase L1 ..... UCHL1
United States of America .....USA
Vinyl sulfonic acid .....VSA

ABSTRACT

NANOTECHNOLOGY-ENHANCED BLOOD AND URINE ANALYSIS FOR THE IDENTIFICATION OF BIOMARKERS RELATED TO SEVERE TRAUMATIC BRAIN INJURY AND ACUTE RESPIRATORY DISTRESS SYNDROME

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George Mason University, 2018

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Traumatic brain injuries (TBIs) are physical damages to cerebrum tissue, resulting in temporary or permanent debilitation of brain function. Each year, TBIs contribute to 135,000 deaths and cases of permanent disability in the United States. Acute Respiratory Distress Syndrome (ARDS) is a life-threatening lung condition, which is usually identified by symptoms, including dyspnea, severe hypoxemia, decreased lung compliance, and diffuse bilateral pulmonary infiltrates. ARDS occurs in patients who are significantly ill or are hospitalized due to severe injuries, one of which is TBI. Researchers have been on a quest to find biomarkers for brain injury and its complications in different biofluids. Still, clinically validated TBI biomarkers in urine

and serum are lacking sensitivity and specificity. In this study, we applied an affinity nanotechnology and mass spectrometry to discover biomarker candidates related to TBI and ARDS in urine. We analyzed 75 samples (52 = TBI patients, 10 = ARDS patients and 13 = Controls including patients that underwent trauma but did not develop TBI or ARDS). 8 biomarkers related to TBI and 4 biomarkers related to ARDS were selected using Receiver Operating Characteristic analysis. Candidate biomarkers were related to the following biological functions: acute inflammation, cell death, anti-oxidation, endothelial cell repair and regeneration, pulmonary fibrosis and amyloid-beta plaque formation. TBI biomarkers detected in urine are compared with serum TBI biomarkers observed in the preliminary study. Comparison of biomarkers identified in urine and serum revealed that urine yielded a higher number of TBI and ADRS candidate biomarkers (4 and 8, respectively)

INTRODUCTION

Traumatic Brain Injury

Traumatic brain injury (TBI) is physical damage to cerebrum tissue, resulting in temporary or permanent debilitation of brain function. Road accidents comprise nearly 60% of TBIs globally, in which alcohol is best documented as the major cause (1–3). While mild TBI can cause significant functional morbidity, severe TBI can lead to either long-term hospitalization or high mortality. According to the United States Centers for Disease Control and Prevention, TBI affects over 2 million people every year, in which severe TBI accounts for as many as 50,000 deaths and 85,000 long-term disability cases. Data from the World Health Organization also indicate that by the year 2020, TBI will surpass numerous diseases as the major reason for death and disability.

Acute Respiratory Distress Syndrome

ARDS refers to the syndrome of acute bronchogenic injury, identified by symptoms, including dyspnea, severe hypoxemia, decreased lung compliance, and diffuse bilateral pulmonary infiltrates. ARDS is a common complication developed in patients who suffer from severe TBI. In the USA, the syndrome causes a great impact on roughly 200,000 cases annually, leading to significant patient morbidity and health care burden

(4–6). Severe limitations exist in the accuracy of ARDS diagnosis; ARDS related mortality in severe TBI patients remains very high and might be preventable with the timely diagnosis. Thus, an advanced, accurate prediction of ARDS incidence in severe TBI patients is crucial for clinicians to perform suitable clinical trials in the most appropriate patient population.

Biomarkers And Nanotechnology

Generally, neurological examinations (Glasgow coma scale, GCS) and neuroimaging (computed tomography - CT and magnetic resonance imaging - MRI) are the two most commonly used techniques to analyze and classify TBI. However, these techniques are liable to various critical constraints. Examples are the potential inaccurate early evaluation of the neurological seriousness of severe TBI patients and the inability to detect the potential pulmonary complications triggered by TBI. These limitations in these conventional techniques have led to calls for alternative methods of assessing the injury to improve the diagnosis of TBI. Among various alternative strategies, a blood or urine test that can diagnose TBI with high specificity and sensitivity would be of great value. Unfortunately, such a test is not currently available. We propose to use affinity nanoparticle technology and mass spectrometry to identify protein biomarkers associated with TBI and ARDS that can supplement functional and imaging-based assessments and might become a potentially useful tool for TBI-related research (7–9).

Recently, nanoparticle technology has emerged as a novel tool in providing sensitive and specific detection of low-abundance biomarkers in diluted concentration

(10–13). Biomarkers related to TBI and ARDS exist in the blood and urine at a concentration below the detection limit of current analytical techniques for protein identification and characterization such as mass spectrometry. Nanoparticle technology can overcome this barrier and increase the effective analytical sensitivity of mass spectrometry. Hydrogel nanoparticles composed of N-isopropylacrylamide backbone and functionalized with chemical dye baits are introduced into the sample solution. The open network, spherical nanoporous particles are in open exchange with the aqueous surrounding. The internal dye acts as a chemical bait, capturing the low-abundance proteins inside the core nanoparticles. Trapped protein biomarkers sequestered by the particles can be amassed in small volumes, thus increasing their concentration and improving the sensitivity of mass spectrometry. This provides an innovative method to encapsulate the low-molecular weight protein biomarkers that exist in body fluids in extremely low concentrations.

HYPOTHESIS

The overarching hypothesis of this study is that proteins deriving from the brain and involved in biological processes underlying TBI and ARDS are present in serum and urine and detectable with a nanotechnology-enhanced mass spectrometry workflow.

In this study, we propose 1) to identify proteins associated with severe TBI/ARDS in urine, 2) to identify candidate biomarkers that are associated with ARDS in severe TBI patients, and 3) to compare the candidate protein biomarkers in urine versus serum in a TBI/ARDS study group. This might contribute to further understanding of TBI and to develop more effective diagnostic and prognostic assays for this neurotrauma condition.

STUDY DESIGN

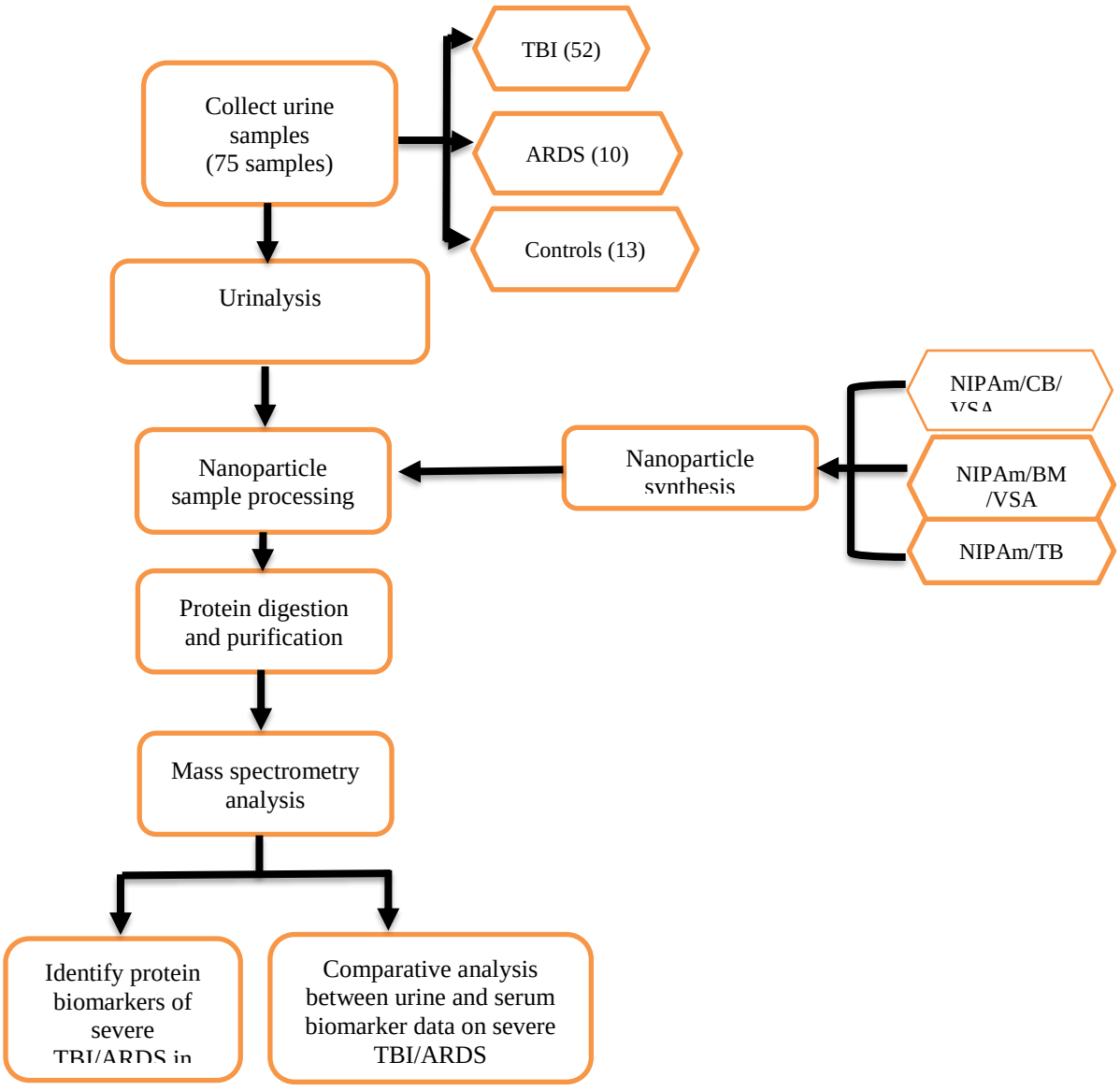

Figure 1: Study Design. NIPAm = N-isopropylacrylamide, BM = Bismarck Brown Y, TB = Trypan Blue, CB = Cibacron Blue F3G-A, VSA = vinyl sulfonic acid

STUDY AIMS

1. Utilizing affinity nanoparticles and mass spectrometry, analyze 62 urine samples and 13 urine controls to identify protein biomarkers associated with severe TBI, ARDS-specific candidate protein biomarkers in severe TBI patients. Peptides will be identified with the following stringent parameters: LC-MS/MS parameters (flow-dependent and compound-dependent parameters) and ROC parameters ($AUC \geq 0.7$, $P < 0.05$).

2. Urinary proteome associated with TBI and ARDS will be correlated to matched serum proteome on the same patients. Define which biofluid is preferable for future diagnosis and investigation.

METHODOLOGY

Materials

Cibacron Blue F3G-A dye (CB), Bismarck Brown Y dye, Trypan Blue dye, N-isopropylacrylamide (NIPAm), N-N'-methylenebis(acrylamide) (BIS), allylamine (AA), acrylic acid (AAc), potassium persulfate (KPS), N-(3 dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS) were purchased from Sigma-Aldrich, Inc, USA. Internal standards for the mass spectrometry, including lysozyme chloride form from chicken egg white (25ng/ml) and human SDF1-beta, were purchased from Sigma-Aldrich, Inc, the USA and Antigenix America, Inc, USA, respectively. Urine for the experiments was collected by medical personnel at INOVA Health System under informed consent at time 24 and 48 hours. Samples fall into three categories: severe TBI patients (N=52), ARDS patients (N=10) and patients from severe accidents with no detection of TBI and ARDS as the trauma controls (N=13). Out of 44 patients, the majority were Caucasian male, the overall mean age was 46.52 +/- 20.34 years, with the average percentage of death rate at 49%. A T-tests were performed to evaluate if the age factor caused significant difference among three groups. Null hypothesis was that s there is no significant difference in the age.

Table 1. Demographics of sample cohort based on clinical data.

Parameters	TBI (Mean+/ St. Dev)	ARDS	Control	Overall
Age (Mean +/- St.dev)	45.59 +/- 20.42	51.60 +/- 14.48	46.75 +/- 24.77	46.52 +/- 20.34
P value of Age (to Control)	0.45	0.35		
Gender % (Male)	70% (N=21)	60% (N=3)	88% (N=7)	72% (N=31)
Races % (White)	77% (N=23)	100% (N=5)	88% (N=7)	81% (N=25)
Morgue %	63% (N=19)	20% (N=1)	13% (N=1)	49% (N=21)

NIPAm-AA, containing CB in the core and vinyl sulfonic acid (VSA) in the shell

NIPAm (0.6 g, 5.30 mmol) and BIS (0.0185 g, 0.018 mmol) were dissolved in 15 mL of MilliQ water and filtered. The solution was purged with nitrogen for 30 min at room temperature under medium stirring rate before AA (45 μL, 0.003 mmol) was added. The solution was then purged with nitrogen for another 15 min and then heated to 75°C for 30 min. KPS (0.0065 g, 0.024 mmol) in 5.0 mL of MilliQ water was added to the solution to initiate the polymerization. The reaction was maintained at 75°C under nitrogen for 6 hours, then allowed to cool to room temperature and stirred overnight. Particles were then washed repeatedly to remove excess monomer by centrifugation (19,000 rpm, 45 min). After centrifuging the supernatant from each tube was removed and the particles were resuspended in MilliQ water.

To covalently bind the CB dye to NIPAm-AA core, the following procedure was followed. CB (0.212 g, 0.25 mmol) was dissolved in 16 mL of 0.125 mol/L aqueous Na2CO3 and filtered. 20ml of NIPAm-AA particle suspension was stirred at room temperature in a 100 mL three-neck round-bottom flask for 15 min. The CB solution was then added to the NIPAm-AA particle suspension, and the activated particles solution was incubated with CB dye for 12 hours and was purged with nitrogen for 1 hour at 70°C before VSA shelling.

To add a vinyl sulfonic acid containing shell to NIPAm/CB cores, NIPAm (0.156 g, 1.38 mmol), BIS (0.020 g, 0.13 mmol), KPS (0.092 g, 0.33 mmol), and VSA (26 μL, 23.72 mmol) were dissolved in 20 mL of MilliQ water, filtered and then transferred into a three-neck round-bottom flask. The solution was purged with nitrogen at medium stir rate for 15 min at room temperature. Then the solution was added to the NIPAm/CB suspension. The reaction was maintained at 70 °C under nitrogen for 3 h. The NIPAm/CB/VSA particles were washed at least four times by centrifugation (19,000 rpm, 45 min) and then resuspended in 20ml of MilliQ water.

NIPAm nanoparticle functionalized with AAc

NIPAm (0.032 g, 2.8 mmol), BIS (0.023 g, 0.173 mmol) were dissolved in 15 mL of MilliQ water, filtered and transferred to a three-neck round-bottom flask. The solution was purged with nitrogen for 30 min at room temperature with a medium stirring rate before AAc (0.035 μL, 0.487 mmol) was added. The solution was purged with nitrogen for another 15 min and then heated to 75°C for 30 min. KPS (0.018 g, 0.025 mmol) in 5.0

mL of MilliQ water was added to the solution to initiate the polymerization. The reaction was maintained at 75°C under nitrogen for 6 hours, allowed to cool to room temperature and stirred overnight. Particles were washed repeatedly to remove excess monomer by centrifugation (19,000 rpm, 45 min) and resuspended in 20 mL of MilliQ water.

NIPAm-AAc functionalized with chemical dye baits

Bismarck Brown Y (BM) and Trypan Blue (TB) dye were coupled by condensation to AAc in the NIAm-AAc particles as described below. 20 mL of the NIPAm-AAc particle suspension was centrifuged (13.2 rpm, 30 min), the supernatant was discarded and the particle pellet was resuspended in 10 mL of 0.2 M NaH2PO4. The particle suspension was transferred to a three-neck round-bottom flask, and 2 mL of 0.2 M NaH2PO4, 466 mg EDC with 280 mg of NHS were added to the NIPAm-AAc solution. The reaction was held at room temperature and medium stirring rate for 15 min. Then, the reaction was stopped by aliquoting out the solution into Eppendorf tubes and centrifuging at 13.2 rpm in 15 min. The supernatant was discarded, the particle pellet was quickly resuspended in 10 mL of 0.2 M Na2HPO4. After this activation step, 0.2 g of dye (BM or TB) was dissolved in 200 mL of 0.2 M Na2HPO4, filtered, and added to the activated particles. The reaction was held at room temperature and medium stirring rate for 12 hours. The NIPAm/dye particles were washed at least 4 times with MilliQ water (19,000 rpm, 45 min) to eliminate the excess dye. Supernatants were discarded, and particles were resuspended in 20 mL of MilliQ water.

NIPAm-Bismark Brown Y (NIPAm/BM) core with VSA shell preparation

To add a VSA containing shell to NIPAm/BM cores, NIPAm (0.156 g, 1.38 mmol), BIS (0.020 g, 0.13 mmol), KPS (0.092 g, 0.33 mmol), and VSA (26 μL, 23.72 mmol) were dissolved in 20 mL of MilliQ water, filtered and then transferred into a three-neck round-bottom flask. The solution was purged with nitrogen at medium stir rate for 15 min at room temperature. Then the solution was added to the NIPAm/BM particle suspension. The reaction was maintained at 70 °C under nitrogen for 3 hours. The NIPAm/BM/VSA particles were washed repeatedly to remove excess monomer by centrifugation (19,000 rpm, 45 min) and then resuspended in 20ml of MilliQ water.

Incubation of nanoparticles with urine samples

Aliquots of 40 mL of human urine sample were tested using urinalysis Multistix, spiked with 800 ng of lysozyme from chicken egg white and 300 ng of human SDF1-beta. 100 μL of NIPAm/TB particles was added to the aliquot, resuspended and incubated for 30 min at room temperature. 50 μL of NIPAm/CB/VSA and 100 μL of NIPAm/BM/VSA particles were later added to the mixture and incubated for another 30 min. Particles were then separated by centrifugation (13.2 rpm, 15 min) and washed three times with MilliQ water. The particle pellet was resuspended in 300 μL elution buffer (0.019M Acetonitrile, 0.025M Ammonium hydroxide) and incubated for 15 min at room temperature, then centrifuged (13.2 rpm, 10 min). The pellet was removed, and the eluate was dried under nitrogen.

Protein digestion by Trypsin and LC-MS/MS analysis

Proteins were reconstituted in Urea (10 μL, 8M), add ammonium bicarbonate reduced by dithiothreitol DTT (1 μL, 1M) and incubated at 37^0C for 15 min. Iodoacetamide (IAA) (6 μL, 0.5M) was then added and let incubate in the dark for 10 min at room temperature. NH4HCO3 (10 μL, 0.5M), trypsin (1 μL, 0.5ug/μL) and 12 μL MilliQ water were added to the samples. Samples were incubated at 37^0C overnight; after the incubation period, 2 μL of acetic acid 1M was added to stop the digestion. Peptide samples were further purified using Pierce C18 Spin Columns (Thermo Fisher, USA), analyzed by mass spectrometry. LC-MS/MS experiments were performed on an Orbitrap Fusion (ThermoFisher Scientific, Waltham, MA, USA) equipped with a nanospray EASY-nLC 1200 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were separated using a reversed-phase PepMap RSLC 75 μm i.d. × 15 cm long with 2 μm, C18 resin LC column (ThermoFisher Scientific, Waltham, MA, USA). The mobile phase consisted of 0.1 % aqueous formic acid (mobile phase A) and 0.1 % formic acid in 80% acetonitrile (mobile phase B). After sample injection, the peptides were eluted by using a linear gradient from 5% to 50 % B over 90 min and ramping to 100 % B for an additional 2 min. The flow rate was set at 300 nL/min. The Orbitrap Fusion was operated in a data-dependent mode in which one full MS scan (60,000 resolving power) from 300 Da to 1500 Da using quadrupole isolation, was followed by MS/MS scans in which the most abundant molecular ions were dynamically selected by Top Speed and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. "Peptide Monoisotopic Precursor Selection" and "Dynamic Exclusion" (8 sec

duration), were enabled, as was the charge state dependency so that only peptide precursors with charge states from +2 to +4 were selected and fragmented by CID. Tandem mass spectra were searched against the NCBI human database using Proteome Discover v 2.1 with SEQUEST using tryptic cleavage constraints. Mass tolerance for precursor ions was 5 ppm, and mass tolerance for fragment ions was 0.5 Da. Data were analyzed with oxidation (+15.9949 Da) on methionine as a variable post-translation modification, and carbamidomethyl cysteine (+57.0215) as a fixed modification. A 1 % false discovery rate (FDR) was used as a cut-off value for reporting peptide spectrum matches (PSM) from the database.

Data Analysis

Graphs were created using Microsoft Excel. Data analysis was performed using Receiver Operating Characteristic (ROC) in MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium). ROC was performed on two diagnostic sets of sample data: ARDS/Control and TBI/Control. Continuous variables were expressed as PSM value. Categorical variables were presented as dichotomous variable indicating diagnosis (0 = negative or Control, 1 = positive or TBI/ARDS). The area under the curve (AUC) was used to select candidate biomarkers with a threshold of AUC $\geq$ 0.7 and P-value < 0.05. The AUC and the corresponding P-value show that a protein is significantly associated with TBI/ARDS in this experimental dataset. Biomarker comparison between TBI and ARDS in urine was analyzed and visualized by BioVenn (14) and GraphPad Prism 7.04 (GraphPad Software Inc., La Jolla, USA).

RESULT

Candidate urinary biomarkers for TBI and ARDS

Affinity nanoparticles were produced by precipitation polymerization and validated using light scattering and SDS PAGE analysis. Nanoparticles had on average a hydrodynamic diameter of 600 nm. Nanoparticle batches pass the SDS PAGE quality control if, incubated with serum, exclude >85% of serum proteins whose size is larger than 30 kDa. For this study, a total of 500 mL of nanoparticles at a 5 mg/mL dry weight concentration were produced. Nanoparticle-enhanced proteomics analysis was performed on urine samples collected from 75 patients with the following characteristics: severe TBI patients (N=52), ARDS patients (N=10) and patients from severe accidents with no detection of TBI and ARDS as the trauma controls (N=13). TBI was diagnosed using neurological imaging and clinical assessment (Glasgow Coma Scale < 8). The diagnosis of ARDS was based on symptoms, vital signs and chest X-ray in patients. Internal protein controls including chicken lysozyme and human SDF-1 were spiked in the urine of patients to assess that the overall yield of the nanoparticle/mass spectrometry workflow. A total of 7878 proteins were identified by mass spectrometry. ROC AUC was calculated for each protein to assess the protein diagnostic ability in binary classification

problems (TBI versus controls and ARDS versus controls). A protein was selected when its ROC AUC was ≥ 0.7 and the associated p-value was less than 0.05 (280 selected proteins). A scientific literature analysis was performed for the 280 selected proteins. Information related to the association with diseases, biological process, and tissue specificity was recorded. 40 were selected with the following criteria: protein not belonging to the 70 most abundant serum proteins, scientific evidence that the protein is expressed in the brain, and scientific evidence that the protein is involved in brain-related conditions and biological processes. Figure 2 shows a Venn diagram of urinary candidate protein biomarkers associated to TBI and ARDS. The percentage of TBI-only proteins was 51.28%, the percentage of ARDS-only proteins was 11.11%, and the percentage of proteins in common between the two groups was 37.61%. The proteins belonging to this Venn diagram intersection area can be a source of potential biomarker candidates associated to ARDS in TBI patients. These proteins were reviewed according to the main pathways they reflect (Supplementary Table) and the proteins associated with ARDS were summarized with their reviewed studies in Table 2. In addition, three proteins, lipopolysaccharide binding protein (LBP), angiotensinogen (AGT) and growth/differentiation factor 15 (GDF-15) were chosen and visualized to demonstrate the potential of these proteins as low-abundance biomarkers for ARDS. As shown in Figure 3, ARDS was present in LBP, AGT and GDF-15 at 12.79 ± 2.588 (mean ± SEM), 20.38 ± 3.253, 13.37 ± 2.612, respectively. In general, ARDS in all three proteins represented a significant value compared to the Control but still expressed smaller mean and lower distribution among patients in comparison with TBI.

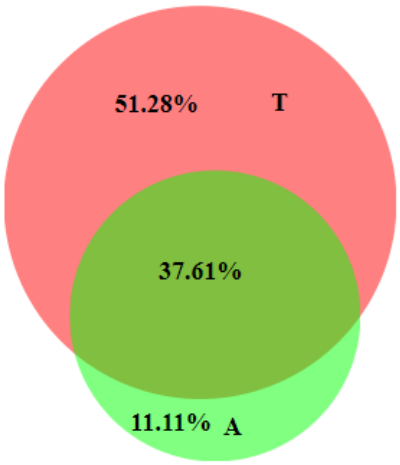

Figure 2: Venn diagram of candidate urinary protein biomarkers for TBI (T) and ARDS (A). Circle areas are proportional to the number of proteins contained in the subset. TBI-only protein and the ARDS-only group have a value of ROC AUC ≥ 0.7 in only TBI patients and only ARDS patients, respectively.

Table 2: Candidate ARDS urinary protein biomarkers and scientific literature showing correlation with biological processes involved in lung diseases.

Author	Protein	Study population	Study outcome
Bhargava *et al.*(15)	AGT	Human bronchoalveolar lavage fluid Minnesota, USA	AGT has differential expression between ARDS survivors and non-survivors.
Takaoka *et al.*(16)	GAPDH	Mice serum Tokyo, Japan	GAPDH pre-injection prolongs survival in an LPS-induced, sepsis-related severe ARDS mouse model
Clark *et al.*(17)	GDF-15	Human plasma in patients with severe chronic obstructive pulmonary disease Maryland, USA	Higher levels of GDF-15 are significantly associated with poor outcome in ARDS patients
Lorenz *et al.*(18)	S100A8	Human serum in ARDS patients Ohio, USA	Elevated levels of S100A8 are associated with acute inflammation in ARDS patients.

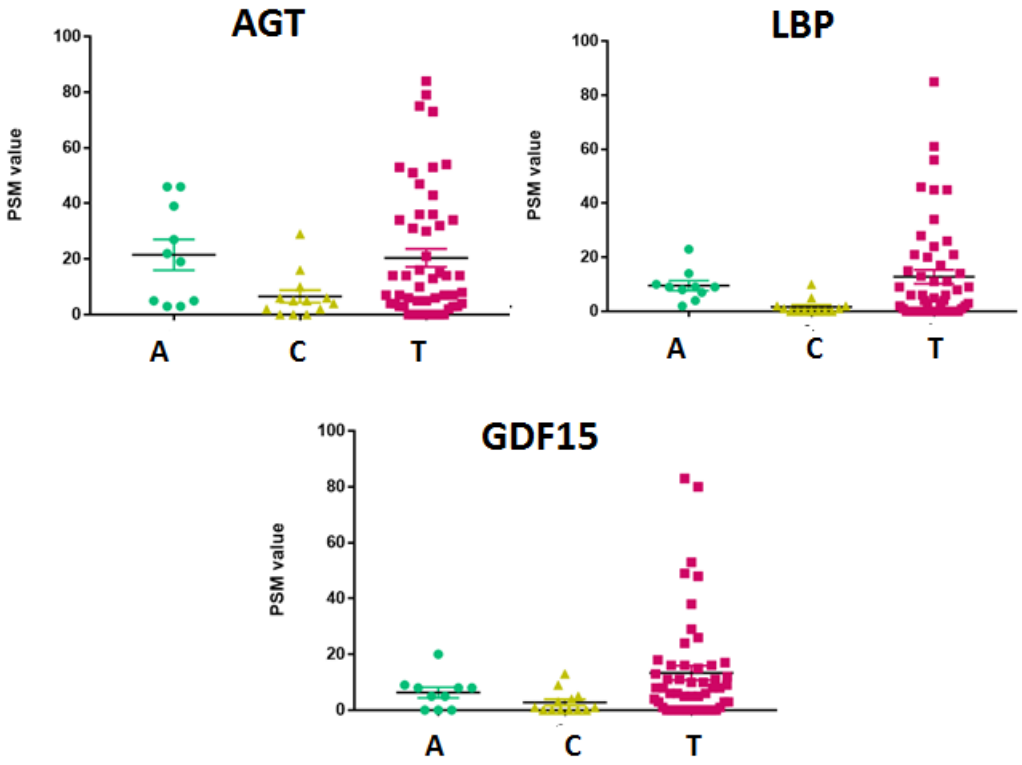

Figure 3. Peptide spectrum matches of three ARDS-associated proteins selected in this study. Raw data are represented as a scatter plot where every dot is the value of peptide spectrum matches of the protein indicated in the graph title and the horizontal line is the median of the protein abundance values in the class. A: ARDS, C: Control, T: TBI.

Candidate TBI biomarkers in urine

To identify the protein as potential biomarkers, the following criteria for candidate protein selection were used: (1) The protein has a ROC AUC value greater or equal to 0.7 in TBI sample. (2) The protein was correlated with neurological disorders and/or neuronal injury in published studies. Among over 7000 proteins detected in urine samples, 40 proteins were selected (Table 3), of which 9 proteins were directly linked to TBI in previous literature studies (Table 4). The number of peptide spectrum matches of five TBI candidate protein biomarkers selected with this algorithm are reported in Figure

5 and 6. The average values of peptide spectrum matches in TBI patients was 1.578 ± 0.4039, 3.525 ± 0.6361, 9.578 ± 1.385 for COL18A1, UCHL1, PRDX6, respectively; the average values of peptide spectrum matches in controls were 0.077 ± 0.0769, 0.308 ± 0.2371, 4.231 ± 0.7857 for COL18A1, UCHL1, PRDX6, respectively. Similarly, the average values of peptide spectrum match in TBI patients were 16.55 ± 18.73, 16.91 ± 27.96 for CRYAB and ANXA4, respectively, while their mean control levels were 8.00 ± 11.31, 5.8 ± 7.497, respectively.

Table 3: Forty selected TBI protein biomarkers, with biological processes and clinical conditions associated

ASSOCIATED CONDITION / BIOLOGICAL PROCESS	PROTEIN
TBI	COL18A1, UCHL1, APOE, PDHA1 Isoform X2, MME, CTSB, ACE, PRDX6
Oxidative stress	GAPDH
Ischemia	AGT, TTR
Alzheimer	MME, PCK1, CKB, PKM, ANXA4, CBR1, CRYAB, CTSD, ENO1, MIF, HSPA8, SERPINA1, SERPINA3, YWHAZ, CLU, PSAP
Parkinson	ALDH1L1, PARK7
Frontotemporal lobar degeneration	EEF1A1, GRN, HSPB1, MDH1, SLC3A2, ATP6V1H
Brain tumor	LGALS3, LDHA, PKM, RAC1
Polyneuropathy	CD59

Table 4: Candidate TBI urinary protein biomarkers and scientific literature showing correlation with biological processes involved in TBI.

Authors	Protein	Study population	Study method	Study outcome
Dardiotis *et al.*(19)	ACE	TBI patients Thessaly, Greece	Logistic regression analyses, five tag single nucleotide polymorphisms (SNPs) across the ACE gene.	Genetic variation in a specific region of the ACE gene influence outcomes of TBI patients.
Brichtova *et al*(20).	APOE	ApoE genotyped children with history of TBI, Brno, Czech Republic	Polymerase chain reaction/restriction fragment length polymorphism	ApoE ε4 genotype might cause severe clinical symptomatology and unfavorable neurological outcome after TBI
Chen *et al.*(21)	COL18 A1	Human cerebrospinal fluid (CSF) from severe TBI patients Shanghai, China	Enzyme-linked immunosorbent assay in the cerebrospinal fluid	Endostatin/collagen XIII is increased in CSF after severe TBI
Hook *et al.*(22)	CTSB	CTSB-deficient male mice Freiburg, Germany	E64d treatment, by oral administration	Knock-out of the CTSB gene in mice resulted in amelioration of TBI
Johnson *et al.*(23)	MME	Human brain tissue from acute, severe TBI patients Glasgow, U.K.	IHC and genotyping	Genetic variants in MME influence acute amyloid deposition after TBI and define which TBI patients will rapidly form A*B* plaques.
Robertson *et al.*(24)	PDHA1 Isoform X2	Immature rats after controlled cortical impact (CCI) Maryland, USA	Mitochondria isolation	Pyruvate dehydrogenase complex activity is lower in mitochondria in the acute TBI immature rats.
Buonora *et al.*(25)	PRDX6	Rat serum Maryland, USA	Autoimmune profiling	PRDX6 levels are acutely elevated in mild-to-moderate TBI compared with normal human plasma
Brophy *et al.*(26)	UCHL1	Human CSF and serum from	ELISA	UCH-L1 CSF and serum maximum concentrations

		severe TBI patients Florida, USA		were significantly greater than in control

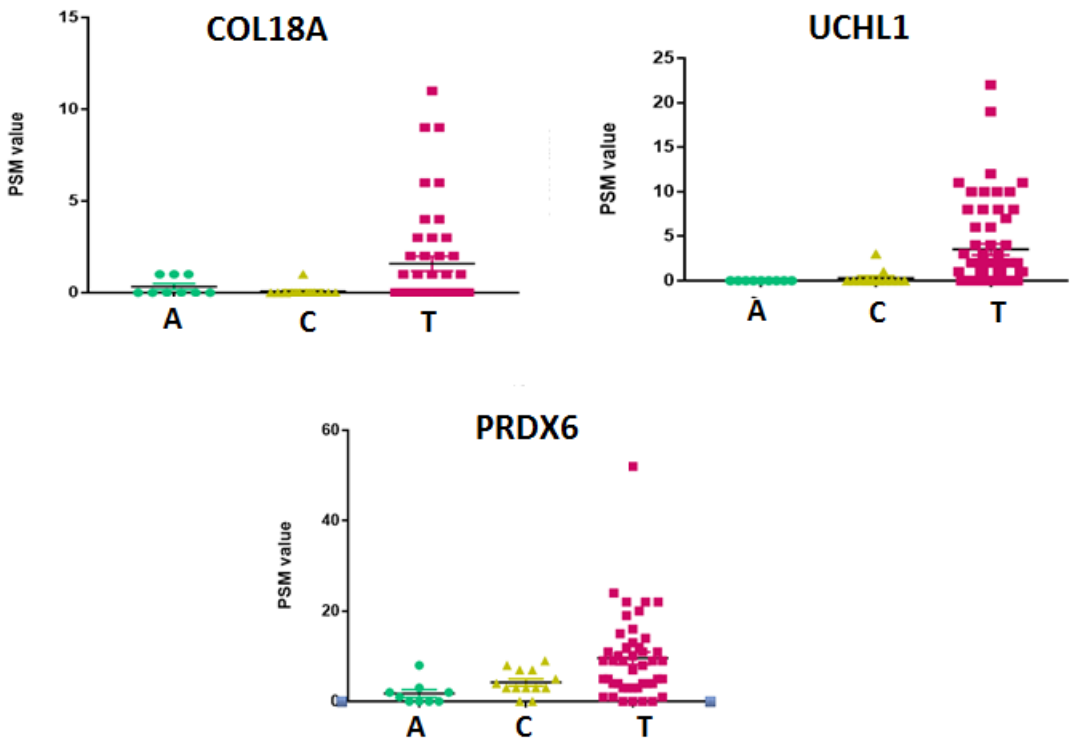

Figure 4. Peptide spectrum matches of three TBI-associated proteins selected in this study and that are known serum biomarkers for TBI. Raw data are represented as a scatter plot where every dot is the value of peptide spectrum matches of the protein indicated in the graph title and the horizontal *line* is the median of the protein abundance values in the class. A: ARDS, C: Control, T: TBI.

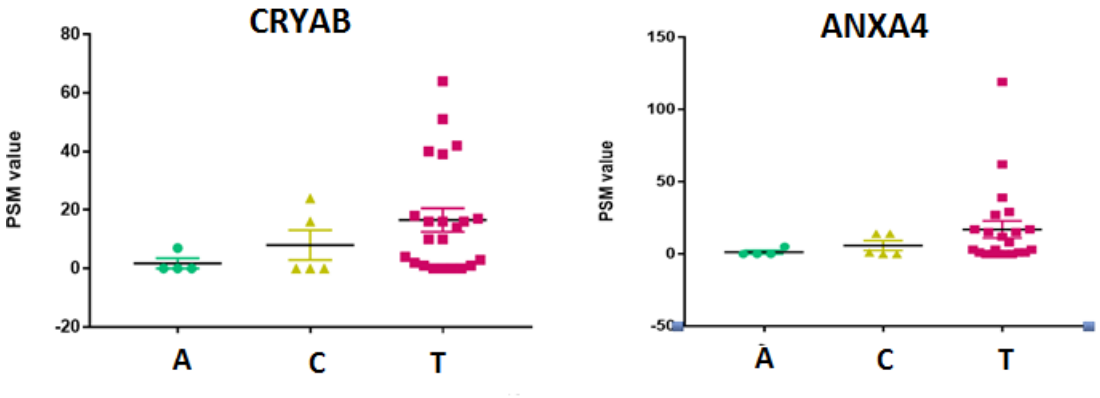

Figure 5. Peptide spectrum matches of two TBI-associated proteins selected in this study that were previously unknown as biomarkers for TBI. Raw data are represented as a scatter plot where every dot is the value of peptide spectrum matches of the protein indicated in the graph title and the horizontal *line* is the median of the protein abundance values in the class. A: ARDS, C: Control, T: TBI.

Comparative analysis of biomarker candidate in urine and serum

The first aim of this study is to identify candidate biomarkers for TBI and ARDS in the urine of well-characterized human samples. Results are reported in the previous sections. The second aim of this study is to compare candidate protein biomarkers identified in the urine with candidate protein biomarkers identified in the serum of the same TBI and ARDS patients in comparison to controls. To achieve this goal, protein biomarkers described in the previous section were compared with protein biomarkers identified, according to the same parameters, in the serum of the same patients. Serum was collected at INOVA health system at the same time as urine collection from patients. Serum was cryopreserved until the time of analysis. Serum was concentrated with affinity nanoparticles and then subjected to mass spectrometry analysis. Total of 1286 proteins was identified in the serum data set. Analogously to the urinary proteins, ROC analysis on serum proteins was conducted for the following two binary classification problems:

TBI patients versus controls and ARDS patients versus controls. ROC AUC was calculated for each serum protein. Proteins that had AUC ≥ 0.7 were selected. As shown in Figure 6, the number of proteins selected in serum was much lower than the number of proteins selected in urine. We also found no significant correlation between urine and serum in all three subgroups. Moreover, the overlap of biomarkers in the serum versus urine dataset is minimal. Specifically, in the TBI group, there was no overlap between serum and urine (Figure 6b). In ARDS and ARDS/TBI-overlapped group (Figure 6a,c), the number of proteins in common between serum and urine was small compared to the actual size of each group. The most promising candidate biomarkers for ARDS or TBI identified in urine on the basis of the literature search confirming protein involvement in relevant biological processes and of brain/lung tissue origin were not identified in the serum.

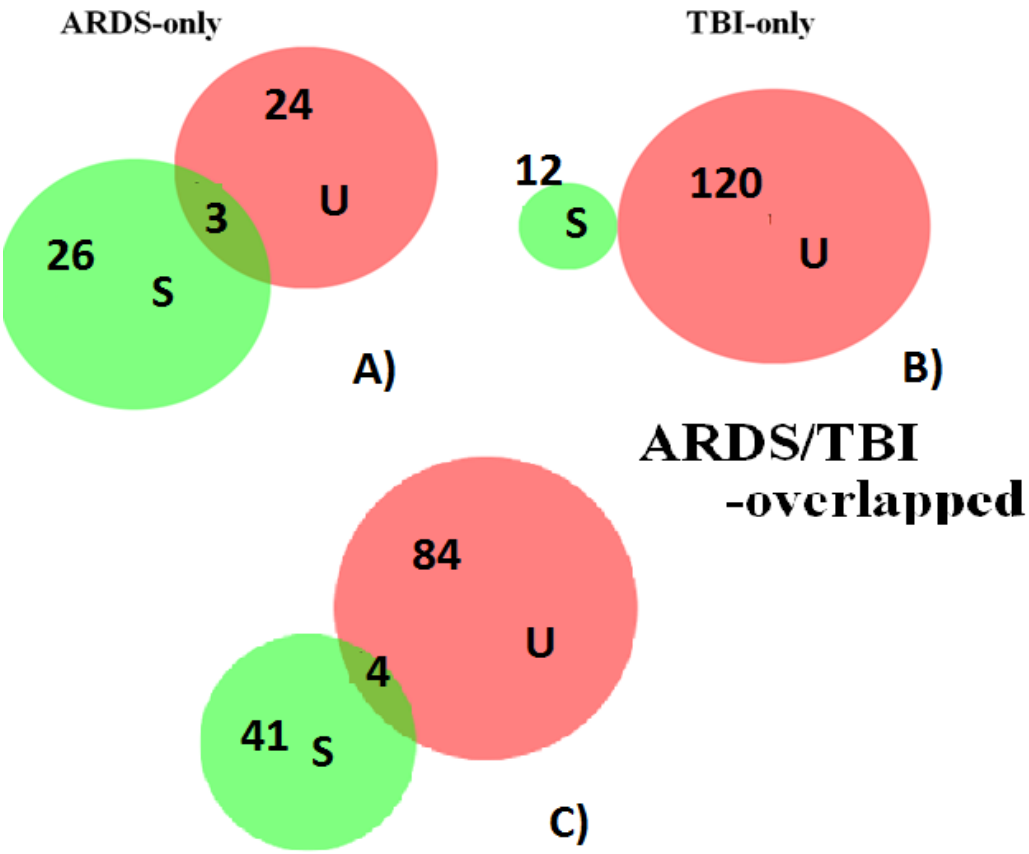

Figure 6. Venn diagram of candidate protein biomarkers related to TBI/ARDS found in serum and urine. S: ARDS-only proteins in serum, U: ARDS-only proteins in urine. (b) TBI-only group in serum and urine. S: TBI-only proteins in serum, U: TBI-only proteins in urine. (c) ARDS/TBI-overlapped group in serum and urine. S: ARDS/TBI-overlapped proteins in serum, U: ARDS/TBI-overlapped proteins in urine.

DISCUSSION

This study demonstrates how an affinity concentration method and mass spectrometry analysis can be used to successfully identify TBI and ARDS candidate biomarkers, of relevant biological function and low abundance, in the urine and serum of patients. Upon direct comparison of urine and serum deriving from the same patient and collected at the same time, the results obtained in this study suggest that urine is the biofluid that yields a higher number of total proteins and TBI/ARDS candidate biomarkers. Urine yielded a much higher number of proteins (over 7000 proteins were detected) compared to over 1000 proteins detected by tandem mass spectrometry in serum. This is important information because, historically, the biofluids of choice for discovery and verification of TBI and ARDS related biomarkers were always the serum and the cerebrospinal fluid (Table 2, 4). The fact that urine outperforms serum in this study involving a cohort of 75 well-characterized TBI and ARDS patients can be explained based on physiological and technical concepts.

The blood-brain barrier (BBB) can lose its integrity upon brain injury and permit the permeation of molecules into the blood (27). Importantly, small molecular weight proteins of size inferior to 60 kDa are passively transported from the blood to the urine by

glomerular filtration in the kidneys and are concentrated in the bladder. This is a physiological concentration effect that supports our experimental data. Another important aspect related to the comparison of serum and urine for proteomics studies is the technical characteristics of mass spectrometry. When operated in discovery mode, the mass spectrometer selects the ions that show the 5 most abundant peaks in the first fragmentation in each scan to proceed to the second fragmentation. This operating mode determines the dynamic range of the analysis that is currently 4 orders. This means that the mass spectrometer is able to detect proteins whose concentration is 4 orders of magnitude lower than the most abundant protein present in the sample. Therefore, the concentration range spanned by proteins in the biological sample is a very important consideration. Serum proteins have a much larger concentration range with respect to urinary proteins. It is important to note that the nanoparticle affinity concentration method is effective in reducing the dynamic range of protein concentrations in the sample due to size sieving properties and the fact that albumin and larger, high abundance proteins are excluded. Nevertheless, the concentration effect is based on the volumetric ratio between the initial sample volume and the final analytical volume. The fact that the volume of urine samples is generally much larger than serum samples yields higher concentration factors. Importantly, urine is a noninvasive, readily attainable biofluid. Both non-invasiveness and ease of collection are the preferable attributes for longitudinal monitoring in rehabilitative care (including outpatient) or in selected populations, such as pediatric TBI (9). Hence, the results showed in this study corroborated by technical and physiological characteristics of the human circulation and the proteomics instruments

suggest that urine is a very promising biofluid to conduct studies of TBI and it deserves a greater attention.

In this study, we demonstrate how our experimental approach enables the detection of known importance in relation to TBI diagnosis (Table 4). Importantly, we also discovered novel proteins that were not previously associated with TBI but that are very promising candidate biomarkers based on the tissue specificity and involvement in relevant biological processes (Table 3).

Additionally, four candidate biomarkers for ARDS were identified in the urine of patients with severe TBI that progressed to ARDS (Table 2). These biomarkers, once validated, can be clinically important to improve management of TBI patients. It is important to highlight how urine proteomic analysis can capture a protein molecular signature related to events that lead severe TBI patients to develop ARDS.

A limitation of this study is the relatively small number of samples analyzed. An extensive literature search has been performed to highlight the functional significance of the findings.

The findings of this study provide evidence supporting larger cohort studies in TBI and ARDS, separately or together. Follow-up studies can focus on the stability and specificity of different individual TBI/ARDS candidate and evaluate a more efficient comparative analysis of that between urine and serum or other biofluids.

The functionally relevant proteins identified in this study related to TBI and ARDS can provide molecular information and insights for increasing diagnostic

performance, for stratifying patients for therapy and for developing new, more targeted therapies.

CONCLUSION

TBI and its complications, such as ARDS, are an increasing problem in our society. A timely diagnosis of these conditions that could guide an optimal recovery after injury, however, is still out of reach. Our hypothesis was that TBI biomarkers that can be measured in peripheral body fluids, can serve as a new means of definitive diagnosis for brain injuries. This study aimed at identifying urinary biomarkers related to TBI in well-characterized clinical samples. We used an affinity concentration method and mass spectrometry to successfully identify functionally relevant, low abundance candidate TBI biomarkers in urine. Candidate biomarkers were selected on the basis of their performance in distinguishing TBI subjects from non-traumatized controls. We also identified biomarkers related to the insurgence of ARDS in severe TBI patients. Urine and serum were collected from the same patients at the same time from the same patients; proteomics analysis was performed on all samples. The comparison of proteins identified in these biofluids suggests that urine is a superior fluid in comparison to serum. This is an important conclusion and more studies to confirm these results are warranted. We plan on

pursuing further studies to verify and validate the candidate biomarkers for TBI and ARDS.

APPENDIX

Supplementary Table 1: Urinary ARDS/TBI-overlapped proteins according to their pathophysiological process

Pathway	Protein
Blood pressure regulation QTL	**ATP1B1**
Respiratory syncytial virus	**CD14**
Hemolytic anemia,	**CD59**
GM2-gangliosidosis	**GM2A**
Mucopolysaccharidosis type IIIB (Sanfilippo B), Charcot-Marie-Tooth disease	**NAGLU**
Spinal muscular atrophy with progressive myoclonic epilepsy	**ASAH1**
Inflammation and cancer	**S100A8**
Deafness, Baraitser-Winter syndrome 2	**ACTG1**
Glomerulosclerosis	**ACTN4**
Glycogen storage disease XII	**ALDOA**
Aminoacylase 1 deficiency	**ACY1**
Renal tubular dysgenesis, Preeclampsia	**AGT**
Citrullinemia,	**ASS1**
Ceroid lipofuscinosis	**CTSD**
Takenouchi-Kosaki syndrome	**CDC42**
High-density lipoprotein cholesterol level QTL 10, Hyperalphalipoproteinemia,	**CETP**
Bethlem myopathy 1, Ullrich congenital muscular dystrophy 1,	**COL6A1**
Cerebral amyloid angiopathy, Macular degeneration	**CST3**
Homocysteine	**DDAH2**
Enolase deficiency	**ENO1**
Glycogen storage disease XIII	**ENO3**
Glomerulopathy with fibronectin deposits 2, Plasma fibronectin deficiency,	**FN1**
Fructose-1,6-bisphosphatase deficiency,	**FBP1**
Hemolytic anemia	**GPI**
Huntington disease and Dentatorubral-pallidoluysian atrophy	**GAPDH**
Thalassemia	**GDF15**
Legionellosis, Toxoplasmosis, Measles, Influenza A, Epstein-Barr virus infection,	**HSPA8**
Charcot-Marie-Tooth disease	**HSPB1**
Viral carcinogenesis, Systemic lupus erythematosus	**HISTLH41**

Hypercholesterolemia	**ITIH4**
Kininogen deficiency	**KNG1**
Glycogen storage disease XI	**LDHA**
Lactate dehydrogenase-B deficiency	**LDHB**
Salmonella infection, Tuberculosis	**LBP**
Alzheimer's disease	**MME**
Amyotrophic lateral sclerosis 18	**PFN1**
Proteoglycans in cancer, Colorectal cancer, Renal cell carcinoma, Pancreatic cancer	**RAC1**
Microphthalmia	**RBP4**
Emphysema-cirrhosis	**SERPINA1**
Alpha-1-antichymotrypsin deficiency, Cerebrovascular disease	**SERPINA3**
Thyroxine-binding globulin deficiency	**SERPINA7**
Thrombophilia	**SERPINC1**
Bartter syndrome	**SLC12A1**
Cataract	**SORD**
Tibial muscular dystrophy, Myopathy	**TTN**
Amyloidosis, Carpal tunnel syndrome, Dystransthyretinemic hyperthyroxinemia	**TTR**
Hemolytic	**TPI1**
Ceroid lipofuscinosis, Spinocerebellar ataxia	**TPP1**
Hyperuricemic nephropathy, Medullary cystic kidney disease 2, Glomerulocystic kidney disease with hyperuricemia and isosthenuria	**UMOD**
Parkinson's disease, Huntington's disease, Influenza A, HTLV-I infection	**VDAC1**

Only proteins found mentioning in review studies of the corresponding diseases are listed in the table. Abbreviations: ATPase Na+/K+ transporting subunit beta 1, ATP1B1; CD14, CD14 molecule; CD59 molecule CD59; GM2 ganglioside activator GM2A; N-acetyl-alpha-glucosaminidase NAGLU; N-acylsphingosine amidohydrolase 1 ASAH1; S100 calcium binding protein A8 S100A8; actin gamma 1 ACTG1; actinin alpha 4 ACTN4; aldolase, fructose-bisphosphate A ALDOA; aminoacylase 1 ACY1; angiotensinogen AGT; argininosuccinate synthase 1 ASS1; cathepsin D CTSD; cell division cycle 42 CDC42; cholesteryl ester transfer protein CETP; collagen type VI alpha 1 chain COL6A1; cystatin C CST3; dimethylarginine dimethylaminohydrolase 2 DDAH2; enolase 1 ENO1; enolase 3 ENO3; fibronectin 1 FN1; fructose-bisphosphatase 1 FBP1; glucose-6-phosphate isomerase GPI; glyceraldehyde-3-phosphate dehydrogenase GAPDH; growth differentiation factor 15 GDF15; heat shock protein family A Hsp70 member 8 HSPA8; heat shock protein family B small member 1 HSPB1; histone cluster 2 H4 family member a HIST2H4A; inter-alpha-trypsin inhibitor heavy chain family member 4 ITIH4; kininogen 1 KNG1; lactate dehydrogenase A LDHA; lactate dehydrogenase B LDHB; lipopolysaccharide binding protein LBP; membrane metalloendopeptidase MME; profilin 1 PFN1; ras-related C3 botulinum toxin substrate 1 rho family, small GTP binding protein Rac 1RAC1; retinol binding protein 4 RBP4; serpin family A member 1 SERPINA1; serpin family A member 3 SERPINA3; serpin family A member 7 SERPINA7; serpin family C member 1 SERPINC1; sorbitol dehydrogenase SORD; titin TTN; transthyretin TTR; triosephosphate isomerase 1 TPI1; tripeptidyl peptidase 1 TPP1; uromodulin UMOD; voltage dependent anion channel 1 VDAC1

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BIOGRAPHY

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