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Research Paper

# Human Serum Metabolites Associate With Severity and Patient Outcomes in Traumatic Brain Injury

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ABSTRACT

Traumatic brain injury (TBI) is a major cause of death and disability worldwide, especially in children and young adults. TBI is an example of a medical condition where there are still major lacks in diagnostics and outcome prediction. Here we apply comprehensive metabolic profiling of serum samples from TBI patients and controls in two independent cohorts. The discovery study included 144 TBI patients, with the samples taken at the time of hospitalization. The patients were diagnosed as severe (sTBI; $n = 22$), moderate (moTBI; $n = 14$) or mild TBI (mTBI; $n = 108$) according to Glasgow Coma Scale. The control group ($n = 28$) comprised of acute orthopedic non-brain injuries. The validation study included sTBI ($n = 23$), moTBI ($n = 7$), mTBI ($n = 37$) patients and controls ($n = 27$). We show that two medium-chain fatty acids (decanoic and octanoic acids) and sugar derivatives including 2,3-bisphosphoglyceric acid are strongly associated with severity of TBI, and most of them are also detected at high concentrations in brain microdialysates of TBI patients. Based on metabolite concentrations from TBI patients at the time of hospitalization, an algorithm was developed that accurately predicted the patient outcomes (AUC = 0.84 in validation cohort). Addition of the metabolites to the established clinical model (CRASH), comprising clinical and computed tomography data, significantly improved prediction of patient outcomes. The identified 'TBI metabotype' in serum, that may be indicative of disrupted blood-brain barrier, of protective physiological response and altered metabolism due to head trauma, offers a new avenue for the development of diagnostic and prognostic markers of broad spectrum of TBIs.



## 1. Introduction

Traumatic brain injury (TBI) is a major cause of death and disability worldwide (Maas et al., 2008), especially in children and young adults. Classical approaches to grading TBI rely on the Glasgow coma score (GCS) (Saatman et al., 2008) and neuroimaging, but this classification takes no account of mechanistic heterogeneity or addresses emerging approaches to precision medicine (Maas et al., 2015).

TBI is associated with a complex metabolic disruption that results in energy crisis and energy failure, which is the consequence of multiple mechanisms, including classical ischaemia (Coles et al., 2004), diffusion hypoxia (Menon et al., 2004), mitochondrial dysfunction (Lakshmanan et al., 2010) and increased energy needs (from excitotoxicity, seizure activity and spreading depolarization) (Timofeev et al., 2011). Thus far,

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the only approaches to examine this metabolic dysregulation have involved the use of cerebrospinal fluid (CSF), brain microdialysis, arteriojugular venous differences, or advanced metabolic imaging with positron emission tomography (PET) or magnetic resonance imaging and spectroscopy (MRIS), none of which are universally available, and are particularly problematic in less severe TBI. Since metabolic dysregulation is such a fundamental facet of TBI pathophysiology, these metabolic changes may contain prognostic information, and early changes may provide a useful summary of the pre-hospital and early hospital physiological insults experienced by the brain. The broad assumption has been that these metabolic changes are the consequence of metabolic failure – but there is the possibility that some may be causes as well as consequences of the pathophysiology observed. In addition, release of brain specific metabolites (i.e. small molecules with molecular mass under 500 Da) into the systemic circulation may provide information on blood brain barrier (BBB) dysfunction, a fundamental process in TBI (Saw et al., 2014).

Several peptide or protein based biomarkers for TBI have been proposed. These include S100 calcium binding protein B (S100B), neuron-specific enolase, myelin basic protein, creatine kinase brain isoenzyme, glial fibrilary acidic protein, plasma DNA, brain-derived neurotrophic factor, and ubiquitin carboxy-terminal hydrolase-L1 (Berger et al., 2006; Ingebrigtsen and Romner, 2002; Mondello et al., 2011; Papa et al., 2010; Pelinka et al., 2004; Vos et al., 2004). However, most of these biomarkers lack the disease specificity. For example, protein S100B, the most extensively studied biomarker of TBI, has been reported to be released into the serum also after experimental ischemic injury to the liver, kidney, and the gut (Pelinka et al., 2004). Moreover, its levels have been found to have increased also after exercise (Koh and Lee, 2014), extracranial trauma (Savola et al., 2004) and burns (Anderson et al., 2001) and it has also been suggested as a biomarker of melanoma (Torabian and Kashani-Sabet, 2005).

Here we hypothesized that metabolites may be a rich source of circulating TBI biomarkers. We applied comprehensive metabolic profiling of serum samples from TBI patients and controls comprised of acute orthopedic non-brain injuries in two independent cohorts, with the aims (a) to associate metabolite profiles with TBI severity and (b) to predict the outcomes of TBI patients.

## 2. Materials and Methods

### 2.1. Ethics Statement

South-West Finland Hospital District Research Ethics Committee, the Cambridgeshire 2 Research Ethics Committee, and the Norfolk Research Ethics Committee approved the protocol. All patients or their next of kin were given both oral and written information about the study and a written informed consent was obtained. All patients were treated according to standard local guidelines based on the current international guidelines and recommendations (Brain Trauma Foundation et al., 2007).

### 2.2. Study Population and Clinical Assessment

Patient recruitment of this prospective multicenter study was part of the EU funded TBIcare (Evidence-based Diagnostic and Treatment Planning Solution for Traumatic Brain Injuries) project. A total of 389 adult patients with acute TBI and 81 patients with acute orthopedic trauma without acute or previous brain disorders, who served as controls, were prospectively recruited at Turku University Hospital (Turku, Finland) and at Addenbrooke's Hospital (Cambridge, United Kingdom). 211 patients with acute TBI and 55 orthopedic trauma patients had eligible serum samples available (Table 1).

Inclusion criteria were: age ≥ 18 years (≥16 years in United Kingdom), clinical diagnosis of TBI and indications for acute head computed tomography according to National Institute for Health and Care Excellence (NICE) criteria (http://www.nice.org.uk/guidance/cg176). Exclusion criteria were: blast-induced or penetrating injury, chronic subdural hematoma, inability to live independently due to pre-existing brain disease, TBI or suspected TBI not needing head computed tomography, more than two weeks from the injury, not speaking native language, and no consent obtained. The control group comprised patients with acute orthopedic non-trivial trauma without any signs of acute central nervous system involvement, previous central nervous system disease, or previous non-concussional TBI.

Two separate sample cohorts were utilized in this study (Turku and Cambridge studies, respectively). Blood samples for metabolomic analysis (2 ml) were collected within 12 h after hospital admission. After sampling, the samples were allowed to clot for 30 min at +4 °C before centrifugation for 10 min at 10,000 rpm at 4 °C. After centrifugation the serum was divided into five aliquots and immediately frozen at −70 °C. Cerebral microdialysis was undertaken using a CMA71, 100 kDa molecular weight cut-off catheter (M Dialysis AB, Stockholm, Sweden), via a cranial access device (Technicam, Newton Abbot, United Kingdom), perfused at 0.3 ml/min using a CMA106 pump (M Dialysis AB). The perfusate consisted of CNS perfusion fluid (M Dialysis AB).

The severity of TBI was classified on the basis of Glasgow Coma Scale (GCS) (Saatman et al., 2008). GCS 13–15 was considered mild, 9–12 moderate and 3–8 severe. GCS scores in the scene of accident assessed by paramedics or an emergency physician were retrieved from the ambulance sheets. GCS scores at emergency departments were assessed on admission. Lowest recorded GCS before intubation and sedation from the scene of accident or emergency department was used in the statistical analysis.

Outcomes were assessed at 3 months in patients with mild TBI (Cambridge) and at 6–12 months (all other patients) after the injury using Extended Glasgow Outcome Scale (GOSe), which divides patients with TBI into groups allowing the standardized assessment of their

**Table 1**
Clinical characteristics of the study subjects.

| | Study group | # of samples | Age | | | Gender | Mechanism of injury[b] | | | | | | | | CT findings[c] | | Outcome (GOSe score) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | ±SD | (Range) | Male/female[a] | BTH | A/C | V | GLF | FFH | HAO | O | N/A | − | + | Mean | ±SD | (Range) | # with score |
| Discovery group (Turku) | Control | 28 | 51.43 | 20.49 | (20–99) | 13/15 | | | | | | | | | | | | | | |
| | Mild | 108 | 48.37 | 20.18 | (18–91) | 74/34 | 4 | 18 | 11 | 45 | 23 | 5 | 0 | 2 | 48 | 60 | 6.43 | 1.87 | (1–8) | 99 |
| | Moderate | 14 | 59.57 | 17.32 | (32–88) | 8/6 | 0 | 3 | 1 | 6 | 3 | 1 | 0 | 0 | 3 | 11 | 4.79 | 2.01 | (1–7) | 14 |
| | Severe | 22 | 55.05 | 15.25 | (19–77) | 19/3 | 1 | 3 | 0 | 10 | 6 | 0 | 1 | 1 | 0 | 22 | 3.47 | 2.41 | (1–7) | 19 |
| Validation group (Cambridge) | Control | 27 | 42.04 | 15.81 | (18–77) | 14/13 | | | | | | | | | | | | | | |
| | Mild | 37 | 36.84 | 17.95 | (16–84) | 28/9 | 5 | 8 | 0 | 10 | 6 | 5 | 2 | 1 | 16 | 21 | 7.13 | 1.48 | (3–8) | 30 |
| | Moderate | 7 | 41.57 | 20.49 | (19–68) | 7/0 | 0 | 2 | 0 | 1 | 3 | 0 | 1 | 0 | 5 | 2 | 5.71 | 2.06 | (3–8) | 7 |
| | Severe | 23 | 44.87 | 17.71 | (20–69) | 17/6 | 4 | 8 | 0 | 1 | 4 | 6 | 0 | 0 | 0 | 23 | 4.38 | 2.13 | (1–8) | 16 |

[a] The proportion of males is significantly different between the control and TBI groups in both the discovery and validation cohorts ($\chi^2$ test; $p < 0.001$).
[b] Mechanism of injury: BTH, blow to head; A/C, acceleration/deceleration; V, violence; GLF, ground level fall; FFH, fall from height; HAO; head against object; O, other; N/A, not available.
[c] Computed tomography findings (CT findings): −, no visual pathology; +, visual pathology (swelling, contusions, mass lesions).

recovery in eight categories (Wilson et al., 1998). GOSe score 5–8 was classified as favorable outcome, and GOSe score 1–4 was classified as unfavorable outcome.

### 2.3. Metabolomic Analysis

Metabolomic analyses of serum and brain microdialysate samples were performed at VTT Technical Research Centre of Finland (Espoo, Finland). Additional analyses to identify metabolites of interest were also performed at LECO Corporation (St. Joseph, Michigan) and Steno Diabetes Center (Gentofte, Denmark).

Serum samples (30 μl) or microdialysate samples (100 μl) were spiked with 10 μl of internal standard mixture (C17:0 (186,5 mg/l), deuterated valine (37 mg/l) and succinic acid-d4 (63 mg/l). The protein precipitation was done as follows: 400 μl of methanol was added and then the samples were vortexed for 2 min. After centrifugation (5 min at 7800 g at room temperature) and settling for 30 min at −20 °C the supernatant was evaporated to dryness under gentle flow of nitrogen before derivatization. The original metabolites were then converted into their methoxime and trimethylsilyl (TMS) derivatives by two-step derivatization. First, 25 μl of methoxyamine hydrochloride (98%) was added to the residue, and the mixture was incubated for 60 min at 45 °C. Next, 25 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was added, and the mixture was incubated for 60 min at 45 °C. Finally, a retention index standard mixture (*n*-alkanes, 25 μl, c = 8 mg/l) and an injection standard (4,4′-dibromooctafluorobiphenyl, 50 μl, c = 10 mg/l), both in hexane, were added to the mixture.

GC × GC–TOFMS analyses were carried out on an Agilent 6890 gas chromatograph equipped with a split/splitless injector (Agilent Technologies, Santa Clara, CA), cryogenic dual-stage modulator and time-of-flight mass spectrometer (Leco Corp., St. Joseph, MI, USA). In addition, multipurpose sampler with Maestro software (Gerstel, Mülheim an der Ruhr, Germany) was used for derivatization and sample introduction. A 10 m × 0.18 mm I.D. Rxi-5 ms (Restek Corp., Bellefonte, PA, USA) column with film thickness 0.18 μm was used as the first column and a 1.5 m × 0.1 mm I.D. BPX-50 (SGE Analytical Science, Austin, TX, USA) column with film thickness of 0.1 μm as the second column. A phenyl methyl deactivated retention gap column (1.5 m × 0.53 mm I.D.) was installed in front of the first column. The injector was used in the splitless mode at 240 °C for injecting 1 μl of a sample. The splitless period was 90 s. High-purity helium (Aga, Espoo, Finland) was used as the carrier gas in a constant-pressure mode with initial pressure of 276 kPa. The first column oven temperature program was as follows: 50 °C (isothermal for 2 min) then 7 °C/min to 240 °C, and, finally, 25°/min to 300 °C (3 min). The second dimension column oven temperature was maintained 20 °C higher and the programming rate and hold times were similar than in the first dimension. The temperature of the transfer line was maintained at 260 °C and ion source at 200 °C. Modulation time was 4 s. Electron impact ionization was applied at 70 eV, and the mass range from 45 to 700 amu with 100 spectra/s was measured.

Automatic peak detection and mass spectrum deconvolution were performed using a peak width set to 0.2 s. Peaks with signal-to-noise (S/N) values lower than 100 were rejected. The S/N values were based on the masses chosen by the software for quantification. ChromaTOF version 4.32 (LECO Corporation, St. Joseph, MI) was used for the raw data processing. The peak areas from total ion chromatography (TIC) were used for most of the compounds; for compounds that were quantified with the ChromaTOF software, peak areas of selected characteristic *m/z* were used.

Next, the data files obtained by the ChromaTOF software were exported to text files and in-house developed software *Guineu* (Castillo et al., 2011) was used for aligning and normalization of compounds for further analyses. The original GC × GC–TOFMS data includes retention times, retention indices (RI), spectral information (for possible identification), spectral similarity value (S = 0–999), and peak response data. The linear retention indices were calculated based on the total (i.e., sum of the first and the second dimension) retention times of the compounds and the retention times of the retention index standards (*n*-alkanes). The second dimension retention time is so short (1–3.5 s) that its contribution to the retention index is not significant. The alignment of the data was performed based on retention indices, second dimension retention times and spectra. After alignment of the GC × GC–TOFMS data, two filtration criteria was utilized for positive identification: spectral similarity >850 and maximum allowed difference in retention index between experimental and literature values <25. Only the metabolites detected in over 70% of the samples in each of the four study groups were included in the dataset. This procedure resulted in a total of 465 metabolites. The literature values were obtained from NIST 2008 Mass Spectral Library or they were determined experimentally with GC × GC-TOFMS instrument in our laboratory with authentic standards (in-house developed library). In addition, Golm metabolome database (Kopka et al., 2005) was used for further identification of the metabolites. In cases where the metabolite identity could not be determined with available methods, the chemical class was reported based on the MS spectra (Castillo et al., 2011).

The validation and discovery cohorts were analyzed separately. The orders of both sample preparation and analysis were randomized, and a set of controls samples (pooled serum samples), standards and blank samples (solvent blank) was analyzed together with the samples. In the discovery set, the day-to-day variation of internal standards added to all samples was on average 17.3% and the day-to-day variation in control serum samples ($n = 31$) of the quantified metabolites was 18.0%. In the validation set, the variation of internal standards added to all samples was on average 12.3% and the variation of control serum samples ($n = 14$) of the quantified metabolites was 9.2%.

### 2.4. Data Filtering

Most severe TBI patients, as well as several moderate and mild TBI patients and a few controls have been given propofol. Propofol was detected at low concentrations. However, one phase two metabolite of propofol (propofol glucuronide) was detected in relatively high concentrations. The identification was further verified by liquid chromatography-quadrupole-time-of-flight mass spectrometry. Possible correlation of propofol metabolites with other metabolites was also checked using Pearson correlation, showing correlation with unknown compounds that were structurally similar to the identified propofol metabolites, and a few additional metabolites. Three compounds that had similar mass spectra to propofol glucuronide and which showed high correlation ($r > 0.7$, $p < 0.05$) with the compound were removed from the data. Ibuprofen was also detected in several control samples and was removed from the dataset.

### 2.5. Statistical Analyses

All statistical analyses were performed using MATLAB R2014b (The MathWorks, Inc., Natick, MA, USA), applying in-house developed scripts and PLS Toolbox 7.9 (Eigenvector Research, Inc., Wenatchee, WA, USA), and R Statistical Software using *rms* package (Harrell, 2015).

The zero values in the metabolomics dataset were first imputed. For each metabolite, zero value was replaced by half the minimum non-zero value of the metabolite across all samples. Due to the non-normal distribution of data, data were then log-transformed. The mean levels of the four study groups were compared using ANOVA on all 465 metabolites. In order to adjust for multiple testing, False Discovery Rates (FDR) were calculated (Storey, 2002), with FDR q-values <0.05 considered significant. The mean levels of each of the three TBI groups (mild, moderate, and severe) were compared to the control group using two-sided *t*-test (unequal variance).

A principal component analysis (PCA) model was calculated based on the 172 subjects from the Turku cohort and 98 metabolites selected using ANOVA and FDR related to control/mild/moderate/severe subjects. Data were autoscaled and the model consisted of seven principal components. No potential confounding factors, such as age and gender, affected the model in the major components. In the fifth principal component there was a slight age drift. Correcting for this drift by orthogonalization did not change the outcome of the model in relation to the severity status; hence age does not affect the coherence of the metabolites related to the severity of TBI.

### 2.6. Prediction of Patient Outcomes – Multivariate Metabolite Model

1000 prediction models were built using partial least squares discriminant analysis (PLS-DA). However, PLS-DA is sensitive to a skewed dataset and therefore 33 subjects were randomly selected out of the 99 good outcome subjects in each of the 1000 models in order to align with the 33 poor outcome subjects. 49 metabolites were included in the analyses; these were also selected using ANOVA and FDR related to good/poor outcome of the subjects. Data were mean centered. The models were cross-validated using random cross-validation with 10 splits and 10 iterations. During model optimization, metabolites were selected in two runs as being most important based on variables influence in projection (VIP) with values in the first run above 1 and in the second run above 0.75. The most abundant metabolites present in >750 of the 1000 PLS-DA models comprised eight metabolites out of the 49 metabolites included in the analyses.

### 2.7. Prediction of Patient Outcomes – Combined Clinical and Metabolite Model

The additional predictive value of the metabolites over known clinical predictors was assessed with ordinal multivariable regression. Outcomes were assessed at three months in patients with mild TBI (Cambridge) and at 6–12 months (all other patients) after the injury with GOSe. The first model contained the clinical predictors age, pupil reactivity, Glasgow Coma Scale at admission, CT characteristics (Marshall CT classification), and extracranial injury (Injury Severity Score). Selection of these predictors was based on the CRASH prediction model (MRC Crash Trial Collaborators et al., 2008) but limited to the availability of variables in our data.

Model 2 contained the same clinical predictors, and the top ranking metabolites were added (top ranking metabolites based on FDR q-value, comparing favorable (GOSe >4). and unfavorable (GOSe ≤4) outcomes). Both models were fitted on the Turku cohort, internally validated using a bootstrap procedure and subsequently externally validated on the Cambridge cohort. The best combination of metabolites together with the clinical predictors was selected recursively, initially starting with the top ranking metabolite, then adding additional metabolites to the model, until the predictive performance started to decrease due to the model complexity.

Missing clinical predictors were imputed with multiple imputation based on the predictors and outcome, using the *AregImpute* function in R. All subsequent analyses were performed on the 10 imputed datasets and results were pooled in one summary estimate. Models were compared in terms of the AUC curve.

## 3. Results

### 3.1. Serum Metabolites Associate With Severity of TBI

We evaluated serum metabolomic profiles from 144 TBI patients from Turku University Hospital, Turku, Finland. The samples were taken at the time of hospitalization (arrival samples), which was up to 12 h after the injury. The patients were diagnosed as severe (sTBI; $n = 22$), moderate (moTBI; $n = 14$) or mild TBI (mTBI; $n = 108$) according to their lowest recorded pre-intubation Glasgow Coma Scale (GCS) (Saatman et al., 2008) from the scene of accident or emergency department. Additionally, the control group ($n = 28$) comprised of acute orthopedic non-brain injuries (Fig. 1, Table 1). The established metabolomics platform based on two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC × GC-TOFMS) (Castillo et al., 2011) was applied to analyze the serum samples. A total of 465 metabolites were included in the analysis. Among those were amino acids, sugar derivatives, hydroxyl acids, fatty acids as well as sterols and related metabolites.

In the arrival samples, 98 metabolites showed significant differences between the four study groups in the Turku cohort (ANOVA, FDR $q < 0.05$) (Supplemental Table 1). Since the patients could not be controlled for confounding factors such as dietary status, alcohol use and age, we studied the associations of metabolome with severity of TBI only for these 98 top ranking metabolites. Principal component analysis (PCA) showed that the differences between cases and controls were most pronounced for the severe and moderate TBI patients, while the differences were smaller in the mild TBI patients (Fig. 2a, b). None of the potential confounding factors explained the differences between the study groups (Materials and Methods). We also specifically studied the effect of gender on metabolite levels in the control group, due to different gender distributions between the control and TBI groups (Table 1). We found no significant associations between the 98 top ranking metabolites and gender (FDR $q > 0.25$ for all 98 TBI-associated metabolites; two-sided *t*-test, unequal variance).

Metabolite concentrations were highly similar in severe and moderate TBI patients as compared to controls (Fig. 2c). Two medium-chain fatty acids (FA), octanoic acid (OA) and decanoic acid (DA), 2- and 3-hydroxybutyric acids as well as several sugar-derived metabolites were upregulated in sTBI. Among these sugar derivatives, we identified a metabolite 2,3-bisphosphoglyceric acid (2,3-BPG), a key regulator of oxygen release to the tissues (Hsia, 1998), which was strongly associated with severity of TBI. 2,3-BPG was about 100–fold upregulated in sTBI and moTBI as compared to controls. However, the levels of 2,3-BPG was approaching normal levels during the first 24 h, unlike for OA and DA, that remained high in most patients during the first week following the injury (Supplemental Fig. 1). Some metabolites, including the amino acids serine and alanine, as well as indole-3-propionic acid (IPA), were downregulated in TBI (Fig. 2c, Supplemental Table 1).

Metabolite levels in mild TBI patients followed the same pattern as in more severe TBI, but the magnitude of change as compared to controls was clearly less than e.g. in sTBI (Fig. 2d). Taken together, these data suggest that TBI is characterized by a specific 'TBI metabotype', but the degree of metabolite changes as compared to their normal levels is proportional to the severity of TBI.

### 3.2. Administration of Propofol Does not Associate With Observed Metabolic Profiles in TBI Patients

The propofol vehicle contains predominantly long-chain FAs, mainly stearic acid, and it is known that propofol may inhibit mitochondrial entry of long-chain FAs and inhibit the respiratory chain (Wolf et al., 2001). Although the propofol and related metabolites were removed from the dataset and the long-chain FAs were not upregulated in the patients with propofol administration, we examined in more detail the potential association of propofol with the observed changes in medium-chain FA levels. However, the two significantly upregulated FAs (OA and DA) were significantly upregulated also in the severe patients ($n = 8$) who had not been receiving propofol prior to the first sample being taken (4.2 and 3.6-fold, $p = 0.001$ and $p = 0.012$ for OA and DA, respectively). Furthermore, the single control patient who received propofol did not show elevated levels of these two FAs. We therefore concluded that the propofol administration could not explain the observed upregulation of OA and DA in TBI.

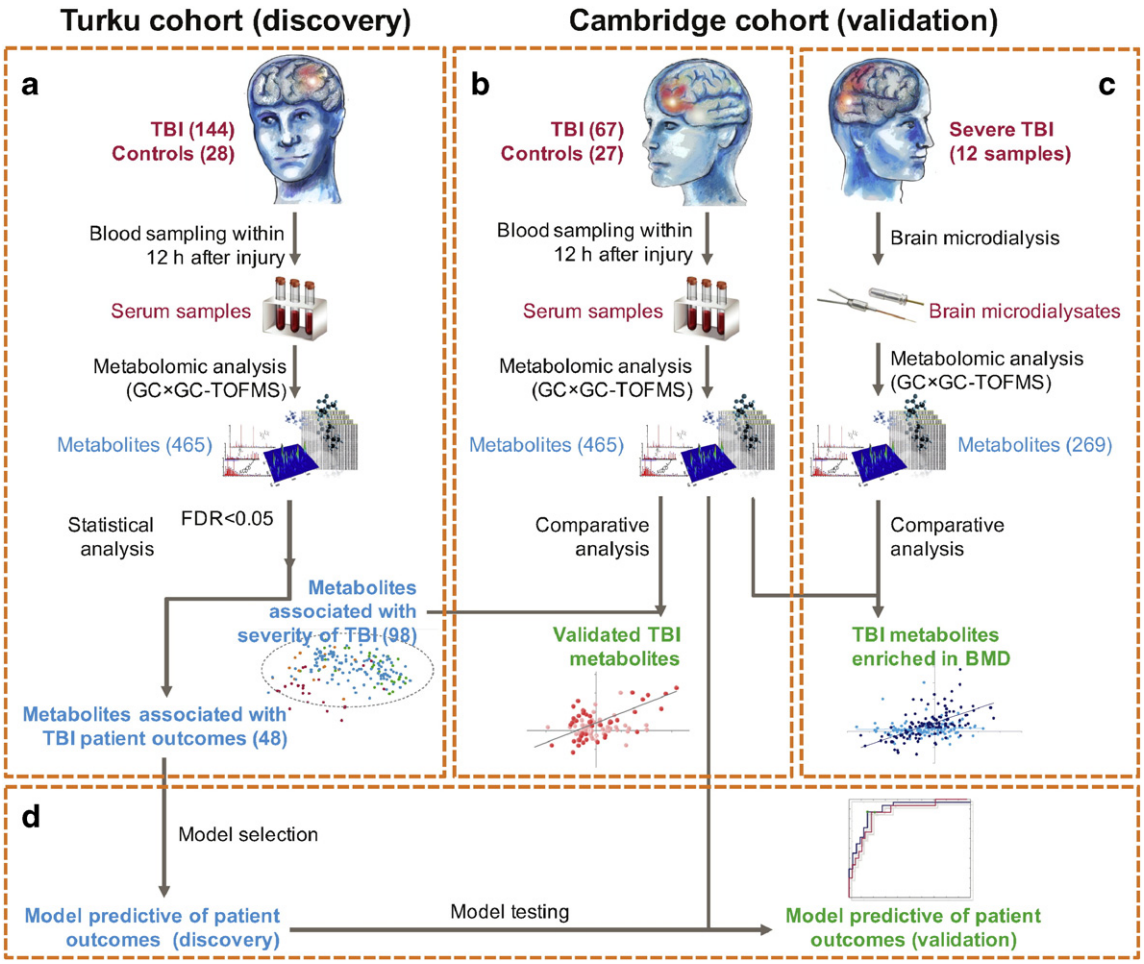


**Fig. 1.** Overview of the workflow to study metabolome in traumatic brain injury (TBI). (a) Serum metabolomics was performed in a series of samples from TBI patients and controls from the city of Turku, Finland. Significant metabolites associated with severity of TBI were identified. (b) The results from the Turku series were compared with metabolomics data from TBI patients and controls from the city of Cambridge, UK. (c) Brain microdialysate (BMD) samples were also analyzed from the severe TBI patients in Cambridge, for possible associations of BMD metabolite concentrations with the changes observed in serum. (d) In order to assess if metabolites can serve as predictor of outcomes in TBI patients, metabolites from the Turku dataset were screened for associations with patient outcomes. Predictive models were developed, which were independently tested in the Cambridge dataset.

### *3.3. Validation of Findings in Independent Studies*

Next, we compared the TBI metabolite profiles from Turku with those from a different population from Addenbrooke's Hospital (Cambridge, United Kingdom). The study group comprised sTBI ($n = 23$), moTBI ($n = 7$), mTBI ($n = 37$) patients and controls ($n = 27$), selected using the same inclusion criteria as in the city of Turku. The metabolomics data was first aligned with the Turku dataset and thus contained a total of 465 metabolites. Out of 98 metabolites found significantly associated with TBI in Turku, 44 were found significantly different also in the Cambridge dataset, when comparing sTBI patients and controls (Supplemental Table 2). There was a high degree of association between the metabolite levels in sTBI when comparing the patients from the two cities (Fig. 3a). No such strong association was found in mTBI, which may have been due to the lack of power in a highly heterogeneous mTBI population (Supplemental Fig. 2). Notably, the metabolites displaying largest differences between TBI patients and controls in the Turku cohort also behaved similarly in the Cambridge cohort, including 2,3-BPG and IPA.

In order to study the potential relevance of the serum metabolic profiles in TBI to brain metabolism, brain microdialysates (BMD) were analyzed from 12 samples acquired from four sTBI patients (Cambridge cohort). The top ranking serum metabolites associated with TBI were found highly correlated with their levels in BMD (Fig. 3b), which suggests possible disruption of the BBB. Among these metabolites were sugar derivatives, metabolites related to energy metabolism as well as several hydroxy-acids. Notably, the medium-chain FAs (C7-C10) including OA and DA were detected at relatively high concentrations in BMD as compared to their corresponding concentrations in blood, while the long-chain FAs had clearly lower levels in BMD than in blood.

### *3.4. Serum Metabolites Predict TBI Patient Outcomes*

We then investigated if the serum metabolome associates with the patient outcomes as assessed by the Glasgow Outcome Scale Extended (GOSe) (Wilson et al., 1998). The metabolite levels from patient arrival samples in the Turku cohort were compared between patients with unfavorable outcomes (GOSe ≤4; $n = 33$) and favorable outcomes (GOSe >4; $n = 99$). Out of 465 metabolites, 49 were significantly different between the two outcome groups, most of them up-regulated in poor outcome patients (FDR $q < 0.05$; Supplemental Table 3), and 26 out of 49 were also significantly different and changed to a similar degree in patients in the two outcome categories in the Cambridge cohort (Supplemental Fig. 3 and Supplemental Table 4). Only 10 out of 49 metabolites were also among the 98 metabolites that were associated with severity of TBI. Interestingly, despite being strongly associated with TBI severity, 2,3-BPG was not associated with patient outcomes.

Based on the metabolomics data from 49 metabolites in the Turku cohort, predictive model was developed using the partial least squares discriminant analysis (PLS-DA) (Supplemental Table 5). When tested

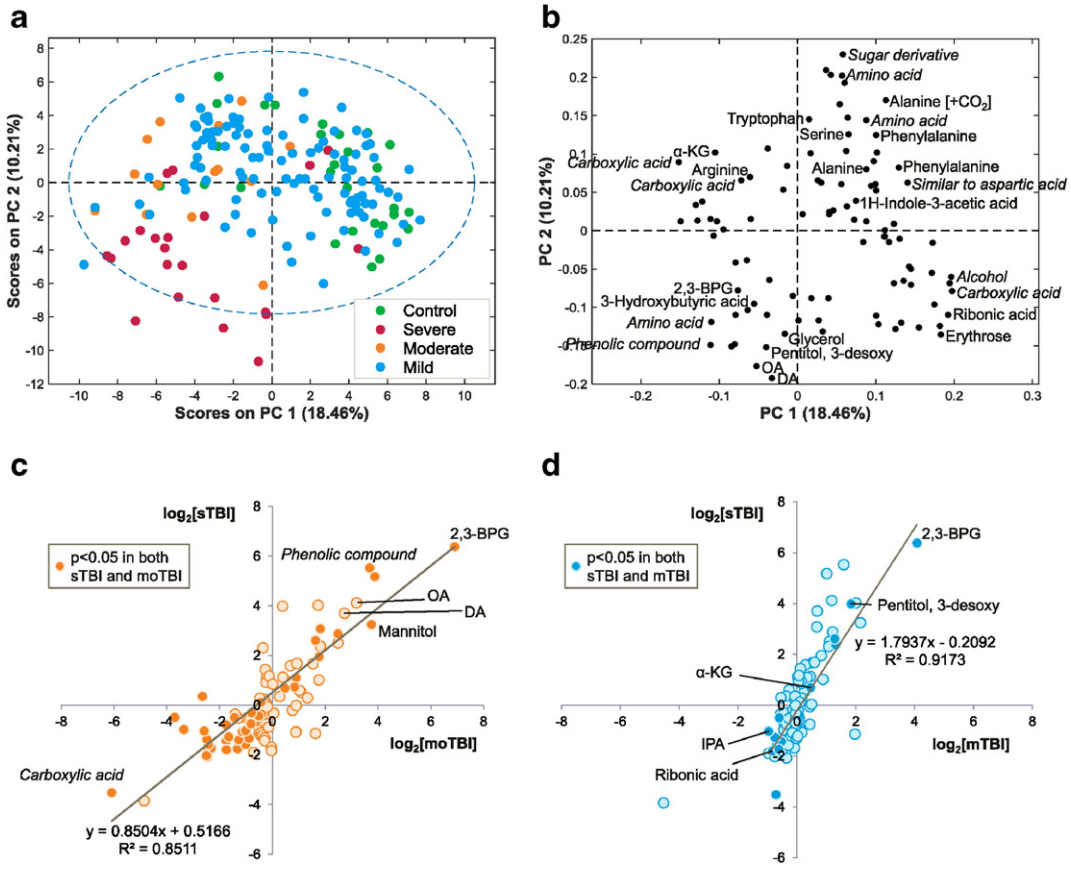


**Fig. 2.** Serum metabolome is associated with severity of TBI. (a) Principal Component Analysis (PCA) scores for the first two principal components (out of seven), using the dataset comprising 98 metabolites with FDR q < 0.05. (b) PCA loadings on the first two principal components reveal which metabolites are associated with specific groups in panel a. (c) Scatter plot of log2 scaled mean metabolite levels in sTBI vs. moTBI patients, with significant metabolites in both groups marked with full circles (*t*-test two sided unequal variance). (d) Scatter plot of log2 scaled mean metabolite levels in sTBI vs. mTBI patients, with significant metabolites in both groups marked with full circles (*t*-test two sided unequal variance). Regression lines in panels c and d are drawn based on significant metabolites in both groups. In panels b-d, the selected unidentified metabolites are listed in *italic* and annotated according to their structural class (Castillo et al., 2011). Abbreviations: 2,3-BPG, 2,3-bisphosphoglycerate; α-KG, alpha-ketoglutarate; DA, decanoic acid; IPA, indole-3-propionic acid; moTBI, moderate TBI; mTBI, mild TBI; OA, octanoic acid; PC, principal component; sTBI, severe TBI.

in the Cambridge patients with outcome values available ($n = 53$), the model predicted the patient outcomes with good accuracy (AUC = 0.84; Fig. 4). We also studied the added value of metabolites over the established clinical prognostic model CRASH (MRC Crash Trial Collaborators et al., 2008) using ordinal multivariable regression. The models were fitted using the Turku data. In external validation in the Cambridge cohort, the AUC of the CRASH model was 0.736. When examining the top ranking metabolites together with the CRASH model (with metabolites selected based on FDR q-value; Supplemental Table 3; i.e. not optimized together as in the PLS-DA model), the best model in external validation setting was achieved for a combination of the clinical model with decanoic acid and pentitol-3-desoxy (AUC = 0.801 in

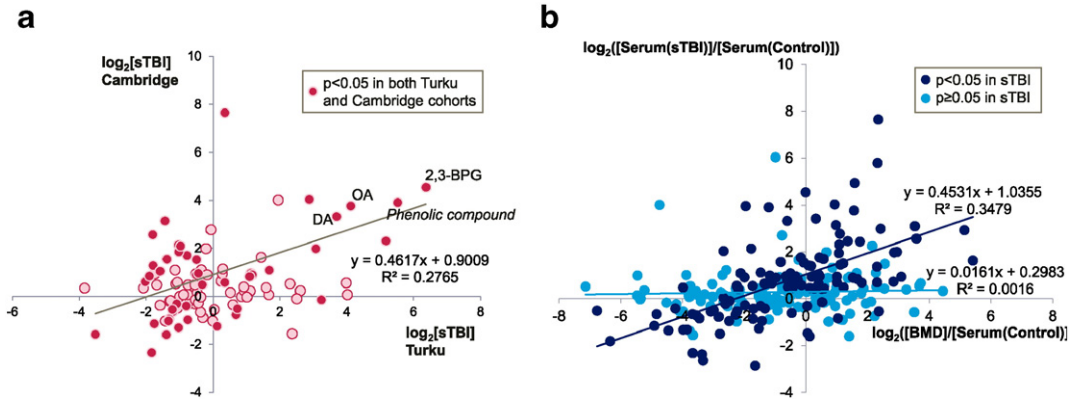


**Fig. 3.** Serum metabolites found associated with TBI display similar associations in an independent cohort and are found enriched in brain microdialysates. (a) Scatter plot of log2 scaled mean metabolite levels in sTBI patients from the cities of Cambridge vs. Turku, with significant metabolites in both groups marked with full circles. Regression line is drawn based on significant metabolites in both groups. (b) Comparison of mean serum metabolite level changes (sTBI vs. Controls; Turku) and mean brain microdialysate (BMD) metabolite levels (normalized to mean serum metabolite levels in Controls from Turku), shown as a scatter plot of all metabolites detected both in serum and BMD. The metabolites that are found significantly associated with TBI in serum (dark blue circles) display a close association with BMD levels, while the non-associated metabolites (light blue circles) do not.

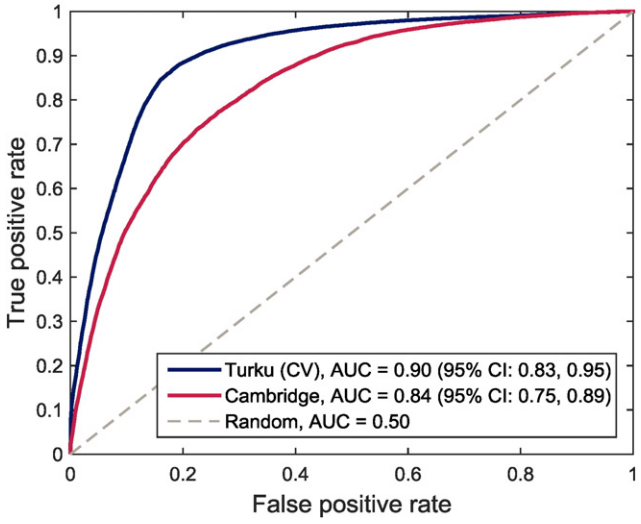


**Fig. 4.** Serum metabolite levels at the time of hospitalization predict the patient outcomes in TBI. Performance of the model to predict patient outcomes in TBI, shown as ROC curves for the training (Turku) and validation (Cambridge) data. Abbreviation: AUC, area under the ROC curve.

external validation; $p < 0.0001$) – significantly improving the predictive performance over the clinical model. The retention indices and mass spectra for the two metabolites are shown in Supplemental Table 6. The predictive performance of a model comprising the two metabolites alone was similar as for the clinical model (AUC = 0.740 in external validation). Using the univariate model fitted to Turku data, individual performance of the two selected metabolites as outcome predictors was assessed in the validation setting. The two metabolites showed good performance, with AUC = 0.648 ($p < 0.0001$) for decanoic acid and AUC = 0.637 ($p < 0.0001$) for pentitol-3-desoxy.

## 4. Discussion

Although several circulating peptide or protein based biomarkers for TBI have been proposed, these generally lack sensitivity and specificity. Here we showed that specific circulating blood metabolites are associated with severity of TBI as well as predict the patient outcomes.

We found that two medium-chain fatty acids (OA and DA) are elevated in TBI as well as associated with poor outcomes in TBI patients. Medium-chain fatty acids readily penetrate the BBB (Oldendorf, 1973) and there is evidence that FAs can be transported in both directions through the BBB (Spector, 1988). A recent study of free fatty acids in plasma and brain, following intravenous human mesenchymal stem cells transplantation into rats that had undergone transient middle cerebral artery occlusion, showed upregulation of several FAs in both blood and brain, including OA, whereas the levels of long-chain FAs were significantly reduced (Paik et al., 2009). Mitochondria play a crucial role in the pathophysiology and energy crisis associated with the brain injury. OA and DA provoke mitochondrial dysfunction by acting as uncouplers and metabolic inhibitors of the oxidative phosphorylation (Schuck et al., 2009b). Several studies have also shown that DA and OA play a role in brain energy metabolism (Ebert et al., 2003) and elicit lipid and protein oxidative damage (Green and Reed, 1998; Reis de Assis et al., 2004; Schuck et al., 2009a).

The observation of increases in plasma 2,3-BPG in the TBI patients, which was unrelated to the patient outcome, is difficult to interpret for two reasons. First, blood concentrations of 2,3-BPG are dominated by the concentration of this metabolite in red blood cells (RBCs), where it acts as an allosteric modulator of oxygen affinity (Hsia, 1998) and is produced through the effect of BPG mutase on 1,3-BPG (a glycolytic intermediate). It is therefore likely that plasma levels may be influenced by the dominant effects of RBC 2,3-BPG, but the relationship between the levels of this metabolite in the two compartments is unclear. Second, levels of 2,3-BPG in RBCs are modulated by physiological influences, including anemia, hypoxia and changes in partial pressure of carbon dioxide (PaCO2), all of which are common in TBI. These factors make it difficult to identify the drivers of changes in serum 2,3-BPG, but may explain why the metabolite had no prognostic relevance.

Several other metabolites, which are present at high concentrations in cerebrospinal fluid (Hartonen et al., 2013) and BMD, were also upregulated in serum of the TBI patients, indicative of disruption of the BBB. These included sugar derivatives, metabolites related to energy metabolism as well as several hydroxyl acids. Elevated levels of sugar derivatives are consistent with earlier studies which have shown that cerebral glucose metabolism is severely disturbed after TBI (Clausen et al., 2011; Glenn et al., 2003; Hovda et al., 1992).

Although multiple amino acids were found to be reduced in the plasma of TBI patients when compared to controls, we were not able to confirm earlier findings that the levels of branched chain amino acids (BCAAs) are reduced in TBI (Jeter et al., 2013); which may have been due to the high variability of BCAA levels due to dietary status not being controlled for. The indole derivative IPA, a deamination product of tryptophan formed by gut microbiota (Wikoff et al., 2009), was downregulated in severe as well as mild TBI patients. IPA has been shown to prevent oxidative stress and death of primary neurons and neuroblastoma cells exposed to the amyloid beta-protein (Chyan et al., 1999). IPA also shows a strong level of neuroprotection in two other paradigms of oxidative stress (Hwang et al., 2009; Karbownik et al., 2001). Our findings thus suggest an intriguing possibility that specific circulating metabolites are transported to the injured brain in order to maintain normal brain metabolism.

The mean age of the patients in Turku cohort was higher than in Cambridge cohort. Consequently, the mean GOSe value was lower in the Turku cohort, which is in accordance with previously published studies reporting that older age predicts poor prognosis in patients with TBI (MRC Crash Trial Collaborators et al., 2008; Takala et al., 2016). The difference in the cohort mean ages likely weakens the total accuracy of the predictive outcome model. However, the added value of two among the top ranking metabolites from Turku cohort significantly augmented the performance of clinical multivariate model (CRASH) including age in Cambridge cohort. Considering the discovery and validation design of the study comprising two distinct TBI cohorts, the metabolite model appears to be potentially valuable in the clinical practice.

As a methodological limitation of the present study, several of the metabolites of interest remained unidentified. However, using our analytical approach (Castillo et al., 2011), we were able to annotate most of them by their chemical class. Based on the acquired spectra and chromatographic information such as retention indices, these metabolites can still be followed-up in the future when better analytical tools and/or spectral library information become available. In order to develop a diagnostic assay based on the biomarker panel identified in our study, the metabolites will also have to be absolutely quantified and then assayed in larger TBI and control populations. This would serve not only to validate the findings, but also to derive a more precise diagnostic model based on the biomarker panel as well as other relevant clinical information such as from the CRASH model.

Taken together, we show that TBI is associated with a specific metabolic profile, which is exacerbated proportionally to the severity of TBI. High degree of concentration changes of TBI metabolites in serum as well as their proportional enrichment in BMD is indicative of multiple underlying mechanisms including potential disruption in BBB as well as protective response and altered metabolism due to head trauma. Our findings also suggest that these metabolites may exhibit diagnostic potential in the full spectrum of TBI. However, further validation studies including metabolic profiles in patients with polytrauma are needed to find out to what extent the observed results reflect systemic responses to severe injury and to what extent they are brain or TBI-specific.

## Declaration of Interests

The authors have no competing financial interest to declare.

## Author Contributions

M.O., O.T. and T.H. devised the study. M.O., D.M., O.T. and T.H. designed the study protocol. Patient recruitment, blood sample collection, and clinical data collection were performed by J.P.P., R.S.K.T., A.J.K., H.A-S., A.K., H.-R.M., J.T., J.P.C., I.H. and J.F. K.L.H.C. and P.J.H. performed brain microdialysis studies. I.M. and S.J. performed metabolomics analyses at VTT Technical Research Centre of Finland (Espoo, Finland). I.M. and T.H. processed the metabolomics data. M.O., M.K.-N., H.F.L. and T.H. analyzed the data. T.H. performed metabolite identification. M.O. and T.H. drafted the first versions of the paper with critical contributions from J.P.P., D.M. and O.T. All authors reviewed, edited and approved the final version.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.07.015.

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