# Metabolomics profiling as a diagnostic tool in severe traumatic brain injury

**Review Article**

**Running title:** Metabolomics in severe traumatic brain injury

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**Abstract**

Traumatic brain injury (TBI) is a complex disease with a multifaceted pathophysiology. Impairment of energy metabolism is a key component of secondary insults. This phenomenon is a consequence of multiple potential mechanisms including diffusion hypoxia, mitochondrial failure and increased energy needs due to systemic trauma responses, seizures or spreading depolarization. The degree of disturbance in brain metabolism is affected by treatment interventions and reflected in clinical patient outcome. Hence, monitoring of these secondary events in peripheral blood will provide a window into the pathophysiological course of severe TBI.

New methods for assessing perturbation of brain metabolism are needed in order to monitor on-going pathophysiological processes and thus facilitate targeted interventions, and predict outcome. Circulating metabolites in peripheral blood may serve as sensitive markers of pathological processes in TBI. The levels of these small molecules in blood are less dependent on the integrity of the blood-brain-barrier and recently characterized glymphatic system, as compared to protein biomarkers. We have recently characterized a specific metabolic profile in serum that is associated with both initial severity and patient outcome of TBI. We found that two medium-chain fatty acids, octanoic and decanoic acids, as well as several sugar derivatives are significantly associated with the severity of TBI. The top ranking peripheral blood metabolites were also highly correlated with their levels in cerebral microdialysates. Based on the metabolite profile upon admission, we have been able to develop a model that accurately predicts patient outcome. Moreover, metabolomics profiling improved the performance of the well-established clinical prognostication model.

In this review, we discuss metabolomics profiling in patients with severe TBI. We present arguments in support of the need for further development and validation of circulating biomarkers of cerebral metabolism and for their use in assessing patients with severe TBI.

## 1. Introduction

The primary brain injury results in a complex series of events, which generate a secondary brain injury process. Different insults belonging to a secondary brain injury are aggravated by impairment of energy metabolism that is a consequence of multiple potential mechanisms including both hypoxia and diffusion hypoxia (1, 2), increased nonoxidative processes (3), mitochondrial failure (4) and increased energy needs due to systemic trauma responses, seizures or spreading depolarization (5, 6). The condition is further convoluted by heterogeneous temporal evolution of brain injury and individual differences between the patients. (7, 8) The degree of disturbance of brain metabolism after TBI is also affected by treatment interventions, which are reflected in clinical patient outcome. Even though the sustained metabolic crisis in the brain is mostly unsolvable by neurotrauma resuscitation and rigorous intracranial pressure (ICP) control (9-11), the monitoring of secondary brain injury events provides insight into early physiological insults experienced by the brain. It also provides an opportunity to treat physiological disturbances and predict the later pathophysiological course of TBI.

Sedation and neuromuscular blockade in the neurocritical care setting limits the ability of clinicians to obtain a reliable neurologic examination. Additionally, clinically observed deterioration often occurs as a late manifestation of secondary brain injury process. Multimodal neuromonitoring helps to detect, and in many circumstances to treat, cerebral ischemia. By monitoring invasively ICP, brain tissue oxygenation (PbtO2), cerebral perfusion autoregulation with the pressure reactivity index (PRx), and continuous electroencephalography (cEEG), it is possible to assess and follow the gross physiology within the brain after severe TBI. (12) Cerebral microdialysis provides a window into the underlying cellular metabolism of injured neurons by assessing the lactate/pyruvate ratio. An increase in this ratio reflects a decrease in the oxidative mitochondrial metabolism and mitochondrial failure. Thus, the basis for obtaining tissue metabolic data rests on the need for detecting a transition from

aerobic to anaerobic metabolism. The switch to anaerobic metabolism is associated with poor neurological outcome in patients with TBI. (5, 13, 14)

Cerebral microdialysis can be regarded as a type of targeted metabolomics study, but there are also other means of assessing a vast spectrum of endogenous compounds with small molecular mass that serve as substrates and intermediates of biochemical pathways in the human body. In the continuum of expansion of 'omics' in biomedical research, the global study of metabolism at the molecular level, metabolomics, has enabled simultaneous determination of thousands of small molecules at various levels of cellular function due to the advances in systems biology. There is an on-going paradigm shift towards knowledge-based systemic 'omics' studies leading to comprehensive metabolite profiling and fingerprint diagnostics in contrast to current hypothesis-driven research. (15, 16)

Herein we discuss metabolomics approach as a diagnostic and biomarker discovery tool for the injured brain after severe TBI.

## 2. Metabolomics

### 2.1 Clinical need for new blood-based biomarkers of severe TBI

TBI has been a clinically challenging problem for several reasons, including poorly understood complex pathophysiology that behaves unpredictably, and vast patient and injury heterogeneity. There are a number of sensitive organ-based biomarkers in clinical use for medical emergencies (17) and oncology diagnostics (18). Accordingly, similar markers for TBI have been searched for in order to assess the nature and severity of the injury and patient outcome. (19, 20)

For an ideal universal molecular biomarker of TBI, the compound should be readily measurable in peripheral venous blood or non-invasively collected biological fluid, as diagnostics from cerebrospinal fluid or cerebral microdialysates are too invasive methods in evaluating mild or moderate TBI. Moreover, severe TBI sets special requirements for biomarkers. The usefulness of a

biomarker in severe TBI is theoretically based on its ability to distinguish initially subtle changes, such as transition to mild cerebral energy crisis or regional swelling, before converting to global cerebral energy failure and uncontrollable ICP elevation. Depending on the nature of the diagnostic aim, a biomarker of TBI should be able to confirm the presence or absence of TBI, assess the severity and nature of TBI, monitor treatment effects, and predict outcome. Furthermore, validation of a biomarker will require that it needs to be linked to established clinically relevant indicators of disease severity, e.g., Glasgow coma scale (21), acute imaging findings (such as acute head computed tomography or magnetic resonance imaging), brain tissue fate as assessed with different methods (22-24), or outcome (25).

It appears highly unlikely that a single biomarker could accurately describe these different clinical needs in a case of severe TBI at the emergency department and intensive care unit. This is because patients and injuries are highly heterogeneous and there is significant uncontrolled variability even within the same category of TBI severity, merely as assessed by rough clinical measures. In the case of an extremely complex disease, such as severe TBI, also the inherent variability needs to be taken into account, because the molecular biomarkers might not be fundamentally related to TBI but rather to normal and reactive physiological processes and protective responses, such as those related to age, gender, diet, CNS comorbidities, and extracranial injuries. Therefore, instead of measuring a single TBI-sensitive biomarker, there is a need for comprehensive injury-sensitive biochemical profiling and individual fingerprint diagnostics.

### 2.2 Metabolomics as an opportunity for TBI diagnostics

Metabolomics is a global approach to study the structure, function, and interactions of low molecular weight metabolites in cells, tissues, and biofluids. (26) Unlike in the setting of protein diagnostics, the metabolic profile is a snapshot that provides a window into the *in vivo* enzymatic activity of the brain and body, because free metabolite concentrations affect and are affected by the global metabolic activity. (27, 28) Metabolites can be studied and compared between physiological and

pathophysiological conditions, allowing better and more comprehensive understanding of disease processes.

As simultaneous determination of a plethora of molecules has become possible due to the new analytical technologies in systems biology, metabolomics enables a conception of biological organism as network of interacting cells and their metabolites. Given the highly complex nature of the human brain, metabolomics can be utilized to address the biomolecular interaction networks of the brain in health and disease. (16, 29)

Protein-based biomarkers have partially failed to fill the expectations in diagnostics of TBI. Their problems have been one-dimensional diagnostic perspectives, sensitivity and specificity for TBI and brain, and the inability to pass an intact blood-brain-barrier (BBB). The variability in dysfunction of the BBB as a result of TBI strongly affects the performance of proteins to serve as reliable biomarkers of intracranial events. Small molecules with molecular mass under 1000 Da are more readily able to pass an intact BBB and are thus much more independent from fluctuating and immeasurable confounders related to BBB dysfunction, which is one of the cornerstones of TBI pathophysiology. (30) As metabolic profiling can detect and measure a large number of substances, it may enable accurate characterization and stratification of the TBIs for targeted therapies.

Intracranial dynamics is efficiently monitored by the current methods such as invasive monitoring of ICP, PRx and $PbtO_2$, reflecting global and regional changes in patients with severe TBI. The validated methods for monitoring metabolic crisis in the brain following severe TBI have been brain microdialysis, arterio-jugular venous differences, and positron emission tomography, of which the first-mentioned is not universally available and the latter is neither universally available nor suitable for patients with unstable or intractable ICP.

### 2.3 Technology and statistical methods used in metabolomics diagnostics

Several techniques for metabolomics have been applied in discovery and analysis of different biomarkers. Most methods are based on mass spectrometry (MS), typically combined with chromatographic separation techniques, such as gas or liquid chromatography (GC or LC). Also proton nuclear magnetic resonance ($^1$H-NMR) has been widely applied. However, because its poorer sensitivity (micromolar concentration range), it is not so useful for biomarkers of TBI which are typically present at lower levels (picomolar to nanomolar) than measurable by NMR. The advantage of NMR over MS based methods is the relative simplicity of the sample preparation required. Additionally, $^1$H-NMR suffers less from batch-to-batch variation observed in global MS based approaches. However, another key limitation to $^1$H-NMR is the resolution of the resultant spectra. Typically, metabolomic profiling occurs on high field systems (600 MHz and above) but there is still significant overlap of the peaks. (31) This leads to problems with metabolite identification, even if two-dimensional experiments are performed. (32) Furthermore, it makes interpreting the increase in NMR signals difficult as it is often unclear which metabolite causes the increase in signal if multiple metabolites overlap. The MS based methods require sample preparation, which is typically based on extraction or protein precipitation.

In metabolomics, there are basically two types of methodologies used, namely untargeted and targeted analyses. Untargeted analyses are typically applied in biomarker discovery studies, and they correspondingly aim at analyzing of comprehensive metabolic profiles. These methods are usually semi-quantitative, i.e., relative concentrations of metabolites are determined between the study groups. The targeted analyses are generally quantitative, and they are limited to the analysis of specific target metabolites. To achieve quantification in a MS based assay you need to run a standard curve of the know metabolite and then relate the area or peak height detected in the MS back to a known concentration. When profiling all metabolites in a bio fluid it is not possible to have a complete set of pure compounds to generate these standard curves. Therefore, class based internal standards are used which allows a relative concentration to be calculated. This is known as semi-quantification.

In the more targeted approach where the number of metabolites being analyzed is smaller it is possible to generate standard curves for all metabolites. You can then use an appropriate internal standard to correct for matrix effects during the run.

In an untargeted approach, it is still not possible to analyze the whole metabolome with a single method, because of the large diversity of the metabolites, both in terms of chemical diversity and concentration range. First of all, it is not possible to extract both hydrophilic and hydrophobic metabolites with a single method in a robust manner. In untargeted analysis, the most common approach is to use either GC or LC combined with MS. GC based systems are suitable for volatile and semi-volatile metabolites, while LC can in principle be used for all types of metabolites. The advantages of GC based systems are good separation efficiency, capability of analysis of very polar and semi-polar analytes in a single method, and the availability of large commercial mass spectral libraries for the identification of metabolites. The main disadvantage of the GC based methods is the unsuitability for non-volatile metabolites, and that derivatization is needed for the analysis of polar compounds. The main advantage of the LC-MS methods, on the other hand, is the simpler sample preparation, and the applicability to a wide range of metabolites. However, the LC-MS suffers from matrix effects, which make the quantitation more challenging in untargeted methods as compared to GC-based methods. Matrix effects occur when multiple metabolites elute from the column at the same time causing ion suppression in the MS (ref: PMID 11073257). Therefore, depending on the sample and its preparation it is possible to get an apparent reduction in the specific metabolite concentration which prevents absolute quantitation (ref DOI https://doi.org/10.1016/j.trac.2004.11.021). To minimize these matrix effects it requires the presence of a heavy isotope standard which elutes at the same time as the metabolite being studied (ref: Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC− MS/MS). These heavy isotope standards are not always available for the whole metabolome and if they are to have one for each metabolite would be prohibitively expensive. With LC, high-resolution accurate MS systems capable of tandem mass

measurements have been most commonly applied for untargeted metabolomic analyses, particularly using quadrupole time-of-flight MS (QTOFMS) and Orbitrap MS systems. With GC, TOFMS and QTOFMS systems are also widely applied for metabolomics, although the simple quadruple MS systems are still the most commonly employed method.

In untargeted metabolomics approaches, raw MS data first needs to be processed before it can be analyzed by statistical approaches. Several open source software packages have been developed for this purpose, and also, MS vendors currently offer their own solutions for metabolomics data processing. Among the open source tools, MZmine (33) and XCMS (34) have been the most commonly applied for LC-MS based approaches. Once the data processing step is complete and the data is made available, e.g. in the matrix format, the metabolic profiles can be studied by a variety of statistical approaches, depending on the experimental setting. The general statistical considerations for metabolomics (35) or any other high-dimensional 'omics' data (36) need to be applied. The univariate and multivariate methods applicable to metabolomics/lipidomics data analysis have been reviewed extensively. (37) One common pitfall when applying predictive modeling (e.g., for biomarker discovery) from multivariate data is the lack of proper validation. In the literature, the most commonly applied approach for this purpose is the partial least squares discriminant analysis (PLS-DA). (38) However, this approach suffers from the so-called overfitting, and the reported models if developed and tested on the same dataset tend to be over-optimistic, particularly if improper internal validation is applied. Ideally, the PLS-DA model (or those derived from other multivariate methods) needs to be tested and reported on an independent dataset, which has not been used for the model development. A typical workflow of a metabolomics study is demonstrated in Figure 1.

### 2.4 Metabolomics applied to diagnostics of patients with TBI

There are two studies that have utilized a lipidomics approach in TBI research: in a murine model (39) and in humans (40), while two studies have employed metabolomics approach in humans with TBI (41, 42).

Daley and colleagues reported that in adolescent male hockey players who had sustained a concussion, a set of metabolites relying notably on glycerophospholipids accounted for 82 % of the variance between 12 concussed and 17 non-concussed athletes. The group utilized $^{1}$H-NMR and a method using both direct injection and liquid chromatography combined with tandem MS. The two methods together cover amino acids, acyl carnitines, specific lipids and some amines (FIA/LC-MS) as well as glucose, specific hydroxyl acids and ketone bodies ($^{1}$H-NMR). The method combining multivariate statistical analysis and machine learning exhibited 92 % accuracy rate in diagnosing a concussion. (41) However, the possible cofounding factors, such as diet, time from last meal or BMI were not accounted for in the statistical analyses, and the results have not been independently validated in another study group.

TBIcare investigators and Turku Centre for Biotechnology Systems Medicine research group applied comprehensive metabolic profiling of serum samples from two large independent cohorts of patients with full spectrum of TBI and orthopedic injuries. (42) Serum metabolomic profiles from 144 patients with mild, moderate or severe TBI were investigated. A control group comprised of 28 patients with acute orthopedic injuries without an acute or earlier TBI. The samples were taken upon admission to emergency department (<12 h after the injury). Two-dimensional GC coupled to time-of-flight MS was utilized to analyze the serum samples. The metabolite profiles of the four patient groups were compared to an independent validation cohort from Addenbrooke's Hospital (Cambridge, UK) comprising of 67 patients with TBIs of all severities and patients with orthopedic injuries. Decanoic and octanoic acid, which are medium-chain fatty acids, and sugar derivatives including 2,3-bisphosphoglyceric acid were strongly associated with the severity of TBI. Brain microdialysates were also analyzed from 12 samples acquired from patients with severe TBI in the validation cohort, in order to compare the significant serum metabolites with brain extracellular metabolites. The levels of top-ranking serum metabolites associated with TBI correlated highly with their levels in brain microdialysates, thus suggesting disruption of the BBB. As a second main aim of the study, a

prognostic model was developed to discriminate patients with favorable (Glasgow Outcome Scale extended 5-8) and unfavorable (Glasgow Outcome Scale extended 1-4) outcome. In the discovery cohort, the performance of the model reached an area under curve (AUC) of 0.90 (95% CI 0.83-0.95) and in validation cohort an AUC of 0.84 (95% CI 0.75-0.89). The added value of the prognostic model was also studied together with the established CRASH prognostic model (43), comprising of clinical variables. The stand-alone AUC of the CRASH model was 0.74 in the validation cohort. When the top-ranking metabolites (decanoic acid and pentitol-3-desoxy) from prognostic metabolomic model were combined to CRASH model, AUC reached 0.80. The results demonstrate that TBI is associated with a specific metabolic profile, which is exacerbated proportionally to the severity of TBI.

**3. Concluding remarks**

A new era is emerging in the diagnostics of TBI. There is a paradigm shift towards comprehensive 'omics' studies leading to proteomics and metabolomics profiling and fingerprint diagnostics, in contrast to current clinical diagnostics with non-specific and unreliable clinical markers. In the future, a confluence of multi-time-point proteomics and metabolomics diagnostics and advanced imaging studies will highly likely offer precise stratification and outcome prediction, while individual point-of-care biomarker monitoring of the injured brain will provide means for assessment of intervention efficacy. The identified serum 'TBI metabotype' offers a new avenue for the development of next generation diagnostic, prognostic, monitoring and surrogate markers of broad spectrum of TBIs. The challenges in this work lie in validating the different metabolite panels for different clinical needs and at variable time points, in this vastly heterogeneous patient population.

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Figure 1. Overview of analytical strategies for metabolomics: There are three key phases required for the development of metabolomics biomarkers if they are to be used in a clinical setting. The initial discovery phase can capture a large number of metabolites using a combination of GC and LC based techniques. High separation efficiency preferably combined with high mass accuracy is important at this stage due to the high number of metabolites with similar masses. These techniques generate semi-quantitative measurements, which makes them unsuitable for clinical practice. These techniques can also identify unknown compounds, which potentially require time and extra experiments to identify. The validation stage is key to any biomarker study. Once the metabolites of interest have been identified from the discovery phase a more targeted method must be developed aimed at only quantifying these. This method needs to be applied to the original data as well as new independent samples to avoid overmodeling. Finally, a fully quantitative assay must be developed for use in the clinic. The analysis needs to be streamlined and user friendly to ensure cost efficiency. Key: GC – Gas chromatography; GCxGC – Two-dimensional gas chromatography; LC – Liquid chromatography; Q – Single quadrupole; QqQ – Triple quadrupole TOF – Time-of-flight mass spectrometry.