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Cerebrospinal fluid purinomics as a biomarker approach to predict outcome after severe traumatic brain injury

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Abstract

Severe traumatic brain injury (TBI) is associated with high rates of mortality and long-term disability linked to neurochemical abnormalities. Although purine-derivatives play important roles in TBI pathogenesis in preclinical models, little is known about potential changes in purine levels and their implications in human TBI. We assessed cerebrospinal fluid (CSF) levels of purines in

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[11]Author's contributions:

NRS, MAS, JPO, DHS, and LVP built the study concept. MAS and AEB were involved in the patients' clinical management and collected the samples and data. LVP, NRS, JPO, and MAS designed the study and investigation. MSR, AK, GH, VGO, JVP, and ETS prepared samples, performed the HPLC procedures, analyzed the data, and created the figures. NRS and LVP wrote the first draft of the manuscript, and all authors reviewed and edited the final manuscript. DHS and LVP acquired funding. An early preprint version of this manuscript was submitted to MedRxiv on October 26th, doi: https://doi.org/10.1101/2021.10.20.21265297.

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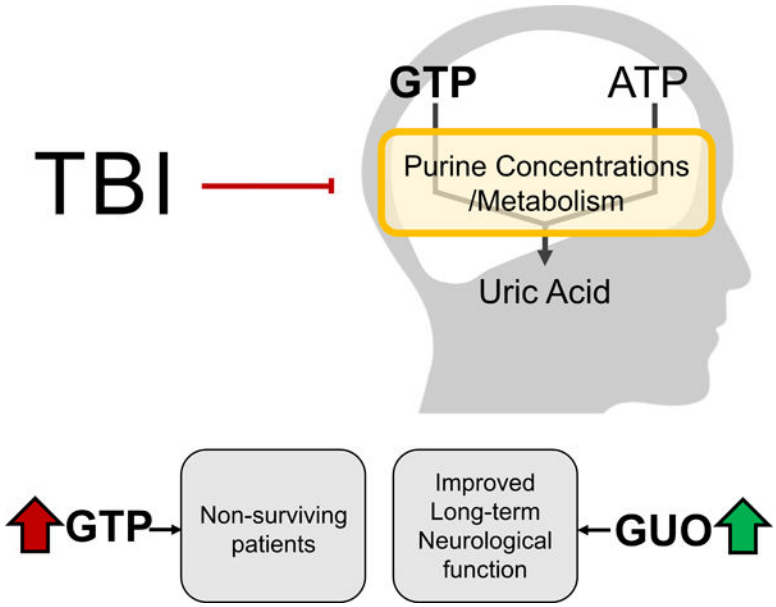
severe TBI patients as potential biomarkers that predict mortality and long-term dysfunction. This was a cross-sectional study performed in 17 severe TBI patients (Glasgow Coma Scale < 8) and 51 controls. Two to four hours after admission to ICU, patients were submitted to ventricular drainage and CSF collection for quantification of adenine and guanine purine-derivatives by HPLC. TBI patients survival was followed up to 3 days from admission. A neurofunctional assessment was performed through the modified Rankin Scale (mRS) two years after ICU admission. Purine levels were compared between control and TBI patients, and between surviving and non-surviving patients. Relative to controls, TBI patients presented increased CSF levels of GDP, guanosine, adenosine, inosine, hypoxanthine, and xanthine. Further, GTP, GDP, IMP, and xanthine levels were different between surviving and non-surviving patients. Among the purines, guanosine was associated with improved mRS ($p=0.042$; $r= -0.506$). Remarkably, GTP displayed predictive value (AUC=0.841, $p=0.024$) for discriminating survival vs. non-survival patients up to three days from admission. These results support TBI-specific purine signatures, suggesting GTP as a promising biomarker of mortality, and guanosine as an indicator of long-term functional disability.

Graphical Abstract



Severe Traumatic Brain Injury (TBI) is a major cause of death worldwide and is also associated with long-term neurological deficits. The availability of complementary prognostic tools to identify TBI patients at risk of death, or delayed unfavorable neurological outcomes is scarce. We identified that TBI altered the concentration of specific purines in the CSF, which represents a neurobiological signature with potential clinical and metabolic relevance. Particularly, increased GTP level, a guanine-derived purine, displayed value as a biomarker for mortality after TBI, and the nucleoside guanosine, also derived from guanine, was associated with impaired two-years neurological function.

Keywords

CSF nucleotides derivatives; GTP; guanosine; IMP; biomarkers; severe traumatic brain injury

1. Introduction

Traumatic brain injury (TBI) is considered a major health concern with a high incidence of disability worldwide, and it is the leading cause of mortality among young adults from low- and middle-income countries (Nguyen *et al.* 2016). In severe TBI, immediate mechanical damage to brain tissue can be life-threatening (Kuenzler *et al.* 2015) and can trigger a series of secondary neurochemical cascades that further damage the brain. Some of the most well-characterized of these processes include excitotoxicity, formation of proteotoxic aggregates, neuroimmune dysfunction, and changes in brain metabolism (Doran *et al.* 2019; Stefani *et al.* 2017; Johnson *et al.* 2013; Johnson *et al.* 2012; Lyons *et al.* 2018; Henry *et al.* 2020). However, while adenine- and guanine-based purines have been shown to play important roles in the pathogenesis of other brain disorders, little is known about their contribution to the post-TBI sequelae.

Beyond their central role in the formation of nucleic acids and bioenergetics, purines have a wide range of neuromodulatory effects that may be disrupted in various neurological disorders. Indeed, purinergic receptors have been explored as therapeutic targets in animal models of epilepsy, amyotrophic lateral sclerosis, Alzheimer's, and Parkinson's disease (Burnstock 2020; Burnstock 2017). An important feature of normal purine-mediated brain responses is the strict control of their individual concentrations, performed by intra- and extracellular enzymes (Robson *et al.* 2006). In particular, the Ecto-NTPDases are a family of enzymes responsible for the extracellular degradation of purines, thereby controlling binding to specific purinergic receptors and the magnitude of receptor activation (Robson *et al.* 2006). This includes the stepwise extracellular degradation of ATP and GTP, resulting in the formation of ADP and AMP, or GDP and GMP, respectively. Further catabolism leads to correspondent nucleosides adenosine and guanosine, which consequently generate IMP, inosine, hypoxanthine, xanthine, and uric acid (Pelligrino *et al.* 2010; Cunha 2016; Zeng *et al.* 2018). Purine-derived metabolites also cross plasma membranes through specific transporters, influencing their intra- and extracellular levels (Beal *et al.* 2004). Remarkably, from the extracellular triphosphate forms to their downstream metabolites, each purine derivative may exert characteristic physiological roles in the brain mediated by purinergic receptors and GTPase-activating proteins, neuroinflammatory effectors, and glutamatergic neurotransmission. Hence, changes in purine levels in the brain after TBI could play an important role in TBI-related outcomes.

While clinical studies on purines in TBI have been limited, Headrick and colleagues demonstrated that acute increases in extracellular adenosine levels were associated with impaired neuroenergetic and neurological functions in a preclinical rat TBI model (Headrick *et al.* 1994). In addition, inhibition of adenosinergic signaling with caffeine has been shown to decrease the mortality rate of rats submitted to severe TBI (Lusardi *et al.* 2012). Furthermore, intraperitoneal administration of guanosine after experimental TBI attenuated cognitive and mitochondrial dysfunction (Gerbatin *et al.* 2017; Dobrachinski *et al.* 2019). Finally, *in vivo*, extracellular levels of adenosine, inosine, and hypoxanthine were found to increase soon after TBI, which was mechanistically linked to energy failure mirrored by the rapid ATP catabolism to adenosine (Bell *et al.* 1998). Although preclinical studies have

already unveiled associations between TBI and purine metabolism regarding neuroprotective properties, the potential clinical relevance of extracellular purine levels and interconversion, as prognostic biomarkers have received little attention in the TBI settings..

In this study, we assessed cerebrospinal fluid (CSF) levels of purines in severe TBI patients in order to identify specific signatures associated with mortality and long-term neurological dysfunction.

2. Subjects and Methods

2.1. Study population and clinical management

This is a single-center cross-sectional study, carried out in the Emergency Unit of the Cristo Redentor Hospital (Porto Alegre, RS, Brazil). This study included a total of 21 consecutive severe TBI patients, presenting on hospital admission both Glasgow Coma Scale (GCS) ≤ 8 and an abnormal brain CT scan (Böhmer *et al.* 2011; Stefani *et al.* 2017), and due to the exploratory nature of this study, no sample size calculation was performed, and the sample size was based in previous studies exploring TBI biomarkers (Stefani *et al.* 2017; Böhmer *et al.* 2011). Power analysis performed *a posteriori* through G*Power 3.1 software for our primary outcome, CSF purines as biomarkers of mortality in TBI (namely GTP), showed a power of 0.9851 (98.5%). Our secondary outcome, purines as prognostic biomarkers of long-term neurological function (namely guanosine), showed a power of 0.8411 (84.1%). TBI Patients were predominantly young (mean [SD] age, 29 [13] years; M/F ratio, 9:1), with an average Glasgow Coma Scale upon admission of 6, and Intracranial pressure of 12.89 [11.92] (mean [sd]). Inclusion criteria were an isolated severe TBI, with no previous history of comorbidities that could influence the biomarkers' concentrations and clinical outcomes. Exclusion criteria and clinical management were previously reported (Stefani *et al.* 2017; Böhmer *et al.* 2011). CSF was collected between 2 and 4 h after hospitalization through an intraventricular catheter.

Additionally, 51 healthy subjects (ASA I status) scheduled for elective urological, gynecological, general, or vascular procedures were selected as age and sex-matched controls (Böhmer *et al.* 2011). Control subjects were predominantly young (mean [sd] age, 27.60 [6] years, M/F ratio, 8:1). Experienced anesthesiologists collected the CSF after successful subarachnoid puncture and before the intrathecal injection of anesthetics or analgesics. The anesthetic procedure was standardized, all patients received a combined spinal injection, at the level of L 3⁄L 4, of hyperbaric bupivacaine 0.5% (10 to 20 mg), associated with fentanyl 20 μg and/or morphine 100 μg, depending on the surgical procedure. The first 0.5 mL of CSF aspirated was discarded, and the subsequent 0.5 mL sample was collected and inspected visually for blood contamination.

Immediately after CSF collection, the samples from controls and patients were centrifuged at 10,000 x g for 5 min to obtain a supernatant free of cells and cellular debris. The supernatant was then transferred to sealed plastic tubes and stored at −70°C within 30 min of collection. Up to 48 hours from collection, CSF samples were thawed over ice for purines assessment.

Written informed consent for participating in this study was obtained from patients' family members and directly from healthy individuals, according to the Declaration of Helsinki. Concomitantly, family members were questioned about the patient's lifestyle and pre-existent diseases. This study was not pre-registered and was approved by the local institutional Ethics Committee approved this protocol (project number 0038.0.164.165–05).

2.2. Outcome measures

The primary patient outcome was defined accordingly: deterioration to brain death (non-survival n = 6) or survival (survival, n = 11), within 3 days after hospital admission. Deterioration to brain death occurred up to 3 days after admission to the ICU in non-surviving patients. The ICP, hemodynamic, and metabolic variables including mean arterial blood pressure and cerebral perfusion pressure were assessed daily and reported previously (Böhmer *et al.* 2011). Two years after discharge, telephone calls were placed to confirm survival, and investigate the level of long-term functional disability. TBI patients were then assessed for the modified Rankin Scale (mRS), the secondary outcome, and scored by an experienced neurologist. The mRS ranks disability following stroke and cerebral injuries, ranging from 6 (dead) to 0 (fully independent), and is considered a reliable endpoint for clinical neurological studies.

2.3. CSF Purinomics

We carried out measures in 10μL CSF aliquots at a Shimadzu Class-VP high-performance chromatography system (HPLC) in which the separation of the adenine- and guanine-based purines was achieved with a Supelcosil™ LC18 250mm, 4.6 mm diameter size (Sigma Aldrich, 2011, Catalog number: 58298), as previously described by our group and others (Schmidt *et al.* 2015; Oses *et al.* 2007; Domanski *et al.* 2006). Absorbance was read at 254 nm, and quantification of all purines from each subject was obtained in a single run.

The following purines and metabolites were determined: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), guanosine (GUO), inosine monophosphate (IMP), inosine (INO), hypoxanthine (HXN), xanthine (XAN), and uric acid (UA). HPLC procedure was performed simultaneously between control and TBI samples in a randomized manner. Collection of samples from control and TBI patients was performed concomitantly, patient information from each sample was stored, anonymized, and sequentially assigned an internal identification number that did not indicate grouping or outcome, randomly assigning sequential numbers to control and TBI patients. HPLC procedure was performed utilizing the sequential identification numbers by a researcher blinded to patient information. The acquired data was stored in a data bank for future comparisons.

2.4. Statistical analysis

Experimental outline, with included patients, and a step by step schematic of data analysis is presented in Figure 1. Before performing statistical analysis, obtained data was submitted to Kolmogorov-Smirnov testing for normality, and purine levels of controls and patients were screened for mathematical outliers. Subjects that presented purines value above 1.5-fold

of interquartile range from the median, or 3-fold of standard deviation from mean were excluded from all analyses (Mowbray *et al.* 2019). A total of 4 patients were excluded from all analyses due to outlier levels of purines. Excluded patients frequently presented outlier values for more than one purine concurrently, with the purines that most diverted from average values being AMP (Patient #4: 11.85μM), Inosine (Patient #3 33.10μM and Patient #7: 23.70μM), and Uric acid (Patient #14: 314μM). A complete report of purine concentrations of excluded patients is available as supplementary table 1." Data were analyzed using RStudio (1.2.5001), and associated packages were further indicated. Data are presented as mean ± SD, or median ± IQR and the graphical representation was made using GraphPad Prism 8. Comparison between groups was performed by two-tailed Student's independent t-test or Mann-Whitney test. Receiver Operating Characteristic (ROC) curves were created to explore the ability of biomarkers to predict survival, indicated by the Area Under the Curve (AUC). The cutoff value was calculated by the Youden method, using the R package "*OptimalCutpoints"* (López-Ratón *et al.* 2014). Multivariate correlations between all purines and clinical data were assessed through the Spearman correlation test, with corrections by Bonferroni, using the R package "*Psych"* (Revelle 2018) for statistical testing, and "*Corrplot"* (Wei and Simko 2017) for graphical representation.

The multivariate correlation of purine derivatives was utilized to create a model, that superimposes correlations with the classical cascade of purine degradation obtained from the KEGG database (Kanehisa 2019; Kanehisa and Goto 2000).

Briefly, based on the significant purine correlations present in controls, but absent in TBI patients, we obtained a list of correlations that were impaired by TBI. Within this list of correlations impaired by TBI, we selected only the correlations between purines that both presented altered concentration in TBI patients (Highlighted in the model by arrowheads). We then graphically present these impaired correlations over the theoretical enzymatic degradation cascade of purines. The concept behind this model is to provide a topographical overview of the impact of TBI on the purine interconversion and profile.

For all tests, the statistical significance was considered when $p \leq 0.05$.

3. Results

3.1. Severe TBI triggers specific changes in CSF purine metabolites

Among guanine-derived purines, GDP and guanosine were significantly increased in TBI patients, compared to controls (Figure 2A and B, and Table 1), while no difference was observed in GTP and GMP levels (Supplemental Figure 1A and B). In non-surviving TBI patients, there were increased CSF concentrations of GTP, and GDP compared with surviving patients (Figure 3A and B, respectively). Concentrations of GMP and guanosine did not differ between surviving and non-surviving patients (Table 2, supplemental figures 2A and B).

Adenine-based purines, ADP and adenosine, were increased in TBI patients, relative to controls (Figure 2C and D, respectively, and Table 1), whilst no difference was observed in ATP and AMP levels (Supplemental figure 1C and D). Further, ATP, ADP, AMP,

and adenosine were not different between surviving and non-surviving patients (Table 2, supplemental figure 2C to F).

The downstream guanine and adenine purine metabolism converge to the formation of IMP, inosine, hypoxanthine, xanthine, and uric acid. While CSF levels of IMP were reduced in TBI patients, inosine, hypoxanthine, and xanthine were increased (Figure 2E to H, respectively, also Table 1). No significant difference was observed in uric acid CSF levels (supplemental figure 1E). Among TBI patients, IMP and xanthine levels were increased in non-surviving compared with surviving patients (Figure 3C and D). Similar levels of inosine, hypoxanthine and uric acid were found in both surviving and non-surviving patients (supplemental figures 2G, H, and I, respectively).

3.2. CSF purinomics yield prognostic signatures for neurological outcomes

Considering that four different purine metabolites presented statistical differences between surviving and non-surviving TBI patients, we investigated the accuracy of these metabolites as prognostic biomarkers for mortality due to severe TBI.

The AUROC indicates how well GTP, GDP, IMP, and xanthine can discriminate patients that will survive from patients that will die up to 3 days from the admission to ICU (Figure 4A to D). With a cut-off value of > 2.780, GTP provided 66.67% of sensitivity, and 100% of specificity (AUROC: 0.841, $p = 0.0237$, 95% CI, CL: 95.45% to 100%). GDP, IMP, and xanthine did not present statistically significant predictive values ($p > 0.05$). However, the AUROC values for IMP barely reached significance (p = 0.06).

Also, we performed bivariate correlations between CSF purine levels and clinical neurological outcomes. We found that the modified Rankin Scale (mRS) did not correlate with TBI severity at admission (GCS) (r: −0.32, p = 0.207). Only guanosine correlated with mRS (r: −0.50, p = 0.042) (Figure 3 A and B, respectively), with all remaining purines and derivatives not presenting statistical correlation with long-term neurological outcomes (supplementary figure 3). Clinical parameters measured at ICU such as the medium arterial pressure, and cerebral perfusion pressure were not statistically correlated to mRS (supplementary table 2).

3.3. Severe TBI disrupts CSF purinomic networks

Correlations between purines were assessed to estimate functional purinomic networks. Briefly, a stepwise metabolism of ATP generates ADP and AMP, and downstream metabolites, meaning that correlations between these molecules represent physiological interconnections. Here we show that TBI patients displayed a distinct profile of associations from controls, thereby suggesting a rupture in the physiological purinomic networks (Figure 6A and B).

Additionally, we propose a model based on the classical purine metabolism cascade, as described in the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. The model displays in the double-headed arrows correlations between purines present in controls, but absent in TBI patients (the color scales represent Spearman r correlation values). Here, we

intended to explore how TBI impairs interconversion of purines within the topographical map of purine metabolism (Classical degradation and purine salvage pathways).

In this model, we primarily showed that correlations between purines in controls subjects match the stepwise cascades of the classic purine degradation and salvage pathways. For instance, a negative correlation between ADP and GDP with INO is observed in control subjects. This finding fits the physiological degradation of ADP and GDP driving the formation of INO.

However, in TBI patients such correlations are lost, as indicated in our model by the colored dashed lines (Figure 6C). For instance, the negative correlation between guanosine (GUO) and xanthine (XAN) is not present in TBI patients, suggesting that this classical degradation step may be impaired following injury. Similarly, the absence of the positive correlation between hypoxanthine (HXN) and IMP may suggest an impairment in the salvage of HXN to IMP.

4. Discussion

This study provides the first evidence that examination of purine levels in CSF in the acute setting after TBI may serve as a promising biomarker in predicting outcomes. Such “purinomics” approach showed that CSF GTP levels assessed within a short time after ICU admission appears to be a prognostic indicator of death and survival outcomes. Also, guanosine levels were associated with the impairment in a functional neurological score (mRS) two years after ICU admission. Correlations between ATP- and GTP-derived purines, resulted in a mechanistic model that highlighted the impact of TBI on the enzymatic degradation of purines.

There is rapidly increasing interest in examining body fluids in search of biomarkers that diagnose pathological changes and predict outcomes after TBI (Atkinson *et al.* 2001; Zetterberg *et al.* 2013). The purinergic system has established components in the brain that share specific localization and functions with glutamatergic tripartite synapse, microglia, and oligodendrocytes (Burnstock 2017; Burnstock 2020). Among those components, are transporters, receptors, and Ecto-NTPDase (E-NTPDase) enzymes, which at the mechanistic level control the balance of extracellular purine levels, and consequently, their effects on specific extrasynaptic and synaptic players.

We have previously shown that after a single convulsive seizure in a rat model, CSF adenine and guanine E-NTPDase activities increased in a similar time profile to classical biomarkers of neuronal (NSE) and astrocyte (S100B) death, implying that such increased activities also reflected neural cell damage (Oses *et al.* 2004; Cruz Portela *et al.* 2002; Oses *et al.* 2007). Similarly, pentylenetetrazol-kindling rats showed alterations in adenine and guanine enzymatic degradation along with increased CSF GTP, GDP, ADP, and uric acid levels (Oses *et al.* 2007). Notably, it has been described that TBI promotes disturbances in purinergic signaling that may favor the development of epilepsy (Boison 2008; Fedele *et al.* 2005; Englander *et al.* 2003; Jackson *et al.* 2016). These findings suggest that an acute alteration in extracellular purine concentrations after TBI could influence neurological outcomes.

The findings of the present study demonstrate that several extracellular purines are altered after a severe TBI in patients, and may serve as brain biomarkers of both short- and long-term injury caused by mechanisms associated with glutamatergic excitotoxicity (Stefani *et al.* 2017). Remarkably, increased extracellular purine catabolism has the potential to generate endogenous neuroprotective anti-glutamatergic responses through adenosine and guanosine (Dunwiddie and Masino 2001; Schmidt *et al.* 2000), and an antioxidant defense through the uric acid. Accordingly, exogenous administration of guanosine after mild TBI in rats displayed neuroprotective properties (Courtes *et al.* 2020). However, considering the extravasation of active biomolecules caused by the rupture of cell membranes and axons (Johnson *et al.* 2013) in the first hours after TBI, it is unlikely that these endogenous neuroprotective mechanisms are ready-to-use and overcome the biochemical components of secondary damage (Stefani *et al.* 2017).

While clinical studies investigating purine levels in biological fluids after TBI are scarce, previously published reports in rodent models of TBI (Verrier *et al.* 2012; Bell *et al.* 1998; Marklund *et al.* 2006) have demonstrated acute increases in the levels of ADP, adenosine, inosine, and hypoxanthine, which are consistent with the concentration profile found in our current study (Fig 1E, F, H and I respectively). However, we did not find reports of changes in GDP, guanosine, and xanthine levels in the biological fluids of patients, or in preclinical models of TBI. Hence, this work primarily demonstrates that CSF levels GDP, guanosine, ADP, adenosine, inosine, xanthine, and hypoxanthine increased above the control levels within the first 3 hours after TBI, whereas IMP was consistently decreased. Although this composite of purinergic abnormalities likely reflects damage to the brain cells, it further requires associations with clinical and neurological endpoints to be featured as a feasible biomarker in the ICU (Atkinson *et al.* 2001).

Among the adenine- and guanine-derived purines, GTP, GDP, IMP, and xanthine were significantly increased in non-surviving compared to surviving patients. Despite IMP being decreased in the TBI group relative to control, comparison between non-surviving and surviving patients within the TBI group displayed differences (see Figures 2 E and 3 C). The discriminatory profile of these particular purines may encompass features with potential validity to be explored as predictive biomarkers (Cristofori *et al.* 2005; Laketa *et al.* 2015).

A previous case report investigating energy failure and oxidative damage in one severe TBI patient has shown that the CSF levels of adenosine, hypoxanthine, xanthine, and uric acid increased up to 100 h after ventricular catheter insertion. The concentrations of hypoxanthine, xanthine, and uric acid increased 4- to 6-fold 72 h after catheter insertion and before brain death, which also suggests a putative prognostic value (Cristofori *et al.* 2005). Albeit the time between the catheter insertion and CSF collection differs from our work, we found similar increments in xanthine levels before brain death. Also, our work further highlighted the discriminatory profile of GTP, GDP, IMP, and xanthine, expanding the opportunity to identify candidate biomarkers and clinical associations.

Regarding the sensitivity and specificity of GTP, GDP, IMP, and xanthine in determining the short-term neurological prognosis of severe TBI patients, interestingly, only GTP levels displayed predictive value relative to mortality within 3 days after TBI (sensitivity of

66.67% and specificity of 100%). A potential mechanism underlying these properties has been identified in a post-mortem examination of the brains of TBI patients. The GTP binding receptors RhoA and RhoB expression levels were acutely increased after TBI, which has been sustained for months after the head impact (Brabeck *et al.* 2004). These receptors participate in cerebral responses to injuries, regulating cerebrovascular tonus, astrocytic and microglial activation, axonal regeneration, and neuroplasticity (Stankiewicz and Linseman 2014). In animal models of TBI, it has been reported an increase in the downstream GTP-binding receptor pathways, such as the RhoA-ROCK cascade, which is known to exacerbate neuronal death (Sabirzhanova *et al.* 2013; Labandeira-Garcia *et al.* 2017; Dubreuil *et al.* 2006; Rikitake *et al.* 2005). Such role is apparently corroborated by the increased GTP levels observed in non-surviving patients in our study. In addition, we sought associations between purine levels and long-term neurologic disability. Our data demonstrate that only guanosine levels at admission in the ICU were inversely associated with mRS scores two years after TBI. Exogenous administration of guanosine has well-recognized effects against glutamatergic excitotoxicity and mitochondrial dysfunction, important components of secondary damage following TBI (Gerbatin *et al.* 2017; Dobrachinski *et al.* 2019; Gerbatin *et al.* 2019). Based on these properties, we posit that even a build-up of endogenous levels of guanosine at admission may attenuate the progression of neuronal damage and functional decline following severe TBI.

Here, we further attempted to uncover the mechanism of purine degradation after TBI using a modeling based on the multivariate correlations analysis between purines. Primarily, we showed that purine metabolism in controls is tethered to the classical enzymatic degradation steps (KEGG pathways). The correlation network, present in controls, reflects an integrated enzymatic degradation, endorsing the biological plausibility of these proposed associations. However, many of these correlations present in the controls disappear in the TBI group, suggesting an uncoupling of the enzymatic steps, leading to the accumulation of specific purine derivatives. For instance, in the TBI group, negative correlations (Figure 6C, red double-headed arrows) between inosine and ADP, guanosine and xanthine are lost, as well as the positive correlations (Figure 6C, blue double-headed arrows) between IMP and both, hypoxanthine and xanthine. The loss of negative correlations was accompanied by the accumulation of GDP, ADP, inosine, xanthine, and guanosine (indicated in the model by the arrowheads), likely suggesting the impairment of some enzymes, including the E-NTPDases.

Noteworthy, the loss of the positive correlations between IMP and both, hypoxanthine and xanthine paralleled with a decrease in IMP levels, and consequent accumulation of hypoxanthine and xanthine. A previous study demonstrated that changes in ectonucleotidase activity in the rodent cortex, hippocampi, and caudate nucleus caused reduced AMP hydrolysis, but not ATP hydrolysis 4 hours after injury, emphasizing that purine degradation enzymatic activities are actually altered by TBI (Bjelobaba *et al.* 2009). Also, accumulation of xanthine and hypoxanthine in both CSF and serum, and alterations in xanthine oxidase activity have been previously reported in preclinical studies of TBI, supporting the mechanistic involvement of purine metabolism (Laketa *et al.* 2015; Solaroglu *et al.* 2005; Tayag *et al.* 1996). Overall, our mathematical model in CSF of patients suggests that the imbalance of purine metabolism was likely mediated by altered enzymatic activity caused

by TBI. These collective findings support further investigations of key effectors mediating purine degradation after TBI, as well as the role of specific enzymes on clinical outcomes.

Our study presents limitations that should be disclosed. This is a unicentric study, that enrolled primary 17 patients with severe TBI. The low number of TBI patients limits the power of the clinical associations with purine levels. Nonetheless, this cohort has been previously explored for well-established biomarkers, including NSE, GFAP, and S100B (Böhmer *et al.* 2011) and replicates studies with large TBI populations. Also, a *a posteriori* power analysis suggests that our results present appropriate power with the current study population, for our two main outcomes; a. GTP as an indicator of mortality (98.5%), and to a lesser extent b. GUO as a prognostic indicator of long-term neurofunctional status (84.1%). Also, this is a hypothesis-generating work and was not designed to determine GTP or GUO as definitive biomarkers. Therefore, considering the primary nature of this exploratory study, and the novelty of these findings, the results presented here reveal potentialities that need to be better explored in further studies. Also, the comparison between TBI and controls could be somewhat biased considering the location of the CSF collection. Whereas the TBI patients had their CSF samples collected from external ventricular drains, controls had a lumbar puncture for CSF collection, which could affect the composition and content of various purines and their metabolites (Hegen *et al.* 2018; Brandner *et al.* 2013; Podkovik *et al.* 2020).

5. Conclusion

In the present study, severe TBI imprinted particular purine-derived signatures in the CSF of patients. We identified GTP as a sensitive and specific predictive biomarker of mortality, and guanosine as an indicator of long-term functional disability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation List

ADO	Adenosine
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate

AUC	Area Under the Curve
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CL	Confidence Levels
GUO	Guanosine
GDP	Guanosine Diphosphate
GMP	Guanosine Monophosphate
GTP	Guanosine Triphosphate
HPLC	High Performance Liquid Chromatography
HXN	Hypoxanthine
INO	Inosine
IMP	Inosine Monophosphate
ICU	Intensive Care Unit
ICP	Intracranial Pressure
KEGG	Kyoto Encyclopedia Of Genes And Genomes
mRS	Modified Rankin Scale
NSE	Neuron-Specific Enolase
TBI	Traumatic Brain Injury
UA	Uric Acid
XAN	Xanthine

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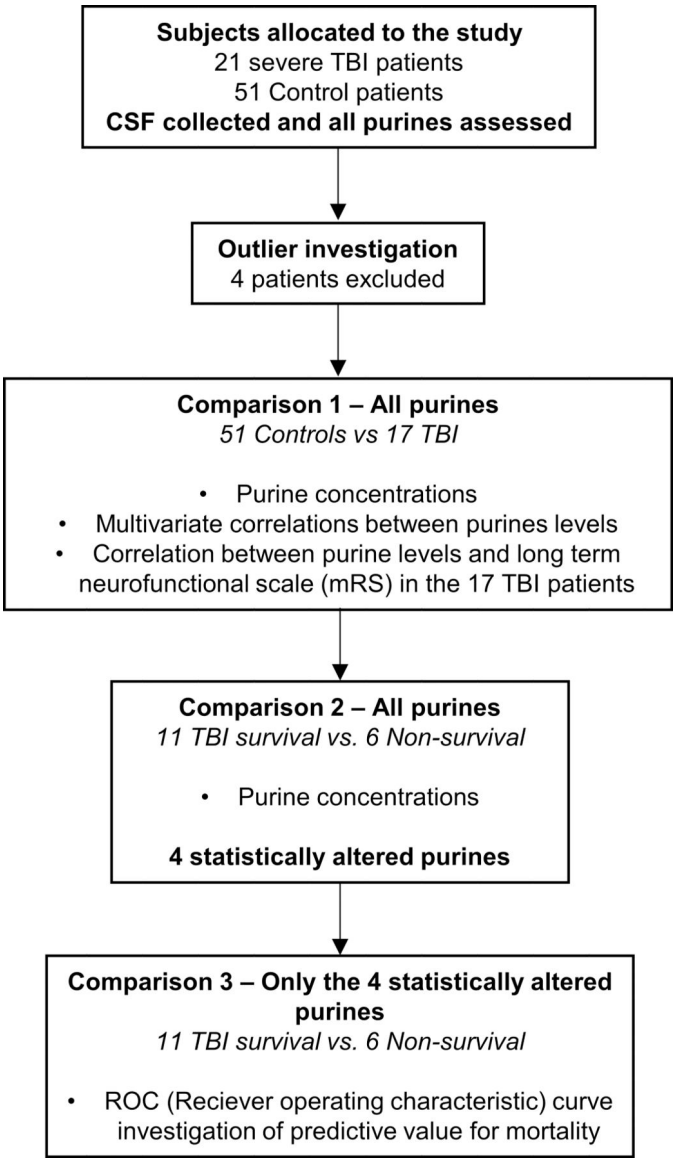

Figure 1: Flow diagram of patient inclusion and data analysis.

Following eligibility criteria, 21 TBI patients and 51controls were initially included in the study, with CSF collected and purines quantified. Homogeneity of data analysis led to exclusion of 4 subjects from all further analyses. Step by step description of data analysis shows that first, all purines were investigated regarding TBI patients relative to controls, to assess the impacts of TBI over physiological purine profiles, and associate purine levels impact over a long-term neurofunctional score. Posterior analysis investigated differential purine profiles within surviving and non-surviving TBI patients, identifying only 4 altered purines. Finally, the 4 altered purines in non-surviving patients were investigated relative to their predictive values of mortality through a receiver operating characteristic curve.

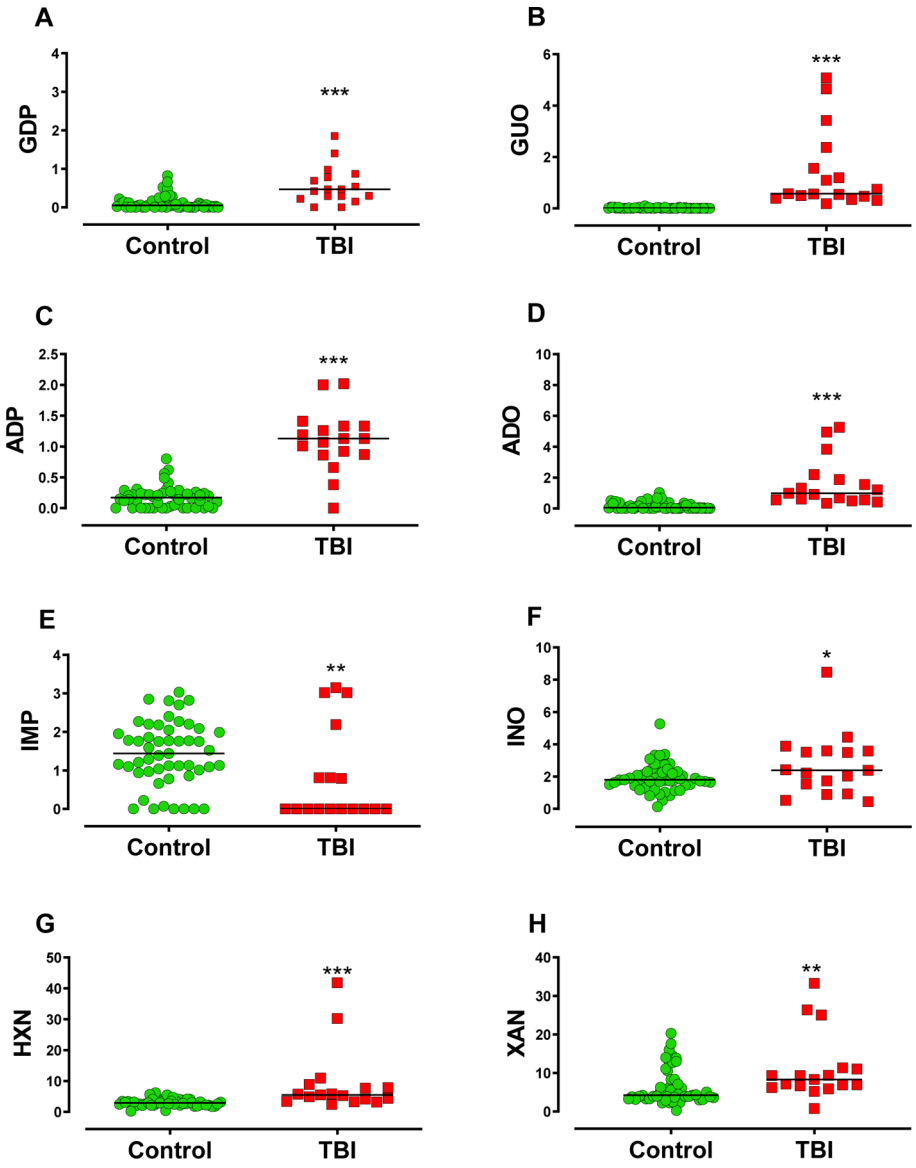

Figure 2. Purine profile is altered in the CSF of TBI patients.

This setting displays the composite of purines with statistically significant differences between TBI patients and controls. **(A)** Guanine derivatives GDP, **(B)** and guanosine (GUO) presented increased levels in TBI patients compared to controls. **(C)** Adenine derivatives ADP and **(D)** adenosine (ADO) were increased in TBI patients compared to controls. **(E)** CSF IMP levels were reduced in TBI patients compared to control, **(F)** whilst inosine (INO), **(G)** hypoxanthine (HXN), and **(H)** xanthine (XAN) were increased. Horizontal lines indicate the median value. Statistical significance was assessed through Mann-Whitney or Student's independent T-test, with *, **, *** indicating $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively (Controls n= 51 patients, TBI n = 17 patients

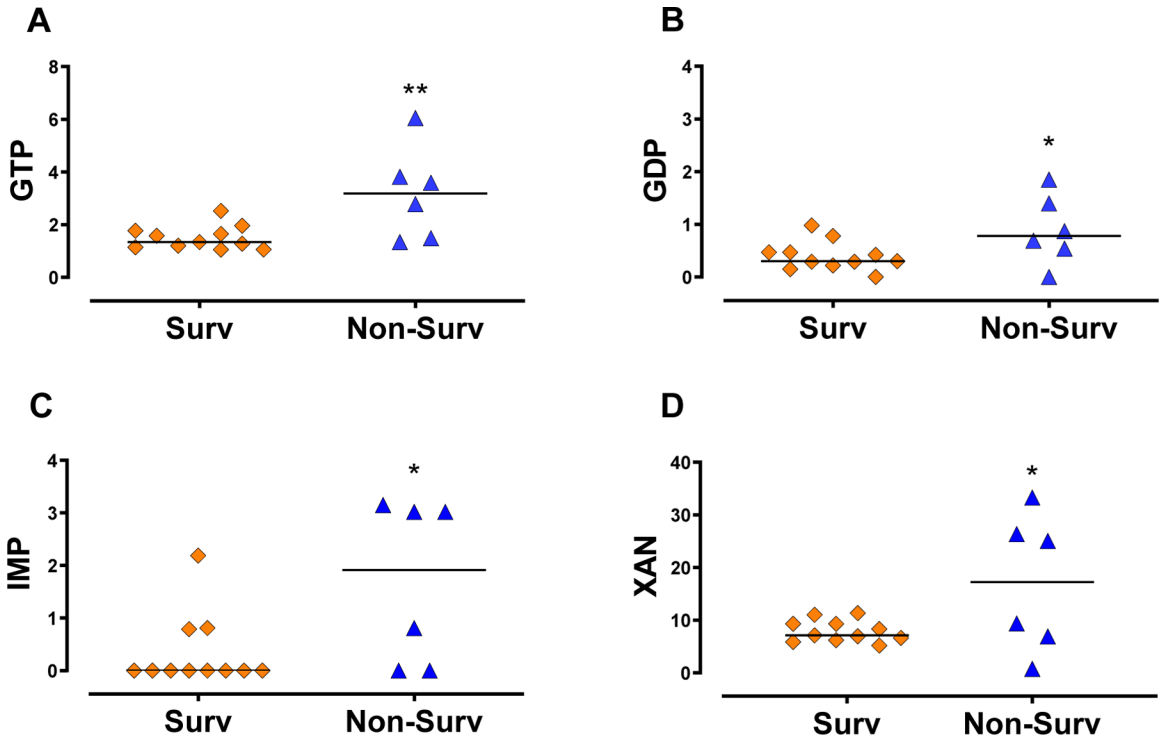

Figure 3. Increased concentration of CSF purines is observed in non-surviving TBI patients.

(A) GTP **(B)** and GDP concentrations were increased in non-surviving (Non-Surv), relative to surviving TBI patients (Surv). **(C)** Non-surviving patients also presented increased IMP **(D)** and xanthine **(L)** CSF levels compared to surviving patients. Horizontal lines indicate the median value. Statistical significance was assessed through Mann-Whitney or Student's independent T-test, with *, **, *** indicating $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively (Surv n =11 patients, Non-Surv n=6 patients).

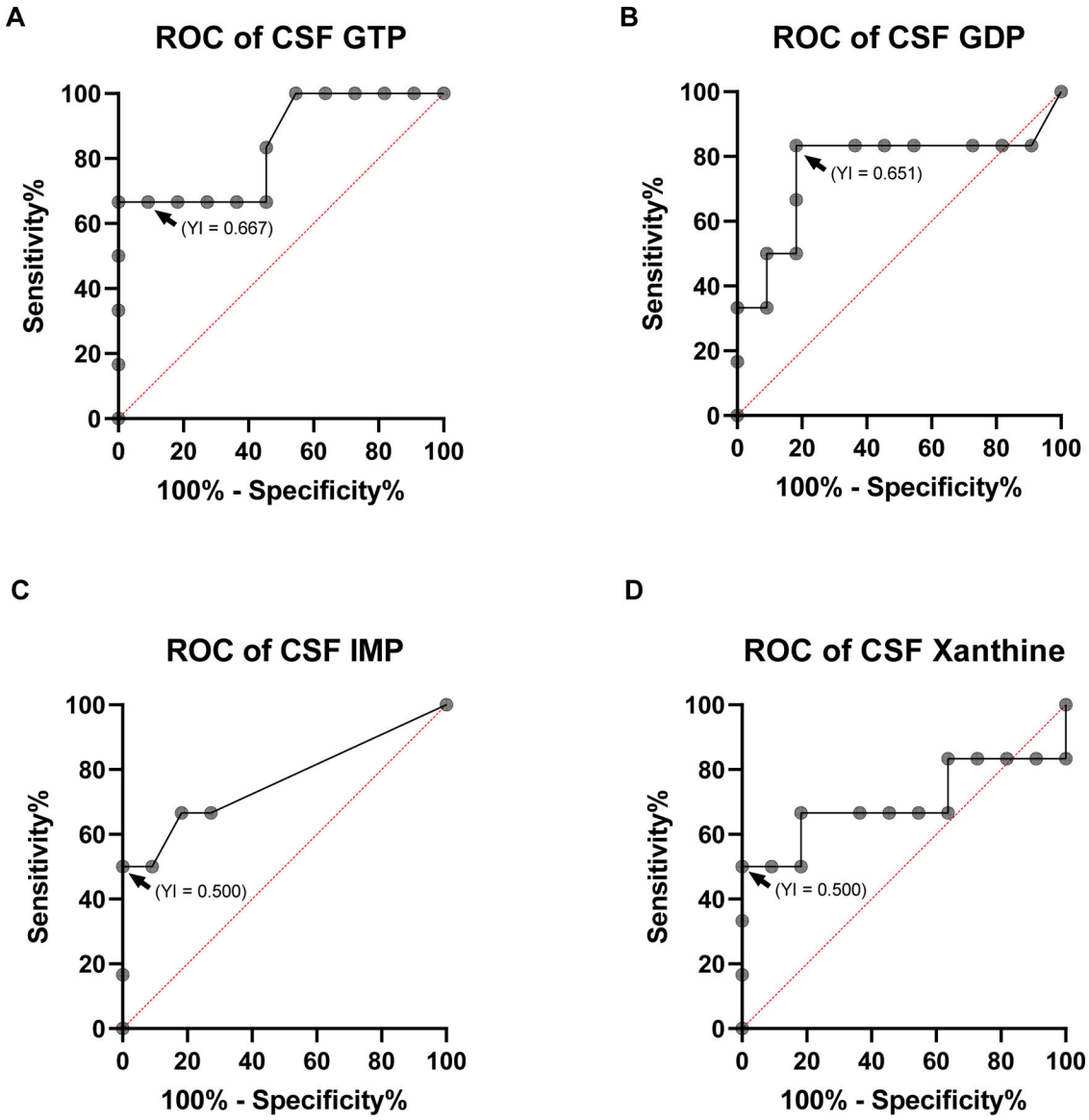

Figure 4. Predictive accuracy of CSF purines relative to death up to 3 days from ICU admission.

The area under the curve of the receiver operating characteristic curve (AUROC) for GTP **(A)**, GDP **(B)**, IMP **(C),** and Xanthine **(D)**. **(A)** GTP presented at a cut-off value of >2.780 μM (indicated by arrowhead) a sensitivity of 66.67%, and 100% of specificity (AUROC: 0.841, $p = 0.0237$, 95% CI, CL (Confidence Levels): 62.26% to 99.53%, Youden Index (YI) for >2.780 μM = 0.667). CSF GDP **(B)**, IMP **(C)** and xanthine **(D)** levels presented AUROC with lower predictive value ($p > 0.05$). Arrowheads indicate the optimal cut-off point indicated by the Youden criteria, followed by the respective Youden Index (YI) obtained, presented within parenthesis. (Survival n =11 patients, Non-Survival n=6 patients)

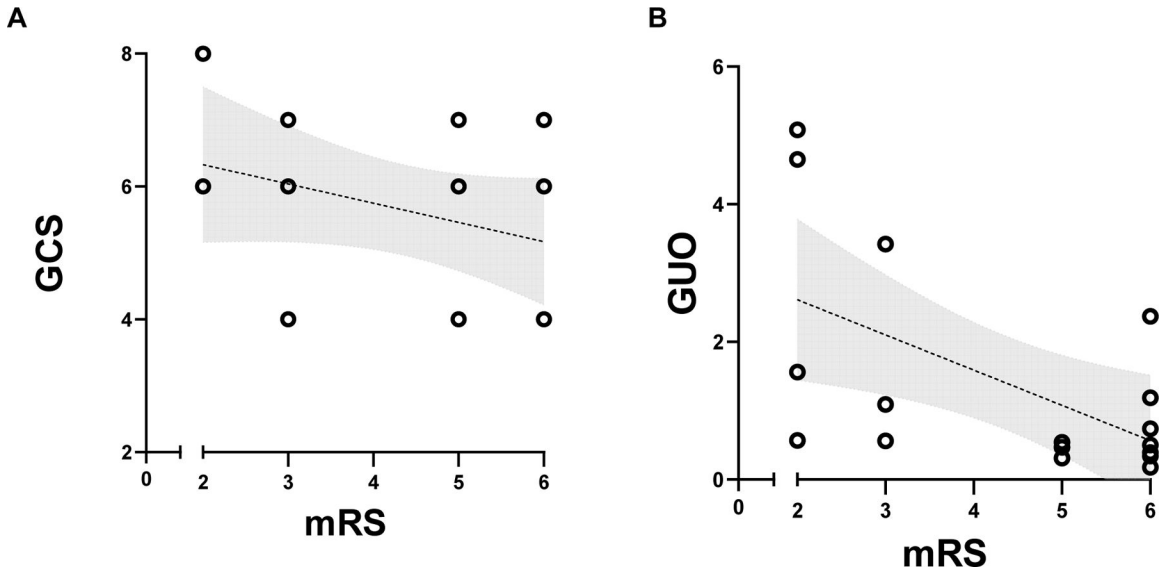

Figure 5. Spearman bivariate correlation between CSF purine levels and clinical neurological outcomes.

(A) Glasgow Coma Scale (GCS) at admission did not present a statistically significant correlation with two years modified Rankin Scale (mRS) ($p = 0.2070$, $r = -0.302$, n= 17 patients). **(B)** Guanosine (GUO) levels at admission were statistically correlated with disability 2 years after discharge (mRS) ($p = 0.0428$, $r = -0.506$, n = 17 patients) (Survival n =11 patients, Non-Survival n=6 patients). Lines indicate linear regression model, and shaded area indicates the of 95% confidence intervals.

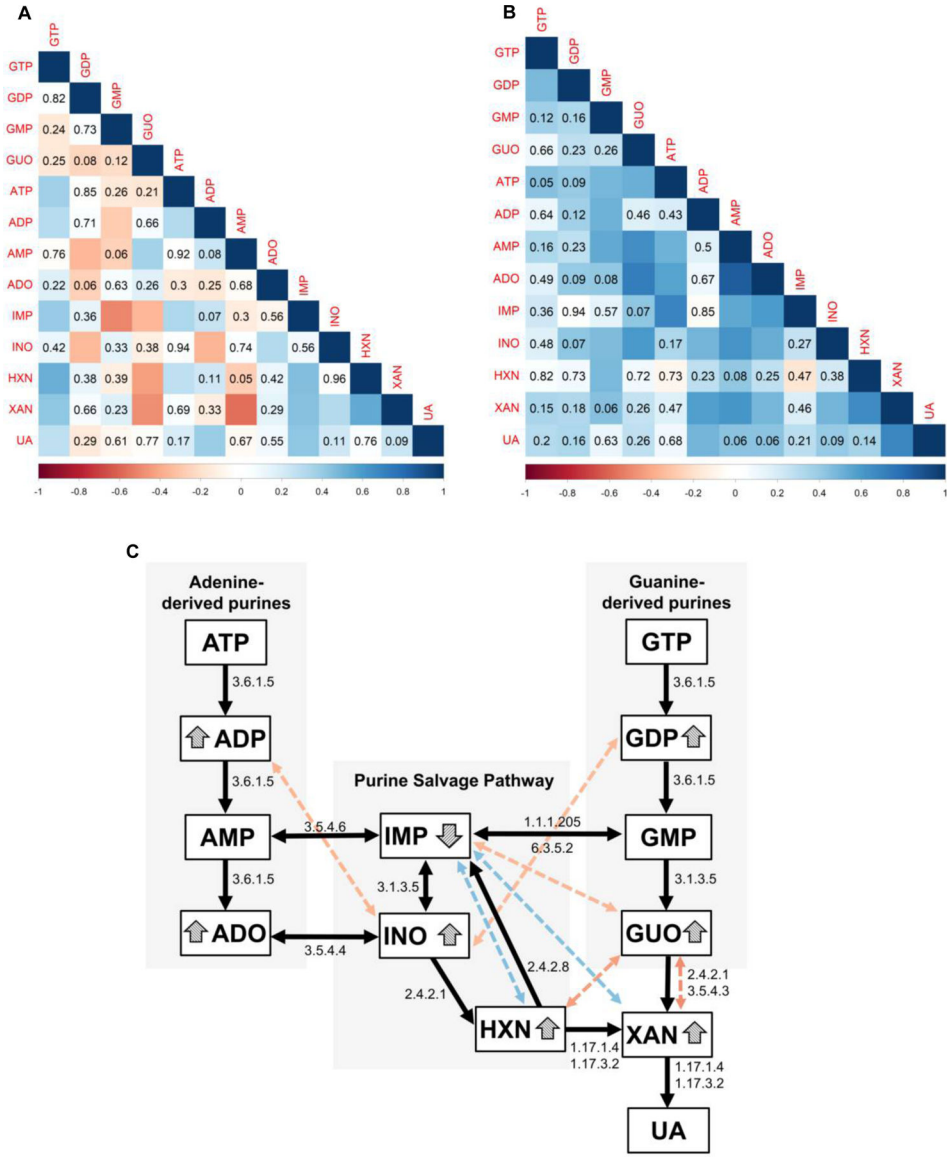

Figure 6. Multivariate correlations between purine derivatives in the CSF.

(A), and **(B)** report Spearman's *r* values adjusted by Bonferroni, indicated by color scale ranging from −1 (red) to +1 (blue), and obtained p-values are displayed for non-statistically significant correlations in both panels. Squares with no indication of p-value represent correlations with $p<0.05$. **(A)** Spearman r correlations between purines in controls. The positive and negative correlations between adenine and guanine derivatives and their products of downstream degradation suggest an integrated physiological metabolic network. **(B)** Only positive correlations between purine derivatives were observed in TBI patients, with a limited association between adenine- and guanine-derived metabolites and a rupture of the proposed physiological negative correlations.

(C) Integrative model based on classical metabolic pathways of purine degradation indicated by the black flowchart, accompanied by the E.C. (Enzyme Commission) numbers of the enzymes related to the respective metabolic steps leading from GTP and ATP to uric acid

(UA) formation. The colored dotted double-headed arrows indicate correlations in purine metabolism of controls, that are lost in TBI patients. The color of double-headed arrows indicates Spearman r values, following the color scale displayed in (A) and (B). The specific purine derivatives modified by TBI are highlighted by the arrowheads.(Controls n= 51 patients, TBI n = 17 patients).

Table 1:
Descriptive data of purine profile, comparing TBI patients and matched controls.

Descriptive statistics are presented as mean ± SD, or median and interquartile range (IQR) (Q1 – Q3). Purine levels are shown as μM concentration. Statistical values are reported for independent t-test, or Mann-Whitney U test, with the respective t-values or U-values, degrees of freedom (Df), and obtained p-values for the tests.

	Gaussian distribution				
	Controls (mean ± sd)	**sTBI Patients (mean ± sd)**	**t-value**	**Df**	**p-value**
Inosine	1.930 ± 0.855	2.711 ± 1.933	2.308	66	*0.0241
	Non-Gaussian distribution				
	Controls (Median (IQR))	**sTBI Patients (Median (IQR))**	**U-value**	**Df**	**p-value**
GTP	2.140 (1.60 – 2.34)	1.580 (1.24 – 2.65)	347	66	0.2241
GDP	0.050 (0.000 – 0.120)	0.470 (0.255 – 0.825)	136.5	66	***<0.0001
GMP	0.000 (0.000 – 0.000)	0.000 (0.000 – 0.000)	454	66	0.9408
Guanosine	0.000 (0.000 – 0.0200)	0.570 (0.430 – 1.965)	0	66	***<0.0001
ATP	0.000 (0.000 – 0.000)	0.000 (0.000 – 0.040)	353	66	0.1073
ADP	0.170 (0.000 – 0.240)	1.130 (0.865 – 1.330)	50.50	66	***<0.0001
AMP	1.710 (0.000 – 2.680)	0.830 (0.520 – 2.370)	403	66	0.6883
Adenosine	0.050 (0.00 – 0.290)	0.990 (0.560 – 2.040)	37	66	***<0.0001
IMP	1.440 (0.970 – 2.050)	0.000 (0.000 – 1.500)	252	66	**<0.01
Xanthine	4.250 (3.290 – 8.340)	8.330 (6.430 – 11.19)	232	66	**0.0037
Hypoxanthine	2.890 (2.210 – 3.660)	5.510 (3.740 – 8.335)	116	66	***<0.0001
Uric Acid	24.35 (18.03 – 33.76)	22.41 (15.13 – 31.08)	386	66	0.5091

* Indicates p-values <0.05,

** indicates p-values <0.01 and

*** indicates p-values < 0.001. (Controls n = 51 patients, TBI n = 17 patients.

Table 2:

Descriptive data of purine profile, comparing surviving and non-surviving severe TBI patients.

Descriptive statistics are presented as mean ± SD, or median and interquartile range (IQR) (Q1 – Q3). Purine levels are shown as μM concentration. Statistical values are reported for independent t-test, or Mann-Whitney U test, with the respective t-values or U-values, degrees of freedom (Df) and obtained p-values for the tests.

Gaussian distribution					
	Surviving (Mean ± sd)	**Non-surviving (Mean ± sd)**	**t-value**	**Df**	**p-value**
GTP	1.505 ± 0.452	3.178 ± 1.74	3.075	15	**0.0077
GDP	0.397 ± 0.28	0.819 ± 0.654	2.209	15	*0.0431
ADP	1.138 ± 0.362	1.008 ± 0.720	0.5017	15	0.6232
Inosine	3.124 ± 2.200	1.953 ± 1.100	1.210	15	0.2449
Xanthine	7.943 ± 2.076	16.96 ± 12.97	2.315	15	*0.0352
Non-Gaussian distribution					
	Surviving (Median (IQR))	**Non-surviving (Median (IQR))**	**U-value**	**Df**	**p-value**
GMP	0.000 (0.000 – 0.000)	0.000 (0.000 – 1.170)	27.5	15	0.353
Guanosine	0.740 (0.540 – 1.560)	0.485 (0.338 – 3.048)	26	15	0.524
ATP	0.000 (0.000 – 0.040)	0.000 (0.000 – 0.088)	32.50	15	>0.999
AMP	0.830 (0.570 – 2.220)	0.880 (0.298 – 2.620)	30	15	0.7885
Adenosine	1.210 (0.560 – 1.880)	0.655 (0.545 – 5.028)	35.20	15	0.9812
IMP	0.000 (0.000 – 0.790)	1.915 (0.000 – 3.053)	15.5	15	*0.0438
Hypoxanthine	5.260 (3.340 – 7.660)	6.805 (3.705 – 33.15)	24	15	0.4043
Uric Acid	25.95 (15.58 – 27.95)	20.01 (12.50 – 129.7)	32	15	0.9612

* indicates p-values <0.05,

** indicates p-values <0.01 and

*** indicates p-values < 0.001 (Surviving n = 11 patients, Non-surviving n = 6 patients).