„Doctoral (Ph.D.) thesis”

Metabolomic and biomarker analyses in aneurysmal subarachnoid hemorrhage

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1. INTRODUCTION

aSAH is a severe and life-threatening neurological event that accounts for approximately 5– 10% of all strokes worldwide. Its global incidence averages between 10–13 per 100,000 person-years, with a rate of 8.3 per 100,000 in Hungary. Despite advances in neurocritical care and surgical or endovascular treatments, aSAH still results in high morbidity and mortality rates. About 15% of patients die before reaching medical care, and another 18–20% die within the first three days of hospitalization. The overall one-month mortality rate is nearly 50%. Importantly, mortality is not mainly caused by the hemorrhage itself but by secondary complications such as EBI, cerebral vasospasm, and DCI, which remain leading factors in poor neurological outcomes. Although extensive research has focused on these processes, their pathophysiological mechanisms remain only partially understood. As a result, reliable prognostic indicators for early risk assessment and outcome prediction are lacking. In recent years, the focus has increasingly shifted toward innovative molecular profiling methods to discover new biomarkers associated with aSAH progression. Among these, metabolomics and lipidomics have emerged as promising high-throughput analytical techniques that enable the simultaneous measurement of multiple metabolites in biological samples. These technologies provide a detailed "molecular snapshot" of systemic and cellular metabolism, capturing the dynamic biochemical changes that occur during disease progression. By analyzing altered metabolic pathways, metabolomics can identify novel biomarkers and mechanistic targets that might not be detectable using traditional biochemical tests. However, despite their widespread application in other neurological and systemic disorders, metabolomic studies specifically investigating aSAH remain scarce and heterogeneous. Existing data offer valuable insights into cerebral energy dysfunction, amino acid metabolism, and oxidative stress after hemorrhage; however, findings are sometimes inconsistent and not sufficiently validated in larger groups. This highlights the need for integrative approaches that combine metabolic profiling with additional systemic markers to understand the complex cascade following aneurysmal rupture better. 28 Alongside central nervous system–focused research, increasing evidence highlights a significant role for the gut–brain axis and intestinal barrier dysfunction in cerebrovascular diseases. Recent studies suggest that gut dysbiosis and increased intestinal permeability may impact inflammation, vascular reactivity, and ultimately aneurysm stability. Several gut-derived biomarkers, such as FABP-I, LBP, and

soluble CD14 (sCD14), have been identified as sensitive indicators of enterocyte damage and systemic inflammatory activation. These molecules reflect disruption of the intestinal epithelial barrier, facilitating translocation of microbial products that can exacerbate systemic and neuroinflammatory processes. Therefore, integrating serum metabolomic profiling with intestinal barrier and inflammatory biomarkers may provide a more comprehensive understanding of the systemic pathophysiology underlying aSAH. Such combined biomarker panels hold promise for identifying novel prognostic indicators, improving early risk assessment, and potentially guiding individualized therapeutic strategies in this complex and devastating disease.

2. AIMS

The underlying pathophysiological mechanisms of the DCI and EBI remain incompletely understood. Given the complexity of systemic and cerebral interactions in aSAH, identifying biomarkers and metabolic signatures that indicate disease severity and prognosis is especially important in clinical practice. This doctoral research, therefore, aimed to achieve two complementary goals related to the systemic effects of aSAH. The primary goal was to examine indicators of intestinal barrier integrity and systemic inflammatory activation—specifically, serum levels of FABP-I, LBP, and soluble CD14 (sCD14)—in patients with aSAH. Special emphasis was placed on excluding the confounding effects of hospital-acquired infections, so the observed biomarker changes could be mainly interpreted as reflections of central nervous system injury and its associated systemic responses. Additionally, we aimed to assess whether these biomarkers were linked to the development of delayed cerebral ischemia and long-term clinical outcomes. The second objective was to describe how serum metabolomic profiles change over time during the early (24 hours) and late (7 days) stages of aSAH using LC–MS/MS analysis. By measuring a wide range of metabolites simultaneously, we aimed to identify altered metabolic pathways and examine their potential links to patient outcomes, thereby emphasizing metabolites with prognostic value. Overall, the primary objective of this work was to integrate biomarker and metabolomic data related to the gut–brain axis, providing new insights into the pathophysiology of aneurysmal subarachnoid hemorrhage and facilitating the development of future diagnostic and prognostic tools.

3. MATERIALS AND METHODS

3.1.Study design and population

This single-center, prospective observational study was conducted at a tertiary neurovascular referral center following approval by the institutional review board (IV/8468-1/2021/EKU). Written informed consent was obtained from all patients or their legal representatives prior to enrollment. Adult patients (>18 years) diagnosed with spontaneous aneurysmal subarachnoid hemorrhage (aSAH) between February 2021 and November 2023 were consecutively included. The diagnosis of aSAH was established by non-contrast head computed tomography performed within 24 hours of the ictus, and the presence of an intracranial aneurysm was confirmed using CT angiography or digital subtraction angiography. Patients were excluded if they had traumatic SAH, pregnancy, hospital admission later than 24 hours after symptom onset, absence of aneurysm treatment, bleeding from an arteriovenous malformation, lack of written informed consent, rerupture after the initial ictus, early clinical deterioration during treatment, or evidence of acute or chronic infection on admission, including SARS-CoV-2 infection. Additional exclusion criteria comprised relevant systemic comorbidities, such as malignancies, chronic liver or renal insufficiency, chronic lung disease, inflammatory bowel disease, or other chronic gastrointestinal disorders. According to institutional treatment protocols, all eligible aneurysms were treated endovascularly within 24 hours of diagnosis. Following aneurysm occlusion, patients were admitted to the neurointensive care unit for a minimum duration of 12–14 days to ensure continuous neurological monitoring and early detection of anticipated complications, including symptomatic cerebral vasospasm and delayed cerebral ischemia. Standard intensive care management included early enteral nutrition administered orally or via nasogastric tube to ensure adequate caloric intake, as well as prophylactic oral nimodipine therapy (60 mg six times daily) initiated on the first day of admission for vasospasm prevention. Symptomatic macrovascular vasospasm was suspected in cases of neurological deterioration, defined as the occurrence of new focal neurological deficits or a decrease of at least two points on the Glasgow Coma Scale. Diagnostic confirmation was obtained using magnetic resonance imaging and MR angiography targeting the major intracranial vessels. If non-invasive imaging was inconclusive, digital subtraction angiography was performed, and intra-

arterial nimodipine was administered when indicated. Demographic characteristics, vascular risk factors (including hypertension, diabetes mellitus, and smoking status), admission clinical parameters, laboratory results, radiological severity scores (WFNS and modified Fisher scale), and intensive care interventions were systematically recorded. The occurrence of infectious complications, delayed cerebral ischemia, and other in-hospital events were documented prospectively. Delayed cerebral ischemia was diagnosed according to established consensus criteria and required the agreement of at least two experienced neurointensivists after exclusion of alternative causes of neurological deterioration. Isolated large-vessel vasospasm without clinical symptoms or new ischemic lesions was not classified as DCI. Clinical outcome was assessed at 3 months following the hemorrhagic event using the modified Rankin Scale (mRS), obtained via structured telephone interviews or personal follow-up visits. A favorable outcome was defined as an mRS score of 0–3. Outcome assessments were performed by independent, trained professionals blinded to the study data. For baseline comparisons, a control group consisting of patients with unruptured intracranial aneurysms not requiring interventional treatment, or who declined treatment, was included. In these individuals, serum samples were obtained after exclusion of active infections or systemic conditions that could influence laboratory measurements.

3.2.Sample collection and processing protocol

Arterial blood samples were collected from all eligible patients at predefined time points following the ictus. Serum samples were obtained on the first day after hemorrhage (D1) and during the subacute phase (D7 or D9), depending on the specific analysis. Blood samples were collected into plain glass tubes and centrifuged within 30 minutes of collection. The resulting serum was aliquoted into polypropylene tubes and stored at −80 °C until further processing. For metabolomic analysis, serum samples were thawed on ice and prepared by protein precipitation. Briefly, 100 µL of serum was mixed with 400 µL of acetonitrile, vortexed, and centrifuged at 20,000×g at 4 °C for 10 minutes. The supernatant was collected and analyzed by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Measurements were performed using a Q-Exactive Focus mass spectrometer coupled with a Dionex Ultimate 3000 UHPLC system. Chromatographic separation was achieved on an amide column under gradient elution

conditions. Data acquisition was primarily conducted in MS1 scan mode using negative ionization, covering a mass range of 100–800 m/z. Pooled quality control samples were injected repeatedly throughout the analytical batch to ensure data stability and reproducibility, in accordance with established metabolomics guidelines. Additional MS/MS analyses were performed for metabolite confirmation using data-dependent acquisition in both positive and negative ionization modes. For targeted biomarker analysis, serum levels of gut permeability–associated markers, including soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), and intestinal fatty acid–binding protein (FABP-I), were quantified using commercially available ELISA kits according to the manufacturers' instructions. Colorimetric reactions were developed using tetramethylbenzidine and measured at 450 nm with a microplate reader.

3.3.Statistical analysis

Statistical analyses were performed using SPSS software and GraphPad Prism. Categorical variables are presented as frequencies and percentages, while continuous variables are expressed as means with standard deviations or medians with interquartile ranges, as appropriate. Group comparisons were conducted using the chi-square or Fisher's exact test for categorical variables and the independent t-test, Mann–Whitney U test, or Kruskal–Wallis test for continuous variables. Correlations between serum gut permeability markers were assessed using Spearman's rank correlation coefficient. Metabolomics data were processed and normalized using established pipelines, and multivariate statistical analyses were performed with MetaboAnalyst. Orthogonal partial least squares discriminant analysis (OPLS-DA) was applied to explore global metabolic differences between outcome groups at different time points, and model validity was assessed using permutation testing. Differential metabolites were selected based on variable importance in projection (VIP) values, false discovery rate–adjusted p-values, and fold change thresholds. Receiver operating characteristic (ROC) curve analysis was used to evaluate the discriminative performance of selected metabolic biomarkers. Pathway enrichment analysis was conducted to identify biologically relevant metabolic pathways associated with outcome differences. A p-value < 0.05 was considered statistically significant.

4. RESULTS

4.1. The Difference in Serum Metabolomic Profiles between the Good and Poor Outcome Groups at 3 Months in the Early and Late Phases of Aneurysmal Subarachnoid Hemorrhage

We analyzed serum samples obtained from aSAH patients (n = 46) with good outcomes (n = 16) and poor outcomes (n = 30) using untargeted LC–MS metabolomics. The two outcome groups were age- and gender-matched ($p > 0.05$). The distribution of mRS scores was as follows: mRS 0: 1 (2%), mRS 1: 11 (24%), mRS 2: 4 (8.7%), mRS 3: 7 (15%), mRS 4: 11 (24%), mRS 5: 8 (17%), mRS 6: 4 (8.7%). Compared with the good outcome group, patients with poor outcomes had higher mFisher and WFNS scores, and more patients required mechanical ventilation and extraventricular drainage (all $p < 0.001$). The admission CRP ($p = 0.027$), lymphocyte count ($p = 0.023$), and serum glucose level ($p = 0.028$) were significantly higher in the group with poor 3-month outcomes compared to the favorable outcome group. Similarly, there were significantly more infections ($p = 0.001$) and patients requiring nasogastric enteral feeding in the poor outcome group ($p < 0.001$).

A total of 92 serum metabolites were detected. Serum metabolomic changes between D1 and D7 were explored by comparing the metabolomic profiles from different sample time points with OPLS-DA (cumulative Q2:0.505, R2Y: 0.777). T-test and fold change (FC) analysis were used to screen differential metabolites. The criteria for screening differential metabolites were VIP (variable importance in projection) > 1.0, Log2 FC ≥ 1.2 or <0.8333, and p-value <0.05. A total of 14 metabolites were identified, of which 4 were up-regulated, and 10 were down-regulated (Table 1). Only homocysteine had a high enough AUC value (AUC = 0.838), which signifies good discriminative ability between early and late sampling time points.

Metabolites	*p*-Value [a]	FDR [b]	FC [c]	VIP [d]	AUC
Homocysteine	<0.0001	0.0000	0.54299	2.5649	0.838
Pantothenate	<0.0001	0.0022	0.72975	2.0931	0.736
Theobromine	<0.0001	0.0022	0.27842	2.3236	0.732
Homogentisate	<0.0001	0.0022	1.4076	1.3739	0.736
Pregnenolone Sulfate	<0.0001	0.0023	0.27842	2.1133	0.727
Paraxanthine	0.0005	0.0054	0.16176	2.1269	0.706
Glycocholate	0.0006	0.0054	1.9743	1.7525	0.670
Methyl Galactoside	0.0014	0.0116	0.62255	1.8354	0.691
Quinate	0.0019	0.0149	0.72407	1.6022	0.685
Biliverdin	0.0032	0.0218	0.75389	1.7025	0.677
Xanthurenate	0.0042	0.0257	1.4583	1.6431	0.672
Glycochenodeoxycholate	0.0046	0.0267	2.0485	1.3549	0.670
Bilirubin	0.0051	0.0278	0.74985	1.6738	0.668
Glutamate	0.0071	0.0361	0.72695	1.4018	0.662

Table 1. List of differential metabolites between D1 and D7 in aSAH patients**:** The p-values were calculated using two-tailed Student's t-tests. False Discovery Rate (FDR) was calculated using the Benjamini– Hochberg method. Metabolites with FDR values ≤ 0.05 were considered as significant. Fold change (FC) was calculated based on the arithmetic mean value of each group. Metabolites were obtained when the Log2 FC ≥ 1.2 or <0.8333. Variable importance in the projection (VIP) was obtained based on OPLS-DA with a value > 1.0.

To accurately identify the differential metabolites in the serum of aSAH patients with good and poor outcomes, the samples were further analyzed using the OPLS-DA model at both time points (D1 and D7). The OPLS-DA score plots showed moderate discrimination between the metabolomic profiles of the good and poor outcome groups at the D1 sampling time point (Figure 1A, cumulative R2Y = 0.852, Q2 = 0.353). Slightly better discrimination is observed when applying the model between the two groups at the D7 sampling time point (Figure 1B, cumulative R2Y = 0.895, Q2 = 0.421).

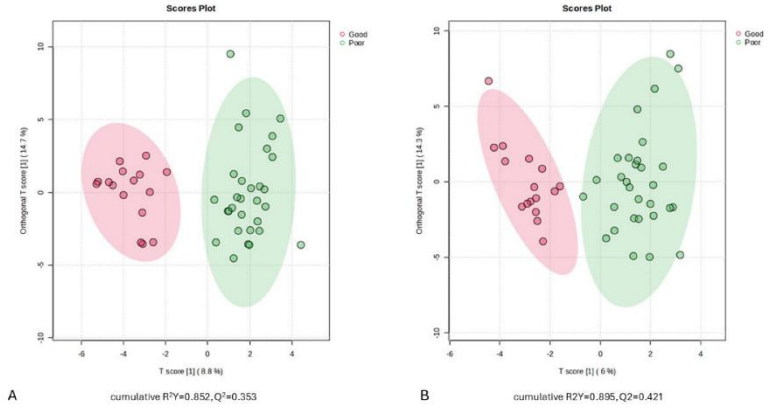


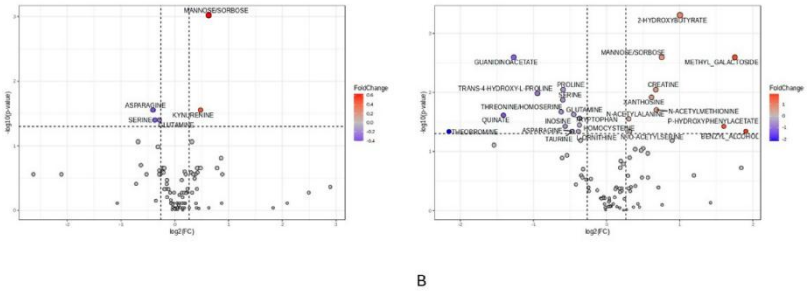
Figure 1. The score plots of the OPLS-DA models: (A) (D1), (B) (D7). The horizontal axis in the figure is the first principal component, and the vertical axis is the second principal component. The number in parenthesis is the score of that principal component, which indicates the percentage of the overall variance explained by the corresponding principal component. D1, sampling time 24 h after injury; D7, sampling time 168 h after injury.

T-test and fold change analysis (FC analysis) were used to screen differential metabolites. The criteria for screening differential metabolites were VIP (variable importance in projection) ≥ 1, Log2 FC ≥ 1.2 or <0.8333, and p-value <0.05; they are summarized in Table 2. Based on these criteria, a total of 5 differential metabolites were identified on D1, and 19 differential metabolites were identified on D7, of which 2 (D1) and 9 (D7) were up-regulated, and 3 (D1) and 10 (D7) were down-regulated. The unfavorable outcome was characterized by an increase in mannose/sorbose and kynurenine and a decrease in asparagine, serine, and glutamine on day 1 after injury (D1). On day 7 (D7) after injury, there was an increase in 2-hydroxybutyrate, methyl-galactoside, mannose/sorbose, creatine, xanthosine, N-acetyl-methionine, P-hydroxyphenylacetat, benzyl alcohol, and N-acetylalanine in patients with poor outcome vs. the patients with good outcome at 3 months. We detected a decrease in the following metabolites in patients with a poor prognosis on D7: guanidinoacetate, proline, serine, trans-4-hydroxy-l-proline, threonine, glutamine, quinate, tryptophan, inosine, homocysteine. Volcano plots show

differences in these metabolites between the good and poor outcome groups on D1 and D7 (Figure 2A, B).

Metabolites	*p*-Value [a]	FDR [b]	FC [c]	VIP [d]	AUC
		D1			
Mannose/Sorbose	<0.0001	0.0009	1.5782	2.2981	0.868
Kynurenine	0.0006	0.0278	1.3946	2.2316	0.820
Asparagine	0.0009	0.0278	0.7551	1.8846	0.800
Serine	0.0020	0.0400	0.7720	1.7817	0.758
Glutamine	0.0022	0.0400	0.8195	1.7818	0.768
		D7			
2-Hydroxybutyrate	<0.0001	0.0014	2.0034	2.2096	0.856
Methyl_Galactoside	0.0002	0.0050	3.3747	2.0167	0.820
Guanidinoacetate	<0.0001	0.0000	0.4144	2.0029	0.917
Mannose/Sorbose	0.0001	0.0031	1.6853	1.9738	0.835
Creatine	0.0004	0.0063	1.5934	1.8054	0.808
Proline	0.0003	0.0062	0.6618	1.7326	0.812
Serine	0.0015	0.0142	0.6596	1.6987	0.779
Trans-4-Hydroxy-L-Proline	0.0012	0.0121	0.5188	1.6620	0.785
Threonine/Homoserine	0.0067	0.0327	0.6503	1.6316	0.741
Xanthosine	0.0022	0.0183	1.5301	1.6144	0.770
Glutamine	0.0046	0.0282	0.7317	1.5320	0.752
N-Acetylmethionine	0.0006	0.0073	1.6020	1.4859	0.802
Quinate	0.0042	0.0279	0.3762	1.4790	0.754
P-Hydroxyphenylacetate	0.0039	0.0277	3.0389	1.4328	0.756
Benzyl_Alcohol	0.0067	0.0327	3.7385	1.3918	0.741
Tryptophan	0.0010	0.0113	0.7737	1.3769	0.789
Inosine	0.0105	0.0481	0.6753	1.3634	0.729
N-Acetylalanine	0.0031	0.0235	1.2340	1.3633	0.762
Homocysteine	0.0063	0.0327	0.7728	1.3288	0.743

Table 2. List of differential metabolites between good and poor outcomes at sampling times D1 and D7 in aSAH patients: The p-values were calculated using two-tailed Student's t-tests. FDR was calculated using the Benjamini– Hochberg method. Metabolites with FDR values ≤ 0.05 were considered as significant. Fold change was calculated based on the arithmetic mean value of each group. Metabolites were obtained when the Log2 FC ≥ 1.2 or <0.8333. Variable importance in the projection (VIP) was obtained based on OPLS-DA with a value > 1.0. D1, sampling time 24 h after injury; D7, sampling time 168 h after injury; FDR, false discovery rate; FC, fold change; AUC, area under the curve.



A B

Figure 2. Differential metabolites screening. Volcano plots: (A) (D1) and (B) (D7). Red represents up-regulated metabolites, and blue represents down-regulated metabolites. Grey represents neither up- or down-regulated metabolites. D1, sampling time 24 h after injury; D7, sampling time 168 h after injury.

A receiver operating characteristic (ROC) curves were generated to evaluate the predictive value of differential metabolites for poor outcomes in aSAH patients. Metabolomic markers that met the criteria for differential metabolites (VIP ≥ 1, Log2 FC ≥ 1.2 or <0.05) outperformed clinical biomarkers in predicting poor outcomes at an early sampling time point (D1), as shown in Figure 3. Among the D7 metabolites, only those with a p-value <0.001 were included in the model. Subsequently, the various metabolic pathways were analyzed using MetaboAnalyst 5.0. We selected clinical markers for outcome prediction based on binary logistic regression analysis. On univariate analysis, age, admission CRP, mFisher score, and WFNS showed associations with the outcome, binary logistic regression identified age (OR: 1.112, 95% CI:1.019–1.214, p = 0.017), and WFNS (OR:3.462, 95%CI:1.238–9.513, p = 0.018) as independent predictors of the outcome. Therefore, we used their combined predictive probability in the clinical predictive model. The general overview of metabolic pathways in D1 and D7 is shown in Figure 4.

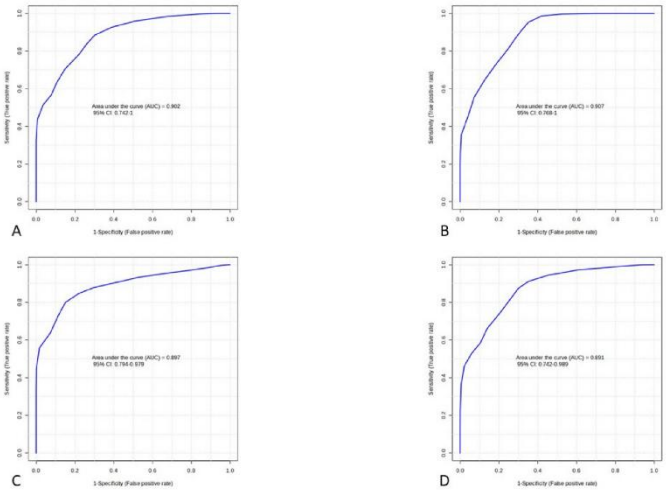


Figure 3. Power of the biomarker panel to distinguish between good and poor 3-month outcome groups: (A) clinical markers on D1, Area under the curve (AUC) = 0,902 95% CI: 0,742-1. (B) metabolomic markers on D1, Area under the curve (AUC) = 0,907 95% CI: 0,768-1. (C) clinical markers on D7, Area under the curve (AUC) = 0,897 95% CI: 0,794-0,979. (D) metabolomic markers on D7, Area under the curve (AUC) = 0,891 95% CI: 0,742-0,989. AUC, area under the curve, CI, confidence interval. The clinical markers were found to be independent predictors (age, WFNS) in binary logistic regression analysis, and their combined predicted probabilities were used. D1, sampling time 24 h after injury; D7, sampling time 168 h after injury.

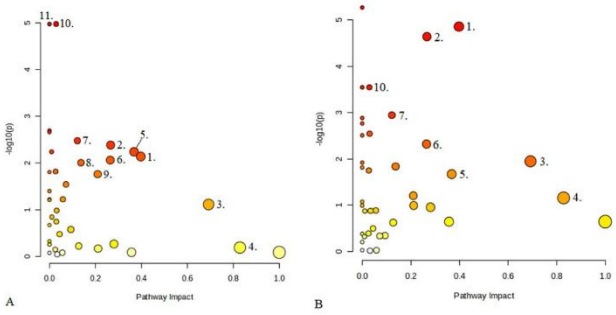


Figure 4. Metabolic pathway analysis: (A) D1; (B) D7. The horizontal coordinate indicates the pathway impact value, the vertical coordinate indicates the −log10(*p*) of the pathway, a dot in the figure represents a metabolic pathway, the size of the dot is proportional to its impact value, and the color of the dot represents the size of the pathway *p*-value, where the color change from yellow

to red represents the change in the *p*-value from large to small. Metabolic pathways: 1. arginine and proline metabolism; 2. glycine, serine, and threonine metabolism; 3. caffeine metabolism; 4. taurine/hypotaurine metabolism; 5. tryptophan metabolism; 6. cysteine and methionine metabolism; 7. glyoxylate and dicarboxylate metabolism; 8. arginine metabolism; 9. alanine metabolism, asparagine, and glutamate metabolism; 10. galactose metabolism; 11. amino sugar and nucleotide sugar metabolism.

4.2. Effects of Aneurysmal Subarachnoid Hemorrhage in Patients Without In-Hospital Infection on FABP-I, LBP, and sCD-14

A total of 206 patients with first-ever aSAH underwent initial evaluation. Among them, 24 patients were excluded because of the following reasons: severe underlying systemic diseases (11), aneurysm rebleeding (2), severe kidney failure (2), loss of follow-up (5), and unavailable biomarker measurements (9). Finally, 177 subjects with aSAH met the eligibility criteria and completed the entire study protocol. Among these 177 patients, 128 (72%) were females, 56 (32%) smoked cigarettes. The mean age was 57.8 years (SD, 12 years). A total of 100 healthy controls with unruptured intracranial aneurysm were recruited. All patients included in the study underwent endovascular treatment; no patients who received open surgery were included in the study. Among these 100 controls, 71 were females and their mean age was 59.6. years (SD, 12 years). There was no significant difference in age or the proportion of females between the aSAH patient and control groups.

The serum FABP-I level was significantly lower in the aSAH patient group (FABP-I levels of D1 and D9 samples) compared to the control group (p <0.05). Within the patient group, the FABP-I levels in the late samples (D9) were significantly lower ($p <$ 0.001) compared to the early samples (D1), Figure 5A. In the case of LBP, we observed significantly higher ($p <$ 0.001) serum levels in the patient group compared to the control group; however, no difference was found between the levels in the early and late samples, Figure 5B. We observed similar results in the examination of serum sCD-14 levels, with significantly higher ($p <$ 0.001) serum sCD-14 levels in the aSAH group compared to the control group. However, there was no substantial difference between the D1 and D9 serum sCD-14 levels.

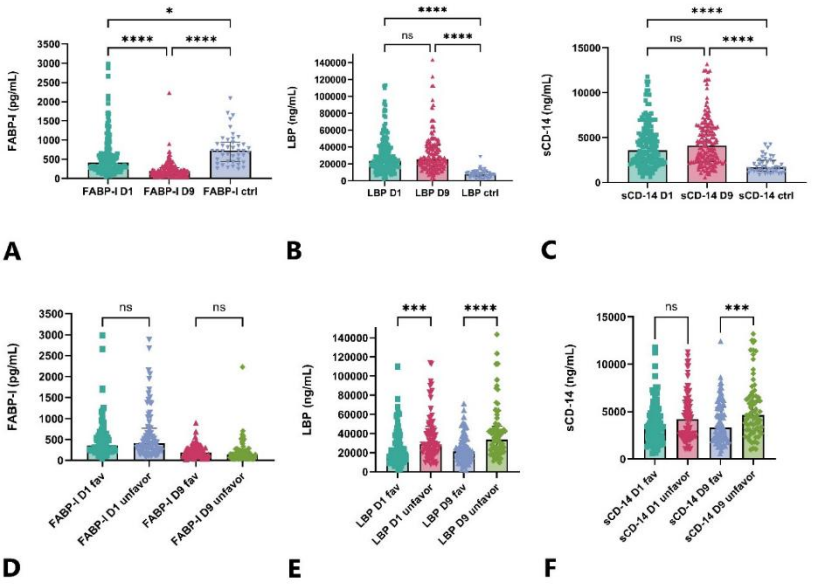


Figure 5. Serum level of FABP-I, LBP and sCD-14 in aSAH patients versus controls (**A**–**C**) and in different outcome groups (**D**–**F**). FABP-I, fatty acid-binding protein-intestinal, LBP, lipopolysaccharide-binding protein, D1, sampling time 24 h after ictus, D9, sampling time 9 days after ictus, fav, favorable (modified Rankin score 0–3) outcome, unfavor, unfavorable (modified Rankin score 4–6) outcome, ns, non-significant, * denotes p <0.05, *** denotes p <0.001, **** denotes p <0.0001, number of control subjects: 100, number of patients with favorable outcome: 94, number of patients with unfavorable outcome: 83.

In the analysis of FABP-I levels, no differences were observed in the serum levels of either the early or late samples between the two outcome groups (Figure 5D). In the group with unfavorable outcomes, significantly higher LBP serum levels were observed compared to the favorable outcome group at both measurement time points (D1, D9) (Figure 5E). A significant difference in serum sCD-14 levels between the two outcome groups was observed only at the D9 sampling time point, while no difference was detected in the early sample taken 24 h after the ictus (Figure 5F). None of the three examined metabolites showed any correlation in the early phase (D1) of aSAH with the occurrence of delayed cerebral ischemia. In contrast, both LBP and sCD-14

reached significantly higher serum levels in the DCI subgroup in the later phase (D9) (Figure 6).

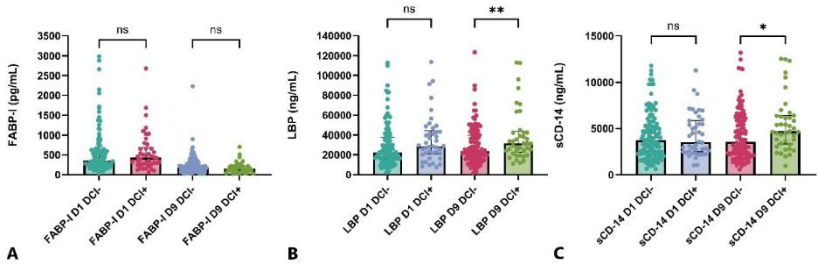


Figure 6. Serum level of FABP-I (A), LBP (B) and sCD-14 (C) in patients with and without delayed cerebral ischemia. DCI delayed cerebral ischemia, +, patients with DCI, −, patients without DCI, FABP-I, fatty acid-binding protein-intestinal, LBP, lipopolysaccharide-binding protein, ns, non-significant, * denotes p <0.05, ** denotes p <0.01, number of patients with DCI: 47, number of patients without DCI: 130.

We also examined the serum levels of LBP in the context of hospital-acquired infections and found that in patients who did not develop an infection (n = 116) during hospitalization (median [IQR] serum level of CRP during hospitalization: 5.7 [2–12]), LBP reached significantly higher levels in the unfavorable outcome group compared to those with favorable outcomes at both measurement time points (Figure 7A). We found a similar but weaker correlation in the case of sCD-14 as well (Figure 7B). In patients who developed an infection (n = 61) during hospitalization (median [IQR] serum level of CRP during hospitalization: 73.4 [53–112]), we did not observe any difference in serum levels of LBP or sCD-14 between the favorable and unfavorable outcome groups at any measurement time point. In the group without infection (n = 116), we performed a correlation analysis between serum CRP levels and early (D1) and late (D9) LBP and sCD-14 values. We did not observe a significant correlation for any marker in either the early or late phase.

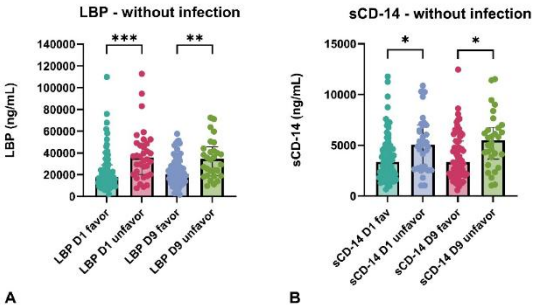


Figure 7. Serum levels of LBP and sCD-14 in aSAH patients without in-hospital infection according to 3-month outcome. The serum LBP level in the aSAH group without infection (A), the serum sCD-14 level in the aSAH group without infection (B). D1, sampling time 24 h after ictus, D9, sampling time 9 days after ictus, favor, favorable (modified Rankin score 0–3) outcome, unfavor, unfavorable (modified Rankin score 4–6) outcome, * denotes p <0.05, ** denotes p <0.01, *** denotes p <0.001, number of patients in each group, favorable outcome: n = 61, unfavorable outcome: n = 55.

In addition, very strong positive correlations were observed between LBP and sCD-14 in both the early (D1) and late (D9) phases of aSAH (Figure 8). In addition, we did not observe any significant correlation among the three metabolites and factors such as age, gender, smoking, diabetes, and hypertension.

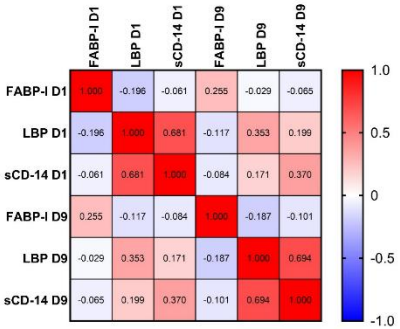


Figure 8. Heat map showing correlation matrix of FABP-I, LBP and sCD-14. In heatmap, red indicates positive, while blue indicates negative correlation. The darker the color, the stronger the correlation. The Spearman rank correlation test was used. A p-value <0.05 was accepted as significant. p > 0.10 are presented in white squares. FABP-I, fatty acid-binding protein-intestinal, LBP, lipopolysaccharide-binding protein, D1, sampling time 24 h after ictus, D9, sampling time 9 days after ictus.

5. DISCUSSION

In this study, we utilized the UPLC-MS/MS system to examine the changes in the metabolomic profile of aSAH patients between day 1 and day 7 post-aSAH, as well as differences between groups with good and poor 3-month outcomes. The most important findings can be summarized as follows: (1) At 24 h post-injury, the group with poor 3-month outcomes exhibited significantly elevated levels of mannose/sorbose and kynurenine alongside reduced levels of amino acids. (2) At 168 h following the injury, significantly higher levels of 2-hydroxybutyrate, methyl galactoside, mannose/sorbose, creatine, xanthosine, N-acetylmethionine, N-acetylalanine, and p-hydroxyphenylacetate were detected in the serum of patients with poor 3-month outcomes. (3) At 168 h post-aSAH, the poor outcome group exhibited significantly lower levels of guanidinoacetate. Moreover, certain essential amino acids were also found at reduced levels during both the early and late sampling times in this group. Conversely, concentrations of specific N-acetyl amino acid derivatives were elevated in the poor prognosis group at the late sampling time. (4) Between the two sampling times, homocysteine showed significantly lower serum levels in the late samples compared to the early ones. After 24 h following initial SAH, based on metabolomic measurements, we observed a significant increase in kynurenine levels in the group with poor outcomes. Kynurenine serves as a sensitive marker of neuroinflammation in the acute phase of neurological disorders associated with inflammation. Previous research has shown that activation of the kynurenine pathway is associated with poor outcomes in critically ill patients, as well as with higher mortality and larger infarct volumes in early-phase ischemic stroke patients. Additionally, it is significantly overactivated during inflammation in traumatic brain injury. These prior findings are consistent with those observed in the current study. During the acute phase, patients with aSAH (aneurysmal subarachnoid hemorrhage) and poor outcomes exhibit heightened inflammatory responses. In a metabolomic study conducted on critically ill patients, higher levels of kynurenine, sucrose, and p-hydroxyphenylacetate showed an association with higher mortality rates. The elevated kynurenine levels observed in the poor outcome group in this study underscore the presence of significant acute neuroinflammation. Guanidinoacetate (GAA) is a naturally occurring amino acid derivative that serves as a direct precursor of creatine and is produced from arginine and glycine through the catalytic action of l-arginine: glycine amidinotransferase (AGAT), primarily in the kidneys and liver. Later, it is converted into creatine

and homocysteine, predominantly by guanidinoacetate N-methyltransferase (GAMT), mainly in the liver but also in the brain. Accumulation of GAA in the brain and body fluids (serum levels > 16 μmol/L) is associated with muscle weakness, epilepsy, and intellectual disability in individuals with GAMT deficiency. Conversely, GAA depletion (serum levels <1.9 μmol/L) has been observed in patients with chronic kidney disease and diabetes mellitus. GAA may act as an agonist for gamma-amino butyric acid (GABA)-A receptors and could potentially modulate GABA metabolism in the brain and peripheral tissues. It also exhibits a vasodilatory effect and can function as a phosphocreatine mimetic and an alternative energy donor, particularly when creatine availability is limited. Its vasodilatory effect is achieved through two mechanisms: inhibiting norepinephrine methylation, thus affecting its half-life and sympathetic activity, and indirectly inducing vasodilation by sparing arginine and promoting arginine-mediated nitric oxide (NO) production. In our current study, we found that on the 7th-day post-SAH, serum samples from the group with poor outcomes showed GAA depletion, higher creatine levels, and decreased homocysteine levels compared to the group with good outcomes. The lower GAA levels in the poor prognosis group could be attributed to impaired GAA production or increased utilization. One possible explanation for GAA depletion is the enhanced conversion to creatine, while the conversion to homocysteine is less pronounced. The decrease in available GAA levels could also be attributed to liver and kidney dysfunction resulting from severe conditions, which are prevalent in a significant portion of patients with severe conditions and poorer outcomes. The low level of guanidinoacetate definitely draws attention to severe energy metabolism disturbance in the late stage of SAH. Furthermore, the lower GAA levels observed in the group with poor prognosis theoretically limit its vasodilatory and GABA agonist effects. However, the clinical significance of this observation remains uncertain and warrants further investigation. In our study, significantly higher levels of 2-hydroxybutyrate were observed in serum samples taken on day 7 in the group with poor 3-month outcomes. During brain ischemia, the liver produces β-hydroxybutyrate, which is then consumed by the brain. Clinical studies have demonstrated a linear correlation between the arterial concentration of ketone bodies (KB) and cerebral KB uptake. In human studies, elevated KB values can appear as early as 72 h after the injury, depending on the degree of starvation. 2-hydroxybutyrate is a ketone body that accurately predicts diabetic ketoacidosis in children and adolescents, and ketosis may represent an intermediate state of metabolic dysregulation rather than being associated with a more severe acute illness. However, high levels of ketone bodies have

numerous favorable effects on central nervous system functions. 2-hydroxybutyrate has been shown to improve neurological function in focal ischemia, reduce outcomes of global ischemia and stroke, and prevent neuronal death in models of Alzheimer's and Parkinson's disease. It exerts multiple activities in the brain, including interaction with ion channels, inhibition of histone deacetylation, indirect antioxidative activity, and inhibition of neuroinflammation. The production of β-hydroxybutyrate during brain ischemia may offer advantages as it can serve as an energy source for the brain, bypassing the need for glucose and reducing the risk of harmful lactate formation. β-hydroxybutyrate also reduces the periinfarct glucose-metabolism-driven production of reactive oxygen species and astrogliosis, leading to improved neurogliovascular and functional recovery after ischemic insult. BHB serves as an alternative carbon source, fueling oxidative phosphorylation (OXPHOS) and the production of bioenergetic amino acids and glutathione, which are crucial for maintaining redox balance. Additionally, BHB supports T cell responses during infections and may indicate impaired ketogenesis. Ultimately, elevated levels of 2-hydroxybutyrate may signal metabolic dysregulation due to acute subarachnoid hemorrhage. Alternatively, this increase could represent a compensatory response, indicative of a shift in energy production towards ketosis, which offers several beneficial effects on nervous system function. In our study cohort, all patients received adequate enteral nutrition in accordance with clinical guidelines regarding both quantity and quality. The primary objective of enteral feeding in critically ill patients is to provide sufficient nutrition and support to satisfy their metabolic requirements and either maintain or enhance their nutritional status. In the group with poor outcomes, the higher levels of ketone bodies observed in the late phase are likely not a consequence of inadequate enteral nutrition but rather attributable to changes in energy metabolism independent from nutritional circumstances. Overall, the presence of significantly higher levels of 2-hydroxybutyrate in serum samples taken on day 7 in the group with poor 3-month outcomes might suggest alterations in metabolic processes, potentially reflecting changes in energy metabolism or metabolic stress in individuals with adverse outcomes. In our current study, we observed significantly lower amino acid levels in the poor outcome group compared to the good outcome group at both sampling time points, except for the concentrations of N-acetylmethionine and N-acetylalanine. Sjöberg et al. observed an increase in amino acid levels in the blood during the first week following subarachnoid hemorrhage and described the moderate predictive power of serum myoinositol for a one-year favorable outcome. In a study

involving 29 patients, amino acid levels were higher in the poor outcome group compared to the favorable outcome group, but the significance of the difference was very slight and was only observed in the early stage (0–3 days). In another study where plasma samples of aSAH patients were examined, no differences were found in the levels of amino acids between early and late samples, nor between the plasma amino acid levels of good and poor outcome groups; in fact, certain amino acid levels decreased in the plasma of aSAH patients compared to controls. Tuoho et al. observed a hypermetabolic state in patients with ICH and aSAH undergoing surgery; similar results were observed in another study, additionally demonstrating that increased amino acid infusion had no effect on amino acid exchange. Based on the literature data and our study, it could be concluded that the changes in amino acid metabolism detectable in the blood following aSAH are complexly influenced by numerous factors (severity, postoperative nutrition, etc.). During critical illness, the metabolism of macronutrients is altered at multiple levels. The utilization of energy substrates predominantly relies on the mobilization of endogenous stores, which is governed by a range of regulatory mechanisms. The oxidation of carbohydrates is globally more increased during the early phase than the oxidation of lipids and proteins in critically ill patients. Moreover, when increasing nitrogen intake while maintaining calorie load or changing only the composition of nitrogen carriers, the supply of essential amino acids could become inadequate. Our results support this previous observation, as we found significantly increased mannose/sorbose levels and decreased amino acid levels in patients with poor outcomes, both in the early and late stages of aSAH.

In the second study, we found the following key results: (i) the serum levels of LBP and sCD-14 were significantly higher in the aSAH cohort compared to the unruptured control group, while the serum level of FABP-I was significantly lower in the patient group compared to control, (ii) the serum level of FABP-I did not differ significantly, whereas LBP and sCD-14 levels (specifically in the D9 samples) were significantly different between the outcome groups, (iii) LBP and sCD-14 showed a difference between the favorable and unfavorable outcome groups in patients without in-hospital infections, while (iv) LBP and sCD-14 were significantly higher in the DCI group during the later phase, (v) LBP and sCD-14 exhibited a strong positive correlation in both the early and late phases of aSAH. First, we found that FABP-I was significantly lower in the aSAH cohort than in the control group, which was contrary to our expectations. Moreover, the decrease between the

early and late phase values within the patient group was also significant. This result is unexpected, considering that several studies involving patients with ischemic or inflammatory gastrointestinal conditions, or those who had experienced traumatic brain injury, reported higher levels compared to the healthy control group. Similarly, elevated serum FABP-I levels have also been observed in certain psychiatric disorders and in ischemic stroke. Our results may have several explanations. Theoretically, a reduced level of FABP-I may reflect diminished enterocyte mass or altered intestinal function. In our previous study, we found that in aSAH patients, metabolism shifts towards ketosis and alternative energy sources in both the early and late phases, even with adequate enteral nutrition, particularly in patients with poor outcomes. Other studies also have highlighted that the nutritional status of aSAH patients, both at the time of admission and throughout their hospitalization, plays a crucial role in affecting their clinical outcomes. It is well-established that energy expenditure significantly increases in patients with aSAH following the onset of the condition, but we also know from an international, multicenter ICU study that aSAH patients were routinely underfed, receiving less than 60% of both total caloric and protein intake. Additionally, Hernandez et al. found that a short period of enteral fasting was associated with significant duodenal mucosal atrophy and abnormal gut permeability in critically ill patients. Based on this, we can assume that inadequate intake of nutrients can impair intestinal health and reduce enterocyte proliferation, and the early malnutrition of aSAH patients may lead to decreased synthesis of FABP-I due to a reduced number of functional enterocytes. In our study, we also found that LBP and sCD14 levels were significantly higher in the poor outcome group compared to the favorable outcome group. LBP is a soluble protein that binds to LPS and delivers it to CD14, triggering the activation of target cells via toll-like receptors (TLRs), which play a crucial role in regulating innate immunity. CD14 also exists in a soluble form (sCD14). LBP levels in the serum reach their highest shortly after the onset of bacteremia or endotoxemia and remain elevated for up to 72 h. Once in the bloodstream, LBP binds to LPS, facilitating its interaction with CD14 receptors. Membrane-bound CD14 is linked to TLR4, which transmits the signal from the CD14-bound LPS to the cell's nucleus, initiating a cascade that leads to the release of inflammatory cytokines. The LBP level has been proposed as a clinical indicator of "active endotoxemia". Elevated LBP levels may indicate exposure to bacterial components, particularly LPS, and could serve as a predictor of disease progression and unfavorable outcomes. The increase in LBP concentration has been observed in various metabolic diseases, which are linked to changes in

gut permeability and alterations in the gut microbiome. An increase in plasma endotoxin activity was associated with higher levels of LBP and sCD14. Elevated concentrations of both LBP and sCD14 can inhibit the bioactivity of LPS both in vitro and in vivo. The rising blood levels of LBP and sCD14 may serve as a compensatory mechanism to prevent excessive TLR4 stimulation and to limit systemic inflammation in patients with ischemic stroke. In the study by Klimiec et al., the source of circulating LPS could not be determined, and there was no difference in serum levels between patients with and without infection, which raised the possibility of bacterial translocation as a contributing factor. This suggests that LPS might enter the bloodstream from the gut rather than from an overt infection. In our study, the serum levels of LBP and sCD-14 were significantly higher in the unfavorable outcome group among aSAH patients without in-hospital infection. Based on this, it can be stated that in our current study, although the molecules examined are well-documented serum markers of gut permeability, they do not clearly prove that the appearance of LBP in the systemic circulation is definitely a product of microorganisms related to the gut microbiome. Nevertheless, the body's microbiome systems (e.g., lungs, gonads, etc.) may also be affected by the pathophysiological stress caused by aSAH. Therefore, endotoxins could be released from these microbial compartments as well. Thus, our results primarily indicate that while the precise origin of the LBP and sCD-14 appearing in the circulation and reaching significantly higher levels in the unfavorable outcome group cannot be confirmed, one potential source could be the human gut microbiome, whose endotoxins may enter the systemic circulation during the early phase of aSAH due to impaired gut permeability.

Our current study also found that the serum levels of LBP and sCD14 were significantly higher in the subgroup with DCI. Since DCI typically occurs between days 7 and 14, the difference observed in our study between the DCI-positive and DCI-negative groups in the late phase regarding LBP and sCD-14 serum levels may indicate a correlation between these two markers and DCI. Our findings suggest a connection between DCI and elevated levels of such permeability markers. Importantly, patterns of certain cytokines observed in the sera of patients with aSAH are linked to the development of DCI. Considering the previously outlined relationship between the LPS–LBP–sCD14 interaction in activating the inflammatory response, we hypothesize that the endotoxins entering the circulation during aSAH may play a direct role in the development of DCI by initiating and sustaining inflammatory processes. In conclusion, while the studied markers do not aid in the early prediction of DCI, LBP in the early phase (D1) is indicative of an unfavorable

prognosis. Notably, both sCD-14 and LBP significantly differentiate between outcome groups in the infection-free cohort, even during the early phase. These findings highlight the potential of these biomarkers for early outcome stratification, though further studies are needed to clarify their causative role.

6. CONCLUSION

This dissertation integrates the results of two complementary studies investigating metabolic alterations and systemic biomarker changes following aneurysmal subarachnoid hemorrhage. Together, these findings highlight the dynamic and multifactorial nature of secondary brain injury and its impact on clinical outcome.

The metabolomics analysis identified distinct, time-dependent serum metabolic profiles that differentiated patients with favorable and poor neurological outcomes at three months. These alterations predominantly affected pathways related to energy metabolism, amino acid turnover, and oxidative stress regulation. In patients with poor outcomes, metabolic changes observed in the later phase after hemorrhage—characterized by elevated 2-hydroxybutyrate levels and reduced guanidinoacetate and amino acid concentrations—suggest a shift toward ketone-based energy metabolism, despite adequate enteral nutrition during intensive care. These findings indicate that systemic metabolic adaptations may reflect disease severity and influence neurological recovery, and they raise the possibility that targeted nutritional strategies could play a role in patient management. However, the precise pathophysiological role of ketosis in the late phase of aSAH requires further investigation.

The biomarker study extended these observations by demonstrating early alterations in serum markers of gut permeability and innate immune activation. Elevated levels of lipopolysaccharide-binding protein and soluble CD14 were associated with disease severity and clinical outcome. In patients without clinically confirmed infection, these changes suggest the potential involvement of gut-derived inflammatory signaling, possibly mediated by endotoxin translocation, contributing to neuroinflammation and secondary brain injury.

Taken together, the results of these two studies provide a more comprehensive view of the interconnected metabolic and inflammatory responses following aSAH. Combined metabolomic and biomarker profiling may improve

prognostication and offers a promising approach for identifying novel therapeutic targets and supporting more personalized treatment strategies in the future.

7. FURTHER AIMS AND PERSPECTIVES

Future investigations should aim to validate these findings in larger, multicenter cohorts by using standardized sample collection and harmonized analytical protocols. Long-term analyses across multiple time points can give a more complete understanding of metabolic and inflammatory patterns, helping to detect delayed cerebral ischemia and predict long-term functional outcomes. Combining metabolomic data with imaging, genomic, and clinical information may further aid in developing strong multimodal prediction models and support precision medicine strategies in aSAH care.

From a clinical perspective, translating key biomarkers—such as LBP and FABP-I—into rapid or bedside tests could greatly improve early assessment and monitoring of systemic complications. Simultaneously, mechanistic studies exploring how metabolism, inflammation, and gut barrier function interact are needed to identify new therapeutic targets that reduce early brain injury. Overall, this work lays a foundation for future translational research that connects metabolic insights to personalized clinical management in aneurysmal subarachnoid hemorrhage.

8. LIST OF PUBLICATIONS

Publications related to the thesis:

Orban B, Tengölics R, Zavori L, Simon D, Erdo-Bonyar S, Molnar T, Schwarcz A, Csecsei P. The Difference in Serum Metabolomic Profiles between the Good and Poor Outcome Groups at 3 Months in the Early and Late Phases of Aneurysmal Subarachnoid Hemorrhage. Int J Mol Sci. 2024 Jun 15;25(12):6597. doi: 10.3390/ijms25126597. PMID: 38928303; PMCID: PMC11203497.

IF: 4,9

Orban B, Simon D, Erdo-Bonyar S, Berki T, Molnar T, Zavori L, Schwarcz A, Peterfi Z, Csecsei P. Effects of Aneurysmal Subarachnoid Hemorrhage in Patients Without In-Hospital Infection on FABP-I, LBP, and sCD-14. Int J Mol Sci. 2025 Jan 8;26(2):485. doi: 10.3390/ijms26020485. PMID: 39859200; PMCID: PMC11764490.

IF: 4,9

Cumulative impact factor related to the thesis: **9,8**

Other publications:

Csecsei P, Takacs B, Pasitka L, Varnai R, Peterfi Z, **Orban B**, Czabajszki M, Olah C, Schwarcz A. Distinct Gut Microbiota Profiles in Unruptured and Ruptured Intracranial Aneurysms: Focus on Butyrate-Producing Bacteria. J Clin Med. 2025 May 16;14(10):3488. doi: 10.3390/jcm14103488. PMID: 40429482; PMCID: PMC12111819.

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