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Original Article

Comparative Metabolomic Profiles of Vascular Involvement in Behçet's Disease

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Abstract

Background: Behçet's disease is a systemic, inflammatory disease affecting multiple organs. Vascular involvement is the main cause of morbidity and mortality in Behçet's disease patients. Though clinically well-defined, there is limited information related to disease pathogenesis and vascular incidence in this patient group. The aim of this study is to investigate the unique metabolic signatures of Behçet's disease patients with vascular involvement.

Methods: Metabolomic profiling was performed on serum samples of 48 Behçet's disease patients (18 with vascular involvement) and 40 healthy controls using gas chromatography-mass spectrometry-based untargeted metabolomics analysis. Multivariate and univariate statistical analyses were performed to find altered metabolites and pathways.

Results: Untargeted metabolomics results showed that a total of 168 metabolites were identified. The comparison between the groups of Behçet's disease, vascular involvement in Behçet's disease, and the healthy control group showed that altered amino acid and oxidative stress pathways, especially with glutathione synthesis, could be an important stage for developing Behçet's disease.

Conclusion: In the present work, the untargeted metabolomics approach provided new molecular insights for a better understanding of Behçet's disease pathogenesis and also developing vascular involvement in Behçet's disease at the metabolite level. The results showed that vascular involvement in Behçet's disease could be highly linked with amino acid metabolism and also the antioxidant system, and these disease-related pathways could be evaluated with further experiments for diagnosis and prognosis of Behçet's disease and also for vascular involvement in Behçet's disease.

Keywords: Behçet's disease, vascular involvement, metabolomics

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Introduction

Behcet's disease (BD) is a recurrent inflammatory vasculitis mostly seen in young adults. The disease is a chronic, systemic disease, and it affects different tissues and organs such as oral and genital mucosa (aphtheous ulcers), skin, joints, vascular structures, central nervous system, gastrointestinal system, and ocular tissues.[1] The disease is basically distributed among Mediterranean countries and is seen with a frequency of nearly 300 cases per 100 000 population. The etiology and pathogenesis of BD are not still clearly known. Genetic susceptibility and immunological abnormalities are the most commonly stated possible causes of BD development.[2] Vascular involvement is seen in around 7%-45% of all BD cases. Vascular incidences like venous thromboembolism and arterial aneurysm are risk factors for mortality and morbidity in BD patients.[3] Although clinically well presented, BD diagnosis remains to be a challenge due to the lack of specific biomarkers for diagnosis. The metabolic basis of BD is unknown yet, and it hampers the improvement of laboratory markers or specific parameters to support clinical findings, diagnosis, and treatment.

Metabolomics is a branch of the post-genomic era and gives crucial information about the phenotype of organisms. In recent years, metabolomics has been used to observe disease mechanisms, find markers at the early phase to diagnose and monitor the prognosis of diseases and new therapeutic targets. In the literature, there are several studies about metabolomic profiles of different autoinflammatory diseases, including BD, rheumatoid arthritis (RA), and systemic lupus erythematosus.[4,5]

In the present work, we focused on several points regarding BD. First, we analyzed BD and vascular involvement in BD (VBD) to find differences from healthy control groups to understand the molecular basis of BD. Moreover, we analyzed the altered metabolome structure of the VBD group according to BD for understanding the mechanism of VBD.

Material and Methods

Study Population

The study is a cross-sectional study including 48 BD patients, of whom 18 had vascular BD, and 40 age- and gender-matched healthy participants who were admitted to rheumatology outpatient clinics. Participants having any coexistent autoimmune or inflammatory diseases, any type of infection, anemia, and any chronic metabolic illnesses (such as diabetes mellitus type 2, dyslipidemia, hypertension, and metabolic syndrome), any cardiovascular disease, any abnormal renal or hepatic function, thyroid disease, malignancy, and women who were pregnant or postpartum for 6 months were not included in the study.

Compliance with Ethical Standards

Medical approval was received from the Gülhane Training and Research Hospital Ethics Committee with the decision number 2020/15. The research protocol follows the 2000 Declaration of Helsinki, and written informed consent was received from all subjects.

Clinical Evaluation

A detailed medical history and physical examination were carried out for all participants. Behcet's disease diagnosis was made based on the criteria from the International Study Group. Demographic and clinical data were also recorded.[6] Disease activity was evaluated with the Turkish version of the Behcet Disease Current Activity Form (BDCAF).[7] The items included in the form are as follows: headache, oral and genital ulcers, erythema, skin pustules, arthralgia and arthritis, and involvements in intestinal, ocular, nervous systems, and also in major vessels. The BDCAF score was calculated by summing up each item score, and the scale was between 0 and 12 points. Patients who had vascular involvement were the ones who had active vasculitis lesions during sample collection (basically deep vein thrombosis, aortic plaques, stenosis, occlusions, and aneurysms). Clinical features of BD patients are summarized in Table 1.

Table 1. Clinical Features of Patients with Behçet's Disease

Parameter	Values
Age at diagnosis [years], mean ± SD	32.3 ± 4.9
Disease duration [months], median (IQR)	94.8 (71.7)
Active disease, n (%)	28 (58.3)
BDCAF, median (IQR)	2.0 (0.0-3.0)
Disease symptoms in patient population, n (%)	
Oral ulcers	48 (100)
Genital ulcers	36 (75)
Erythema nodosum	24 (50)
Papulopustular lesions	28 (58.3)
Positive pathergy test	12 (25)
Arthritis	21 (43.7)
Uveitis	24 (50)
Vascular involvement	18 (37.5)
Venous thrombosis	16 (89)
Arterial aneurysm	1 (5.5)
Pulmonary aneurysm	1 (5.5)

Metabolomics Analysis

Sample Collection

Blood samples were collected from the antecubital vein from all participants and centrifuged at 3000 rpm for 15 minutes. Separated sera were elicited into Eppendorf tubes to be stored at −80°C until sample analysis.

Metabolomics Sample Preparation Analysis

Eight hundred fifty microliters of methanol : water mixture (9 : 1, v/v) was added to 150 µL of sample. The mixture was thoroughly vortexed for 1 minute, and then centrifugation was performed at 15 000 rpm for 10 minutes. Four hundred microliters of supernatant was evaporated overnight at 4°C using a vacuum centrifuge. After drying, samples were methoxylated and derivatized as described previously.[8,9] After the addition of 20 µL methoxyamine hydrochloride (20 mg/mL in pyridine), all samples were incubated for 90 minutes at 30°C. Finally, N-methyl-N-(trimethylsilyl)-trifluoroacetamide solution including 1% trimethylchlorosilane was added, and then the samples were incubated at 37°C for 30 minutes.

Gas Chromatography-Mass Spectroscopy Analysis of Plasma Metabolites

Metabolomic analysis based on gas chromatography-mass spectroscopy (GC-MS) was performed as previously detailed.[9] Metabolomic profiling was carried out by a GC-MS analyzer (Shimadzu GCMS-QP2010 Ultra, Kyoto, Japan) with a DB-5MS stationary phase column (30 m + 10 m DuraGuard × 0.25 mm i.d.; 0.25 µm film thickness). Sample injections were done in splitless mode. The oven temperature was fixed at 60°C for 1 minute, then elevated gradually to 325°C by an increase of 10°C each minute, and held for 10 minutes at 325°C. The separation time was 37.5 minutes in total. Electron-impact ionization was carried out at 70 eV. Data acquisition was performed in full scan mode, and the mass range was 50-650 m/z.

Data Analysis

Complex chromatograms were deconvoluted and the peaks were aligned, and a data matrix was constructed using MS-DIAL (v.2.56, http://prime.psc.riken.jp/compms/msdial/main.html). In the MS-DIAL setting, the range for mass detection was 50-650 Da, and the minimum peak height for trustable component detection was chosen to be 1000 amplitude. The preferred tolerance limit for retention time was 0.05 minutes. The recognition cut-off score was 70%. The Fiehn Retention Index database was used to identify the metabolites.

The data matrix normalization was established based on the sum of the total peak area for individual samples. In the matrix, metabolites that had traits with over 50% of the values missing were not included. Missing values in the data matrix were filled with the half value of the lowest concentration in the metabolite group. The Partial least squares-discriminant analysis (PLS-DA) analysis in Metaboanalyst 4.0 platform was used for class separation, simplified interpretations, and searching for candidate biomarkers. The variable importance in projection (VIP) value is used to discriminate the most significant metabolites for stratified groups. Student's unpaired *t*-test was used for comparison of changes in mean expression per metabolite among groups. $P < .05$ was accepted to be statistically significant. Altered metabolites were evaluated in pathway analysis within the Metaboanalyst 4.0 platform.

Results

In this study, we focused on dynamic plasma metabolome structure to understand the physiological fundamentals of BD and VBD using GC-MS-based metabolomics analysis. Untargeted metabolomics analysis covered a

Main Points

- This work is a pioneer study as it comparatively evaluates the metabolic profiles of Behcet's disease (BD) patients with and without vascular involvement.
- The results of the study have shown that the most significant difference in BD patients is seen in amino acid metabolism and antioxidant pathways.
- The results of the study can bring out candidate diagnosis markers in terms of metabolites.

comparison of metabolome between healthy and BD and VBD groups. In metabolomics analysis, we totally identified 168 metabolites.

Metabolomics results of BD and healthy control groups were compared to understand physiological changes in BD (Figure 1). A total of 11 metabolites decreased and 3 metabolites increased in the BD group, and these metabolites have a molecular function in various pathways. Most significantly decreased metabolites in BD patients are α-ketoglutarate, alanine, glutamine, glutamate, malic acid, citramalic acid, cysteine, homoserine, methyl myristate, shikimic acid, and oxaloacetic acid. There were increases in levels of trehalose, 2-hydroxybutyric acid, and melibiose in BD patients.

Vascular involvement in Behçet's disease is a rare type of BD (Behçet's Disease), and there is limited information about this condition. First, we compared the metabolomes of VBD, and healthy control groups and altered metabolites and their pathways are given in Figure 2.

Vascular involvement in Behçet's disease and BD comparison is another important goal in understanding the mechanism of vascular involvement in BD. Results represented in Figure 3 show that various metabolites involved in amino acid metabolism were significantly downregulated in VBD groups. Seven metabolites decreased in the VBD group, and only hydroxyphenylacetic acid increased in the VBD group. These metabolites are involved in amino acid metabolism, sulfur metabolism, and also methionine metabolism.

Discussion

Behçet's disease is a chronic, systemic autoinflammatory vasculitis presented with oral and genital ulcers, uveitis, and arthritis.[10] Vascular involvement in Behçet's disease is known to be seen in nearly 10%-40% of BD patients and is a major cause of morbidity in BD patients.[11] The diagnosis of BD disease is challenging because of the lack of specific or serological biomarkers. Especially, the detection of vascular involvement in BD is presented by clinical vascular incidences like deep vein thrombosis, pulmonary embolism, or arterial aneurysms. Unfortunately, there is no predictive or identifying biomarker for early detection and prevention of disease.[12,13] Metabolomics is a new and promising method for the detection of disease-specific biomarkers, and the metabolomic approach has come to the front for finding biomarkers in various rheumatic diseases like lupus, RA, ankylosing spondylitis, and also BD.[14-17]

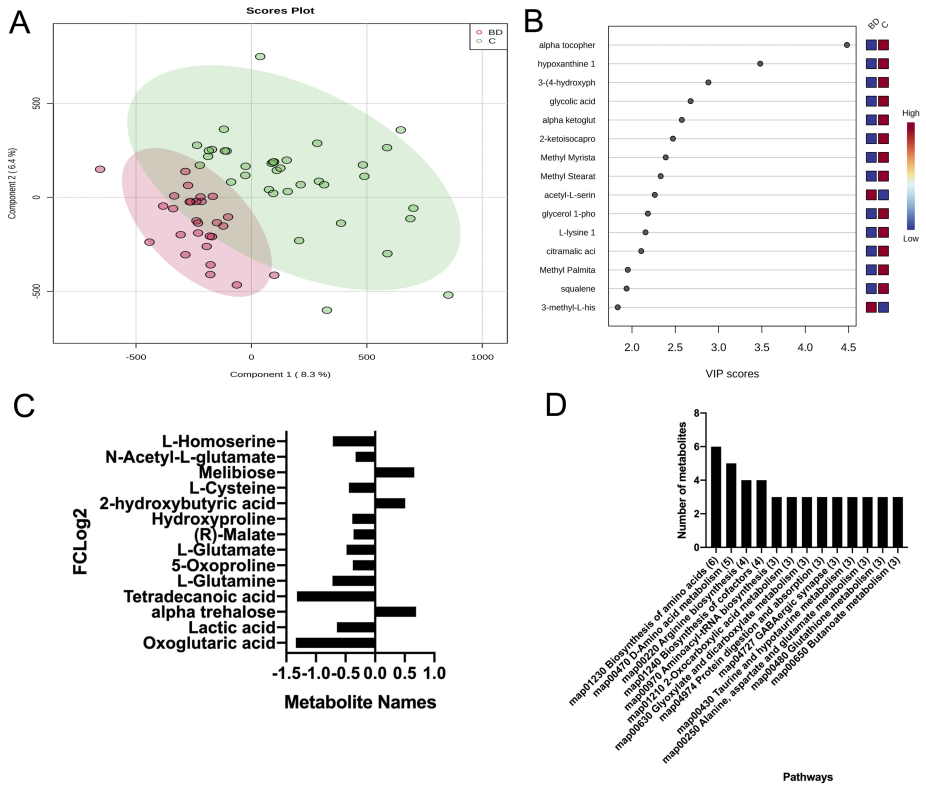

Figure 1. (A-D) PLS-DA analysis of BD and healthy control (C) groups (A), VIP scores of PLS-DA analysis (B), statistically altered metabolites in BD group (C), and pathway analysis of altered metabolites (D). BD, Behçet's disease; VIP, variable importance in projection.

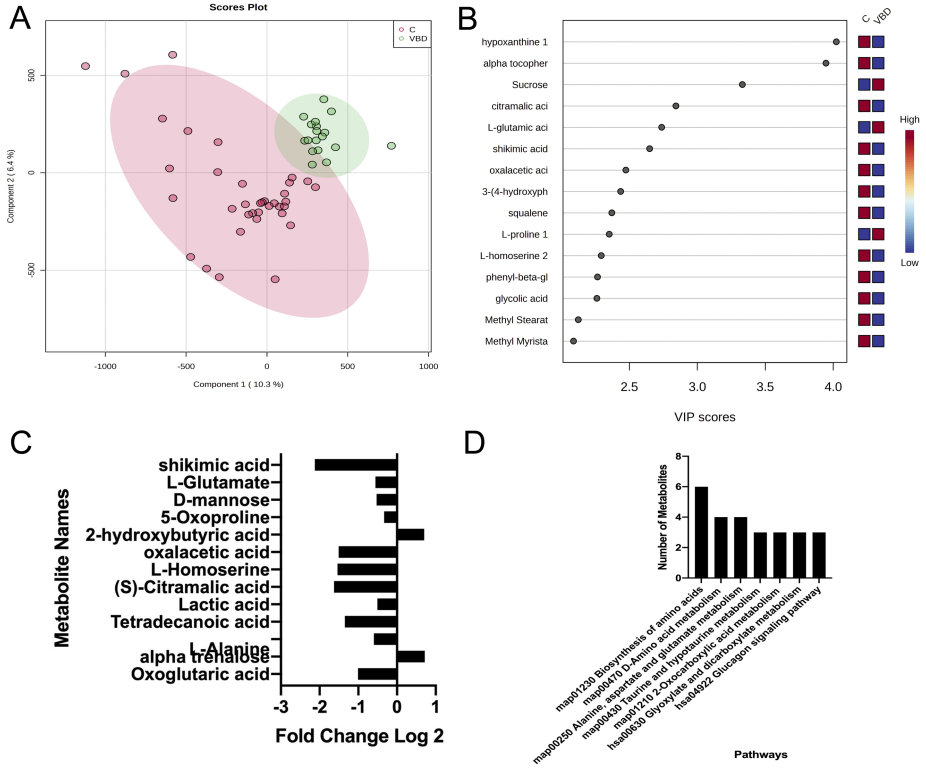

Figure 2. (A-D) PLS-DA analysis of VBD and healthy control (C) groups (A), VIP scores of PLS-DA analysis (B), Statistically altered metabolites in VBD group (C) and Pathway analysis of altered metabolites (D). VBD, vascular involvement in Behçet's disease; VIP, variable importance in projection.

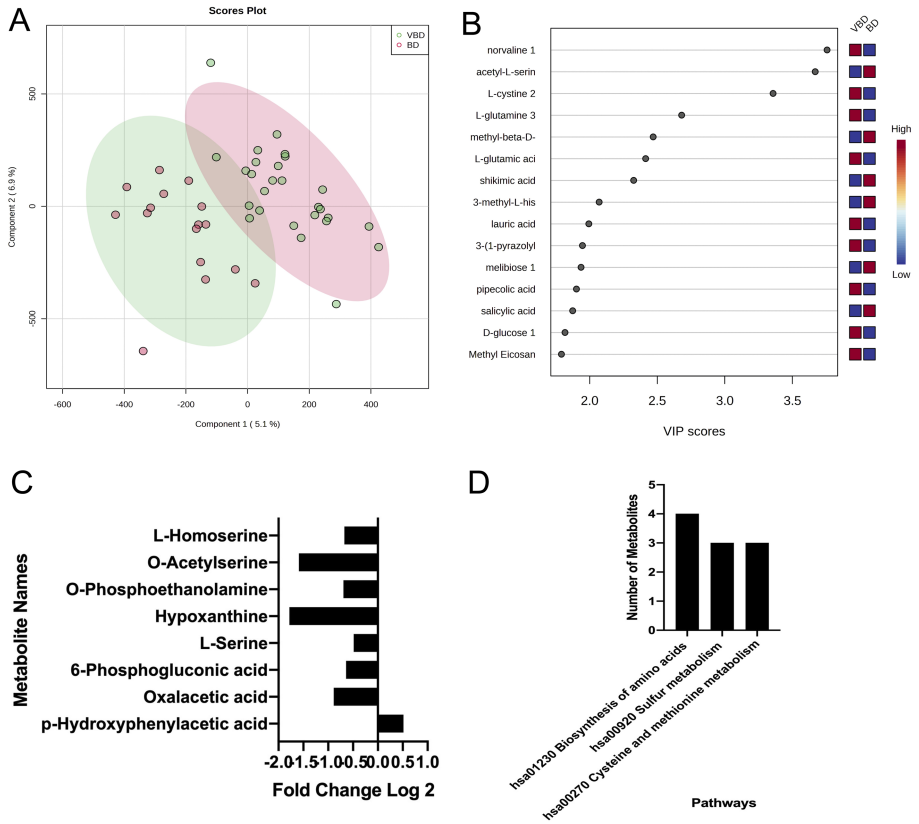

Figure 3. (A-D) PLS-DA analysis of VBD and BD groups (A), VIP scores of PLS-DA analysis (B), statistically altered metabolites in VBD group (C), and pathway analysis of altered metabolites (D). BD, Behçet's disease; VBD, vascular involvement in Behçet's disease; VIP, variable importance in projection.

In this study, we examined the metabolomic profile changes in BD patients who did or did not have vascular involvement. A total of 168 metabolites in the serum samples of 3 study groups (BD, VBD, and healthy controls) were identified. In total, 29 amino acids and biogenic amines were detected among participants.

This study revealed significant amino acid metabolic disturbances among BD patients and control group. When we compared the overall metabolomic profiles of BD patients and VBD patients with healthy controls in terms of amino acid biosynthesis and utilization pathways, we observed that the most common changes have occurred in glutamate, glutamine, alanine, α-ketoglutarate, cysteine, oxaloacetate, and homoserine levels (Figure 1). The levels of all these amino acids and amino acid derivatives were higher in the control group and decreased in BD patients. The decrease in levels of these amino acids and their derivatives in BD patients may suggest the utilization of these amino acids in this patient group. The common amino acids affected both in BD and VBD patients are glutamate, α-ketoglutarate, and homoserine. Besides being a major amino acid in protein synthesis, glutamate is also involved in various metabolic processes in a variety of organisms. Glutamate is produced by the conversion of α-ketoglutarate by glutamate dehydrogenase in the citric acid cycle, which is the key element of oxidative phosphorylation in the human body. This can be a sign of decreased aerobic respiration in BD patients, which may be caused by tissue hypoxia.[18] Another key function of glutamate is that it is a building block of glutathione, which functions in redox homeostasis of the cell and protects the cell against oxidative stress.[19] In previous studies, it has been shown that serum antioxidant status decreases in patients with BD.[20] The findings of our study are in concordance with these results. The fold changes in both α-ketoglutarate and glutamate were similar in both BD and VBD groups when compared to healthy controls. L-cysteine levels were also found to be lower in BD patients without vascular involvement when compared to healthy controls. As cysteine is another amino acid found in the structure of glutathione, this also supports the idea that antioxidant mechanisms are diminished in BD patients. Homoserine was another commonly decreased metabolite in both BD and VBD patients compared to healthy controls. Homoserine is the first-step precursor of methionine synthesis. A decrease in levels of both cysteine and homoserine in BD patients can be accepted as a sign of interrupted amino acid synthesis in these patients. Another commonly decreased substance in all BD patients was 5-oxoproline (L-pyroglutamic acid). This natural amino acid derivative is, in fact, a metabolite in the glutathione cycle that is converted to glutamate by 5-oxoprolinase.[21,22] This reaction is also helpful in the regulation of cellular redox potential.[23] The lower levels of oxoproline together with cysteine and glutamate in BD patients once again point out the altered glutathione metabolism, reduced redox potential, and increased oxidative stress in this patient group.

Methyl myristate levels were decreased in all BD patients, regardless of vascular involvement. Myristic acid (tetradecanoic acid) is a commonly known saturated fatty acid that is known to serve as a lipid anchor in biological membranes. In the body, myristic acid may have positive effects on high-density lipoprotein (HDL) cholesterol and so improves the HDL/total cholesterol ratio.[24] It is known that BD is a disease that affects vascular structures, and in one of our previous studies, we also focused on increased atherogenic risk in patients with vascular BD.[25] Decreased levels of HDL increase the incidence of cardiovascular events. The findings of the present study about decreased methyl myristate in BD patients are in concordance with previous studies, indicating that it will cause a lowering in circulating HDL levels and increase atherogenic risk.

The glutamine level decreased in BD patients, while the alanine level decreased in VBD patients. Both glutamine and alanine are synthesized in the skeletal muscle from the essential branched-chain amino acids (BCAAs). Glutamine is the most abundantly found amino acid in free form within the human body, and it comprises nearly 20% of the free amino acid pool in circulation. Glutamine is also important for fast-dividing cells, and it also functions in nucleotide synthesis. Alanine is also important in gluconeogenesis.[26,27] In the disease state, protein turnover is increased and BCAAs are degraded, which causes an increase in glutamine and alanine levels. However, the demand for alanine and glutamine in catabolic illness increases, so their use exceeds their production, and there occurs starvation in alanine and glutamine in plasma and tissues.[28] Several reports have shown that alanine–glutamine have protective roles toward tissue injury and ischemia–reperfusion injury by elevating GSH (glutathione) levels in the liver.[29,30] So,

these findings are in concordance with the decreased GSH levels and increased oxidative stress in BD patients we previously mentioned earlier.

We observed a decrease in malic acid and citramalic acid levels in BD and VBD groups, respectively. Citramalic acid is a malic acid analog with an extra methyl group. These organic acids are basically found in fruits and vegetables, but in recent years, they were found to have pharmacological effects such as anti-inflammatory, anti-oxidant responses, anti-platelet aggregation, and direct cardioprotective effects.[31,32] We can conclude that these organic acids may function as protection during ischemia/reperfusion injury. The decrease in levels of malic and citramalic acid in BD patients also supports our hypothesis that these patients are more prone to inflammation-based atherosclerotic and cardiovascular diseases.

Shikimic acid levels were lower in VBD patients than in the control group. Shikimic acid pathway is the common pathway for aromatic amino acid synthesis and metabolites like folic acid cofactors and coenzyme Q10, together with vitamins E and K. This biosynthetic pathway is found in bacteria in the human gut flora.[33] The disruption of gut microflora may result in decreased antioxidant activity in VBD patients in our study.

2-hydroxybutyric acid, an intermediate produced during threonine metabolism and glutathione synthesis, was higher than in control subjects in both patient groups. It is known to be elevated in patients with energy metabolism deficits or in lactic acidosis. Its increase may be attributed to increased oxidative stress in BD patients.[34]

Trehalose levels were also found to be elevated in both patient groups. Trehalose is a non-reducing disaccharide of glucose. In recent years, it has been found to be a protective agent against reactive oxygen species and chronic inflammation. An increase in trehalose levels can be explained as a compensatory response to increased oxidative stress and inflammation.[35]

The citramalic acid, oxaloacetic acid, homoserine, and shikimic acid levels were lower in the VBD group compared to BD. As discussed earlier, the changing amino acid profile can be attributed to inflammatory mechanisms and lower antioxidant capacity. The comparison between the 2 patient groups also supports our previous findings. Especially, lower levels of oxaloacetate and homoserine in the VBD group point to a deeper impairment in amino acid synthesis in these patients. As vascular involvement in BD alleviates the inflammatory processes, our findings are in concordance with previous studies.

The further decreased levels of citramalic acid and scikmic acid in VBD patients indicate that VBD patients are more prone to atherosclerosis as oxidation and inflammation trigger endothelial damage.[25] Since both organic acids have anti-inflammatory and cardioprotective effects.

The most promising metabolites revealed in our study can be listed in glutathione synthesis, which is the major thiol pool of antioxidant mechanisms in the human body. Homoserine, oxaloacetate, citramalic acid, and shikimic acid can be listed as the most prominent metabolites as they can be discriminative not only between BD patients and healthy controls but also between BD and VBD patients.

Our study does pose some limitations. One of which is a single-center study. Also, the patient number is relatively limited. But to our knowledge, there are no published reports on metabolomic profiles in BD patients with and without vascular involvement yet. Our findings propose some potential biomarkers, especially related to oxidative stress pathways in BD patients.
The present study can be accepted as a preliminary report on metabolomic alterations in BD patients with or without vascular involvement. As the etiopathogenesis of this chronic inflammatory disease is still not clearly known, our study can suggest some metabolomic indicators of disease prognosis. Especially for vascular involvement, finding a non-invasive molecule is of critical importance for both diagnosis and treatment of disease. As BD is accepted as a vasculitis and endothelial dysfunction seems to be the pathognomonic signature of the disease, the early detection of candidate oxidative stress markers causing endothelial damage may bring early diagnosis and better clinical management.

Ethics Committee Approval: This study was approved by Ethics Committee of Gülhane Training and Research Hospital (Approval No: 2020/15, Date: August 4, 2020).

Informed Consent: Written informed consent was obtained from the patients who agreed to take part in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – Ç.Y., E.S., A.O., T.Ö.; Design – Ç.Y.; Supervision – Ç.Y., A.O.; Resources – Ç.Y., A.O.; Materials – E.S.; Data Collection and/or Processing – Ç.Y., E.S., A.O.; Analysis and/or Interpretation – E.S., E.K., S.E.K., E.N.; Literature Search – A.O., E.K.; Writing – Ç.Y., E.S., E.N.; Critical Review – T.O., E.N.

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