Korobkova E. O., Kozhevnikova M. V., Ilgisonis I. S., Shakaryants G. A., Appolonova S. A., Kukharenko A. V. , Lartsova E. V., Maltseva A. A., Khabarova N. V., Belenkov Yu. N.
I. M. Sechenov First Moscow Medical University, Moscow, Russia

# Metabolomic Profiling in Patients with Metabolic Syndrome

| | |
|---|---|
| *Objective* | To identify biomarkers, which are most specific for patients with metabolic syndrome (MS) using metabolomic profiling. |
| *Materials and Methods* | Metabolomic profiling of patients with MS and comparison of their profile with the profile of volunteers was performed using high-performance liquid chromatography-mass-spectrometry. |
| *Results* | The metabolomic profile of MS patients differed in several amino acids, including choline, cysteine, and serine and in the acylcarnitine group ($p<0.05$ for all comparisons). |
| *Conclusion* | The metabolites most specific for MS patients were identified. Increased concentrations of a combination of amino acids and carnitines can be considered as possible additional risk factors for cardiovascular diseases. |

| | |
|---|---|
| *Keywords* | Metabolomics; metabolomic profiling; metabolic syndrome; cardiovascular diseases |
| *For citation* | Korobkova E. O., Kozhevnikova M. V., Ilgisonis I. S., Shakaryants G. A., Appolonova S. A., Kukharenko A. V. et al. Metabolomic Profiling in Patients with Metabolic Syndrome. Kardiologiia. 2020;60(3):37–43. [Russian: Коробкова Е. О., Кожевникова М. В., Ильгисонис И. С., Шакарьянц Г. А., Апполонова С. А., Кухаренко А. В. и др. Метаболомное профилирование больных с метаболическим синдромом. Кардиология. 2020;60(3):37–43.] |

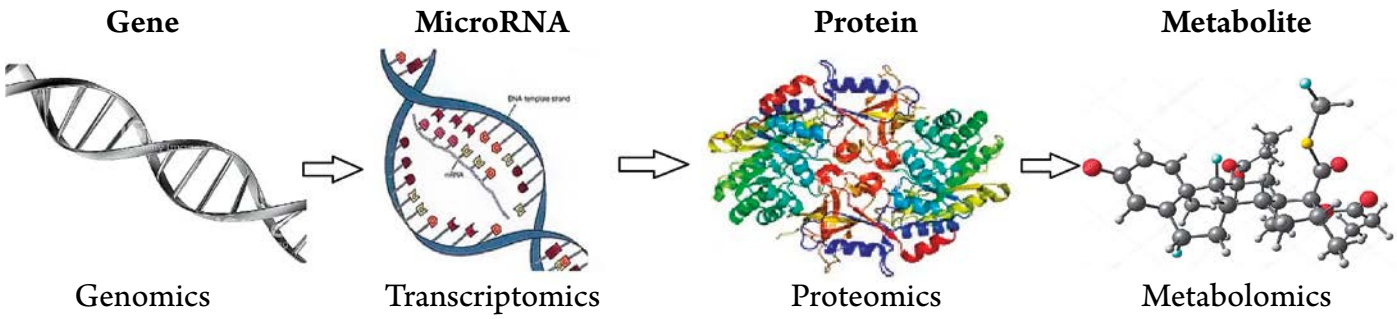
| | |
|---|---|
| *Corresponding author* | Korobkova Ekaterina Olegovna. E-mail: eokor25@mail.ru |

In recent decades, progress in genomics and the use of new technologies have opened an unprecedented era of biomedical research. Advanced visualization techniques and analytical methods have made it possible to more accurately characterize the molecular processes in the cells and tissues of the body, making an invaluable contribution to the rapidly growing knowledge base about biological systems. This technological progress hasa resulted in new fields of research known as «omic» sciences or «omics.» They have formed the basis for the development of a personalized approach in the diagnosis and treatment of various diseases and pathological conditions.

Genomic studies were the first steps in the development of personalized medicine. They were followed by a field of research called transcriptomics: the evaluation of coding and non-coding RNA which characterizes gene expression. As knowledge on the influence of a genotype on phenotype was accumulated, the field of research that studies the totality of the proteins in an organism began to develop. For many years, this field, proteomics played a crucial role in the early diagnosis, prognosis, and monitoring of disease [1]. With the emergence of new high-precision technologies based on mass spectrometry, however, the insight into the processes occurring in cells, tissues, and organs, was greatly enhanced. As a result, another «omic» direction evolved: metabolomics (Figure 1).

Metabolomics is the study of low-molecular-weight compounds, the end products of metabolism. Metabolomic analysis seeks to identify and quantify metabolites in an organism, creating a metabolic profile [2, 3]. Thus, metabolomic studies describe a molecular

**Figure 1.** The basic omic sciences



phenotype of a biological object most accurately and comprehensively. For this reason, metabolomics is the most promising avenue in the search for more accurate markers for detecting disease at an early stage or for predicting the course of a disease. Metabolomic technology makes it possible to define the metabolic pathways characterizing a pathological process, which in turn can guide the development of targeted treatments or lifestyle changes.

Metabolomic analysis uses the methods of separation and detection of low-molecular-weight compounds, such as gas chromatography coupled with mass spectrometric detection (GC–MS); high-performance liquid chromatography coupled with tandem mass spectrometry detection (HPLC–MS/MS); nuclear magnetic resonance spectrometry; and capillary electrophoresis [4–6].

Depending on the purpose of a study, one of two approaches is used: untargeted or targeted analysis. The untargeted approach is used to develop hypotheses and is most suitable for detecting the maximum number of metabolites and identifying new compounds. Hundreds to thousands of metabolites can typically be measured. The principal advantage of this method is the ability to define the relationship between significant metabolites and the construction of metabolic pathways. Targeted analysis is used to confirm discoveries made in research carried out to develop hypotheses. In this approach, a relatively small, specific number of metabolites with known mass, structure, and retention time is analyzed. The target method is used to screen up to 20 metabolites; it has higher specificity and sensitivity than the untargeted method [7].

Both fundamental and clinical studies have shown the significance of metabolomic profiling for developing insight into the pathophysiology of various cardiovascular diseases (CVDs). However, only a few works on metabolomic profiling in patients with metabolic syndrome are described in the literature [8].

The objective of this study was to identify, through metabolomic profiling, biomarkers most specific for patients with metabolic syndrome.

## Materials and Methods

The study included 60 subjects aged 18–80 years from different regions of the Russian Federation: 42 patients with metabolic syndrome (MS) and 18 healthy volunteers. The local ethics committee of I. M. Sechenov First Moscow State Medical University approved this research work. All patients signed written informed consent to participate in the study. Two patients withdrew from the study due to the detection of malignant tumors at baseline. The treatment group consisted of 21 male and 20 female patients with MS diagnosed according to the criteria in the Russian Clinical Guidelines for the Management of Patients with MS [9]. MS was considered significant if one main criterion and at least two additional criteria were met.

### *Criteria for metabolic syndrome*

*Main criterion*

- Central (abdominal) obesity: waist circumference more than 80 cm in female patients and more than 94 cm in male patients.

*Additional criteria*

- Blood pressure (BP) >140/90 mmHg or antihypertensive drug therapy
- Elevated levels of triglycerides (≥1.7 mmol/L)
- Decrease in high-density lipoproteins (<1.0 mmol/L in male patients;<1.2 mmol/L in female patients);
- Increased levels of low-density lipoproteins (>3.0 mmol/L)
- Impaired glucose tolerance
- Impaired fasting glucose

*Exclusion criteria* were severe renal or hepatic dysfunction; administration of systemic glucocorticoids, oral contraceptives; hyper- or hypothyroidism, malignancies, acute cerebrovascular accident or transient ischemic attack within the previous six months, heart failure, unstable coronary artery disease (CAD), alcohol or substance abuse, mental disorders, pregnancy or lactation. The control group consisted of nine female and eight male subjects without clinical and laboratory signs of cardiovascular disorders and criteria for MS. General characteristics of patients included in the study are given in Table 1, and general characteristics of the MS group in Figure 2.

All subjects underwent general clinical examination, which included the collection of complaints, history of disease and life, physical examination, anthropometric measurements (weight, height, waist circumference), calculation of body mass index (BMI) using the formula: weight (kg)/height$^2$ (m$^2$); measurement of office BP; complete blood count; biochemical blood tests (creatinine, uric acid, glucose, glycated hemoglobin, total cholesterol, high-density lipoproteins, low-density lipoproteins (LDL), very-low-density lipoproteins, triglycerides, thyroid-stimulating hormone); urinalysis; electrocardiography; echocardiography; 24-hour monitoring of BP. Venous blood samples were collected on an empty stomach. Additional venous blood samples were obtained on an empty stomach and collected into tubes containing potassium salt of ethylenediaminetetraacetate (EDTA) for analysis of subjects' metabolomic profiles. Four days before the additional blood collection, patients

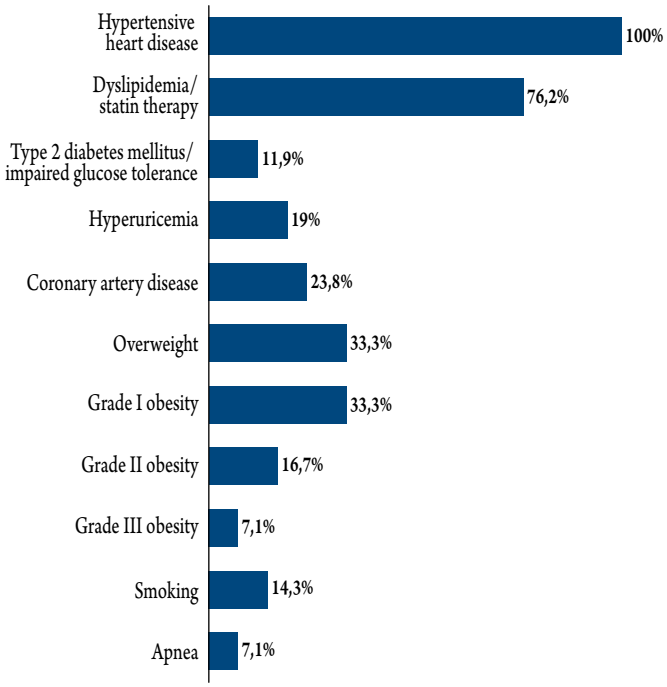

ISSN 0022-9040. Kardiologiia. 2020;60(3). DOI: 10.18087/cardio.2020.3.n903

**Table 1.** General characteristics of the subjects

| Parameter | Patients with metabolic syndrome (n = 41) | Healthy volunteers (n = 17) | p |
|---|---|---|---|
| Male | 21 (51.2) | 8 (47.1) | 0.904 |
| Female | 20 (47.8) | 9 (52.9) | 0.931 |
| Age, years | 58.71 ± 12.20 | 49.78 ± 10.56 | 0.010 |
| **Waist circumference, cm** | | | |
| • Male | 107.77±13.94 | 88.5±8.5 | 0.0007 |
| • Female | 97.35±10.8 | 83.22±11.1 | 0.002 |
| **BMI, kg/m²** | | | |
| • Male | 32.68±5.56 | 27.04±1.78 | 0.0003 |
| • Female | 31.29±4.4 | 26.7±5.56 | 0.011 |
| Glucose, mmol/L | 5.5 ± 0.78 | 5.1 ± 0.57 | 0.015 |
| Total cholesterol, mmol/L | 5.53 ± 1.42 | 5.3 ± 0.85 | 0.420 |
| Uric acid, μmol/L | 349.0 ± 87.1 | 319.0 ± 62.6 | 0.161 |
| SBP, mmHg | 135.5 ± 10.3 | 121.0 ± 9.1 | 0.0004 |
| DBP, mmHg | 82.0 ± 11.5 | 75.0 ± 7.4 | 0.029 |
| GFR (CKD-EPI), mL/min/1.73 m² | 73.21 ± 15.1 | 82.1 ± 5.1 | 0.185 |

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure;
GFR, glomerular filtration rate calculated by the formula CKD-EPI; p, the significance of differences evaluated using the Mann–Whitney test.

**Figure 2.** Characteristics of patients with metabolic syndrome (%)




reduced or stopped, as much as possible, administering all medicines, except for essential drugs; excluded were acetaminophen, all vitamins, minerals, amino acids, and dietary supplements, including sports and energy drinks, carnitine, alpha-ketoglutarate, supplements containing malic acid, salts of citric acid, maleic acid, or any salts of orotic acid. It was recommended if possible to avoid eating or drinking any products containing sweeteners (aspartate, NutraSweet, etc.), monosodium glutamate (MSG), and mono diets for 24 hours before the additional blood sampling.

Immediately after the additional gathering of blood, plasma was separated at 2,000 rpm for 15 minutes. The collected plasma samples were stored at a temperature of –80 °C. The aliquots to be analyzed were delivered to the pharmacokinetics and metabolomic analysis laboratory at the Institute of Translational Medicine and Biotechnology, Sechenov University, under the same temperature conditions, for performing untargeted metabolomic profiling using HPLC coupled with quadrupole-time of flight mass spectrometry (HPLC-qTOF) and targeted metabolomic analysis of the samples using HPLC coupled with tandem mass spectrometry detection (HPLC–MS/MS). The treated sample was analyzed in a gas chromatography-mass spectrometer Maestro with a quadrupole Agilent 5977 analyzer to obtain a metabolomic profile. The assay was performed in positive ionization mode using the electrospray method. Multiple reaction monitoring was used for identification and quantification. Samples were standardized by introducing isotopically labeled standards. The conditions for mass spectrometry were the following: dwell time 0.019–0.025 s and capillary voltage 2 kV; nitrogen was used as the impingement medium, source temperature 150 °C. The import and pretreatment stages for the targeted mass spectral data were performed using the TargetLynx software. The concentration of metabolites was calculated according to the intensity of the signal from analytes and corresponding internal standards. The chromatograms were processed using the XCMS software to obtain the primary information

about the intensity, retention time, and mass spectral characteristics of the peaks. Metabolites were identified from the Golm Metabolome Database. The results were processed using Statistica 8.0, SIMCAR 13.0, and Metaboanalyst 4.0. The Mann-Whitney test was used to detect statistically significant differences between the groups with the significance criterion of $p<0.05$.

## Results

The metabolomic profiling of two study groups (patients with MS and healthy volunteers) was conducted. More than 106 metabolites were identified using the untargeted approach. Using principal component analysis (PCA), no samples were detected that were significantly different from the characteristics of the main study groups (Figure 3A). Minor differences can be associated with comorbidities of a specific subject or the drugs administered. Further discriminant analysis (orthogonal partial least square discriminant analysis [OPLS-DA [) of all metabolite data made it possible to detect components, thereby allowing the division of subjects into two groups, and to show the differences of metabolomic profiles of patients from the treatment group and healthy volunteers (Figure 3B).

The targeted analysis identified statistically significant ($p<0.05$) differences between the groups of patients with MS and healthy volunteers for seven metabolites: amino acids and acylcarnitines (Table 2).

Moreover, the variable significance of all metabolites was compared, and the most significant metabolites were selected to generate a heatmap of the mean concentrations (Figure 4).

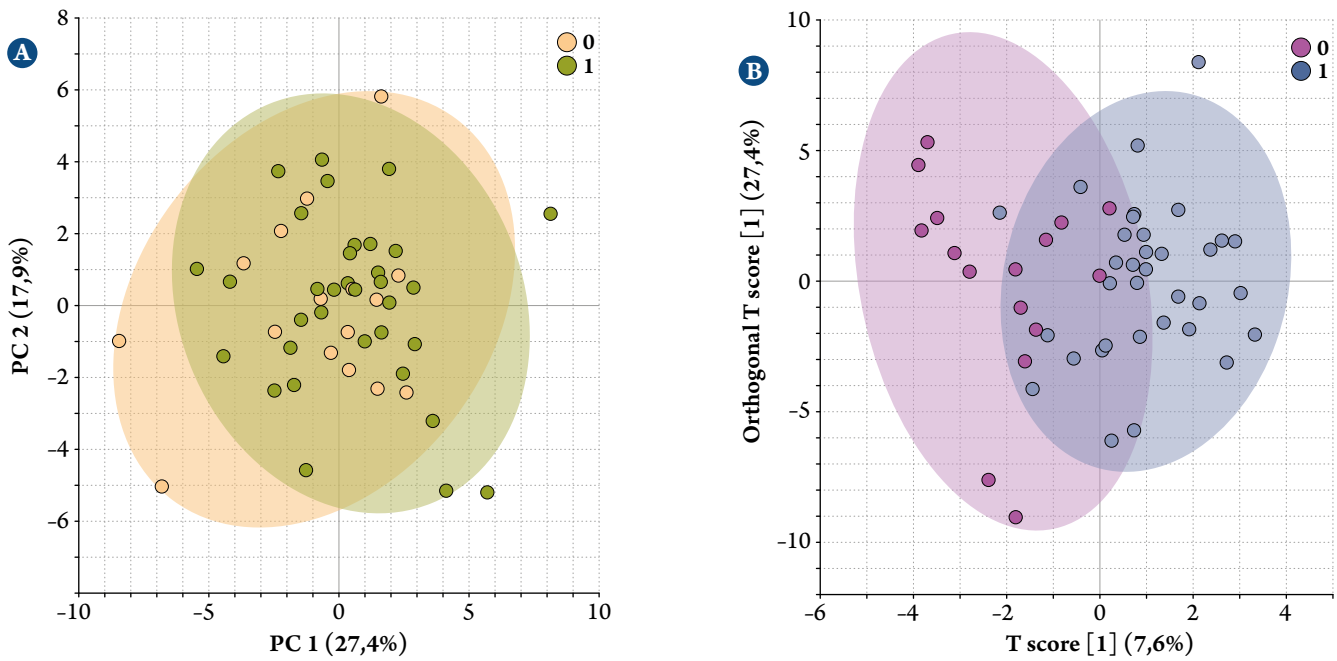
The heatmap shows that increased concentrations of most of the selected metabolites prevail in the group of patients with MS. However, it should be noted that concentration outliers were also observed for the control group. By contrast, samples with a reduced concentration of some metabolites were found in the MS group, which was likely to be associated with drug therapy.

## Discussion

Recent years have seen increased interest in research of the metabolome in patients with various metabolic disorders, including diabetes mellitus type 2 and obesity [10]. However, the pathogenic pathways resulting in changes in these metabolites are understudied. There is still no clear understanding of precisely what mechanisms determine the pattern of development of CVDs in patients with MS.

We performed a comparative analysis of metabolomic profile in patients with MS and healthy volunteers who had no signs of cardiovascular diseases and MS. We found that the blood levels of some amino acids, including choline, cysteine, serine, and acylcarnitines, were significantly higher in patients with MS than in the control group. The obtained results are partially

**Figure 3.** Distribution of patients with metabolic syndrome and healthy volunteers using principal component analysis (PCA) (A) and OPLS-DA (B)




0—healthy volunteers; 1—patients with the metabolic syndrome; PC, principal components;
T score, the first principal component; orthogonal T score, the second principal component orthogonal to the first.

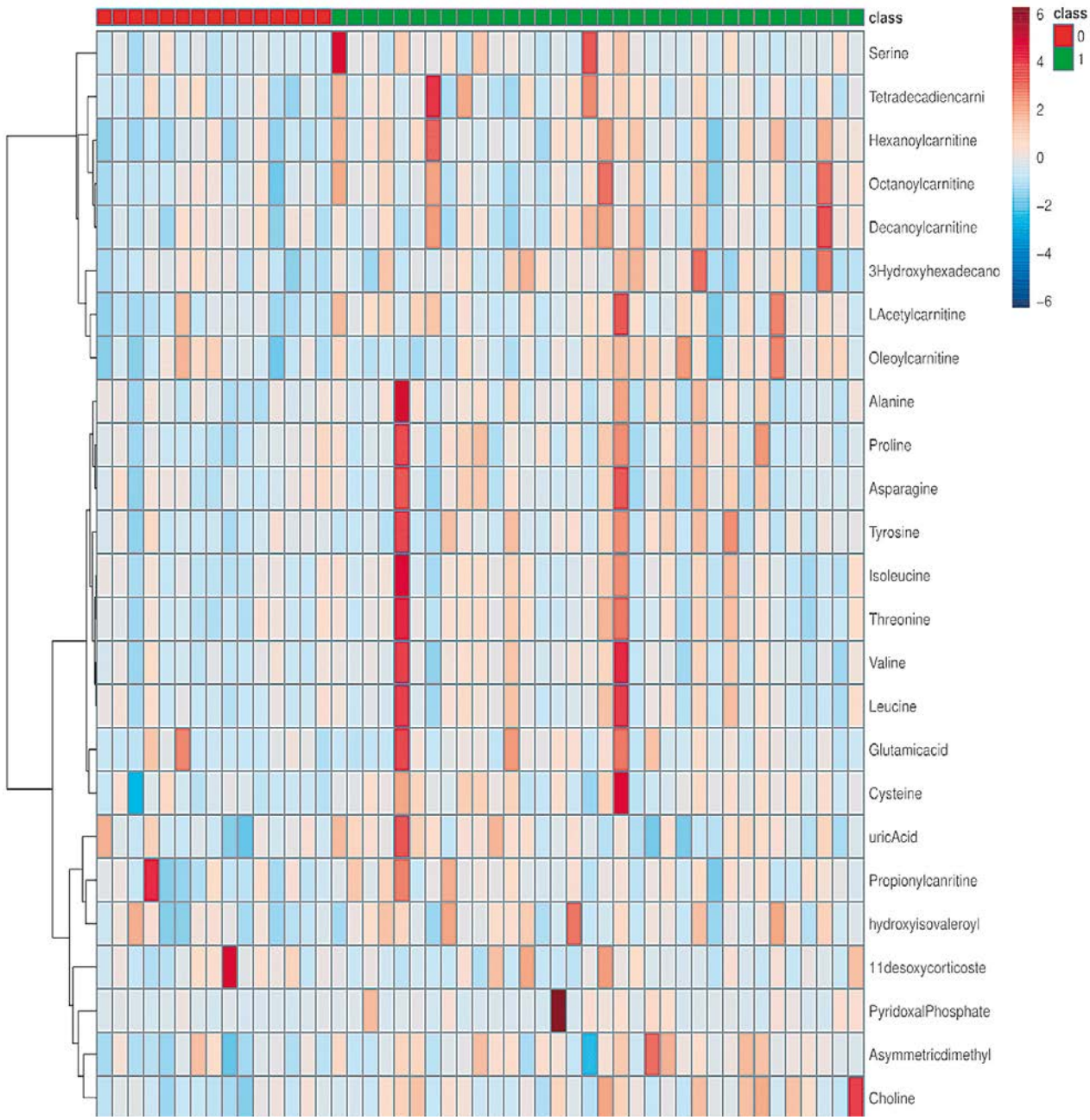

ISSN 0022-9040. Kardiologiia. 2020;60(3). DOI: 10.18087/cardio.2020.3.n903

**Table 2.** Metabolite levels that differ significantly between patients with metabolic syndrome and healthy volunteers

| Metabolite | Patients with MS (n = 41) | Healthy volunteers (n = 17) | p |
|---|---|---|---|
| 3 hydroxyhexanoyl carnitine | 30.234·104 | 23.839·104 | 0.042 |
| O-acyl-L-carnitine | 12.525 | 9.76 | 0.001 |
| Asymmetric dimethylarginine | 31.554·104 | 27.49·104 | 0.022 |
| Hexanoylcarnitine | 64.06·103 | 44.07·103 | 0.001 |
| Serine | 259.844 | 175.335 | 0.005 |
| Choline | 12.689 | 10.171 | 0.007 |
| Cysteine | 390.15 | 306.92 | 0.005 |

p, the significance of differences evaluated using the Mann–Whitney test.

**Figure 4.** Heatmap of mean concentrations of metabolites (heatmap)




0—healthy volunteers; 1—patients with the metabolic syndrome. Red denotes an increase in the concentration of a metabolite; blue is a decrease in the concentration of the metabolite in the selected group. Color intensity reflects the degree of differences in concentrations between the metabolites.

consistent with previous experimental and clinical studies of the metabolomic profile in patients with various cardiovascular diseases. For example, a recent study on a model of spontaneously hypertensive mice showed that a long-term increase in BP results in significant changes in myocardial metabolism even before its remodeling and dysfunction [11]. In this study, excessive concentrations of metabolites, which are not involved in the synthesis of ATP, were found in the heart tissues, including elevated levels of long-chain fatty acylcarnitines. For the first time, data on elevated levels of acylcarnitines in patients with MS and hypertension were obtained. Our study confirms the experimental observations and suggests that it is possible to use the assessment of acylcarnitine levels as an additional cardiovascular risk factor.

Choline is one of the amino acids that has been studied extensively in recent decades in terms of the development and progression of CVDs. It is involved in the synthesis of lipoproteins, phospholipids, and neurotransmitters, and is abundant in dairy products and fish. Elevated plasma levels of choline are identified in patients with metabolic disorders and CAD. They are also associated with an increased risk of myocardial infarction, and, according to Schartum-Hansen et al., can therefore be used to modify the cardiovascular complication risk score [12]. Our results are generally consistent with the previous studies of the relationship of choline with metabolic disorders and the progression of CVDs [13, 14]. We detected statistically significant differences in the levels of choline between the study groups. Moreover, a positive correlation was found between its levels and such criteria of MS as waist circumference and total cholesterol. As choline is involved in the synthesis of blood lipids, these data not only can be used as an additional biomarker of CVDs but can also serve as a basis for a potential new targeted approach for the treatment of dyslipidemia.

Similar results were obtained for cysteine. In 2015, Mohorko et al. [15] found a significant elevation of cysteine levels in patients who did not meet the MS criteria, yet had some metabolic disorders. Our findings fully confirm the theory of cysteine metabolism disorders in patients with MS. Our findings suggest that the levels of cysteine can be used as a biomarker in symptom-free patients and could be included in the standard prognosis of cardiovascular risks. One of the main advantages of metabolomic profiling over the existing biomarkers is the high sensitivity of the method. However, further study of the problem is necessary for the effective use of the knowledge gained and assessment of the opportunities and limitations of metabolomic profiling in diagnosis and risk prognosis.

## Limitations

Our study was limited by a small number of subjects and its cross-sectional design. However, we plan to continue our work, enroll more subjects, and trace the trends in the concentration of metabolites to ensure the higher reliability of data and detect early metabolomic markers of MS onset that can be used as a combination of cardiovascular risk factors over the longer term.

## Conclusion

Currently, there is no doubt that metabolic processes play a significant role in the development and progression of cardiovascular diseases. It is necessary to accumulate a database of all metabolites and metabolic pathways specific to a particular disease. Our study identified metabolites that are the most specific to patients with metabolic syndrome, which is, in most cases, antecedent to the development of cardiovascular diseases. Both well-known metabolites predicting cardiovascular diseases (e.g., asymmetric dimethylarginine, choline, and cysteine) and less explored metabolites (e.g., serine) were identified among them. For the first time, data on elevated levels of acylcarnitines in patients with metabolic syndrome and hypertension were obtained; earlier, this had been explored only in experimental studies. It can be assumed that the higher concentrations of the amino acid and carnitine combination are plausible as additional risk factors for cardiovascular diseases. Moreover, the detected metabolomic pathways are of interest in the search for potential targets for therapeutic intervention.

## Funding

This research work was financed by the Russian Foundation for Basic Research (RFBR) under research project #1831500135.

*The study was supported by the Project for Improving the Competitiveness of the Leading Russian Universities among the World's Leading Scientific and Educational Centers.*

*No conflict of interest is reported.*

**The article was received on 20/10/19**

REFERENCES

1. Jiang Z, Zhou X, Li R, Michal JJ, Zhang S, Dodson MV et al. Whole transcriptome analysis with sequencing: methods, challenges and potential solutions. Cellular and Molecular Life Sciences. 2015;72(18):3425–39. DOI: 10.1007/s00018-015-1934-y



ISSN 0022-9040. Kardiologiia. 2020;60(3). DOI: 10.18087/cardio.2020.3.n903

2. Nicholson JK, Lindon JC. Metabonomics. Nature. 2008;455(7216):1054–6. DOI: 10.1038/4551054a
3. Serkova NJ, Niemann CU. Pattern recognition and biomarker validation using quantitative 1 H-NMR-based metabolomics. Expert Review of Molecular Diagnostics. 2006;6(5):717–31. DOI: 10.1586/14737159.6.5.717
4. Crutchfield CA, Lu W, Melamud E, Rabinowitz JD. Mass Spectrometry-Based Metabolomics of Yeast. In: Methods in Enzymology Elsevier;2010.
5. Coen M, Holmes E, Lindon JC, Nicholson JK. NMR-Based Metabolic Profiling and Metabonomic Approaches to Problems in Molecular Toxicology. Chemical Research in Toxicology. 2008;21(1):9–27. DOI: 10.1021/tx700335d
6. Pan Z, Raftery D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Analytical and Bioanalytical Chemistry. 2007;387(2):525–7. DOI: 10.1007/s00216-006-0687-8
7. Fiehn O. Metabolomics by Gas Chromatography–Mass Spectrometry: Combined Targeted and Untargeted Profiling. Current Protocols in Molecular Biology. 2016;114(1):30.4.1-30.4.32. DOI: 10.1002/0471142727.mb3004s114
8. Wiklund PK, Pekkala S, Autio R, Munukka E, Xu L, Saltevo J et al. Serum metabolic profiles in overweight and obese women with and without metabolic syndrome. Diabetology & Metabolic Syndrome. 2014;6(1):40. DOI: 10.1186/1758-5996-6-40
9. Ministry of Health of the Russian Federation. Recommendations for the management of patients with metabolic syndrome. Clinical recommendations. - Moscow. 2013. - 43p. Av. at: https://mzdrav.rk.gov.ru/file/mzdrav_18042014_Klinicheskie_rekomendacii_Metabolicheskij_sindrom.pdf. . [Russian: Министерство здравоохранения РФ. Рекомендации по ведению больных с метаболическим синдромом. Клинические рекомендации. – Москва, 2013. – 43с. Доступно на: https://mzdrav.rk.gov.ru/file/mzdrav_18042014_Klinicheskie_rekomendacii_Metabolicheskij_sindrom.pdf]
10. Lent-Schochet D, McLaughlin M, Ramakrishnan N, Jialal I. Exploratory metabolomics of metabolic syndrome: A status report. World Journal of Diabetes. 2019;10(1):23–36. DOI: 10.4239/wjd.v10.i1.23
11. Li J, Kemp BA, Howell NL, Massey J, Mińczuk K, Huang Q et al. Metabolic Changes in Spontaneously Hypertensive Rat Hearts Precede Cardiac Dysfunction and Left Ventricular Hypertrophy. Journal of the American Heart Association. 2019;8(4):e010926. DOI: 10.1161/JAHA.118.010926
12. Schartum-Hansen H, Pedersen ER, Svingen GF, Ueland PM, Seifert R, Ebbing M et al. Plasma choline, smoking, and long-term prognosis in patients with stable angina pectoris. European Journal of Preventive Cardiology. 2015;22(5):606–14. DOI: 10.1177/2047487314524867
13. Konstantinova SV, Tell GS, Vollset SE, Nygård O, Bleie Ø, Ueland PM. Divergent Associations of Plasma Choline and Betaine with Components of Metabolic Syndrome in Middle Age and Elderly Men and Women. The Journal of Nutrition. 2008;138(5):914–20. DOI: 10.1093/jn/138.5.914
14. Roe AJ, Zhang S, Bhadelia RA, Johnson EJ, Lichtenstein AH, Rogers GT et al. Choline and its metabolites are differently associated with cardiometabolic risk factors, history of cardiovascular disease, and MRI-documented cerebrovascular disease in older adults. The American Journal of Clinical Nutrition. 2017;105(6):1283–90. DOI: 10.3945/ajcn.116.137158
15. Mohorko N, Petelin A, Jurdana M, Biolo G, Jenko-Pražnikar Z. Elevated Serum Levels of Cysteine and Tyrosine: Early Biomarkers in Asymptomatic Adults at Increased Risk of Developing Metabolic Syndrome. BioMed Research International. 2015;2015:418681. DOI: 10.1155/2015/418681