DETERMINING THE DIAGNOSTIC VALUE OF THE MARKERS OF CONGENITAL METABOLIC DISORDERS BY CHROMATOGRAPHY–MASS SPECTROMETRY

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Thorough investigation of metabolome by mass spectrometry is of great importance for personalized and preventive medicine. It is only timely laboratory diagnosis involving the use of high-tech chromatographic analysis methods that can help identify the patients with disorders of amino acid and acylcarnitine metabolism. The study was aimed to determine the efficacy of conventional and additional markers of metabolic disorders of amino acids and acylcarnitines detected by chromatography–mass spectrometry for the diagnosis of congenital metabolic disorders in children, as well as to create specific panels of the most effective indicators and determine the potential diagnostic efficacy of indentification of the relationships between the levels of amino acids and acylcarnitines in pediatric patients with congenital metabolic disorders. We assessed amino acid and acylcarnitine profiles in blood spots by high-performance liquid chromatography–tandem mass spectrometry in patients aged 6 months to 16 years (48 boys and 32 girls) with suspected aminoacidopathy and organic aciduria/acidemia. The comparison group consisted of 35 children with suspected peroxisomal metabolic disorders, the control group included 40 generally healthy children of various age groups. The data obtained were used to conduct the analysis of correlations between the groups of markers. Strong correlation was revealed for the levels of metabolically most closely related compounds ($r < 0.8$, $p < 0.001$). However, a similar relationship between metabolically not closely related compounds (correlation coefficient 0.45–0.73 ($p < 0.001$)) was revealed for some groups of compounds. Thus, the acylcarnitine profile can be proposed as an additional potential marker to be used in cases of borderline phenylalanine levels, and the sum of normalized acylcarnitine levels (C12+C16) can be a potential secondary marker of phenylketonuria.

Keywords: mass-spectrometry, amino acids, acylcarnitines, hereditary metabolic diseases, correlation analysis, differential diagnosis

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Compliance with ethical standards: the study was approved by the Ethics Committee of the Pirogov Russian National Research Medical University (protocol № 94 dated 14 December 2009). All parents or caregivers of the subjects submitted the informed consent to participation in the study.

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ОПРЕДЕЛЕНИЕ ДИАГНОСТИЧЕСКОЙ ЗНАЧИМОСТИ МАРКЕРОВ НАСЛЕДСТВЕННЫХ БОЛЕЗНЕЙ ОБМЕНА С ПРИМЕНЕНИЕМ МЕТОДА ХРОМАТО-МАСС-СПЕКТРОМЕТРИИ

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Для развития персонализированной и превентивной медицины большое значение приобретает детальное изучение метаболома с применением масс-спектрометрии. Только своевременная лабораторная диагностика с помощью высокотехнологичных методов хроматографического анализа может помочь в выявлении пациентов с нарушениями метаболизма аминокислот и ацилкарнитинов. Целью работы было определить эффективность классических и дополнительных маркеров нарушений обмена аминокислот и ацилкарнитинов, детектируемых хромато-масс-спектрометрическими методами, в диагностике наследственных болезней обмена у детей, создать специфические панели наиболее эффективных показателей и определить потенциальную диагностическую эффективность выявления взаимосвязей между показателями аминокислот и ацилкарнитинов у педиатрических пациентов с врожденными нарушениями метаболизма. Были изучены профили аминокислот и ацилкарнитинов в пятнах крови методом высокоэффективной хромато-масс-спектрометрии у пациентов в возрасте от 6 месяцев до 16 лет (48 мальчиков и 32 девочки) с подозрением на аминоацидопатии и органические ацидурии/ацидемии. Группа сравнения состояла из 35 детей с подозрением на пероксисомные болезни обмена, контрольная группа — из 40 практически здоровых детей разных возрастных групп. По полученным данным, между группами маркеров был проведен корреляционный анализ. Содержание метаболически наиболее близких соединений имело выраженную корреляционную взаимосвязь ($r < 0,8$, $p < 0,001$). Однако такая взаимосвязь проявилась и среди метаболически слабо связанных соединений (коэффициент корреляции варьировал от 0,45 до 0,73 ($p < 0,001$) для некоторых групп соединений. Так, ацилкарнитиновый профиль может быть предложен в качестве потенциального дополнительного маркера при пограничных показателях фенилаланина, а сумма нормализованных показателей ацилкарнитинов (C12+C16) может быть потенциальным вторичным маркером фенилкетонурии.

Ключевые слова: масс-спектрометрия, аминокислоты, ацилкарнитины, наследственные болезни обмена, корреляционный анализ, дифференциальная диагностика

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In the leading countries of the world mass spectrometry is among the newest and most popular laboratory medicine methods. Thorough investigation of metabolome is impossible without the use of mass spectrometry; the datasets acquired by this method are of great importance for the development of innovative personalized medicine technologies ensuring the switch from reactive to predictive and preventive medicine. Since introduction into practice of preventive check-ups and information technology for the development of the disease diagnosis algorithms is the most promising direction in solving the range of problems of personalized medicine, the tandem mass spectrometry is considered to be the method most suitable for fulfillment of these aspects [1–3].

Based on the prevalence, the disorders of amino acid and acylcarnitine metabolism are rather frequent compared to other congenital metabolic disorders. The prevalence of the majority of inherited metabolic disorders, many of which are acidemias that manifest themselves in the neonatal period, is 1:1000 – 1:5000 newborns. Individual pathogenetic pattern of amino acid and acylcarnitine metabolism disorders is complicated by the fact that certain clinical signs in various combinations having varying severity can emerge in individuals with different types of metabolic diseases. As a result, it is only timely laboratory diagnosis involving the use of high-tech chromatographic analysis methods that can help identify the patients with such disorders.

The study was aimed to determine the efficacy of the main (conventional) markers of amino acid and acylcarnitine metabolism disorders detected by chromatography–mass spectrometry for the diagnosis of congenital metabolic disorders, as well as to create specific panels of the most effective indicators and determine the potential diagnostic efficacy of indentification of the relationships between mass spectrometry indicators in children with inherited metabolic disorders of amino acids and acylcarnitines.

METHODS

The study was carried out at the Veltischev Research and Clinical Institute for Pediatrics and Pediatric Surgery of the Pirogov Russian National Research Medical University and the Voino-Yasenetsky Scientific and Practical Center for Specialized Assistance to Children in 2012–2023.

The group of affected individuals included children and adolescents with suspected aminoacidopathy and organic aciduria/acidemia, as well as patients with undifferentiated metabolic disorders. The age of patients included in the group was between 6 months and 16 years. The gender distribution of the group of patients was as follows: 48 boys and 32 girls. Inclusion criteria: children from birth to 18 years of age; history of the following symptoms: psychomotor and physical development retardation, cramps, muscle tone abnormality, ataxia, the combined symptoms including enlarged liver, decreased visual acuity, dermatitis. Exclusion criteria: age over 18 years, severe comorbidities (for example, cerebral palsy, congenital anomalies of the kidney and urinary tract, severe cardiovascular disorders) that could complicate fulfillment of the assessment conditions or cause harm to the patient. The comparison group for this group of affected individuals included 35 children with suspected peroxisomal metabolic disorders. Inclusion criteria for the comparison groups: age under 18 years, history of sharp typical neuronal migration defects, micronodular cirrhosis, kidney cysts, chondrodysplasia punctata, corneal opacity, cataract, glaucoma and retinopathy, congenital heart defects and dysmorphic features. The control group consisted of 40 generally healthy children of various age groups.

The study involved the use of the method for quantification of 12 amino acids and 30 acylcarnitines in dry blood spots by high-performance liquid chromatography–tandem mass spectrometry [4–7] modified in the following way: the mass spectrometry detection parameters for all analytes were optimized to increase sensitivity, the eluent flow rate was increased to reduce the single sample analysis time.

Reference samples: MassChrom®AminoAcids and Acylcarnitines lyophilized mixture of internal standards (Chromsystems; Germany).

Reagents: acetonitrile (LC/MS Grade) (Fisher Scientific; USA), butanol-1 (AR Grade; Chimmed, Russia), n-butyl acetate (AR Grade; Chimmed, Russia), hydrochloric acid (AR Grade; Chimmed, Russia), methanol (AR Grade; Sigma-Aldrich, Germany).

Laboratory glassware and materials: Whatman 903® filter paper for biomaterial sample collection (Whatman; USA), 96-well microplate with protective adhesive film (Eppendorf; Germany).

Laboratory equipment: DSB Puncher (PerkinElmer; USA), ST-3 thermal shaker (ELMI; Latvia), EVA EC-S evaporator (VLM; Germany), Sartorius Biohit Proline single-channel mechanical pipettes (Sartorius Biohit Liquid Handling Oy; Finland) having the volume of 0–100.0 µL, 0–200.0 µL with original disposable tips.

Biomaterial collection

The blood sample drawn from the newborn's heel or finger of the older patient was collected on the special Whatman 903® paper in the form of sheets for capillary blood collection and dried at room temperature until completely dry. Special paper soaked in biomaterial was stored at room temperature for up to one month.

Biomaterial sample preparation

To conduct the analysis, a circle with a diameter of 3.1 mm (corresponding to 3.2 µL of blood sample) was cut out from the dry blood spot with a puncher and placed in the microplate well. To ensure extraction, it was added 200.0 µL of the internal standard mixture (previously dissolved in acetonitrile); then the microplate was covered with protective adhesive film to avoid evaporation and splashing of the sample and mixed on the shaker at 600 rpm for 20 min at room temperature. To ensure evaporation, protective film was removed from the microplate, and the sample was evaporated at 60 °C in the air stream until dry. Sample derivatization was accomplished by adding 60.0 µL of the derivatization reagent (mixture of butanol-1, n-butyl acetate, hydrochloric acid in a volume ratio of 7:2:1) to the sample dry residue in the microplate, then the microplate was covered with the protective film and incubated at 60 °C and 600 rpm for 15 min. To concentrate the sample, the protective film was removed from the microplate, and the sample was evaporated in the air stream until dry. The final phase of sample preparation included dissolving the dry residue in 10.0 µL of methanol. After that the sample was mixed at 600 rpm for 1 min at room temperature. A total of 10.0 µL of the prepared sample were injected in the HPLC system.

Chromatographic conditions

The analysis was performed using the HPLC system consisting of the Agilent 1200 binary gradient pump, vacuum trap, column thermostat, and CTC HTS PAL autosampler connected to the Agilent 6410 QQQ MS detection system (Agilent Technologies; USA). The adaptor coupling was used as a column, and

Table. Diseases found in the group of patients with suspected aminoacidopathy and organic aciduria/acidemia based on the dry blood spot analysis by high-performance liquid chromatography–tandem mass spectrometry

Disease entity	Changes in marker levels	Number of patients
Disorders diagnosed by dry blood spot analysis by HPLC-MS/MS		
Phenylketonuria due to phenylalanine hydroxylase deficiency	Elevated phenylalanine levels, decreased tyrosine levels	7
Homocystinuria due to cobalamin metabolism disorders	Decreased methionine levels, presence of homocysteine	5
Argininemia	Elevated arginine levels	4
Cobalamin A type (cblA) and cobalamin B type (cblB) methylmalonic acidemia (MMA)	Elevated propionylcarnitine (C3) and methylmalonylcarnitine (C4DC) levels	5
Medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency	Elevated levels of medium-chain acylcarnitines (C6, C8, C10)	3
Carnitine uptake defect (CUD)	Decreased free carnitine levels (C0)	1
Glutaric acidemia type I (GA I)	Elevated glutarylcarnitine levels (C5DC)	3
Tyrosinemia type I	Elevated levels of tyrosine, phenylalanine, and methionine	3
Tyrosinemia type II	Elevated tyrosine levels	1
Hyperammonemia due to N-acetylglutamate synthase deficiency	Elevated alanine levels	5
Citrullinemia	Elevated citrulline levels, decreased arginine levels	2
Isovaleric acidemia/aciduria (IVA)	Elevated isovalerylcarnitine levels (C5)	2
Propionic acidemia (PA)	Elevated propionylcarnitine levels (C3) and the C3/C2 ratio	2
Nonketotic hyperglycinemia	Elevated glycine levels	5
Maple syrup urine disease (leucinuria) (MSUD)	Elevated total indicator (leucine + isoleucine)	6

acetonitrile was used as a mobile phase and solution used to wash the injector needle. The HPLC system settings were as follows: injection volume — 10.0 µL, analysis time — 1.7 min, mobile phase flow rate during system equilibration — 0.5 mL/min.

Data processing

The data obtained were processed in MassHunter® (Agilent Technologies; USA).

The analytes were quantified by the internal standard method. The concentration of each analyte in the sample was calculated using the formula (1) as a ratio of its analytical signal intensity in the sample to the analytical signal intensity of the corresponding internal standard in the same sample multiplied by the concentration of this internal standard in the mixture of internal standards (calibration mixture):

$$c\_(A, \mu mol/L) = ([\![A\_A]\!]\_)/A\_IS \times c\_(IS, mmol/L), \quad (1)$$

where c_(A, µmol/L) was the concentration of analyte in the sample (µmol/L); A_A was the analyte analytical signal intensity in the sample; c_(IS, mmol/L) was the concentration of internal standard in the calibration mixture (µmol/L); A_IS was the internal standard analytical signal intensity in the sample.

The concentrations of all internal standards in the MassChrom®AminoAcids and Acylcarnitines mixture (Chromsystems; Germany, registration certificate № RZN 2018/7415 dated 27.07.2018) were provided in appropriate supporting documents.

Method validation characteristics

Depending on the analyte, the rate of amino acid and acylcarnitine extraction from dry blood spots was 69–97%; the detection limit for amino acids varied between 2.0 and 15.6 µmol/L, while that for acylcarnitines was 0.1–1.6 µmol/L; the coefficients of variation for all analytes were within the range of 3.4–15.6%; the linearity range of amino acids was up to 2000 µmol/L, while that of acylcarnitines was up to 200 µmol/L.

To demonstrate the diagnostic value, ROC curves were plotted [8]. Hierarchical cluster analysis and the heatmaps based on Spearman's rank correlation were also used. The correlation analysis was performed using a programming language R, and the median values were compared with the interquartile ranges. The results were processed using the Morpheus statistical tool and the SPSS Statistics 23® (IBM Corporation; USA), Statistica 6.0® (StatSoftInc.; USA), Excel'2007® (MicroSoft Corp.; USA) software packages.

RESULTS

Based on the analysis of dry blood spots collected from 80 children in the first group with suspected aminoacidopathy and organic aciduria/acidemia and clinical and laboratory characteristics, a total of 54 patients with the following monogenic disorders were identified: aminoacidopathies, organic acidemia, fatty acid oxidation defects, and carnitine transport defect. These diagnoses were later verified by molecular genetic methods (Table). Based on the analysis of dry blood spots collected from 35 individuals in the comparison group (patients with suspected peroxisomal disorders), low free carnitine levels (C0) in blood were revealed in five patients (within the range of 10–16 µmol/L, while the reference range was 19–45 µmol/L), which could be indicative of other metabolic disorder, such as secondary carnitine deficiency. In other patients, all the indicators were within reference ranges or at their high ends. The analysis of dry blood spots collected from 40 children in the control group confirmed the fact that the group included generally healthy children, since all the studied indicators were within reference ranges in all children.

When comparing the median values with the interquartile ranges of marker metabolites in the subjects of the comparison

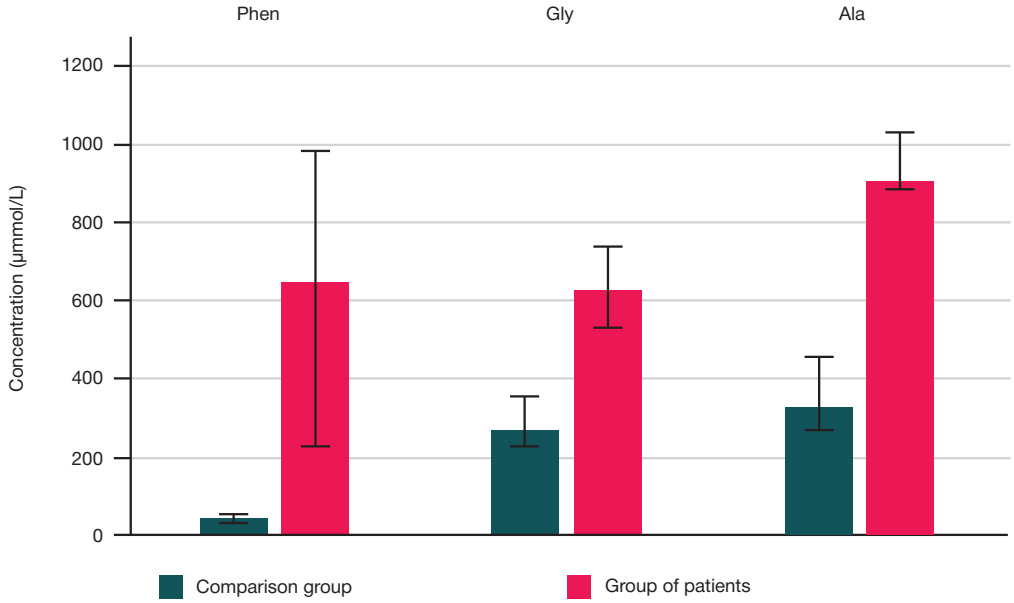

Fig. 1. Comparison of marker metabolite levels in surveyed individuals of the comparison group and the patients with phenylketonuria (diagnostic marker — phenylalanine (Phe)), nonketotic hyperglycinemia (diagnostic marker — glycine (Gly)), and hyperammonemia (diagnostic marker — alanine (Ala))

group and the patients diagnosed with metabolic disorders, significant (10–100-fold) differences become evident. This is exemplified by comparison of marker metabolite levels in surveyed individuals of the comparison group and the patients diagnosed with congenital amino acid metabolism disorders: phenylketonuria (more than 100-fold), nonketotic hyperglycinemia, and hyperammonemia (Fig. 1).

It is noteworthy that the levels of propionylcarnitine (C3) being the main marker of the disease are significantly higher in patients with methylmalonic acidemia than in surveyed individuals in the comparison group; the opposite trend is observed in patients with homocystinuria: the levels of the main marker, methionine (Met), are lower in affected patients than in individuals in the comparison group (Fig. 2).

The above examples confirm the diagnostic value of the biochemical markers determined by chromatography–mass spectrometry in this study when used to diagnose metabolic disorders of amino acids, acylcarnitines.

Fig. 3 presents a heatmap with a dendrogram for patients with identified metabolic disorders of amino acids and acylcarnitines, in the rows of which the data on each patient with identified disorder are provided, and the columns of which provide data on the tested metabolytes; potential markers are clustered by cluster analysis.

The more detailed review of the data provided in the upper right corner of the heatmap shows the decreased blood levels of short-chain and long-chain acylcarnitines in patients with phenylketonuria compared to subjects in the comparison group, along with preserved levels of medium-chain acylcarnitines. These results have been confirmed for acylcarnitines C12, C14, C14:1, C16, C16:1, C18, C18:1, C5, C5OH using the nonparametric Mann–Whitney U test at $p < 0.05$. Thus, the acylcarnitine profile can be proposed as an additional potential marker to be used in cases of borderline phenylalanine levels.

A downward trend in different acylcarnitine profile indicators (short-, medium-, and long-chain acylcarnitines) is also observed

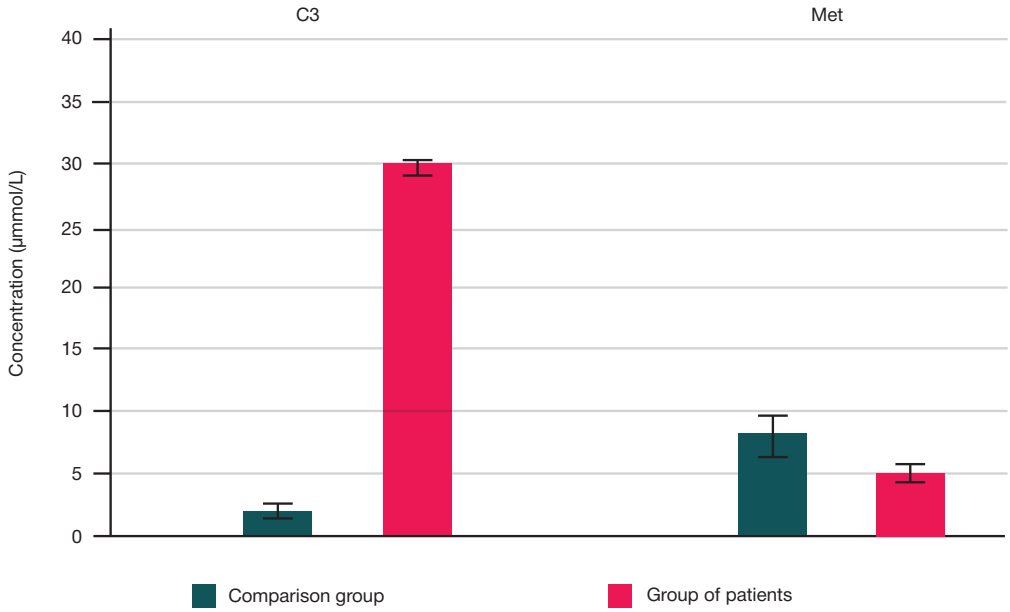

Fig. 2. Comparison of marker metabolite levels in surveyed individuals of the comparison group and the patients with methylmalonic acidemia (diagnostic marker — propionylcarnitine (C3)) and homocystinuria (diagnostic marker — methionine (Met))

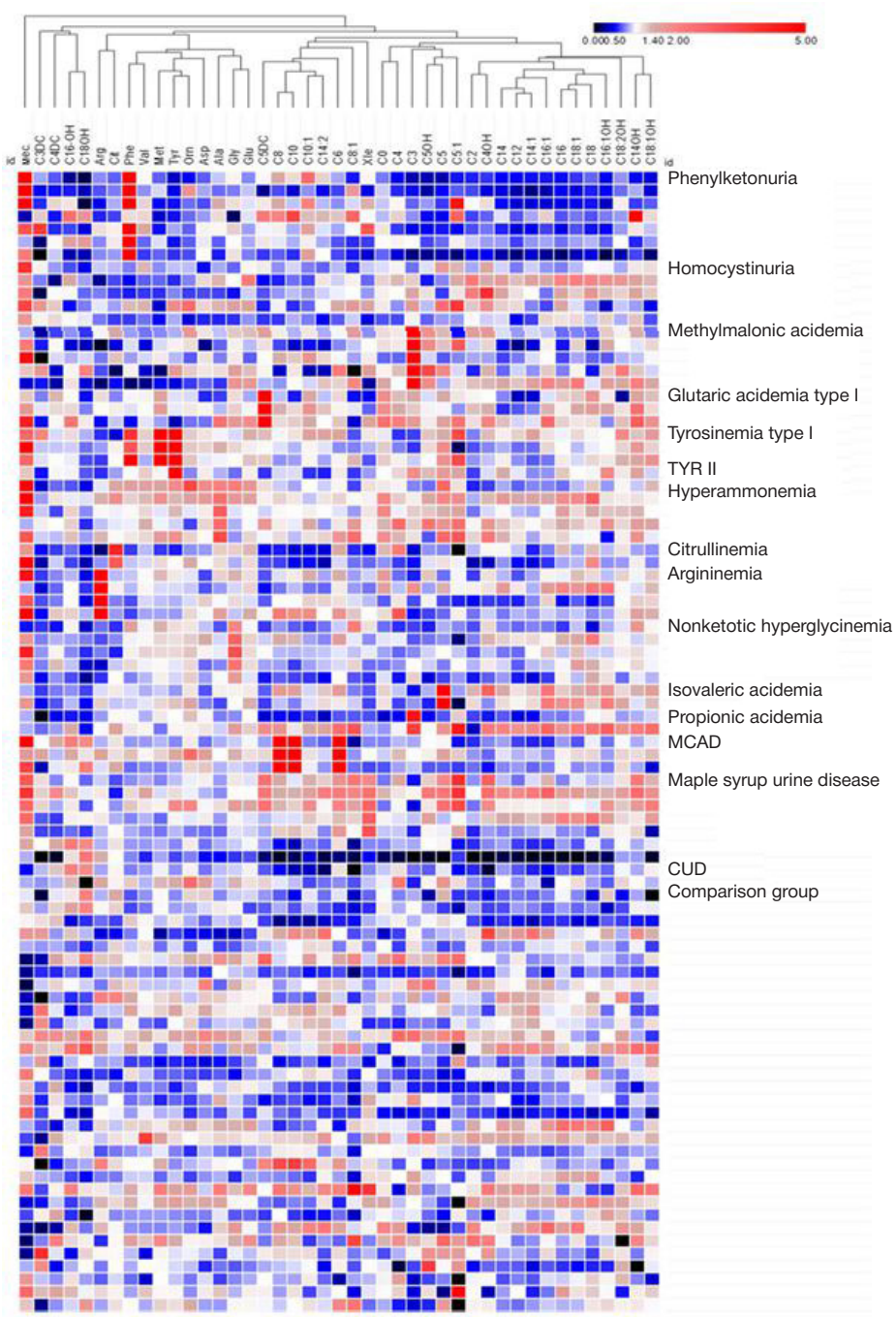

Fig. 3. Heatmap with a dendrogram of amino acid and acylcarnitine profile in patients with disturbed metabolism of these substances (the ratio of the diagnostic marker concentrations to the average concentration value in the control groups corresponds to the color chart)

in patients with citrullinemia and nonketotic hyperglycinemia. Despite the fact that these data are not enough to make statistically significant conclusions, the data definitely constitute the grounds for further research focused on identification of the groups of marker metabolites for the diagnosis of metabolic disorders of amino acids.

Specificity represents a far more important parameter for rare disorders than sensitivity, therefore, the tests having higher specificity show higher diagnostic efficacy [9]. Fig. 4 presents the ROC curve for the sum of normalized acylcarnitine levels (C12+C16). High values of area under the curve (> 0.9), specificity (almost 100%), and sensitivity (above 80%) make it possible to propose this parameter as a potential secondary marker of phenylketonuria.

It is obvious that phenylalanine levels represent one the most important diagnostic markers of phenylketonuria compared to others, but further study of the acylcarnitine profile will make it possible to differentiate phenylketonuria from other hyperphenylalaninemias with its help.

These examples allow us to say with certainty that hierarchical cluster analysis can be a reliable tool for physicians to be used in differential diagnosis of congenital metabolic disorders in children.

The analysis of correlations between certain markers and groups of markers selected based on the cluster analysis results was performed. The correlation analysis results are presented as the figures, in which the divisions mark the abscissa and ordinate axes for histograms; the concentration distribution graph for each marker metabolite is arranged diagonally; the graphs showing correlations between two variables are located under the diagonal, while the correlation coefficients and significance levels are located above the diagonal.

Predictably, the closest metabolically related substances, such as short-chain acylcarnitines, show the highest degree of correlation (Fig. 5).

However, the correlation between not metabolically closely related amino acids, such as methionine and tyrosine, was also observed (r = 0.73); the ornithine levels rather strongly correlated with the levels of aspartic acid, glycine, and glutamic acid (r = 0.55 and 0.43). The levels of the latter, in turn, were correlated to the levels of glycine, alanine, and ornithine (r = 0.47, 0.45, and 0.48, respectively) (Fig. 6).

High correlation of free carnitine (C0) with acetylcarnitine (C2) and butyrylcarnitine (C4) is confirmed by the correlation analysis results: r = 0.46 and 0.48, respectively (p < 0.001). High correlation between all markers in the group of long-chain acylcarnitines (C12, C14, C16, C18) enables the complex use of this group of markers, i.e. in the form of the common profile: the correlation coefficient for these markers vary between 0.69 and 0.88 (p < 0.001).

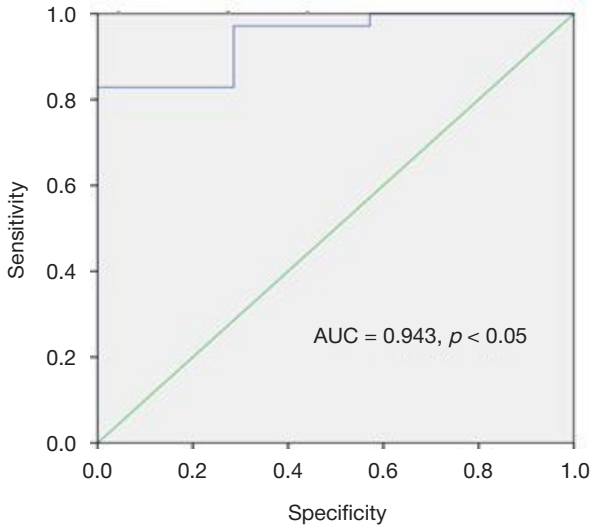

Fig. 4. ROC curve for the sum of normalized acylcarnitines (C12+C16)

DISCUSSION

In our country, chromatography–mass spectrometry has been introduced and used in clinical laboratory diagnosis for more than 10 years, and the reports published reflect the practical relevance of using the method by physicians of various specialties for the diagnosis of different disorders and disease entities, especially congenital metabolic disorders [10, 11]. The published papers contain information about the use of various developed qualitative and quantitative metabolic disorder marker determination methods for the diagnosis of different congenital and acquired metabolic disorders, however, no correlation analysis of indicators of various marker groups was performed, and different marker groups were not compared with each other. The published studies involved no big data analysis and no search for new statistically significant markers [12, 13]. At the same time, the tactics of big data analysis is currently being actively used in laboratory diagnosis. Large samples make it possible to create the datasets on their basis, allowing one to apply parametric tests used in statistical (cluster, correlation) analysis of various types. This represents an extra tool to be used to search for new laboratory markers and assess their diagnostic efficacy. Several papers were published, in which assessment of the entire set of data on the concentrations of amino acids and acetylcarnitines and the search for correlations between these groups of metabolytes provided additional information and made it possible to determine the associations and develop the algorithms for the diagnosis of various metabolic disorders, such as type II diabetes mellitus and metabolic syndrome [14, 15]. Thus, a prognostic model containing a panel of acetylcarnitines and amino acids improved the classification of diabetes mellitus cases compared to the model comprising the identified risk factors only [15]. At the same time, no studies focused on assessing the sets of data on the concentrations of amino acids and acetylcarnitines in pediatric population were conducted; these groups of metabolites were always considered separately from each other.

CONCLUSIONS

The experimental data analysis confirmed the efficacy of conventional markers of the disorders of amino acid and acetylcarnitine metabolism when used for the diagnosis of congenital metabolic disorders. The statistical processing applied enabled identification of new markers and marker profiles, which would help ensure a more thorough differential diagnosis of congenital metabolic disorders. The identified

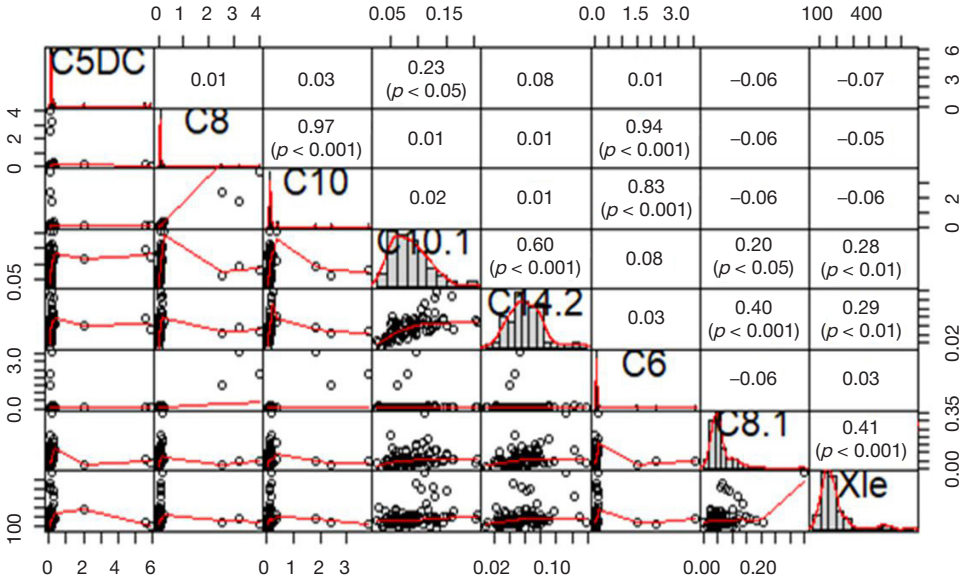

Fig. 5. Results of the correlation analysis performed in the group of marker short-chain acylcarnitines (p < significance level)

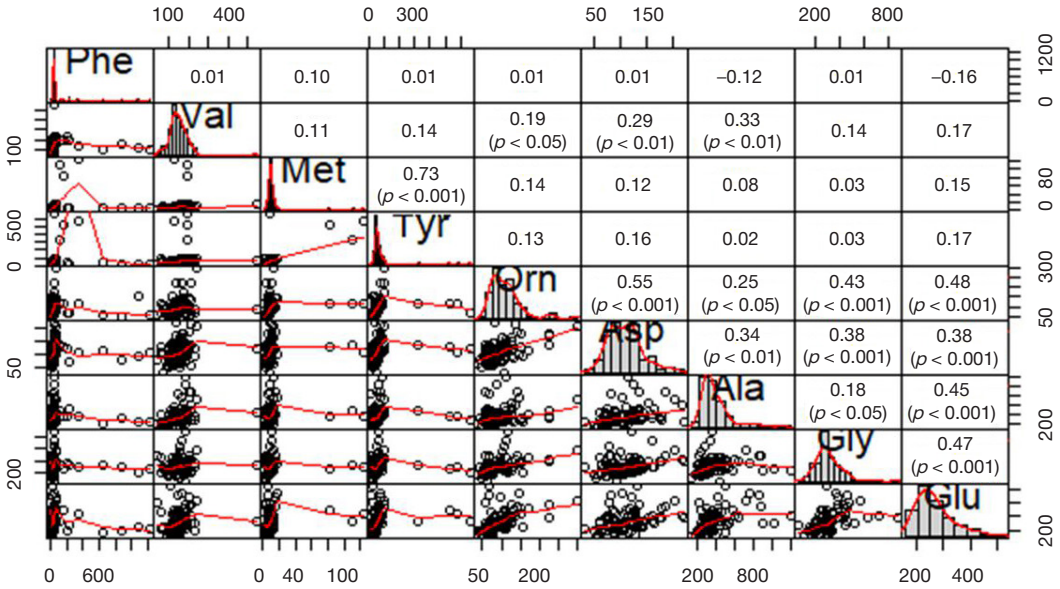

Fig. 6. Results of the correlation analysis performed in the group of marker amino acids (p < significance level)

relationships between mass spectrometry indicators of different groups (amino acids and acylcarnitines) demonstrate some potential diagnostic efficacy when testing children for congenital metabolic disorders. The acylcarnitine profile can be proposed as an additional potential marker to be used in cases of borderline phenylalanine levels, in patients with citrullinemia and nonketotic hyperglycinemia; the sums of normalized acylcarnitine levels (C12+C16) can be proposed as a potential secondary marker of phenylketonuria, and high correlation between all markers belonging to the group of long-chain acylcarnitines (C12, C14, C16, C18) enables the complex use of this group of markers, i.e. as a common profile.

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