AMINO ACID PROFILE IN DIMINISHED OVARIAN RESERVE

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Diminished ovarian reserve (DOR) represents a relevant issue of reproductive medicine that is often associated with infertility and reduced efficacy of IVF programs. The changes in amino acid metabolism can play a role in the DOR pathogenesis as manifestations of the folliculogenesis and oogenesis epigenetic alterations. The study was aimed to assess alterations of amino acid metabolic pathways in blood plasma and follicular fluid and estimate their clinical significance in DOR. A total of 115 infertile women aged 25–42 years were included in the study. Groups were formed based on the ovarian reserve and age. Amino acid levels in blood plasma and follicular fluid were assessed by high performance liquid chromatography–mass spectrometry (HPLC-MS); bioinformatics analysis of amino acid metabolic pathways was performed. We revealed significant changes in the phenylalanine, tyrosine and tryptophan biosynthesis (effect = 0.5; p = 0.026), alanine, aspartate and glutamate metabolism (effect = 0.114; p = 0.013), and arginine biosynthesis (effect = 0.289; p < 0.001) pathways playing a role in folliculogenesis, oogenesis, and embryogenesis. The detected differences in the amino acid levels in various body fluids made it possible to construct the logistic regression models confirming DOR with the 88% probability based on the amino acid levels in follicular fluid (sensitivity 88%, specificity 84%) and 82% probability based on plasma levels (sensitivity 65%, specificity 91%). The findings can be used for further research focused on the pathogenesis of infertility associated with DOR and for selection of the most optimal diagnostic and treatment tactics.

Keywords: amino acid profile, metabolism, metabolic pathway, childbearing age, infertility, diminished ovarian reserve, IVF, HPLC-MS

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АМИНОКИСЛОТНЫЙ ПРОФИЛЬ ПРИ СНИЖЕННОМ ОВАРИАЛЬНОМ РЕЗЕРВЕ

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Сниженный овариальный резерв (СОР) является одной из актуальных проблем репродуктивной медицины и часто ассоциирован с бесплодием и уменьшением эффективности программ ЭКО. Изменение метаболизма аминокислот может играть роль в патогенезе СОР как проявление эпигенетических нарушений в процессах фолликуло- и оогенеза. Целью исследования было проанализировать изменения метаболических путей аминокислот в плазме крови и фолликулярной жидкости и оценить их клиническое значение при СОР. В исследование вошли 115 женщин в возрасте 25–42 лет с бесплодием. Группы были сформированы в зависимости от овариального резерва и возраста. Выполнено исследование уровней аминокислот в плазме крови и фолликулярной жидкости методом высокоэффективной жидкостной хроматографии с детектированием на масс-спектрометре (ВЭЖХ-МС) и проведен биоинформатический анализ их метаболических путей. Обнаружено статистически значимое изменение путей биосинтеза фенилаланина, тирозина и триптофана (влияние = 0,5; p = 0,026), метаболизма аланина, аспартата и глутамата (влияние = 0,114; p = 0,013) и биосинтеза аргинина (влияние = 0,289; p < 0,001), играющих роль в процессах фолликуло-, оогенеза и эмбриогенеза. Обнаруженные различия в содержании аминокислот в различных биологических жидкостях позволили разработать модели логистической регрессии, подтверждающие СОР с вероятностью 88% по уровням аминокислот в фолликулярной жидкости (чувствительность — 88%, специфичность — 84%) и 82% по уровням в плазме крови (чувствительность — 65%, специфичность — 91%). Эти результаты могут быть использованы для дальнейших исследований патогенеза бесплодия при СОР и выбора наиболее оптимальной тактики диагностики и лечения.

Ключевые слова: аминокислотный профиль, метаболизм, метаболический путь, репродуктивный возраст, бесплодие, сниженный овариальный резерв, ЭКО, ВЭЖХ-МС

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Diminished ovarian reserve (DOR) represents a relevant issue of reproductive medicine. The reduced antral follicle counts (AFC), decreased anti-mullerian hormone (AMH) levels and elevated basal follicle-stimulating hormone (FSH) levels in blood serum result in infertility, poor ovarian response, deterioration of oocyte quality, the decrease in fertilization and pregnancy rates in in vitro fertilization programs (IVF/ ICSI), and the increase in the rate of early pregnancy loss [1].

The hormone and amino acid metabolism that can reflect epigenetic alterations of the folliculogenesis and oogenesis processes [2] is characterized not only by changes in the levels of certain metabolites, but also in their interaction in the form of complex metabolic "networks" [3]. Considering the cumulative contribution of the endocrine and metabolomic alterations of blood and follicular fluid to the cell metabolism and their impact on the outcomes of IVF programs, we can uncover some pathogenetic mechanisms of DOR and determine metabolites that can serve as potential biomarkers for estimation of ovarian reserve [4, 5].

In our previously published study, a significant decrease in plasma levels of such amino acids, as sarcosine and tryptophan, decreased levels of phenylalanine, tryptophan, methionine, asparagine, arginine, and lysine in follicular fluid, and the correlation of those with the indicators of folliculogenesis, oogenesis, and early embryogenesis in the IVF program were found in women with infertility and diminished ovarian reserve [6].

The study was aimed to assess alterations of amino acid metabolic pathways in blood plasma and follicular fluid and their correlation with age, as well as to estimate their clinical significance for the DOR pathogenesis.

METHODS

The prospective observational study involved infertile women of childbearing age, who contacted the Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology for IVF program.

The total sample consisted of 115 women, who were divided into groups with DOR (anti-mullerian hormone (AMH) < 1.2 ng/mL, antral follicle counts (AFC) < 5)) and normal ovarian reserve (AMH ≥ 1.2 ng/mL, AFC ≥ 5). To clarify the effect of age on the amino acid profiles of infertile women, the studied groups were further stratified by age: under the age of 35 years and over the age of 35 years. Inclusion criteria: childbearing age (25–42 years); not getting pregnant for at least one year of regular sexual activity without birth control; consent to study participation. Exclusion criteria: contraindications to ART; history of ovarian surgery; immunodeficiencies; systemic connective tissue disorders and rheumatic diseases; cancer of any etiology; chromosomal and genetic abnormalities; the use of donor oocytes or embryos, surrogacy.

All the patients underwent mandatory assessment prior to entering the assisted reproductive technology (ART) programs in accordance with the regulatory documents [7]. The levels of amino acids and their metabolites in blood plasma and follicular fluid were determined by high performance liquid chromatography–mass spectrometry (HPLC-MS) using the Agilent 1260 II liquid chromatography system (Agilent; USA) and the Agilent 6460 mass spectrometer (Agilent; USA). The parameters of mass spectrometry and chromatographic separation were set in accordance with the guidelines provided in the JASEM manual on amino acid analysis (JASEM; Türkiye).

Prior to statistical processing the HPLC-MS data were normalized to the composite signal of all analytes and converted into the standardized format using the following formula [9]:

$$z_i = \frac{x_i - \bar{x}}{\text{stddev}(x)}$$

where z_i — standardized parameter value, x_i — initial parameter value, $\bar{x}$ — average parameter value, stddev(x) — standard deviation for the population.

The search for metabolic pathways potentially involved in the DOR pathogenesis was performed using MetaboAnalyst via analysis of the involvement of amino acids, the levels of which differed significantly between the study groups. The metabolic pathway enrichment was assessed by the over-representation analysis (ORA) based on the hypergeometric test. Statistical significance of the metabolic pathway was determined using the hypergeometric test and the Benjamini-Hochberg procedure. Statistical significance of the pathway (p) corresponded to the probability of the experimental data random intersection with the metabolites of particular metabolic pathway (Fisher's exact test). The disease-associated pathways were considered to be significant at the false discovery rate (FDR) below 0.05.

The logistic regression models were developed to assess the possibility of classifying patients into groups based on the amino acid profiles of blood plasma and follicular fluid. For that all possible combinations of amino acids were considered as independent variables, while the patient's membership in one of the groups was considered as the dependent variable. The quality of models was estimated by ROC analysis and calculation of sensitivity and specificity. From all models, four with the largest area under the ROC curve (AUC) were selected. The Wald test, 95% confidence interval (CI), odds ratio (OR) and its confidence interval were calculated for each model.

The R scripts created using the Rstudio program were used for statistical processing of the HPLC-MS data [8]. The data distribution type was determined before conducting comparative metabolomic data analysis in the studied groups (Kolmogorov–Smirnov test, graphic data analysis).

When the data distribution was normal, the mean and standard deviation M (SD) were determined; Student's t-test was used to assess the differences in the groups. When the data distribution was non-normal, the data were presented as the median and interquartile range Me (Q_1; Q_3), the amino acid levels were compared using the nonparametric Wilcoxon–Mann–Whitney test. The threshold significance level (p) was considered to be 0.05.

RESULTS

The subjects' clinical, anamnestic, and endocrine characteristics are provided in Table. 1. All the patients were matched by age and anthropometric indices, had regular menstrual cycle; their average age was 37.2 ± 5.3 years. No significant differences in the rates of gynecological and somatic disorders were revealed. Patients with DOR had a significantly shorter menstrual cycle, lower antral follicle counts, and lower AMH levels. The endocrine profile analysis revealed an upward trend of FSH levels and a downward trend of androgen (androstenedione and DHEA-S) levels.

Assessment of amino acid profiles in the DOR group revealed a significant decrease in plasma levels of sarcosine and tryptophan and the decrease in the levels of phenylalanine, tryptophan, methionine, asparagine, arginine, and lysine in follicular fluid. The detailed analysis results have been published earlier [6]. The analysis of metabolic pathways involving amino acids with significantly lower levels was performed.

Alterations of the blood plasma and follicular fluid amino acid profiles associated with infertility and DOR have the most

Table 1. Clinical characteristics of women included in the study

Indicators	Group 1 DOR (*n* = 50)***	Group 2 NOR (*n* = 65)***	*p**
Age, years*	38.2 (6.18)	37.4 (4.6)	0.27
Age of menarche, years*	13.2 (1.2)	13.6 (1.4)	0.2
Length of menstrual cycle, days*	27.5 (1.99)	28.8 (1.7)	0.002
AFC*	4.6 (2.9)	13.0 (7.3)	< 0.001
AMH, ng/mL**	0.65 (0.32; 0.92)	2.7 (1.9; 4.6)	< 0.001
LH, mIU/mL**	5.8 (4.1-13.4)	4.1 (3.4-9.4)	0.2
FSH, mIU/mL**	8.3 (7.9-12.6)	6.5 (5.1-7.6)	0.1
DHEA-S, μmol/L**	3.8 (1.9; 5.4)	5.1 (4.1; 6.9)	0.14
Androstenedione, nmol/L**	4.5 (2.8; 7.8)	9.2 (7.9; 9.9)	0.09

Note: * — M (SD), *t*-test; ** — Mann–Whitney *U* test; *** — median (interquartile range).

prominent effect on the biosynthesis of phenylalanine, tyrosine, and tryptophan (effect = 0.5, p = 0.026). We also revealed significant impact on the arginine biosynthesis (effect = 0.289, p <0.001), aspartate metabolism (effect = 0.25, p = 0.027), alanine, aspartate, and glutamate metabolism (effect = 0.114, p = 0.013) (Fig. 1; Table 2).

The logistic regression models allowing one to distinguish the blood plasma an follicular fluid samples collected from patients with DOR and controls were constructed based on the amino acid profile analysis by HPLC-MS. All possible combinations of amino acids were used to construct the models. ROC analysis was performed for each model, and four models characterized by the largest area under the ROC curve (AUC) were selected.

The model constructed based on the age and the serine, tyrosine, and phenylalanine levels had the largest AUC (0.82). Specificity and sensitivity were 94 and 68%, respectively, and the threshold value was 0.69. All the models constructed included age and phenylalanine levels (Fig. 2; Table 3).

The logistic regression models similar to the earlier reported ones were also constructed for follicular fluid. All models had the same area under the curve (AUC = 0.88). Models 1 and 2 were characterized by higher sensitivity (84%), while models 3 and 4 had higher specificity (88%). All the models constructed included phenylalanine. This was clearly due to the fact that there were the largest differences in the levels of this amino acid between groups (Fig. 2; Table 3).

To clarify the effect of age on the amino acid profiles, patients of both groups were divided into two subgroups: under the age of 35 years and over the age of 35 years. In the late reproductive stage group (over the age of 35 years) with DOR, a significant decrease in plasma levels of lysine, glutamine, serine, glycine, threonine, tyrosine, leucine, tryptophan, glutamic and aspartic acids was revealed, along with the increase in proline levels (Fig. 3A). However, no age-related alterations of the follicular fluid amino acid profiles were revealed in women with infertility and DOR (Fig. 3B). Furthermore, women with normal ovarian reserve showed no significant age-related alterations of both blood plasma and follicular fluid amino acid profiles (Fig. 3C, D).

DISCUSSION

The findings suggest that amino acid metabolism alterations play an important role in the DOR pathogenesis. According to our results, DOR is characterized by alterations of the phenylalanine, tyrosine and tryptophan biosynthesis, i.e. biosynthesis of the aromatic amino acids representing precursors of neurotransmitters, serotonin and catecholamines (dopamine, norepinephrine and epinephrine), the deficiency of which can result in oxidative stress having a toxic effect on the folliculogenesis and oogenesis [10]. Phenylalanine plays an important role in the formation of protein tertiary structure and stabilization of protein structures [11]. The decrease in

Table 2. Involvement of the amino acids characterizing DOR in metabolic pathways

Pathway	Total	Markers	*p*	FDR	Effect
KEGG					
Biosynthesis of phenylalanine, tyrosine and tryptophan	4	1	0.026	0.0429	0.5
Phenylalanine metabolism	10	1	0.063	0.5866	0.357
Biosynthesis of arginine	14	3	< 0,001	0.0029	0.289
Tryptophan metabolism	41	1	0.236	1	0.143
Alanine, aspartate and glutamate metabolism	28	2	0.013	0.0272	0.114
Arginine and proline metabolism	38	1	0.22	1	0.111
Cysteine and methionine metabolism	33	1	0.194	1	0.104
Biosynthesis of aminoacyl-tRNA	48	8	< 0,001	1.68E-09	0
HMDB (Human Metabolome Database)					
Aspartate metabolism	34	2	0.027	1	0.25
Urea cycle	23	1	0.169	1	0.154
Arginine and proline metabolism	48	1	0.324	1	0.082
Ammonia metabolism	25	1	0.18	1	0.033
Biotin metabolism	7	1	0.054	1	0

Note: FDR — false discovery rate.

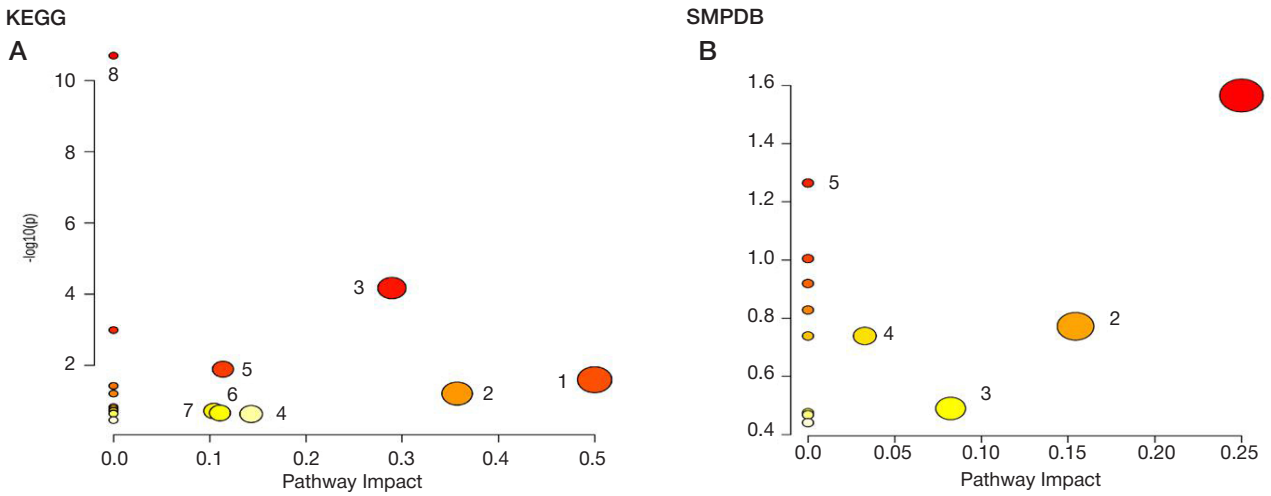

Fig. 1. Map of metabolic pathways involving amino acids showing significant differences in the blood plasma and follicular fluid levels between groups (Kyoto Encyclopedia of Genes and Genomes, KEGG): 1 — biosynthesis of phenylalanine, tyrosine and tryptophan; 2 — phenylalanine metabolism; 3 — biosynthesis of arginine; 4 — tryptophan metabolism; 5 — alanine, aspartate and glutamate metabolism; 6 — arginine and proline metabolism; 7 — cysteine and methionine metabolism; 8 — biosynthesis of aminoacyl-tRNA. Small Molecule Pathway Database (SMPDB): 1 — aspartate metabolism; 2 — urea cycle; 3 — arginine and proline metabolism; 4 — ammonia processing; 5 — biotin metabolism.). The Y-axis and the node color reflect statistical significance of the identified amino acids' involvement in appropriate metabolic pathways; the x-axis and the node radius reflect the studied metabolites' effect of the pathway

phenylalanine levels observed in women with DOR in our study is consistent with the results reported by other authors [12]. The role of phenylalanine in the DOR pathogenesis was been also confirmed by the fact that in our study phenylalanine was included in all logistic regression models with the greatest significance.

The decrease in the levels of phenylalanine being the main substrate for tyrosine synthesis in the body is associated with the

Table 3. Logistic regression models constructed based on the amino acid levels in blood plasma and follicular fluid

Blood plasma				
Model	AUC	Threshold value	Sensitivity	Specificity
Age, serine, tyrosine, phenylalanine	0.82	0.69	0.68 (0.48; 0.88)	0.94 (0.68; 1)
Age, serine, sarcosine, phenylalanine	0.81	0.75	0.65 (0.42; 0.85)	0.94 (0.71; 1)
Age, lysine, histidine, phenylalanine	0.81	0.5	0.8 (0.45; 0.98)	0.76 (0.5; 1)
Age, histidine, serine, phenylalanine	0.81	0.61	0.7 (0.48; 0.85)	0.88 (0.71; 1)
Follicular fluid				
Model	AUC	Threshold value	Sensitivity	Specificity
Methylhistidine, serine, alanine, phenylalanine	0.88	0.45	0.88 (0.62; 0.97)	0.84 (0.69; 1)
Age, ornithine, serine, phenylalanine	0.88	0.47	0.88 (0.59; 0.97)	0.84 (0.69; 1)
Ornithine, serine, alanine, phenylalanine	0.88	0.55	0.84 (0.69; 0.97)	0.88 (0.69; 0.97)
Ornithine, serine, valine, phenylalanine	0.88	0.57	0.84 (0.66; 0.97)	0.88 (0.66; 1)

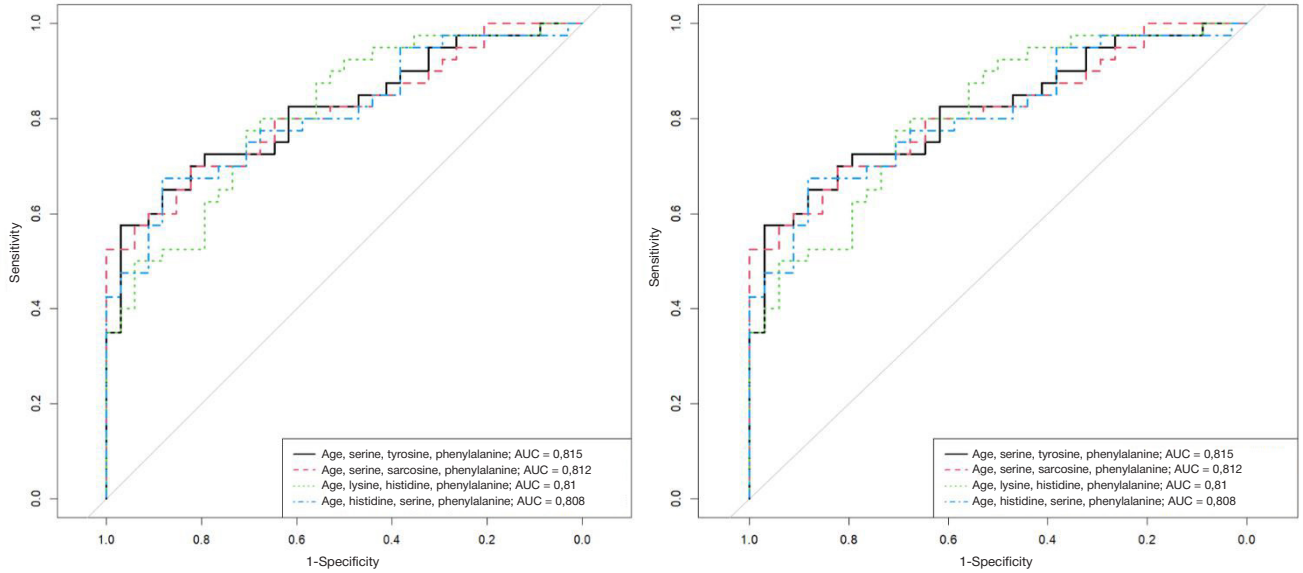

Fig. 2. ROC analysis of the logistic regression models for determination of DOR based on the blood plasma and follicular fluid amino acid composition

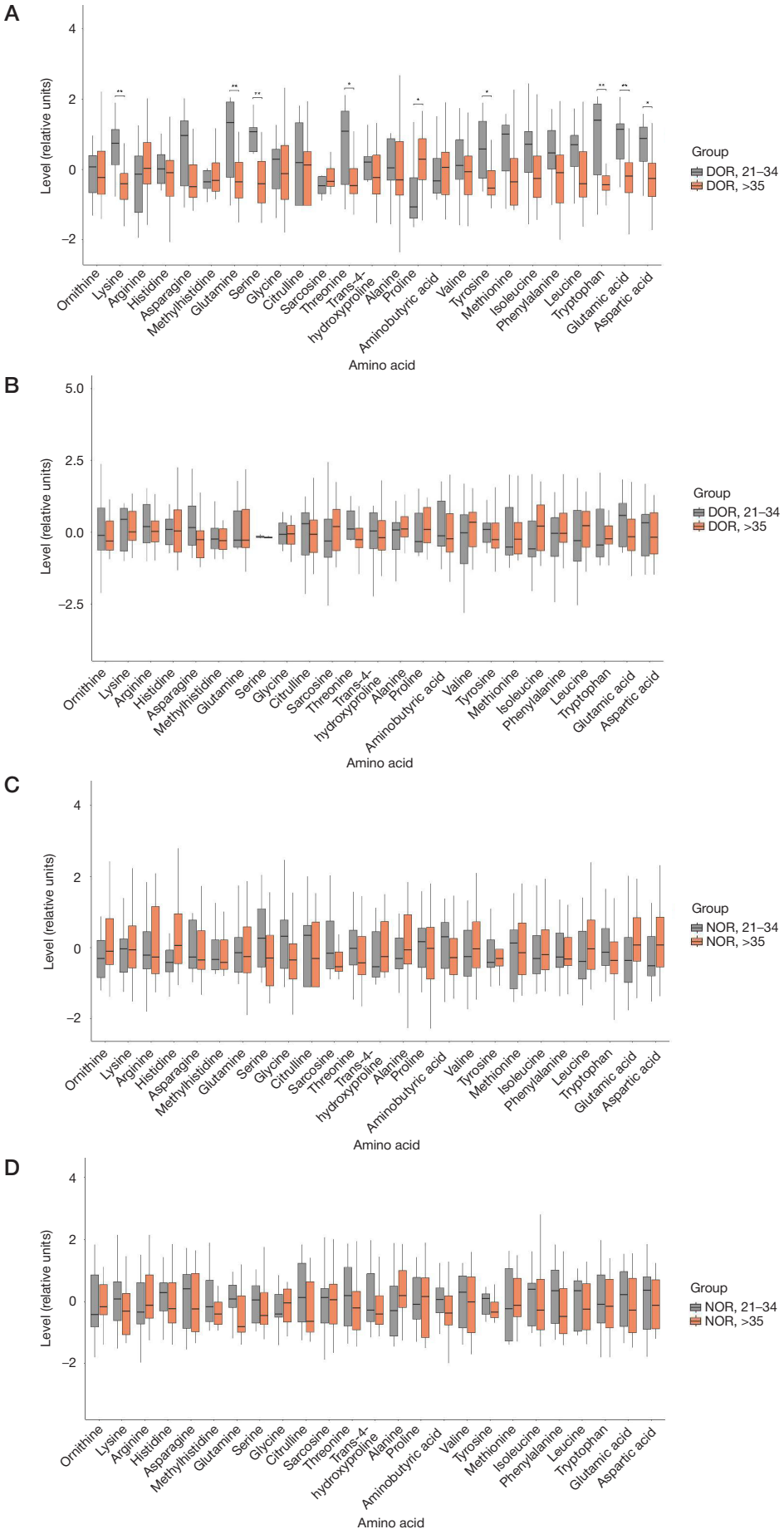

Fig. 3. Comparison of the amino acid profiles of blood plasma **(A, C)** and follicular fluid **(B, D)** in the groups DOR 21–34, DOR >35 **(A, B)**, NOR 21–34 and NOR >35 **(C, D)**

decrease in its availability for production of tyrosine [13] that serves as a basis for the synthesis of amine mediators and hormones: catecholamines, serotonin, melatonin. All tyrosine metabolites contribute to proper and consistent functioning of all body systems in order to ensure fertility. Tyrosine, like its precursor, is essential for synthesis of the benzoquinone structure which is part of the coenzyme Q10 representing an antioxidant capable of neutralizing free radicals, inhibiting lipid peroxidation in biological membranes, and protecting mitochondrial proteins and DNA against oxidative damage; coenzyme Q10 is also involved in ATP synthesis in mitochondria as an electron carrier [14]. The antioxidant effects of tyrosine have been also reported in the study of seminal plasma [15].

The role of tryptophan in many physiological processes, such as maintaining cell growth and regulation of the immune function, synthesis of serotonin and melatonin, the decrease in the levels of which results in disruption of early embryogenesis, is invaluable [16].

In our study, women with DOR of late reproductive age demonstrate the correlation between the decrease in the levels of tyrosine and tryptophan and worse outcomes of IVF programs, which confirms the role of tyrosine and tryptophan in the age-related alterations of oogenesis and early embryogenesis and is consistent with the data reported by foreign colleagues [17].

The arginine biosynthesis that turned out to be significantly altered in our study is of crucial importance for the synthesis of nitric oxide (NO). The latter is a factor of vasorelaxation ensuring optimization of blood flow to the tissues [18] and contributing to normal endometrial growth [19], steroidogenesis and folliculogenesis regulation [20]. Taking drugs with high arginine content by women with poor ovarian response, who are through IVF programs, results in elevation of the arginine, citrulline, and NO levels in blood plasma and follicular fluid and is associated with improved uterine and ovarian blood flow, increased fertilization and pregnancy rates, and reduced rate of pregnancy complications (early pregnancy loss, intrauterine growth restriction, and preeclampsia) [21].

The analysis of amino acid involvement in metabolic pathways has revealed significant changes in the alanine, aspartate and glutamate metabolism. Aspartate and glutamate are excitatory neurotransmitters of the CNS that are involved in the synthesis of purine and pyrimidine nucleotides. According to our research, is higher concentrations of aspartate are found in follicular fluid in women of early reproductive age compared to women of late reproductive age, which has been also confirmed by the positive correlation between the levels of D-aspartic acid in follicular fluid and the oocyte morphology, maturation, percentage of mature oocytes, fertilization rate in one of the studies [22]. Methionine is formed from aspartic acid that is involved in the synthesis of polyamines, post-translational modification of proteins, and regulation of the DNA reading processes. In the animal experiment, dietary methionine restriction decreased the levels of insulin-like growth factor 1, thyroid hormones in blood plasma and reduced fertility [23].

High prognostic potential of follicular fluid in women with DOR, along with blood plasma, was reported in our study, which was in line with the results of other studies confirming the role of amino acid metabolism in follicular fluid as a functional indicator of oocyte quality in women of late reproductive age, who were through the IVF/ICSI programs [24]. The probable decrease in the amino acid availability from follicular fluid can result in the increased amino acid uptake by oocytes in the culture medium in the IVF program.

The important role of antioxidant systems in oocytes and the age-related imbalance between prooxidant activity and the antioxidant defense systems in oocytes [25] trigger the development of mitochondrial dysfunction, contribute to higher rates of aneuploid oocytes/embryos and lower pregnancy rate [26]. Our data on the prominent amino acid profile alterations associated with DOR in women of late reproductive age confirm the overall decrease in amino acid metabolism with age that is also associated with reduction of their antioxidant activity. The data can be used for estimation of ovarian reserve. Further research focused on the use of amino acids in clinical practice for improvement of the outcomes of IVF/ICSI programs both in vitro, involving supplementation of the embryological media, and in vivo, involving taking drugs with high content of essential amino acids, is still relevant [27, 28].

CONCLUSIONS

Significant changes in the amino acid metabolism in blood plasma and follicular fluid associated with infertility and DOR, including metabolism of phenylalanine, tyrosine and tryptophan involved in realization of antioxidant defense in the ovarian tissues, as well as in the synthesis of neurotransmitters, catecholamines and hormones, directly affect the reproductive system by altering the cellular energy metabolism. The models allowing one to confirm DOR based on the amino acid profile of blood plasma with the 82% probability and amino acid profile of follicular fluid with the probability of 88% have been constructed based on the differences revealed using the targeted semi-quantitative metabolomics analysis methods. The data on the age-related alterations of amino acid levels associated with DOR confirm relevance of the studies focused on the use of preliminary treatment with the drugs containing amino acids aimed to improve the IVF program outcomes.

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