# АКУШЕРСТВО И ГИНЕКОЛОГИЯ
# OBSTETRICS AND GYNAECOLOGY

## GUT MICROBIOTA BIODIVERSITY INDICES AS MARKERS OF HYPERANDROGENEMIA IN WOMEN OF REPRODUCTIVE AGE

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## ABSTRACT

***Introduction.*** *Previously, it was shown that the "classic" phenotypes of polycystic ovarian syndrome (PCOS) are associated with significant decrease in gut microbiota alpha diversity as compared with healthy women.*
***The aim of the study.*** *To establish cut-off points for alpha diversity indices, significant in polycystic ovarian syndrome with hyperandrogenism.*
***Material and methods.*** *The manuscript presents a sub-study of Eastern Siberia PCOS Epidemiology and Phenotype Study, conducted in Eastern Siberia (Russia) from 2016 to 2019. All participants (175 women of reproductive age: 26 women with PCOS (according to Rotterdam criteria (2003)) and hyperandrogenemia (increased levels of total testosterone (TT) and/or free androgen index (FAI), and/or dehydroepiandrosterone sulphate (DHEAS)), 149 – without hyperandrogenemia) were recruited during the annual employment medical assessment. Methods included a questionnaire survey, anthropometry and modified Ferriman – Gallwey score, gynecological examination, pelvic ultrasound, and blood serum tests for TT, DHEAS, sex hormone-binding globulin, FAI, prolactin, thyroid-stimulating hormone, and 17-hydroxyprogesterone. Five indices of alpha diversity (amplicon sequencing variant, Shannon index, Simpson index, Chao index, and abundance-based coverage Index) were estimated for the gut microbiota using amplicon metasequencing. Statistical analysis included ROC-analysis for development of cut-off points for the indices, associated with hyperandrogenism in women of reproductive age with PCOS.*
***Results.*** *According to results of ROC-analysis, the greatest sensitivity with moderate specificity, with a high area under the curve was established for the Shannon and Simpson indices with cut-off points classifying women with or without hyperandrogenemia – 5.84 and 0.97, respectively.*
***Conclusions.*** *The developed criteria for assessing alpha diversity using cut-off points for the most significant indices can be useful for monitoring the results of different therapeutic interventions (prebiotics, probiotics, etc.) in hyperandrogenic phenotypes of PCOS.*

***Key words:*** *polycystic ovary syndrome, PCOS, hyperandrogenism, gut microbiota, alpha diversity indices, amplicon metasequencing*

Received: 02.09.2024
Accepted: 10.09.2024
Published: 25.09.2024

**For citation:** Igumnov I.A., Belkova N.L., Atalyan A.V., Klimenko E.S., Sholokhov L.F., Belenkaia L.V., Samarina Ya.M., Danusevich I.N., Lazareva L.M., Nadeliaeva Ia.G., Suturina L.V. Gut microbiota biodiversity indices as markers of hyperandrogenemia in women of reproductive age. *Acta biomedica scientifica*. 2024; 9(4): 12-18. doi: 10.29413/ABS.2024-9.4.2

# ИНДЕКСЫ БИОРАЗНООБРАЗИЯ МИКРОБИОТЫ КИШЕЧНИКА КАК МАРКЕРЫ ГИПЕРАНДРОГЕНЕМИИ У ЖЕНЩИН РЕПРОДУКТИВНОГО ВОЗРАСТА

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## РЕЗЮМЕ

***Введение.*** *Ранее было показано, что «классические» фенотипы синдрома поликистозных яичников (СПЯ) связаны со значительным снижением альфа-разнообразия кишечной микробиоты по сравнению с показателями здоровых женщин.*

***Цель данного исследования****. Установить пороговые значения для индексов альфа-разнообразия, значимых при синдроме поликистозных яичников с гиперандрогенией.*

***Материал и методы.*** *В рукописи представлены результаты, полученные в рамках «Исследования эпидемиологии и фенотипа СПЯ в Восточной Сибири», проведённого в Восточной Сибири (Россия) с 2016 по 2019 г. Все участники (175 женщин репродуктивного возраста: 26 женщин с СПЯ (согласно критериям, принятым в Роттердаме в 2003 г.) и гиперандрогенемией (повышенным уровнем общего тестостерона (Т), и/или индексом свободных андрогенов (ИСА), и/или уровнем дегидроэпиандростерон-сульфата (ДГЭА-С)) и 149 женщин без гиперандрогенемии) были рекрутированы во время ежегодного медосмотра по месту работы. Методы включали анкетирование, антропометрию и оценку по модифицированной шкале Ферримана – Галлвея, гинекологический осмотр, ультразвуковое исследование органов малого таза и анализы сыворотки крови на Т, ДГЭА-С, глобулин, связывающий половые гормоны, ИСА, пролактин, тиреотропный гормон и 17-ОН-прогестерон. Пять индексов альфа-разнообразия микробиоты кишечника (ASV (amplicon sequencing variant), индекс Шеннона, индекс Симпсона, индекс Чао, и ACE (Abundance-based Coverage index)) оценивали с использованием метасеквенирования ампликонов. Статистический анализ включал ROC-анализ для разработки пороговых значений для индексов, ассоциированных с гиперандрогенией у женщин репродуктивного возраста с СПКЯ.*

***Результаты.*** *По результатам ROC-анализа наибольшая чувствительность при умеренной специфичности, с высокой площадью под кривой была установлена для индексов Шеннона и Симпсона с пороговыми значениями, классифицирующими женщин с гиперандрогенемией или без неё: 5,84 и 0,97 соответственно.*

***Выводы.*** *Разработанные критерии оценки альфа-разнообразия с использованием пороговых значений для наиболее значимых индексов могут быть полезны для мониторинга результатов различных терапевтических вмешательств (пребиотики, пробиотики и т. д.) при гиперандрогенных фенотипах СПКЯ.*

***Ключевые слова****: синдром поликистозных яичников, СПЯ, гиперандрогения, микробиота кишечника, индексы альфа-разнообразия, метасеквенирование ампликонов*

**Для цитирования:** Игумнов И.А., Белькова Н.Л., Аталян А.В., Клименко Е.С., Шолохов Л.Ф., Беленькая Л.В., Самарина Я.М., Данусевич И.Н., Лазарева Л.М., Наделяева Я.Г., Сутурина Л.В. Индексы биоразнообразия микробиоты кишечника как маркеры гиперандрогенемии у женщин репродуктивного возраста. *Acta biomedica scientifica*. 2024; 9(4): 12-18. doi: 10.29413/ABS.2024-9.4.2

Статья получена: 02.09.2024
Статья принята: 10.09.2024
Статья опубликована: 25.09.2024

## BACKGROUND

Polycystic ovarian syndrome (PCOS) is the most common type of hyperandrogenism in women and is associated not only with reproductive disorders but also with a high risk of developing metabolic syndrome, diabetes mellitus, cardiovascular diseases, and psychoemotional disorders [1]. The prevalence of PCOS ranges from 2.2 to 19.5 %, depending on the diagnostic criteria used and the characteristics of the population sample [2]. To date, more and more attention is being paid to the role of the gut microbiota in different PCOS pathways: chronic systemic inflammation, digestive hormone imbalance, and decreased insulin sensitivity [3–5]. Nevertheless, the study results are inconsistent and different authors considered the same bacterial species as probiotics or, on the contrary, as pathogens causing polycystic ovary syndrome in model animals. Dysbiosis of gut microbiota correlates with the clinical features of PCOS [6, 7]; however, the role of gut microbiocenosis in the development of hyperandrogenemia has not been definitively established.

Currently, some studies have reported the relationship between PCOS and at least one alpha diversity index [7, 8]. At the same time, in general, data on gut microbiota biodiversity in PCOS and hyperandrogenemia are contradictory and demonstrate decreased [7–9] or comparable diversity with controls values [10].

Previously, we have shown that the "classic" PCOS phenotypes are associated with a significant decrease in the alpha diversity of the intestinal microbiota as compared to healthy women, which was consistent with findings of other studies [11–13]. However, a diagnostic value of biodiversity indices regarding hyperandrogenism is not determined clearly. Therefore, the objectives of this study were to establish cut-off points for the main indices of alpha diversity that are significant in PCOS women with hyperandrogenism.

## METHODS

### Study design

We performed this research as a sub-study of cross-sectional, multicenter, institution-based Eastern Siberia PCOS Epidemiology and Phenotype (ESPEP) Study, conducted in the Irkutsk region and the Republic of Buryatia (Eastern Siberia, Russian Federation) from 2016 to 2019 (ClinicalTrials.gov ID: NCT05194384) [14].

All participants (175 women of reproductive age: 26 women with PCOS phenotypes A, B, C (according to Rotterdam criteria (2003)) and hyperandrogenemia (increased levels of total testosterone (TT) and/or free androgen index (FAI), and/or dehydroepiandrosterone sulphate (DHEAS)), and 149 women without hyperandrogenemia) were recruited during the annual employment medical assessment. The study was conducted in accordance with the WMA Declaration of Helsinki, 1964, with subsequent changes, and approved by the Local Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problems (Protocol No. 2.1, date of approval – February 24, 2016).

The inclusion and exclusion criteria for ESPEP study were previously reported [14]. For sub-study, we also excluded women who had taken antibiotics within one month before recruitment.

For PCOS diagnosis, we used Rotterdam Criteria (2003): a presence of any two of three criteria – hyperandrogenism, oligo/anovulation, and polycystic ovarian morphology – with no conditions with similar symptoms (hyperprolactinemia, hypothyroidism, 21-hydroxylase-deficient non-classic congenital adrenal hyperplasia (NC-CAH), premature ovarian failure) [15].

Methods included a questionnaire survey, anthropometry with body mass index (BMI) calculation (weight (kg)/height ($m^2$)), vital signs, modified Ferriman – Gallwey (mF-G) scoring, gynecological examination, lab tests, and pelvic ultrasound (US). Pelvic US was performed using the portable ultrasound scanner Mindray M7 (MINDRAY, China). Blood serum TT was estimated using liquid chromatography with tandem mass spectrometry. For DHEAS, sex hormone-binding globulin (SHBG), prolactin, luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and 17-hydroxyprogesterone (17-OH-P) we used ELISA. FAI was calculated (i. e. [TT/SHBG] × 100). As previously reported, the upper normal levels (UNL) for androgens (TT, FAI, and DHEAS) were determined from the 98th percentiles for these parameters in the healthy controls [16].

Sampling for high-throughput sequencing was performed according to standard operating procedures (SOP: IHMS_SOP03 V2, IHMS_SOP06 V2) developed during the implementation of the International Human Microbiome Standards (IHMS) project of the international consortium [17]. On the eve of admission, patients received a home fecal collection kit, which included a sterile fecal jar, refrigerant, and instructions. The patients transported the biomaterial in refrigerant to the laboratory within 2 h after defecation. The feces were immediately divided into aliquots for storage at –80 °C until further processing.

Genomic DNA was isolated from feces using the Quick-DNA Fecal/Soil Microbe Kit (Zymo Research, USA). Library preparation and sequencing were carried out in accordance with the manufacturer's recommendations: amplified fragments were indexed using the Nextera XT Index kit v2 (set A-D), and individual libraries were mixed in equimolar amounts and sequenced on an Illumina MiSeq instrument (Illumina, USA) using a MiSeq® Reagent Kit v3 (600 cycle) with double-sided reading (2 × 300 N). Sequencing of the V1–V3 amplicons of the variable regions of the 16S rRNA gene was performed using the equipment of the Core Centrum 'Genomic Technologies, Proteomics and Cell Biology' in ARRIAM, and the primary data were deposited in the international database NCBI SRA (data: PRJNA899143). Amplicon libraries of 16S rDNA were processed using the QIIME2 bioinformatics pipeline to conduct a comparative metagenomic study [18]. Amplicon sequencing variants (ASV) were generated using the DADA2 algorithm, which allows detection, correction,

and filtering of amplicon errors and chimeric sequences. The resulting representative sequences were used to determine their taxonomic classification using the sklearn-based Naive Bayes classifier trained on the SILVA v138 with 99 % 16S full-length database. Diversity analysis was performed to estimate alpha diversity using the "diversity" plugin, and species richness difference analysis was performed using the following indexes: ASV, Shannon, Simpson, Chao, and Abundance-based coverage index (ACE).

The main outcome measures included the cut-off points for the biodiversity indices (ASV, Shannon, Simpson, Chao, and ACE), associated with hyperandrogenemia in premenopausal women with PCOS.

**Statistical analysis**

Sample size calculations for the total population were based on the following formula: $n = [(Z^2_{1-\alpha}\ p(1-p)]/D2$, where $n$ – individual sample size, $Z_{1-\alpha} = 1.96$ (when $\alpha = 0.05$), $p$ – assumed PCOS prevalence according to the previously published data [2], and $D$ – absolute error. The data were collected using Research Electronic Data Capture (REDCap) [19]. Outliers were identified during the Exploratory Data Analysis using the box-plot and 3σ methods. Managing missing data: in our research dataset, there were two types of missing data – missing completely at random (MCAR) and missing at random (MAR). We recorded all missing values with labels of "N/A" to make them consistent throughout our dataset. Pairwise deletion was used when the dataset was analyzed.

To estimate the assumption of the normal distribution of our datasets, we performed a formal statistical test – the Shapiro – Wilk test. Chi-square ($\chi^2$) was used for frequency data. We used a Student's t-test to compare the mean values of the data with an independent sample, which followed a normal distribution, or a Mann – Whitney U-test to compare the ratio between two groups in another case. Statistical analysis included ROC-analysis for cut-offs development.

All data were analyzed using R 3.6.3 (a free software environment for statistical computing and graphics [20] and packages rstatix 0.7.0, ggplot2, and catdap1.3.5.

## RESULTS

The main socio-demographic characteristics of study participants are presented in the Table 1. To determine microbiome-specific markers of hyperandrogenemia using the polycystic ovary syndrome model, all study participants were divided into two groups based on the presence or absence of PCOS with hyperandrogenemia. The criterion for hyperandrogenemia was the presence of an increase above the UNLs of at least one of the following indicators: TT, FAI, DHEAS. As presented in the Table 2, in patients with hyperandrogenemia, we found significantly increased levels of luteinizing hormone and androgens, which confirms the correctness of the model (PCOS) we have chosen.

**TABLE 1**

**SOCIO-DEMOGRAPHIC CHARACTERISTICS OF STUDY PARTICIPANTS**

| Parameters | Total (*n* = 175) |
|---|---|
| Age (years), Mean ± SD | 33 ± 6.2 |
| Ethnicity, *n/N (%)* | |
| Caucasians | 128/175 (73.2) |
| Asians | 28/175 (16) |
| Mixed (Caucasian + Asian) | 19/175 (10.8) |
| Place of residence, *n/N (%)* | |
| City | 105/175 (60) |
| Rural area | 70/175 (40) |
| Marital status, *n/N (%)* | |
| Single | 54/175 (30.9) |
| Married | 82/175 (46.8) |
| Living with another | 23/175 (13.1) |
| Separated | 1/175 (0.6) |
| Divorced | 14/175 (8.0) |
| Widowed | 1/175 (0.6) |
| Would rather not say | 0/175 (0.0) |
| Education, *n/N (%)* | |
| Doctoral degree | 4/175 (2.3) |
| Master's degree | 75/175 (42.8) |
| Bachelor's degree | 29/175 (16.6) |
| Some college | 31/175 (17.7) |
| High school or equivalent | 14/175 (8.0) |
| Middle school only | 2/175 (1.1) |
| Elementary school | 9/175 (5.2) |
| No degree | 3/175 (1.7) |
| Missing data | 8/175 (4.6) |

As presented in the Table 3, the optimal balance of sensitivity and specificity was found for the Shannon and Simpson indices.

**TABLE 2**

**THE MAIN CHARACTERISTICS OF STUDY PARTICIPANTS WITH AND WITHOUT HYPERANDROGENEMIA**

| Parameters | Women with PCOS and HA ($n$ = 26) | Women without HA ($n$ = 149) | $p$ |
|---|---|---|---|
| | Mean ± SD<br>Median (LQ; UQ) | | |
| BMI, kg/m$^2$ | 26.9 ± 6.0<br>25.9 (22.4; 31.3) | 26.4 ± 5.4<br>25.8 (22.0; 29.0) | $p = 0.70^{\#}$ |
| LH, mIU/ml | 13.0 ± 11.2<br>10.0 (6.3; 15.5) | 7.6 ± 7.4<br>5.6 (3.2; 9.2) | $p < 0.001^{\#}$ |
| FSH, mIU/l | 5.9 ± 1.8<br>5.9 (5.0; 7.2) | 5.8 ± 5.4<br>5.1 (3.7; 6.4) | $p = 0.04^{\#}$ |
| Prolactin, mIU/l | 322 ± 161<br>291 (233; 435) | 336 ± 189<br>286 (215; 408) | $p = 0.95^{\#}$ |
| TSH, IU/l | 1.6 ± 0.8<br>1.6 (0.9; 1.9) | 1.8 ± 1.6<br>1.5 (1.1; 1.9) | $p = 0.76^{\#}$ |
| 17-OH-P, nmol/l | 5.4 ± 3.2<br>4.9 (2.7; 7.5) | 5.3 ± 3.5<br>5.0 (2.6; 7.3) | $p = 0.72^{\#}$ |
| Testosterone, ng/dl | 62.8 ± 28.8<br>55.6 (44.5; 81.1) | 27.7 ± 14.7<br>26.0 (17.9; 36.5) | $p < 0.001^{\#}$ |
| SHBG, nmol/l | 65.3 ± 52.9<br>40.3 (31.3; 89.8) | 78.9 ± 51.3<br>69.7 (43.1; 99.3) | $p = 0.03^{\#}$ |
| FAI | 5.3 ± 3.8<br>4.6 (2.2; 6.7) | 1.6 ± 1.2<br>1.3 (0.8; 2.2) | $p < 0.001^{\#}$ |
| DHEAS, μg/dl | 273.6 ± 144.4<br>212.5 (151.0; 389.0) | 172.9 ± 71.8<br>168.0 (116.7; 221.5) | $p < 0.001^{\#}$ |

**Note.** HA – hyperandrogenism; $^{\#}$ – Mann – Whitney U-test.

**TABLE 3**

**THE CUT-OFF POINTS FOR BIODIVERSITY INDICES ASSOCIATED WITH HYPERANDROGENEMIA (ON THE MODEL OF PCOS) IN PREMENOPAUSAL WOMEN**

| Index | The cut-off point | Sensitivity; specificity | 95% CI | AUC | 95% CI |
|---|---|---|---|---|---|
| ASV | 122.5 | 0.80; 0.63 | (103.00; 184.00) | 0.65 | (0.48; 0.82) |
| Shannon | 5.8 | 0.80; 0.68 | (5.20; 6.49) | 0.74 | (0.59; 0.88) |
| Simpson | 1.0 | 0.89; 0.63 | (0.90; 0.98) | 0.73 | (0.58; 0.88) |
| Chao | 135.0 | 0.80; 0.68 | (110.00; 184.50) | 0.69 | (0.53,0.85) |
| ACE | 131.5 | 0.80; 0.63 | (98.50; 168.00) | 0.68 | (0.52; 0.85) |

**Note.** 95% CI – 95% confidence interval; AUC – area under the curve.

## DISCUSSION

The role of the gut microbiota in human metabolic health is crucial. Numerous studies have shown that changes in the abundance and composition of gut microbiota are associated with many diseases, including PCOS [21, 22]. Therefore, the treatment of metabolic diseases using various approaches (probiotics, prebiotics, and fecal microbiota transplantation) is of interest [23–25]. Currently, correction of the gut microbiome disturbances is a promising option in the management of metabolic disorders, associated with PCOS [23, 26, 27]. Nevertheless, there are no strategies for selection of PCOS patients who need the interventions that regulate the gut microbiome correction.

Previously, we showed that diversity indices are an effective biomarker for visualizing differences in the gut microbiota according to PCOS phenotypes [13], which were consistent with data obtained by other authors [28, 29]. In this study, we conducted ROC analysis to develop cut-off values for indices associated with hyperandrogenism in women of reproductive age with PCOS.

The ROC-analysis has been actively used to search for biomarkers for noninvasive diagnostics of certain diseases. Regarding microbiome investigations, ROC curves were used for bac-

terial genera identified by the linear discriminant analysis effect size [30, 31]. Furthermore, the area under the curve (AUC), sensitivity, and specificity were estimated in the study of major depressive disorder patients were accompanied by anorexia [30], and patients with chronic schistosomiasis japonica-induced fibrosis [31], but they did not establish any cut-off points.

According to our results of ROC-analysis, the greatest sensitivity with moderate specificity, with a high AUC was established for the Shannon and Simpson indices with cut-off points classifying women with or without hyperandrogenemia (5.84 and 0.97 for the Shannon and Simpson indices, respectively). Therefore, we consider these values as potential biomarkers of hyperandrogenemia associated with PCOS in premenopausal women.

**Limitations of the study**

The small number of women included in the hyperandrogenemia group is a limitation of the study.

## CONCLUSIONS

The developed criteria for assessing alpha diversity using cut-off points for the most significant indices can be useful for monitoring the results of different therapeutic interventions (prebiotics, probiotics, etc.) in hyperandrogenic phenotypes of PCOS.

**Conflict of interest**

The authors declare no conflict of interest.

**Funding**

This project was supported by the budget of the state scientific programs: No. AAAA-A20-120120790036-3 "Early detection and prevention of metabolic syndrome associated with hyperandrogenism and estrogen-deficient conditions in women of reproductive and postmenopausal age"; No. 123051600030-1 "Prediction of metabolic and psychoemotional disorders in women of different age groups with hyperandrogenic disorders for the development of personalized approaches to prevention and treatment".

The research was performed using the equipment of the Center for the Development of Progressive Personalized Health Technologies at the Scientific Centre for Family Health and Human Reproduction Problems (Irkutsk).

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**Acknowledgments**

Authors express their appreciation to Aleksey Belskikh (Scientific Centre for Family Health and Human Reproduction Problems, Irkutsk, Russian Federation); Daria Lizneva (Icahn School of Medicine at Mount Sinai, NY, USA); the Steering Committee of the ESPEP study: Bulent Yildiz (Hacettepe University School of Medicine, Ankara, Turkey), Richard S. Legro (Hershey Medical Center, Penn State College of Medicine, Hershey, USA), Frank Stanczyk (Keck School of Medicine, University of Southern California, Los Angeles, USA), and Ricardo Azziz (School of Medicine, University of Alabama at Birmingham, Birmingham, USA).