

MEDICAL UNIVERSITY “PROF. DR. PARASKEV STOYANOV” – VARNA

FACULTY OF MEDICINE

DEPARTMENT OF PEDIATRICS

Vilhelm Ruslanov Mladenov, MD

**Polycystic Ovary Syndrome in Adolescence – Contemporary Aspects and Directions for
Diagnosis**

AUTOREFERAT

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Scientific Supervisor:

Prof. Violeta Mihova Iotova, MD, PhD, DSc

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The dissertation comprises a total of 173 pages, illustrated with 25 tables and 16 figures. The bibliography contains 180 literature sources, of which 4 in Cyrillic and 176 in Latin script.

The dissertation has been discussed, accepted, and referred for defense before a scientific jury by the Departmental Council of the Department of Pediatrics at the Medical University “Prof. Dr. Paraskev Stoyanov” – Varna on 08.07.2026.

Scientific Jury:

Chair: Prof. Emil Georgiev Kovachev, MD, PhD, DSc, Medical University – Varna

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Assoc. Prof. Ralitsa Nikolaeva Robeva, MD, PhD, Medical University – Sofia

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Prof. Dimitrichka Ducheve Bliznakova, MD, PhD, Medical University – Varna

Reserve Members:

Assoc. Prof. Margarita Alexieva Arshinkova, MD, PhD, Medical University – Sofia

Assoc. Prof. Krasimira Ivanova Koleva, MD, PhD, Medical University – Varna

The public defense of the dissertation will take place at an open session of the scientific jury, to be held on 20.10.2026 at 1:00 p.m. in UMHAT “Sveta Marina”, floor 5, hall 501.

The materials pertaining to the defense are available at the Research Department of the Medical University – Varna and have been published on the website of the Medical University – Varna.

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ABBREVIATIONS

17-OHP – 17-hydroxyprogesterone

A4 – androstenedione

AMH – anti-Müllerian hormone

AN – acanthosis nigricans

AUC – area under the curve

BG – blood glucose

BMI – body mass index

BP – blood pressure

CAH – congenital adrenal hyperplasia

Chol – total cholesterol

CV – cardiovascular

CVD – cardiovascular disease(s)

CVR – cardiovascular risk

DBP – diastolic blood pressure

E – estradiol

FAI – free androgen index

FGS – Ferriman-Gallwey Score

FH – fasting hyperglycemia

FHI – fasting hyperinsulinemia

HA – hyperandrogenism

HAC – idiopathic hyperandrogenism control group

HC – healthy control group

HTN – arterial hypertension

I – insulin

IFG – impaired fasting glycemia

IGT – impaired glucose tolerance

IR – insulin resistance

LOV – left ovarian volume

MetS – metabolic syndrome

MiRNA – microRNA(s)

OM – oligomenorrhea

OW – overweight

PCOM – polycystic ovarian morphology

PCOS – polycystic ovary syndrome

ROV – right ovarian volume

SBP – systolic blood pressure

SD – standard deviation

T – testosterone

T2DM – type 2 diabetes mellitus

TG – triglycerides

TOV – total ovarian volume

US – ultrasound

WC – waist circumference

PART I. INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders and the most common hyperandrogenic condition among women of reproductive age. From its first description in the scientific literature in 1935 by the American gynecologists Stein and Leventhal to the present day, interest in the disease and the comorbidities and complications associated with it, whose onset is often already in adolescence, remains extremely high.

Despite the high prevalence of PCOS, its etiology and pathophysiology are still not fully understood. Neuroendocrine disturbances leading to elevated luteinizing hormone (LH) levels, tissue-selective insulin resistance and hyperinsulinemia, genetic and epigenetic factors, as well as an imbalance between androgens, follicle-stimulating hormone (FSH) and anti-Müllerian hormone (AMH), mutually reinforce one another and form a self-perpetuating vicious circle.

The clinical picture of PCOS changes with age – from predominantly ovulatory dysfunction and hyperandrogenism in adolescence, often accompanied by obesity and metabolic disturbances, to a predominantly metabolic disorder in adulthood, associated with obesity, insulin resistance, impaired glucose and lipid metabolism, and increased cardiovascular risk. For this reason, over the past several months a group of prominent specialists in the field of PCOS has undertaken a multi-stage process to adopt a new name for the syndrome, reflecting its multisystem characteristics. The scientists' proposal is to rename the disease to polyendocrine metabolic ovarian syndrome (PMOS). The transition to the new name is expected to take place over a period of 3 years.

PCOS is also a significant cause of reproductive problems – impaired fertility, pregnancy complications, preterm births and spontaneous abortions. Women with PCOS also have an increased risk of certain carcinomas (endometrial, ovarian). In addition, mental disorders – anxiety, depression, emotional distress, eating disorders – are common among patients. All these problems lead to a serious impairment of quality of life, which necessitates early diagnosis of the disease already in adolescence and the timely implementation of measures to prevent future complications.

The diagnostic criteria for PCOS in adult women are well established but cannot be applied directly to adolescence. Oligomenorrhea and clinical manifestations of hyperandrogenism

(hirsutism, acne) are physiological phenomena characteristic of the pubertal transition, while increased ovarian volume and a multicystic ovarian appearance on ultrasound are a normal finding in girls of this age. These features create preconditions for both late and inaccurate diagnosis, as well as for overdiagnosis. Therefore, in recent years considerable efforts have been made to discover new biomarkers with high specificity and sensitivity, which would facilitate the elucidation of the pathogenetic mechanism of PCOS, earlier diagnosis, and monitoring of treatment.

PCOS remains one of the complex and multifaceted challenges in contemporary pediatric endocrinology. Its rising prevalence, combined with the long-term reproductive, metabolic and psycho-emotional consequences, transforms PCOS from a purely reproductive disease into a significant medico-social problem. The relevance of the topic is determined by the need to enrich the diagnostic criteria in adolescence with new, highly specific biomarkers. Through timely diagnosis and the application of personalized, early interventions, effective prevention of late complications can be ensured and the quality of life of patients can be durably improved.

PART II. RATIONALE, AIM AND OBJECTIVES

Rationale for choosing the topic

1. Early detection (diagnosis) can serve for the prevention and early treatment of PCOS and its associated comorbidities and complications.
2. Current methods for diagnosing PCOS, especially in adolescence, are imperfect and insufficiently conclusive, creating conditions for overdiagnosis or delayed diagnosis.
3. Some of the criteria for PCOS overlap with physiological phenomena in girls of pubertal age, as well as with manifestations of other diseases presenting with hyperandrogenism.
4. There are no studies among adolescents in Bulgaria concerning the identification of new biomarkers for diagnosing PCOS, nor validation of their suitability for practice.
5. A small number of studies worldwide among adolescents concerning the suitability of promising new diagnostic biomarkers for PCOS.
6. Advances in the clinical laboratory in recent years, which allow the study of new biomarkers.
7. The need to create a national consensus for the diagnosis and treatment of PCOS in adolescents.

Aim

The aim of the present scientific work is to improve and enrich the diagnostic set for adolescent patients with PCOS.

Objectives

1. To assess the clinical and laboratory profile of adolescent patients with PCOS.
2. To investigate some new potential diagnostic biomarkers for PCOS - AMH, FAI and to determine their most specific and sensitive cut-off values for diagnosing PCOS.

3. To establish correlations between hormonal biomarkers, current diagnostic criteria for PCOS (hyperandrogenism, polycystic ovary morphology), as well as with metabolic abnormalities, cardiovascular disorders, the presence of obesity.
4. To determine the frequency of metabolic and cardiovascular disorders (dyslipidemia, impaired glycemic control, insulin resistance, hyperinsulinism, arterial hypertension) in patients with PCOS.
5. To assess the sensitivity and specificity of selected miRNAs as a diagnostic marker for PCOS and to establish correlations with hyperandrogenism, metabolic and cardiovascular disorders, the presence or absence of obesity, and polycystic ovarian morphology.

PART III. DESIGN, PARTICIPANTS AND METHODS OF THE STUDY

1. Design and participants in the study

The scientific study is a case-control type. The target participants were girls up to the age of 18 with a diagnosis of PCOS, established according to the criteria of the International Consortium of Pediatric Endocrinology (ICPE), and an age- and BMI-matched control group of healthy girls. Participants were recruited among girls hospitalized for PCOS or other conditions at the First Pediatric Clinic during the period from 01.11.2021 to 01.12.2023, after obtaining approval from the Research Ethics Committee (KENI) and signing informed consent.

1.1. Inclusion criteria for girls with PCOS:

1.1.1. Girls up to 18 years of age, meeting the criteria for PCOS in adolescents according to the International Consortium of Pediatric Endocrinology (ICPE):

- a. Irregular menstruation/oligomenorrhea;
- b. Hyperandrogenism:
 - Biochemical – T level ≥ 1.7 nmol/l and/or FAI >5 ;
 - Clinical.

1.1.2. Two or more years after menarche;

1.1.3. Absence of other causes of hyperandrogenism and menstrual disorders;

1.1.4. Signed informed consent from a parent and from the patient, following ethical approval from KENI.

1.2. Exclusion criteria:

1.2.1. Less than 2 years after menarche;

1.2.2. Other identified causes of hyperandrogenism;

1.2.3. Other endocrine causes of menstrual disorders;

1.2.4. Severe chronic diseases or diseases that could affect biochemical hyperandrogenism (e.g., type 1 diabetes, Cushing's syndrome, etc.);

1.2.5. Use of contraceptives or other hormonal preparations (with the exception of thyroid hormones) in the preceding 3 months;

1.2.6. Refusal to sign informed consent.

2. Inclusion criteria for the healthy control group:

1. Age up to 18 years;
2. Age and BMI similar to the study group;
3. Two or more years after menarche;
4. Absence of menstrual cycle disorders;
5. Absence of hyperandrogenism and other endocrine disorders;
6. No use of contraceptives or other hormonal preparations (with the exception of thyroid hormones) in the preceding 3 months;
7. Signed informed consent from a parent and from the patient, following ethical approval from KENI.

In some of the girls included in the healthy control group, biochemical hyperandrogenism was found during the investigations, without identifying endocrine causes for it. Since these girls had no menstrual disturbances and could not be included in the group of participants with PCOS, we decided to form a third control group of girls with idiopathic hyperandrogenism.

3. Inclusion criteria for the group of girls with idiopathic hyperandrogenism:

1. Age up to 18 years;
2. More than 2 years after menarche;
3. Evidence of biochemical hyperandrogenism;
4. Absence of menstrual disorders;
5. Signed informed consent from a parent and from the patient.

The protocol was approved by the Research Ethics Committee (KENI) at the Medical University – Varna, with decision No. 107 of 28.10.2021, prior to the start of participant enrollment.

The study of microRNAs was funded through project No. 21019 to the “Science” Fund of the Medical University – Varna.

Participation of all girls in the study was entirely voluntary, and each participant was free to withdraw at any time without stating a specific reason. All participants received oral and written information about the study and the forthcoming procedures, had the opportunity to freely ask questions, which were answered comprehensively by the researcher. For each participant, all study procedures began after the signing of the informed consent.

To comply with ethical requirements for anonymity in data processing, each participant was assigned an individual, anonymous participation code.

2. Study methods

1. Survey method

During their visit to the First Pediatric Clinic of UMHAT “Sveta Marina” – Varna, an interview was conducted with each participant, in the presence of a parent, including detailed collection of anamnestic data regarding:

- history of pregnancy and delivery;
- perinatal history of term status, birth weight and length, and the postnatal period;
- family history of socially significant diseases, hyperandrogenic conditions or diseases among first- and second-degree relatives;
- family history of reproductive failures in first- and second-degree family members;
- history of previous or concurrent chronic diseases in the participants;
- medication history of drug intake by the participants;

- history of clinical symptoms (hirsutism, acne, menstrual disorders, etc.), as well as data on the onset and progression of symptoms and the date of the last regular menstrual period in the participants.

2.2. Anthropometry

All anthropometric measurements were performed in the morning on the day of the examination, by a well-trained researcher and using the same instruments.

- **Body weight (W)** was determined to an accuracy of 0.1 kg using a calibrated digital scale SECA 861 (SECA Ltd, Hamburg, Germany), with each patient weighed without shoes and in light clothing.
- **Height (H)** was determined using a standard certified and standardized medical stadiometer Harpenden 2000 (Hamburg, Germany), to an accuracy of 0.1 cm, with the subject standing upright and the head positioned in the horizontal Frankfurt plane, without shoes and in light clothing.
- **Waist circumference (WC)** was measured with a non-stretch, flexible tape SECA 201 (Seca Ltd., Hamburg, Germany), placed at the horizontal line dividing the right mid-axillary line between the lower edge of the tenth rib and the iliac crest, to an accuracy of 0.1 cm, after removal of all outer clothing, in an upright position, at the end of a quiet expiration.
- To assess the presence of overweight or obesity, **BMI** was calculated using the standard formula $BMI = W \text{ (kg)} / H \text{ (m)}^2$. The presence of overweight and obesity was determined by applying the CDC reference values for BMI for children and adolescents, depending on sex and age. Overweight is defined as $BMI \geq 85\text{th}$

percentile, and obesity as $\text{BMI} \geq 95\text{th percentile}$ for the corresponding sex and age.

2.3. Clinical examination

All participants underwent a complete physical examination, with targeted attention to the presence of manifestations of clinical hyperandrogenism such as hirsutism, acne, hair loss, and marked oiliness of the hair.

- To assess the presence of hirsutism, a modified version of the Ferriman-Gallwey scale was applied, with a score of ≥ 8 points corresponding to the presence of clinical hirsutism.
- Pubertal development was determined through Tanner staging, with additional clarification of the age of menarche onset.
- Blood pressure (BP) was measured with a sphygmomanometer, using a cuff size appropriate to the patient's age. The Korotkoff auscultatory method was applied, with BP measured on the right arm of the participants, in a seated position after 30 minutes of rest, with the middle of the arm at heart level (American Heart Association 2019). The measurement was performed three times at 2-minute intervals, and the arithmetic mean of the last two measurements was used for the analysis, separately for SBP and DBP.
- Pulse rate was measured at the a. radialis with the participant seated, for 1 minute.

2.4. Laboratory biochemical and hormonal investigations

Biochemical and hormonal laboratory parameters were investigated at the Central Clinical Laboratory of UMHAT "Sv. Marina" EAD – Varna. All blood samples were taken from peripheral venous blood under standardized conditions – in the morning, fasting, after a 12-hour food pause, with maximum consideration for the participants and patients. Additionally, for the miRNA analysis, serum was isolated using a gel-separator vacutainer, centrifuged for 15 minutes

at 2500 G. The sera were pipetted into additive-free vacutainers, with subsequent storage of the material in a freezer at -80°C. The following **metabolic** parameters were investigated:

- **Blood glucose** (mmol/l) – the enzymatic hexokinase method was applied, on a biochemistry analyzer ADVIA chemistry 1800, Siemens. The laboratory reference values are 3.3 – 5.6 mmol/l.
- **Lipid profile: total cholesterol** (mmol/l) – measured by enzymatic determination with cholesterol esterase, cholesterol oxidase, peroxidase (CHE-CHOD-POD enzymatic method) and colorimetry (Trinder), with laboratory norms between 2.7 – 5.2 mmol/l. **Triglycerides** (mmol/l) were investigated via enzymatic hydrolysis with lipase and colorimetric determination of glycerol with glycerol kinase, glycerophosphate oxidase and peroxidase (GPO-POD enzymatic method) (Trinder), with reference values from 0.4 to 1.77 mmol/l. **HDL-cholesterol** (mmol/l) – precipitation of LDL and VLDL and determination of cholesterol in the supernatant, with normal value limits between 1.03 – 1.55 mmol/l. **LDL-cholesterol** (mmol/l) – calculated using the Friedewald formula, with a laboratory reference value up to 2.6 mmol/l. All lipid parameters were analyzed on a biochemistry analyzer ADVIA chemistry 1800, Siemens.
- **C-reactive protein (CRP)** (mg/l) was investigated on a biochemistry analyzer ADVIA chemistry 1800, Siemens, applying immunoturbidimetric assay. The laboratory reference values are from 0-5 mg/ml.
- **Insulin** – a commercial kit (DRG Insulin Enzyme Immunoassay Kit) was used for the quantitative determination of insulin in human serum/plasma via sandwich enzyme immunoassay (ELISA). The test registers insulin levels between 0 and 100

μIU/ml, with a method sensitivity of 1.76 μIU/ml, and reference values of 2.0-29.1 μIU/ml.

- Fasting blood glucose and insulin values were used to calculate the **homeostasis model assessment of insulin resistance (HOMA-IR)**. For this purpose, the generally accepted formula was applied: $\text{HOMA-IR} = [\text{fasting blood glucose (mmol/l)} \times \text{fasting insulin (}\mu\text{IU/ml)}] / 22.5$. HOMA-IR values >2.6 are associated with the presence of insulin resistance (IR).

Hormonal parameters were also investigated in the morning, fasting, during the follicular phase (between days 3-5) of the participants' menstrual cycle, and in patients with secondary amenorrhea – after day 90 from the last menstrual period. The following hormones were investigated:

- **Luteinizing hormone (LH)** (IU/l) – chemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 0.05 IU/l. The reference values for the test in the follicular phase are between 1.1-11.6 mIU/ml. An Immulite 2000 Xpi, Siemens immunoassay analyzer was used for the analysis.
- **Follicle-stimulating hormone (FSH)** (IU/l) – chemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 0.1 IU/l. Reference values for the test in the follicular phase are between 2.8-11.3 mIU/ml. An Immulite 2000 Xpi, Siemens immunoassay analyzer was used for the analysis.
- **Estradiol** (pmol/l) – chemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 0.39 pmol/l. Reference values are up to 529 pmol/l. An ADVIA Centaur CP, Siemens immunoassay analyzer was used for the analysis.
- **Testosterone (T)** (nmol/l) – chemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 0.5 nmol/l.

Reference values for the test are between 0-1.38 nmol/l. An Immulite 2000 Xpi, Siemens immunoassay analyzer was used for determination.

- **Androstenedione** (nmol/l) – chemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 0.3 nmol/l. Reference values are between 1.0-11.5 nmol/l. An Immulite 2000 Xpi, Siemens immunoassay analyzer was used for determination.
- **17-OH-progesterone (17OHP)** (ng/ml): NovaTec, Germany, LOD = 0.05 ng/ml. Cross-reactivity: with 11-deoxycortisol (0.846%), with progesterone (0.590%), with pregnenolone (0.250%), with testosterone (0.017%); with aldosterone, androstenedione, cortisol, DHEA, prednisolone, cholesterol: < 0.01%. The method is identical for basal or stimulated serum concentrations. Serum 17OHP levels were reported in ng/ml. Reference values for children are between 0.2 – 0.9 ng/ml, and for women in the follicular phase of the menstrual cycle between 0.2-1.3 ng/ml and between 1.0-4.5 ng/ml in the luteal phase of the cycle.
- **Sex hormone binding globulin (SHBG)** (nmol/l) – chemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 0.02 nmol/l. Reference values for the test are between 18-144 nmol/l. An Immulite 2000 Xpi, Siemens immunoassay analyzer was used for determination.
- **Anti-Müllerian hormone (AMH)** (pmol/l) – electrochemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 0.21 pmol/l. Reference values for the test are between 2.1-84.1 pmol/l. A Cobas e 601, ROCHE immunoassay analyzer was used for determination.

- **Prolactin (μIU/ml)** – chemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 10.6 mIU/l. Reference values are between 40-530 μIU/ml. An Immulite 2000 Xpi, Siemens immunoassay analyzer was used for determination.
- **Cortisol (nmol/l)** – chemiluminescent immunoassay with non-isotopic antibody labeling, LOD = 0.5 nmol/l. Reference values are between 115.7-451.2 nmol/l. An ADVIA Centaur CP, Siemens immunoassay analyzer was used for determination.
- T and SHBG values were used to calculate the **free androgen index (FAI)** according to the formula $FAI = [T \text{ (nmol/l)} / SHBG \text{ (nmol/l)}] \times 100$. FAI values above 5 are associated with androgen excess.

2.5. Study of microRNAs (miRNA)

The study of selected miRNAs was carried out in the laboratory of the Department of Biochemistry, Molecular Medicine and Nutrigenomics, part of the Department of Pharmacy at the Medical University “Prof. Dr. Paraskev Stoyanov”, Varna.

To extract six circulating miRNAs from 200 μl serum samples, the NucleoSpin miRNA Plasma kit (Macherey-Nagel GmbH & Co. KG., Düren, Germany) was used. After assessing RNA concentration and purity, cDNA synthesis was performed on 90 ng of RNA. This process involved the use of stem-loop primers for miR-451a, miR-15a-5p, miR-320-5p, miR-28-5p, miR-103a-5p and miR-194-5p, and reverse U6 primers as an endogenous reference control, using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, US). Luna Universal qPCR Master Mix (New England BioLabs, Ipswich, MA, USA) was used for quantitative PCR (qPCR), together with cDNA templates diluted 3-fold, miRNA-specific forward primers, a universal reverse primer, and U6 forward and reverse primers. All qPCR reactions were run in triplicate. To mitigate batch effects and control experimental variability, the qPCR reactions were divided into two batches: the first batch included 17 samples from patients with PCOS, 6 samples from healthy controls, and 4 samples from hyperandrogenic controls; the second batch included 17 samples from girls with PCOS, 5 samples from healthy controls, and 4

samples from hyperandrogenic controls. The $2^{-\Delta\Delta C_t}$ method was used to determine the comparative expression levels of miRNA. Two separate comparative analyses of miRNA expression were performed: healthy controls versus patients with PCOS, and hyperandrogenic controls versus girls with PCOS. For the first comparison, fold-change values of miRNA in patients with PCOS were calculated using healthy controls as the reference group. For the second comparison, fold-change values of miRNA in patients with PCOS were calculated using hyperandrogenic controls as the reference group. The QuantStudio 5 Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) was used to perform the qPCR analyses.

2.6. Ultrasound examination for the presence of PCOM

Each participant underwent an ultrasound examination of the ovaries and uterus to establish the presence of PCOM. An “ALOKA Prosound SSG-3500” ultrasound scanner with a transabdominal transducer with a floating frequency of 3.5 to 6 MHz was used, with the examination performed by the same researcher. PCOM was defined as the presence of an increase of >2 SD, i.e., above 12 ml, in the volume of one or both ovaries.

2.7. Medico-statistical data processing

A specialized statistical software package for personal computers – SPSS for Windows, version 25.0 (Chicago, IL, USA), GraphPad Prism version 6.01 (GraphPad Software, Inc., Boston, MA, USA) – was used for data processing. The level of statistical significance was set at $p \leq 0.05$. The following analytical methods were applied in processing the data:

- Method of statistical grouping of the data – characteristics were arranged according to their type into variational, interval, categorical, ordinal, and dynamic statistical series.
- Method of statistical estimation:
 - a) point estimates – for calculating the arithmetic mean of continuous variables;
 - b) interval estimates:
 - Confidence probability (significance) – p. At a coefficient $p=0.95$ (95%), the Type I error is 0.05 (5%);

- Confidence intervals (CI). 95% confidence intervals around the point estimate were used, interpreted as the probability that this interval contains the true point value in 95% of cases.

- Graphical method – linear and planar graphical representations and other diagrams were used.
- Descriptive analysis – with determination of measures of central tendency and measures of dispersion of the distribution.
- Variational analysis – when comparing continuous variables, analyses of independent subgroups were used – Student-Fisher t-test for independent samples, and for multiple comparisons of characteristics with several categories, Bonferroni correction of the p-value was applied according to the number of comparisons (ANOVA test).
- Non-parametric analysis – when analyzing categorical characteristics, the Pearson chi-square (χ^2) criterion was used. For some comparisons, the Mann-Whitney and Kolmogorov-Smirnov non-parametric tests, which have greater statistical power, were applied.
- Correlation analysis – univariate coefficients of linear correlation according to Pearson and Spearman (in case of non-normal distribution) were determined. In assessing some characteristics, partial multiple correlation analysis was also used, in which the influence of other factors was accounted for when assessing the relationship between two variables. The correlation coefficient r can take values between 0 and -1 for an inverse relationship, and between 0 and +1 for a direct association. The strength of the relationship between two characteristics is determined by the value of the correlation coefficient r :
 - $r < 0.30$ – weak correlation
 - r between 0.30 and 0.50 – moderate correlation

> > • r between 0.50 and 0.70 – considerable correlation > > • r between 0.70 and 0.90 – strong correlation > > • r > 0.90 – very strong correlation

- Linear regression analysis – for a comprehensive assessment of the independent effect of individual characteristics on a given continuous variable, multifactorial linear regression analysis was applied. The resulting regression models present the dependent variable as a result of various combinations of independent or predictor characteristics, according to the formula: $y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_n x_n$, where y – the continuous variable under study; β_0 – a constant termed the intercept; $\beta_1, \beta_2, \dots, \beta_n$ – calculated regression coefficients of the variables $x_1, x_2, \dots, x_n$ – combination of independent characteristics whose effect is being tested. A backward elimination model was used to remove non-significant variables from the regression equation, after initially including all variables in the regression, with significance levels of 0.05 for inclusion and 0.1 for exclusion from the equation.
- To determine the sensitivity and specificity of the potential diagnostic biomarkers for PCOS, as well as to find cut-off values, ROC curve analysis was used, calculating the area under the curve (AUC), where AUC between 0.6-0.7 indicates a poor model, 0.7-0.8 – a good model, 0.8-0.9 – a very good model, and >0.9 – an excellent model. The Youden index was used to determine sensitivity and specificity, calculated by the formula:

J = Sensitivity + Specificity – 1, where:

- **Sensitivity** = $TP / (TP + FN)$
- **Specificity** = $TN / (TN + FP)$

(TP = true positives, FN = false negatives, TN = true negatives, FP = false positives). Sensitivity and specificity between 70-80% are acceptable, between 80-90% – good, and >90% – excellent.

PART IV. RESULTS

1. General characteristics of the study participants

After signing informed consent and after applying all inclusion and exclusion criteria for participation, 59 participants aged 12.9 to 17.9 years were included in the study.

According to the study design, all participants were divided into three groups:

- i. The group of patients with PCOS. This included girls with a diagnosis of PCOS, according to the selected diagnostic criteria. For brevity, further in the text they will be referred to as “patients” or “PCOS”. This group comprised 61% of the participants (n = 36).
- ii. The group of healthy controls, with age and BMI similar to the patients. Further in the text they will be referred to as “healthy controls” or “HC”. This group included 25.4% of the participants (n = 15).
- iii. The group of controls with idiopathic hyperandrogenism. Further in the text they will be denoted as “hyperandrogenic controls” or “HAC”. This group comprised 13.6% of the participants (n = 8).

The mean age of all study participants was 15.8 ± 1.3 years, with no significant differences between the groups ($p = 0.739$).

Table 1. Age characteristics of the groups

Parameter	PCOS (n=36)	HC (n=15)	HAC (n=8)	p
Age (yrs)	15.9 ± 1.5	15.6 ± 1.0	15.8 ± 1.1	ns

Results are presented as mean $\pm$ SD; ns – not significant.

2. Anthropometric characteristics of the participants

All participants underwent anthropometric measurements according to the study protocol. No significant differences were found between the different groups in the anthropometric parameters,

including the parameters for generalized and abdominal obesity – BMI and waist circumference (WC) (Table 2).

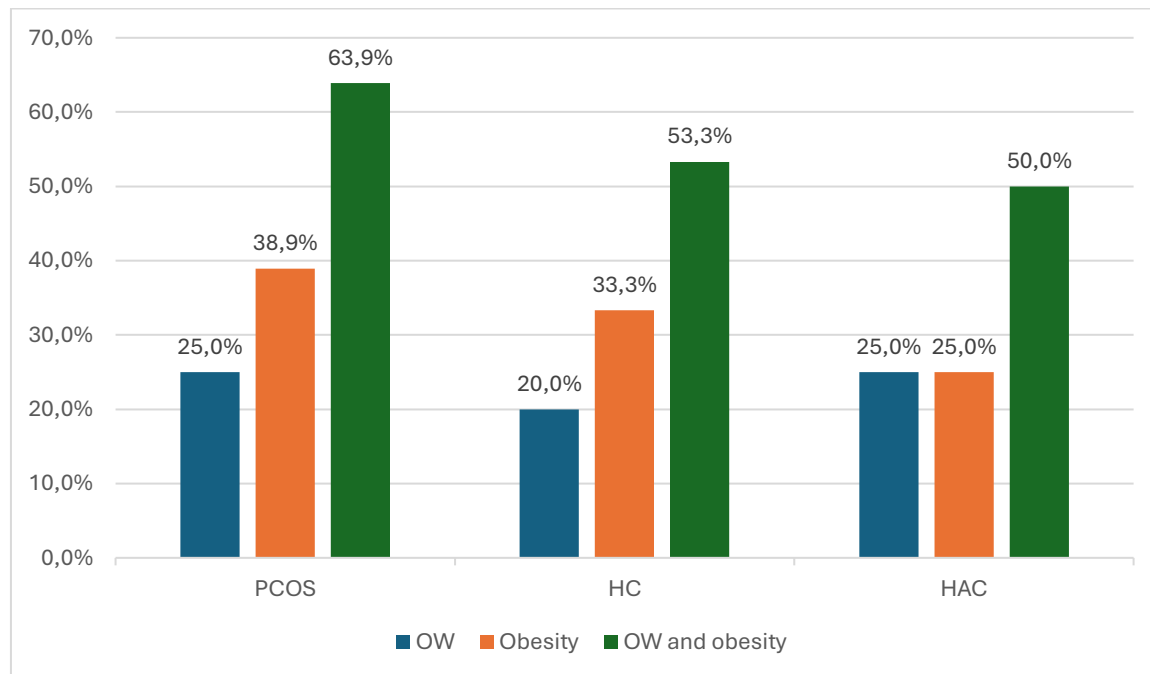
Table 2. Anthropometric characteristics of the participants

Parameter	PCOS (n=36)	HC (n=15)	HAC (n=8)	p
Weight (kg)	73.2±18.6	71.7±17.2	61.1±13.5	ns
Height (cm)	161.7±7.4	165.7±5.3	159.2±9.3	ns
BMI (kg/m ²)	28.1±7.6	26.1±5.7	24.0±4.5	ns
WC (cm)	86.3±17.9	83.0±17.6	78.6±9.6	ns

Results are presented as mean±SD; ns – not significant.

Using the CDC definition of overweight and obesity (OW – BMI >85th percentile, obesity – BMI >95th percentile), based on the sex- and age-specific BMI curves, we determined the frequency of OW and obesity in the three groups (Fig. 1).

Fig. 1. Frequency of overweight and obesity in girls with PCOS, healthy controls and hyperandrogenic controls



We found that 59.3% of all participants were overweight or obese, 23.7% with OW and 35.6% with obesity respectively, but without statistically significant differences between the three groups ($p = 0.663$, $p = 0.926$ and $p = 0.742$). The frequency of overweight and obesity in girls with PCOS was 63.9% ($n = 23$), of whom 25% were overweight ($n = 9$) and 38.9% obese ($n = 14$).

3. Clinical characteristics and manifestations of hyperandrogenism

We used the data from the physical examination performed in all participants to assess the presence of the main symptoms of clinical hyperandrogenism. The clinical profile of the participants from the three groups is presented in Table 3.

Table 3. Frequency of hyperandrogenic symptoms and oligo-amenorrhea in the participants from the three groups

Parameter	PCOS (n=36)	HC (n=15)	HAC (n=8)	p
Hirsutism	88.9%	20.0%	37.5%	<0.001
Acne	69.4%	40.0%	62.5%	ns
Alopecia	8.3%	0.0%	0.0%	ns
Hair loss	25.0%	21.4%	12.5%	ns
Oily skin/hair	72.2%	46.7%	37.5%	ns
Oligomenorrhea	100.0%	0.0%	0.0%	<0.001

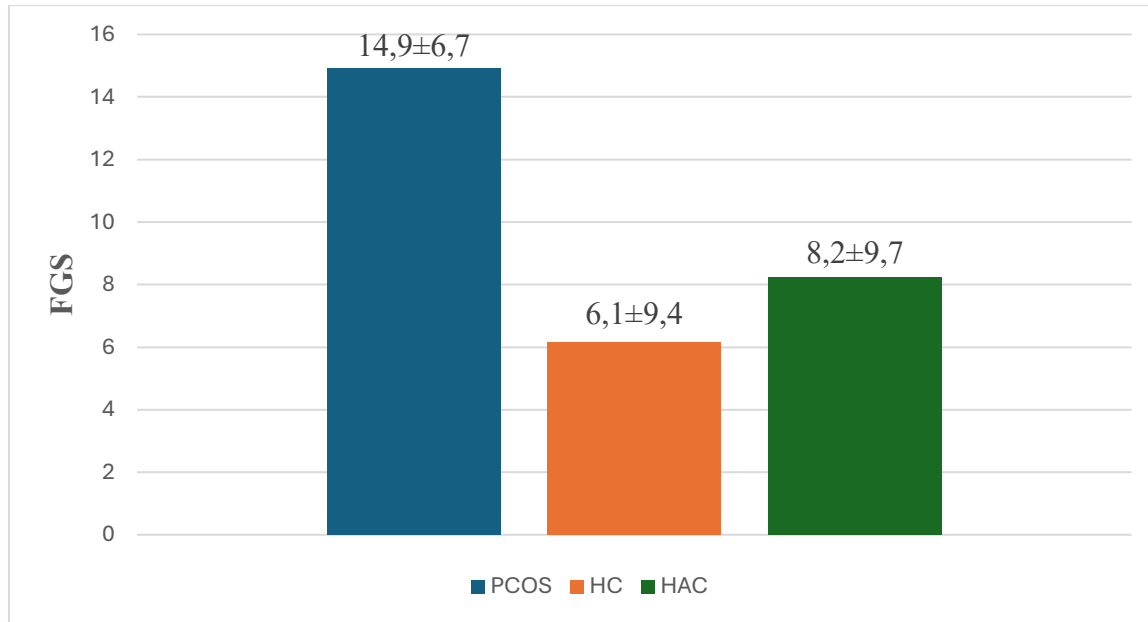
Results are presented as percentages; ns – not significant.

Oligomenorrhea (OM) was found in 100% of the girls with PCOS and 0% in the other 2 groups. This result is because OM is a mandatory criterion for diagnosing PCOS in adolescence. At the same time, an inclusion criterion for participation in the control groups was the absence of menstrual disorders.

Of all the hyperandrogenic symptoms, the only statistically significant difference was found for hirsutism, which was significantly more common in girls with PCOS ($p < 0.001$).

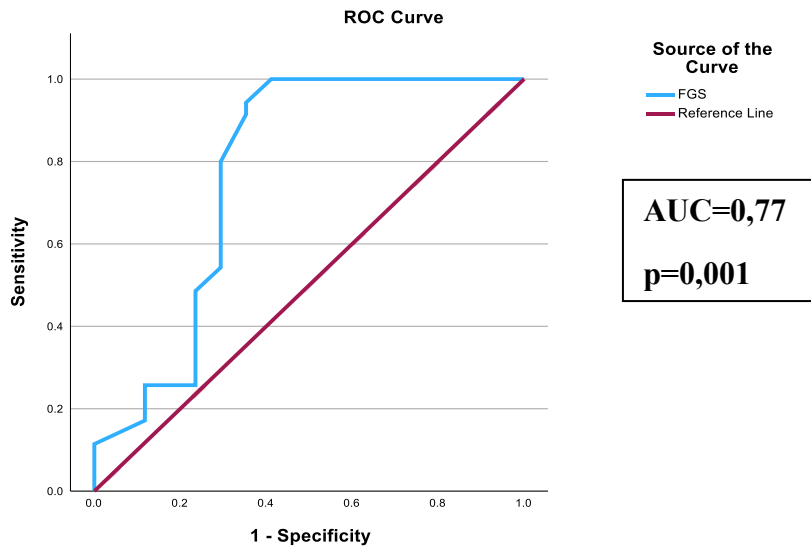
Hirsutism was assessed clinically using the modified Ferriman-Gallwey scale, with a total score ≥ 8 considered indicative of hirsutism. The analysis found a significantly higher mean FGS in girls with PCOS compared with healthy controls ($p < 0.05$). No significant differences were found between the PCOS and HAC groups, nor between HC and HAC. The data are presented in Figure 2.

Fig. 2. Mean value $\pm$ SD of FGS in the three study groups



To determine the sensitivity and specificity of FGS as a diagnostic marker for PCOS, we performed ROC curve analysis (Fig. 3).

Fig. 3. ROC curve for determining the sensitivity and specificity of FGS as a diagnostic marker for PCOS, with AUC and p values



AUC=0.77 was found, corresponding to a good model for diagnosing PCOS ($p = 0.001$). After calculating the Youden index, we found a cut-off value of 6.0 with the best sensitivity and specificity (94% and 65%, respectively) for diagnosing PCOS.

In the correlation analysis performed in the PCOS group, a direct correlation was found between FGS and WC, hirsutism, insulin level and HOMA-IR. When adjusting for age and BMI, the positive correlation of FGS with hirsutism, insulin level and HOMA-IR was retained (Table 4).

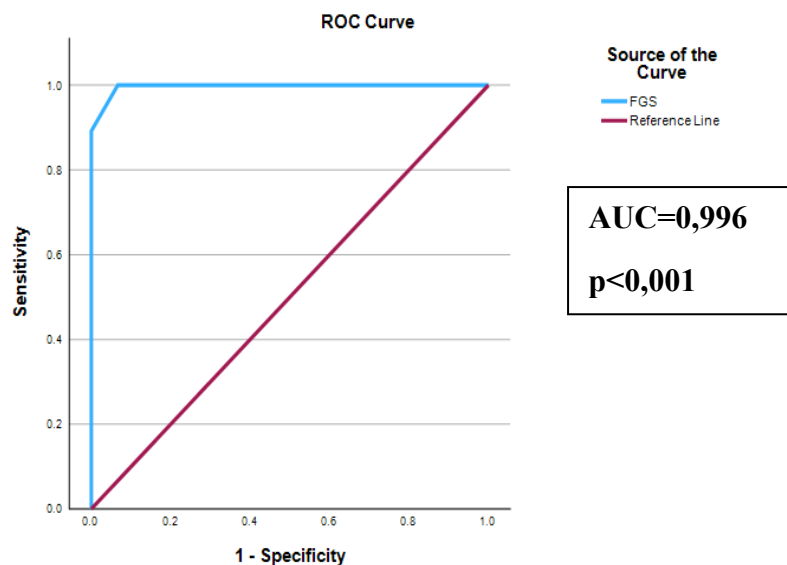
Table 4. Correlation results for FGS

Correlation	r	p
Hirsutism	0.442	0.01
Insulin	0.366	<0.05
HOMA-IR	0.391	<0.05

Results are presented as correlation coefficient r

Because of recommendations that FGS should be adapted to the ethnic characteristics of the population, we decided to evaluate the sensitivity and specificity of the $\text{FGS} \geq 8$ threshold accepted in our country as a cut-off for determining the presence of hirsutism in Bulgarian girls. The ROC curve analysis found that a cut-off FGS score of 7.5 points has 100% sensitivity and 93% specificity for determining the presence of hirsutism in girls from our population (Fig. 4).

Fig. 4. ROC curve of FGS for determining sensitivity and specificity for differentiating hirsutism, with AUC and p values



Linear regression analysis was performed to assess the influence of insulin resistance (HOMA-IR) on FGS. The results show that the model is statistically significant ($F = 8.584$, $p = 0.006$), and insulin resistance is a statistically significant predictor of the severity of clinical hirsutism ($R^2 = 0.206$, $p = 0.006$). The unstandardized coefficient $B = 0.696$ ($p = 0.006$) shows that a 1-unit increase in HOMA-IR is associated with an increase of approximately 0.7 points in the Ferriman-Gallwey Score (FGS). This result confirms the pathophysiological role of hyperinsulinemia in stimulating hyperandrogenism.

We looked for positive correlations of some anthropometric parameters (BMI, WC) with the clinical manifestations of hyperandrogenism, but after adjusting for age and group membership of

the participants, the partial correlation analysis found a positive correlation only between BMI and oily hair ($p < 0.05$).

4. Hormonal profiles of the participants

All participants underwent analysis of a panel of hormonal tests – LH, FSH, Estradiol (E), Testosterone (T), Androstenedione (A4), 17OHP, SHBG, AMH, Prolactin and Cortisol. The results obtained were analyzed using the ANOVA test with Bonferroni correction of the p-value. The results are presented in Table 5.

Table 5. Comparison of hormonal parameters between the three groups

Parameter	PCOS (n=36)	HC (n=15)	HAC (n=8)	p vs HC	p PCOS vs HAC	p HAC vs HC
LH (mIU/ml)	12.1±14.7	11.0±14.9	7.8±6.0	ns	ns	ns
FSH (mIU/ml)	5.4±2.0	5.5±3.3	5.3±1.9	ns	ns	ns
E (pmol/l)	349.6±248.9	468.9±285.7	457.7±361.6	ns	ns	ns
T (nmol/l)	1.7±0.7	0.9±0.3	2.2±0.2	<0.001	ns	<0.001
A4 (nmol/l)	14.3±5.4	6.9±3.7	13.1±3.5	<0.001	ns	<0.05
17OHP (nmol/l)	0.8±0.6	0.5±0.3	0.8±0.5	ns	ns	ns
SHBG (nmol/l)	29.7±23.3	47.1±22.0	54.5±24.7	ns	<0.05	ns
AMH (pmol/l)	47.7±32.4	25.9±15.6	33.6±28.0	<0.05	ns	ns
Prol (ng/ml)	267.5±111.9	330.4±258.2	276.7±77.0	ns	ns	ns
Cort (nmol/l)	398.4±187.7	354.1±154.3	330.3±74.2	ns	ns	ns
FAI	10.6±12.0	2.4±1.9	4.9±2.5	<0.05	ns	ns

Data are presented as mean $\pm$ SD; ns – not significant; Prol – Prolactin; Cort – cortisol

The analysis showed significantly higher levels of T and A4 in the PCOS group compared with the HC group (all $p < 0.001$), but without a significant difference from HAC. SHBG was significantly lower in the PCOS group compared with HAC ($p < 0.05$), but without a statistically significant difference from the HC group. In the HAC group, T and A4 were significantly higher compared with the HC group ($p < 0.001$ and 0.05). AMH and FAI levels were significantly higher in the PCOS group compared with HC (all $p < 0.05$), but no significant differences were found between PCOS and HAC, nor between HC and HAC.

To determine the sensitivity and specificity of the parameters that were significantly higher in the PCOS group compared with HC, ROC curve analysis was performed (Fig. 5, 6, 7 and 8).

Fig. 5. ROC curve of T with AUC and p

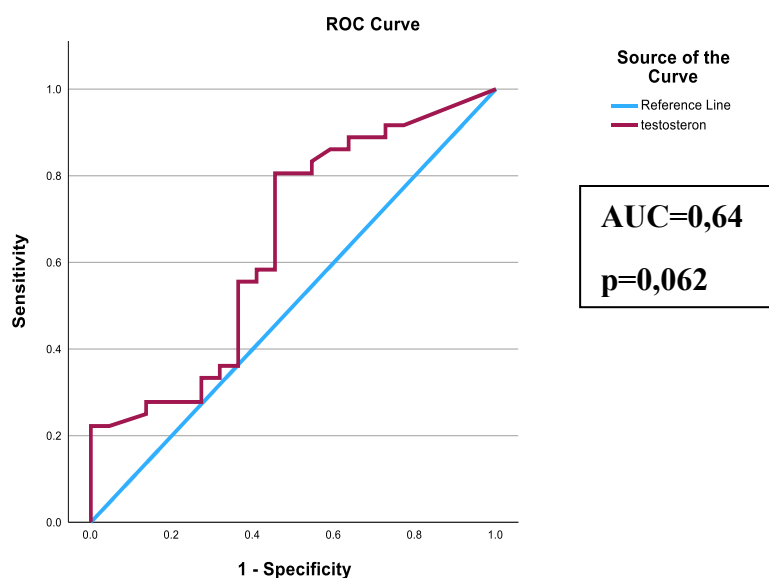


Fig. 6. ROC curve of A4 with AUC and p values

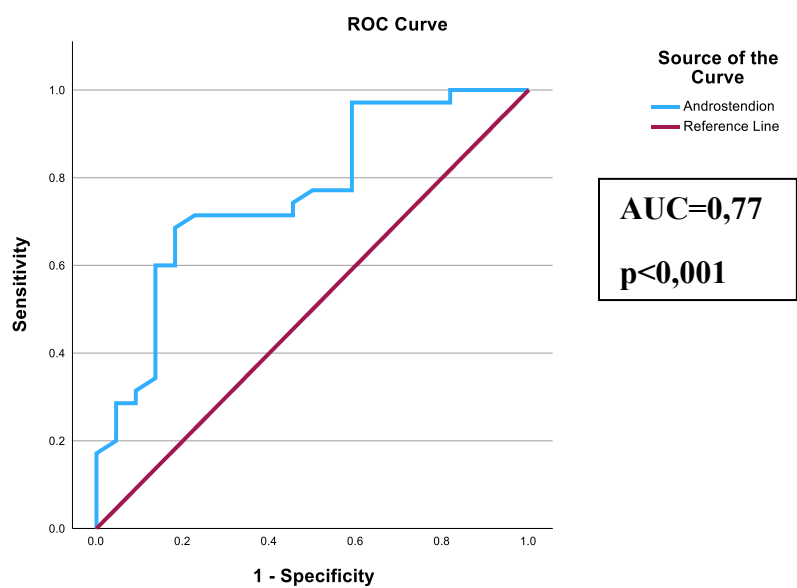


Fig. 7. ROC curve of AMH with AUC and p values

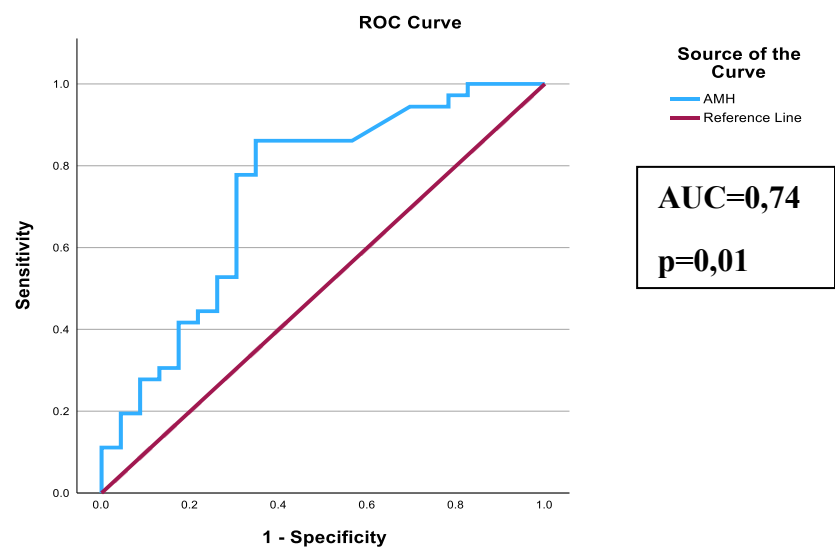
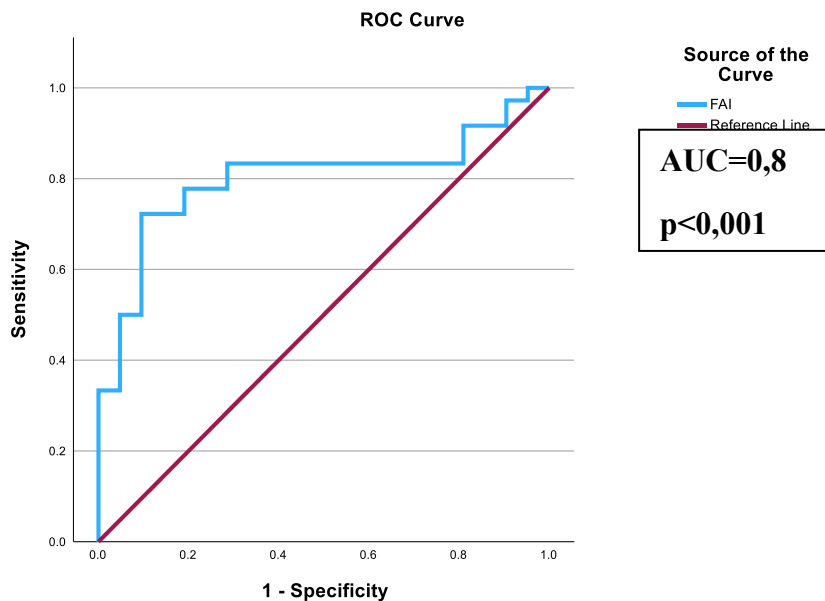


Fig. 8. ROC curve of FAI with AUC and p values



Of the four parameters, A4, AMH and FAI showed good and very good sensitivity and specificity for diagnosing PCOS, whereas T demonstrated good sensitivity but poor specificity. After calculating the Youden index, cut-off values were determined for each of the parameters (Table 6).

Table 6. Cut-off values, sensitivity and specificity of T, A4, AMH and FAI

Parameter	Cut-off	Sensitivity	Specificity
T (nmol/l)	1.13	80.6%	54.5%
A4 (nmol/l)	11.6	69%	100%
AMH (pmol/l)	23.6	86%	65%
FAI	6.2	72%	90%

Sensitivity and specificity between 70-80% – acceptable, between 80-90% – good, and >90% – excellent.

As shown in Table 6, a cut-off value for A4 of 11.6 nmol/l has excellent specificity and acceptable sensitivity; for AMH a value of 23.6 pmol/l – good sensitivity and acceptable

specificity; for FAI a value of 6.2 – acceptable sensitivity and good specificity; and for T – a cut-off of 1.13 nmol/l – good sensitivity but poor specificity for diagnosing PCOS.

Correlation analysis was performed in the PCOS group to detect correlations between the three biomarkers with the best sensitivity and specificity (A4, AMH and FAI) and the known hormonal, metabolic and morphological abnormalities characteristic of PCOS. After adjusting for the influence of age and BMI, the correlations presented in Table 7 were found.

Table 7. Correlation coefficients r between A4, AMH and FAI and hormonal and ovarian morphological parameters, and their statistical significance

	Androstenedione	AMH	FAI
Androstenedione	-	-	$r = 0.636^{***}$
FAI	$r = 0.636^{***}$	-	-
Testosterone (T)	$r = 0.630^{***}$	-	$r = 0.486^*$
SHBG	$r = -0.410^*$	-	$r = -0.602^*$
ROV	-	$r = 0.519^{**}$	-
LOV	-	$r = 0.558^{**}$	-

ROV – right ovarian volume; LOV – left ovarian volume; statistical significance:

** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$*

Significant direct correlations were found between A4, FAI and T levels, as well as a moderate inverse correlation with SHBG levels. For AMH, significant direct correlations with ovarian volume were found, while for FAI – a moderate positive correlation with T, a moderate one with A4, and a significant negative correlation with SHBG.

Linear regression analysis was performed to examine the relationship between the three potential biomarkers and their correlated parameters.

The regression analysis examining the relationship between A4 and FAI found no statistically significant influence of FAI on androstenedione levels ($R^2 = 0.011$, $F = 0.377$, $p = 0.544$). The

reason is probably the low affinity of A4 for SHBG, which is why changes in FAI have no bearing on androstenedione levels.

Linear regression analysis examining the relationship between serum T and A4 levels showed a statistically significant result ($R^2 = 0.336$, $p < 0.001$), with T levels explaining 33.6% of the variation in androstenedione (Table 8).

Table 8. Linear regression analysis with dependent variable Androstenedione

Model	Unstandardized B	p	95.0% CI lower/upper	VIF
(Constant)	7.127	<0.001	3.234/11.020	
Testosterone	4.235	<0.001	2.126/6.343	1.000

The direction of the relationship between T and A4 levels is positive. The relationship is strong and direct, since in steroidogenesis in women (in the ovaries and adrenal glands) A4 is a direct precursor of T. In PCOS, the production of both hormones increases in parallel, which explains why their levels are so closely linked in our model.

Because of the established direct correlation between AMH level and ovarian volumes, multiple linear regression analysis was performed to assess the significance of ovarian volume as a predictor of AMH level. Although the overall regression model was statistically significant ($R^2 = 0.320$, $p = 0.01$), analysis of the individual coefficients showed that neither the right ovarian volume ($B = 1.060$, $p = 0.648$) nor the left ovarian volume ($B = 5.156$, $p = 0.052$) were independent significant predictors of AMH levels. VIF values (1.966) exclude multicollinearity (Table 9).

Table 9. Linear regression analysis with dependent variable AMH

Model	Unstandardized B	p	95.0% CI lower/upper	VIF
(Constant)	-6.570	0.727	-45.017/31.876	
ROV	1.060	0.648	-3.674/5.795	1.966
LOV	5.156	0.052	-0.041/10.354	1.966

To improve statistical significance, a composite variable “Total Ovarian Volume” (sum of the left and right ovarian volumes, TOV) was created. The linear regression analysis performed showed that this model was reliable and statistically significant ($R^2 = 0.304$, $F = 12.236$, $p = 0.002$). TOV explains 30.4% of the variation in AMH levels. The unstandardized coefficient $B = 0.732$ ($p = 0.002$) showed that an increase in TOV by every 1 cm^3 is associated with a linear increase in AMH values of approximately 0.73 pmol/l (Table 10).

Table 10. Linear regression analysis with dependent variable AMH

Model	Unstandardized B	p	95.0% CI lower/upper	VIF
(Constant)	0.732	0.962	-30.339/31.802	
TOV	2.776	0.002	1.150/4.401	1.000

5. Metabolic and cardiovascular parameters of the participants

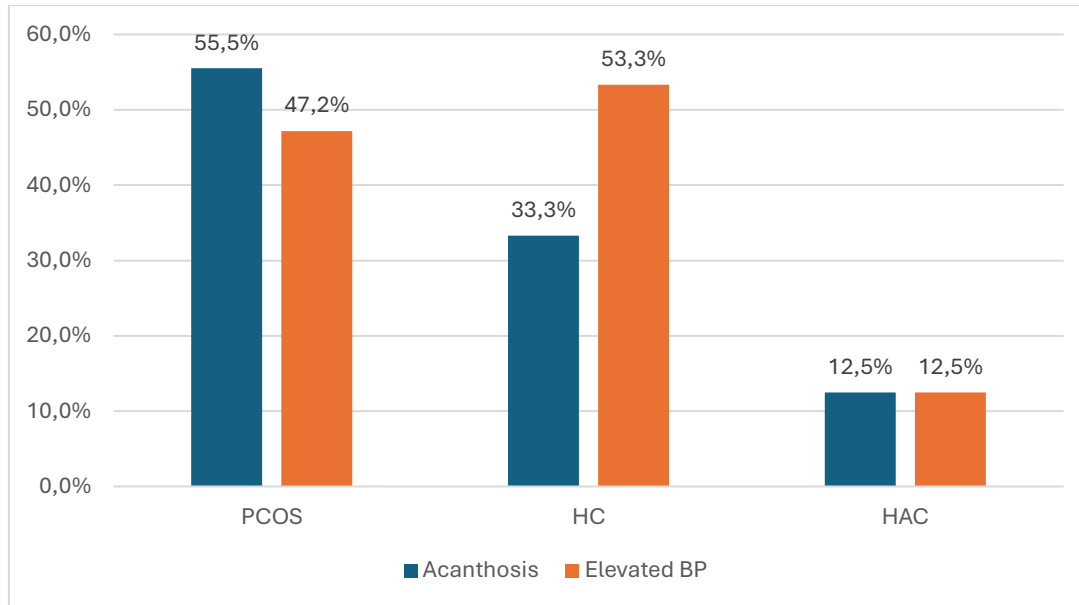
We also assessed the frequency of acanthosis nigricans and elevated blood pressure as manifestations of metabolic and cardiovascular disturbances. To define elevated blood pressure, we used the recommendations of the American Academy of Pediatrics (AAP) for adolescents over 13 years of age – systolic BP ≥ 130 mmHg and/or diastolic BP ≥ 80 mmHg. The data are presented in Table 11 and Figure 9.

Table 11. Data on systolic (SBP) and diastolic (DBP) blood pressure in the three groups

Parameter	PCOS (n=36)	HC (n=15)	HAC (n=8)	p PCOS vs HC	p PCOS vs HAC	p HAC vs HC
SBP (mmHg)	117.5 $\pm$ 12.5	116.4 $\pm$ 11.2	114.1 $\pm$ 4.8	ns	ns	ns
DBP (mmHg)	77.8 $\pm$ 8.9	77.5 $\pm$ 11.9	72.7 $\pm$ 6.5	ns	ns	ns
Elevated BP	47.2%	53.3%	12.5%	ns	ns	ns

Results are presented as mean $\pm$ SD and percentages; ns – not significant.

Fig. 9. Frequency of acanthosis nigricans and elevated BP in all participants



Acanthosis nigricans was found in a total of 44.1% of participants. The frequency was highest in the PCOS group, with the difference from the other groups reaching statistical significance ($p = 0.05$). We performed Pearson correlation analysis to establish the relationship between acanthosis nigricans, metabolic disturbances, and hyperandrogenism. In addition to the expected positive correlation of acanthosis nigricans with HOMA-IR ($r = 0.478$, $p < 0.001$) and insulin level ($r = 0.510$, $p < 0.001$), we found significant positive correlations also with the presence of obesity, the presence of hirsutism and FGS, elevated BP, hypercholesterolemia (defined as total cholesterol ≥ 5.17 mmol/l, NHLBI Guidelines 2011), hypertriglyceridemia (defined as triglycerides ≥ 1.47 mmol/l, NHLBI Guidelines 2011), and a negative correlation with SHBG level. The correlations are presented in Table 12.

Table 12. Correlation results for acanthosis nigricans

Correlation	r	p
HOMA-IR	0.478	<0.001
Insulin	0.510	<0.001
Obesity	0.481	<0.001
Hirsutism	0.303	<0.05

Correlation	r	p
FGS	0.289	<0.05
Elevated BP	0.312	<0.05
Hypercholesterolemia	0.304	<0.05
Hypertriglyceridemia	0.315	<0.05
SHBG	-0.488	<0.001

Results are presented as correlation coefficient r; FGS – Ferriman-Gallwey Score; SHBG – sex hormone binding globulin

The established correlations confirm the importance of IR and its associated hyperinsulinemia both as a factor potentiating hyperandrogenism and as a risk factor for CVD through effects on lipid levels and BP.

SBP and DBP values did not differ significantly between the three groups. Elevated BP was measured in 44.1% of all participants. Despite the differences in frequency between the three groups, statistical significance was not reached ($p = 0.142$). Pearson correlation analysis found a significant positive correlation of elevated BP, in addition to acanthosis nigricans, with the presence of obesity, insulin level, HOMA-IR, and a negative correlation with SHBG level. The results are presented in Table 13.

Table 13. Correlation results for elevated BP

Correlation	r	p
Obesity	0.410	0.001
HOMA-IR	0.405	0.001
Insulin	0.421	<0.001
SHBG	-0.304	<0.05

Results are presented as correlation coefficient r

According to the study protocol, all participants were tested for parameters of glucose metabolism (glucose, fasting insulin and HOMA-IR) and lipid metabolism (total, HDL- and

LDL-cholesterol, triglycerides), as well as CRP as a marker of cardiovascular risk. Data on the parameters of glucose metabolism are presented in Table 14.

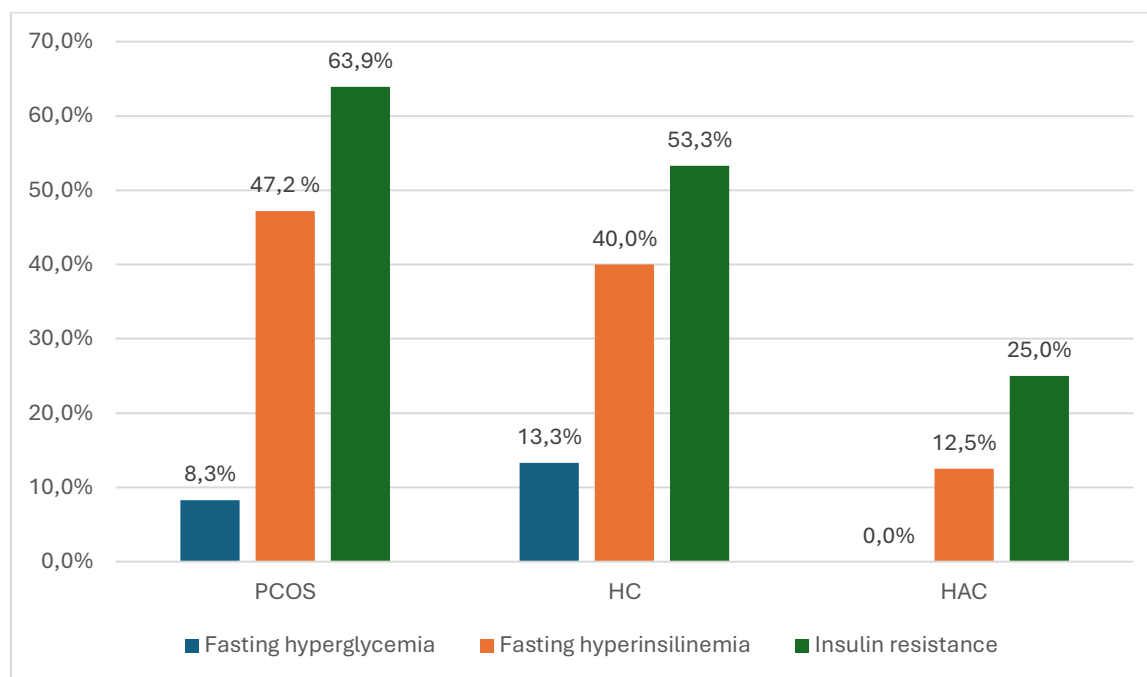
Table 14. Fasting blood glucose (BG), fasting insulin (I) and HOMA-IR values in the three groups

		PCOS	HC	HAC	p PCOS vs	p PCOS vs	p HAC vs
Parameter		(n=36)	(n=15)	(n=8)	HC	HAC	HC
Fasting glucose	(mmol/l)	4.6±0.7	4.7±0.5	4.4±0.8	ns	ns	ns
Fasting insulin	(µIU/ml)	22.9±17.4	17.7±12.4	10.0±6.5	ns	ns	ns
HOMA-IR		4.9±4.3	3.8±3.0	2.0±1.4	ns	ns	ns

Data are presented as mean±SD; ns – no statistical significance.

The analysis found non-significant differences in the mean values of BG, insulin and HOMA-IR in the three groups. Based on the ADA criteria for fasting hyperglycemia (BG ≥5.6 mmol/l and ≤6.9 mmol/l) and fasting hyperinsulinemia (I ≥15 µIU/ml), the frequency of these disorders, as well as of insulin resistance (HOMA-IR ≥2.6), was determined for all participants. The results are presented in Fig. 10.

Fig. 10. Frequency of fasting hyperglycemia (FH), fasting hyperinsulinemia (FHI) and insulin resistance (IR) in the three study groups



Fasting hyperglycemia ≥ 5.6 mmol/l was found in 8.5% of all study participants – 3 girls with PCOS and 2 healthy controls. IR was found in 55.9% of all participants (23 girls with PCOS, 8 healthy controls and 2 hyperandrogenic controls), and fasting hyperinsulinemia in 40.7% of all girls (16 in the PCOS group, 6 healthy controls and 1 in the hyperandrogenic control group). Although the differences between the groups did not reach statistical significance (for FH $p = 0.549$; for FHI $p = 0.245$; and for IR $p = 0.131$), IR and hyperinsulinemia were most common in the PCOS group.

For the PCOS group, a selective analysis of the correlation coefficients between the parameters of glucose metabolism and the levels of lipids, SBP, DBP, CRP and the symptoms of hyperandrogenism was performed. After adjusting for age and BMI, a direct correlation was found between insulin level and fasting hyperglycemia ($r = 0.617$, $p < 0.001$), a direct correlation between HOMA-IR and FGS ($r = 0.436$, $p = 0.013$), as well as between HOMA-IR and fasting hyperglycemia ($r = 0.709$, $p < 0.001$).

To assess the influence of androgen levels on BG, insulin and HOMA-IR, multifactorial linear regression analysis was performed, in which no significant influence of androgens on glucose

homeostasis was found. When assessing the influence of glucose homeostasis parameters on androgens and SHBG, a significant influence of HOMA-IR and insulin level on SHBG was found. Linear regression analysis showed that HOMA-IR has a statistically significant negative influence on SHBG levels ($B = -2.036$, $p = 0.022$). Insulin resistance explains 14.4% of the variation in SHBG levels ($R^2 = 0.144$, $p = 0.022$), with an increase in HOMA-IR of 1.0 predicting a decline in SHBG values of 2.036 nmol/l. Insulin level also has a significant negative influence on SHBG ($B = -0.598$, $p = 0.006$), explaining 20% of the variation in its levels ($R^2 = 0.200$, $p = 0.006$). Each increase in insulin of 1 $\mu\text{IU/ml}$ leads to a decline in SHBG level of 0.598 nmol/l. The decrease in SHBG leads to an increase in free testosterone levels and intensification of the manifestations of hyperandrogenism.

All participants were tested for lipid profile (total, LDL- and HDL-cholesterol and triglycerides) and CRP as a marker of cardiovascular risk. The results are shown in Table 15.

No significant differences in the lipid profile and CRP levels were found between the groups. To determine the frequency of dyslipidemias in all participants, we used the NHLBI adolescent-age (12-19 years) criteria for hypercholesterolemia (total cholesterol ≥ 5.17 mmol/l), hypertriglyceridemia (triglycerides ≥ 1.47 mmol/l), elevated LDL-cholesterol (LDL ≥ 3.36 mmol/l), and low HDL-cholesterol (HDL ≤ 1.03 mmol/l). For elevated CRP, we used the value for high CV risk in adolescence (12-19 years) – CRP ≥ 3.0 mg/l. None of these disorders were found in the group of girls with idiopathic hyperandrogenism. The frequency of dyslipidemia and elevated CRP in girls with PCOS and HC is shown in Fig. 11.

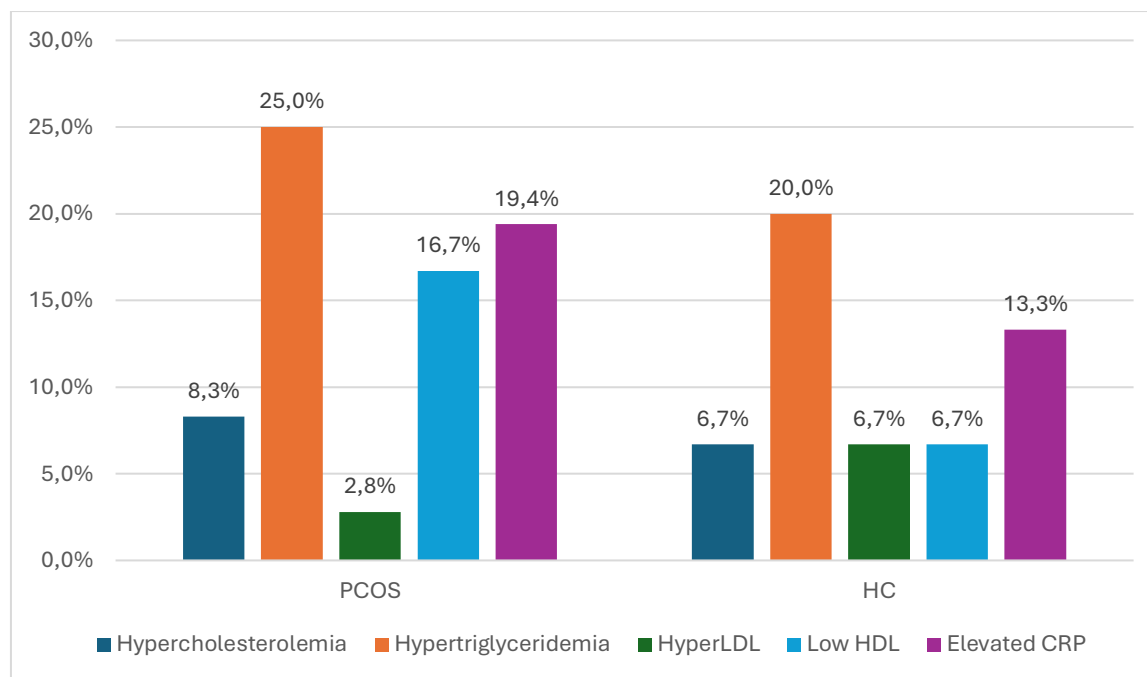
Table 15. Levels of total cholesterol (Chol), LDL-cholesterol (LDL), HDL-cholesterol (HDL), triglycerides (TG) and C-reactive protein (CRP) in the three groups

Parameter	PCOS (n=36)	HC (n=15)	HAC (n=8)	p PCOS vs HC	p PCOS vs HAC	p HAC vs HC
Chol (mmol/l)	4.3 $\pm$ 0.6	4.3 $\pm$ 0.9	4.3 $\pm$ 0.4	ns	ns	ns
LDL (mmol/l)	2.4 $\pm$ 0.6	2.4 $\pm$ 0.8	2.4 $\pm$ 0.5	ns	ns	ns

Parameter	PCOS (n=36)	HC (n=15)	HAC (n=8)	p PCOS vs HC	p PCOS vs HAC	p HAC vs HC
HDL (mmol/l)	1.3±0.3	1.5±0.3	1.5±0.3	ns	ns	ns
TG (mmol/l)	1.2±0.8	0.9±0.5	0.8±0.3	ns	ns	ns
CRP (mg/l)	2.7±4.4	1.3±1.1	1.4±1.0	ns	ns	ns

Data are presented as mean±SD; ns – no statistical significance.

Fig. 11. Frequency of hypercholesterolemia, hypertriglyceridemia, high LDL, low HDL, and elevated CRP in girls with PCOS and healthy controls



Of all the hyperlipidemias, hypertriglyceridemia had the highest frequency in both groups. TG levels ≥ 1.47 mmol/l were found in 9 girls with PCOS and 3 healthy controls. Although girls with PCOS and hypertriglyceridemia were 3 times more numerous than healthy girls with the same lipid disorder, the statistical difference was non-significant ($p = 0.283$). The second most common lipid homeostasis disorder in the PCOS group, found in 6 of the participants, was decreased HDL ≤ 1.03 mmol/l. Among healthy controls, low HDL was found in 1 girl. Again, the statistical difference was non-significant ($p = 0.323$). The difference in the frequency of high

LDL ≥ 3.36 mmol/l between the two groups was also non-significant ($p = 0.666$) – 1 girl with PCOS and 1 healthy control. Elevated total cholesterol was found in 3 girls with PCOS and 1 healthy control, with a non-significant difference ($p = 0.698$). As the second most common marker of elevated CV risk in both groups, after hypertriglyceridemia, elevated CRP ≥ 3.0 mg/l emerged. This was found in 7 girls with PCOS and 2 healthy controls, again with a non-significant statistical difference ($p = 0.373$).

In the PCOS group, correlational relationships were sought between the lipid parameters, CRP, hyperandrogenism, and disorders of glucose metabolism, after adjusting for the influence of age and BMI. The significant correlations found, the correlation coefficient r , and their statistical significance are shown in Table 16.

Table 16. Correlation coefficients r between lipids, androgens, insulin and SHBG in the PCOS group, and their statistical significance

	Cholesterol	TG	LDL	HDL
Cholesterol	-	$r = 0.434^*$	$r = 0.894^{***}$	-
TG	$r = 0.434^*$	-	-	$r = -0.465^*$
LDL	$r = 0.894^{***}$	-	-	
HDL		$r = -0.465^*$		
Insulin	-	-	-	$r = -0.413^*$
SHBG	-	-	-	$r = 0.447^*$
Testosterone	-	-	$r = 0.472^*$	

*Statistical significance: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$*

A direct correlation was found between total cholesterol and triglyceride levels ($r = 0.434$, $p < 0.05$) and total cholesterol and LDL ($r = 0.894$, $p < 0.001$). TG showed an inverse correlation with HDL ($r = -0.465$, $p < 0.05$), while HDL showed an inverse correlation with insulin level ($r = -0.413$, $p < 0.05$) and a direct correlation with SHBG level ($r = 0.447$, $p < 0.05$). Of the lipids, only LDL showed a correlation with androgens (testosterone), and it was direct ($r = 0.472$, $p < 0.05$).

In the multiple regression analysis performed in the PCOS group, no influence of androgens on lipid and CRP levels was found. TG levels were significantly and positively influenced by markers of generalized and abdominal obesity (BMI and WC), as well as insulin level (Table 17).

Table 17. Results of multiple regression analysis with dependent variable TG

	BMI	WC	Insulin
R ²	0.278	0.310	0.133
p	<0.001	<0.001	0.018
B	0.054	0.024	0.017
95.0% CI lower/upper	0.024/0.084	0.012/0.037	0.003/0.032

Multiple regression analysis found that HDL levels were positively influenced by SHBG, and negatively influenced by BMI, WC and insulin (Table 18).

CRP levels were positively influenced by BMI, WC and insulin (Table 19).

Table 18. Results of multiple regression analysis with dependent variable HDL

	BMI	WC	Insulin	SHBG
R ²	0.408	0.426	0.199	0.466
p	<0.001	<0.001	0.006	<0.001
B	-0.026	-0.11	-0.008	0.009
95.0% CI lower/upper	-0.037/-0.015	-0.016/-0.007	-0.014/-0.002	0.006/0.013

Table 19. Results of multiple regression analysis with dependent variable CRP

	BMI	WC	Insulin
R ²	0.193	0.180	0.281
p	0.07	0.01	<0.001
B	0.255	0.105	0.135
95.0% CI lower/upper	0.073/0.437	0.027/0.182	0.060/0.0210

6. Ultrasound examination of the ovaries

Participants in the three groups underwent pelvic ultrasound examination to establish the presence of polycystic ovarian morphology (PCOM), which in girls is defined as an ovarian volume increase >2 SD (>12 ml). The results of the ultrasound examination are presented in Table 20.

Table 20. Ultrasound ovarian volume and presence of PCOM in the participants from the three groups

Parameter	PCOS (n=36)	HC (n=15)	HAC (n=8)	p PCOS vs HC	p PCOS vs HAC	p HAC vs HC
LOV (cm ³)	9.5±3.3	9.3±4.2	8.5±2.0	ns	ns	ns
ROV (cm ³)	9.4±3.5	10.4±3.4	6.7±2.0	ns	ns	ns
TOV (cm ³)	18.9±6.5	20.1±7.8	16.5±3.5	ns	ns	ns
PCOM (%)	23.3%	33.3%	0.0%	ns	ns	ns

LOV – left ovarian volume; ROV – right ovarian volume; TOV – total ovarian volume; PCOM – polycystic ovarian morphology; ns – no statistical significance.

The analysis found no significant difference in ovarian volumes and the presence of PCOM between the groups. In the multiple correlation analysis, adjusting for age, apart from the already-described correlation with AMH, no correlations were found with clinical or biochemical hyperandrogenism, nor with metabolic parameters.

7. Results of the study of selected microRNAs (miRNA)

In a total of 53 girls from all participants (34 from the PCOS group, 11 from HC and all 8 from HAC), selected miRNAs were investigated to assess their suitability as potential diagnostic biomarkers for PCOS. The age and anthropometric characteristics of the participants are presented in Table 21, and the significant clinical and biochemical differences between the groups – in Table 22.

Table 21. Age and anthropometric characteristics of the participants in whom miRNAs were studied

Parameter	PCOS (n=34)	HC (n=11)	HAC (n=8)	p
Age (yrs)	16.0±1.4	15.3±1.5	15.8±1.1	ns
Weight (kg)	71.9±15.8	74.1±16.5	61.1±13.5	ns
Height (cm)	161.8±7.6	165.6±5.9	159.2±9.3	ns
BMI (kg/m ²)	27.5±6.4	27.0±5.4	24.0±4.5	ns
WC (cm)	85.0±16.0	87.4±19.1	78.6±9.6	ns

Data are presented as mean±SD; ns – no statistical significance.

Table 22. Significant clinical and biochemical differences between the groups

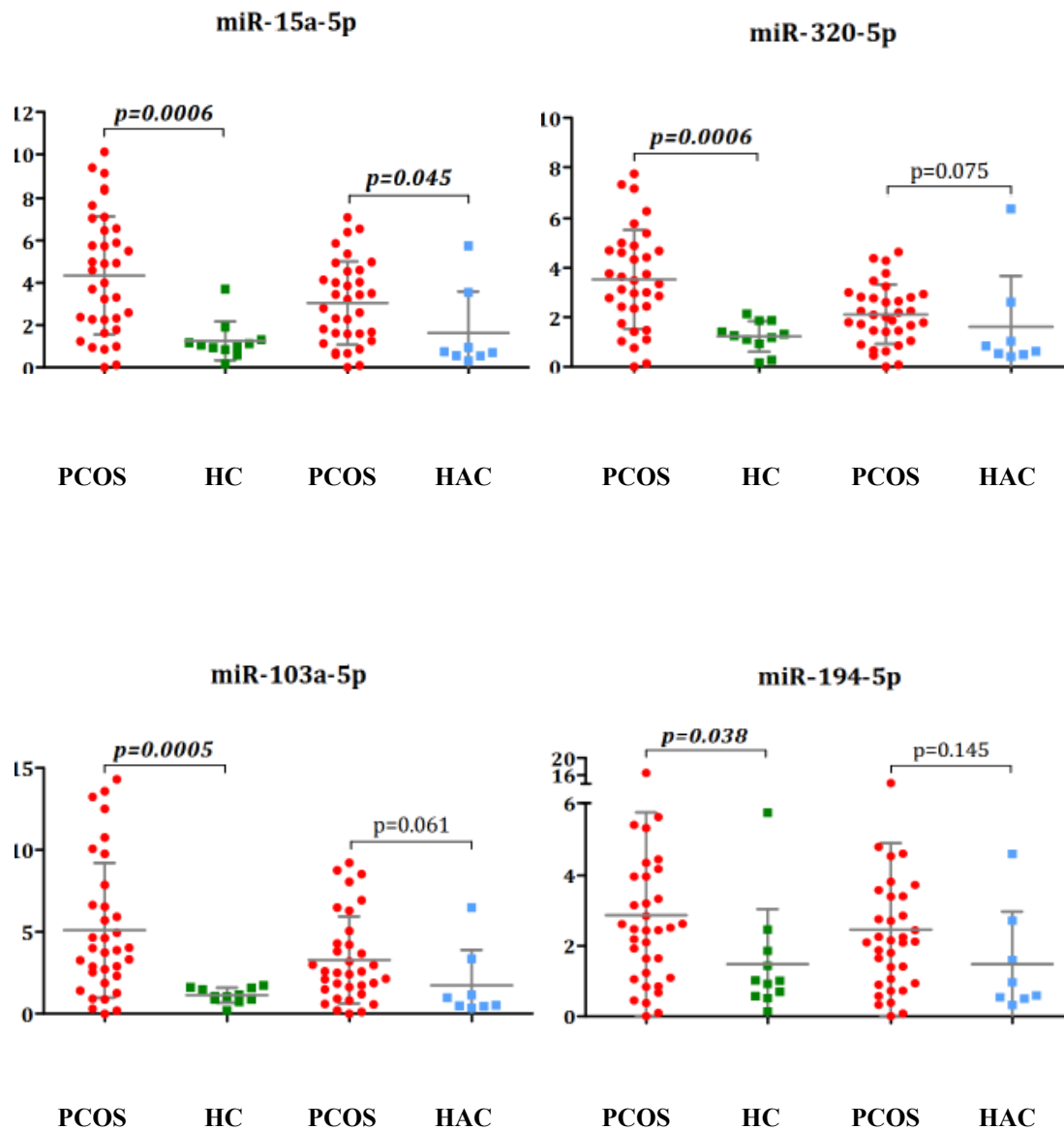
Parameter	PCOS (n=34)	HC (n=11)	HAC (n=8)	p PCOS vs HC	p PCOS vs HAC	p HC vs HAC
Hirsutism	88.2%	10.0%	37.5%	<0.001	<0.001	ns
FGS	15.1±6.8	3.5±7.3	8.2±9.7	<0.001	0.01	ns
Oily hair	74.0%	50.0%	37.5%	ns	0.032	ns
Insulin (μIU/ml)	22.8±17.6	15.5±10.3	10.0±6.5	ns	0.014	ns
T (nmol/l)	1.8±0.8	0.8±0.1	2.2±0.2	<0.001	ns	<0.001
A4 (nmol/l)	14.5±5.4	4.8±2.6	13.1±3.5	<0.001	ns	0.001
AMH (pmol/l)	49.1±32.9	19.3±12.9	33.6±28.0	0.015	ns	ns
SHBG (nmol/l)	28.9±22.6	46.4±21.5	54.5±24.7	ns	0.020	ns
FAI	10.9±12.2	1.9±0.8	4.9±2.5	<0.001	0.028	0.034

Data are presented as mean±SD or percentages; ns – no statistical significance.

Six miRNAs were studied – miR-451a, miR-15a-5p, miR-320-5p, miR-28-5p, miR-103a-5p and miR-194-5p. Most of them have not been studied in patients with PCOS and, with the exception of miR-451a, miR194-5p and miR-15a-5p, have not been studied in girls. The reason for selecting miR-451a is that it has been found to be significantly decreased in girls with PCOS compared with healthy controls, with 100% sensitivity and 100% specificity for diagnosing PCOS (Diaz et al., 2020). MiR-103a-3p and miR-28-3p have also been found significantly decreased in patients with PCOS (Udesen et al., 2023). There are reports that miR-320 is a potential biomarker for disorders of lipid and glucose metabolism, which are often associated with PCOS, and is elevated in these conditions, but decreased in PCOS (Du et al., 2021; Sørensen et al., 2014; Cirillo et al., 2019). MiR-194 has been found to be overexpressed in women with PCOS and impaired glucose metabolism, and to be elevated in granulosa cells of women with PCOS, where it induces apoptosis (Chen et al., 2019; Wu et al., 2021). We decided to investigate whether we would find similar changes in the levels of other members of the miR-103, miR-28, miR-320 and miR-194 families. The reason for selecting miR-15a-5p is that it has been found significantly elevated in patients with endometrial carcinoma and has been proposed as a biomarker for its early detection. Its level also correlates with androgen levels (Zhou et al., 2021).

Four of the six analyzed miRNAs – miR-320-5p, miR-103a-5p, miR-15a-5p and miR-194-5p – were found to show significantly different expression in girls with PCOS. These four miRNAs were significantly elevated in the PCOS group compared with healthy controls (all $p < 0.05$). In addition, miR-15a-5p was significantly overexpressed in the PCOS group compared with the hyperandrogenic controls as well ($p = 0.045$) (Fig. 12).

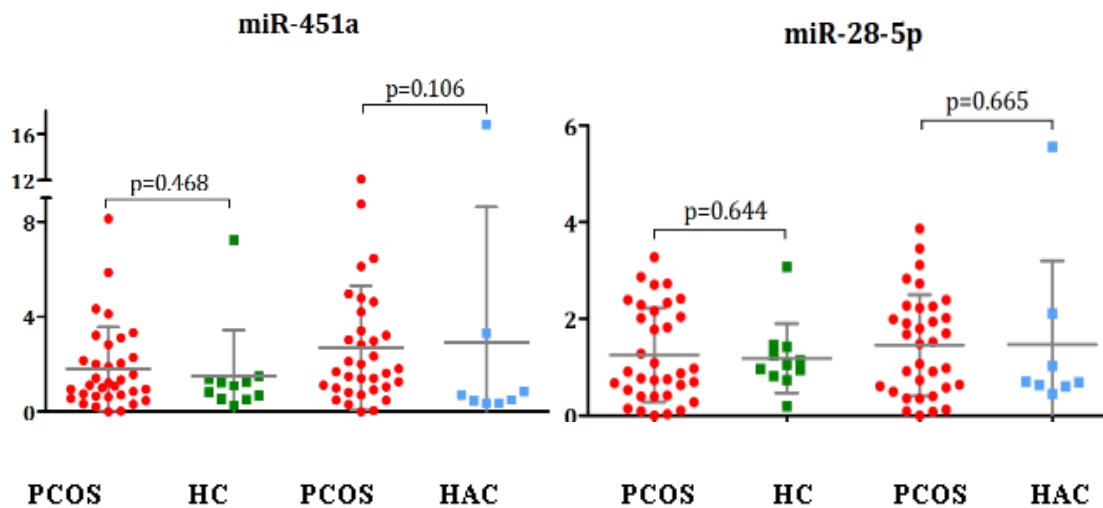
Fig. 12. Comparative expression of the four significantly different miRNAs in patients with PCOS compared with the control groups



HC – healthy controls; HAC – hyperandrogenic controls; significance at $p < 0.05$.

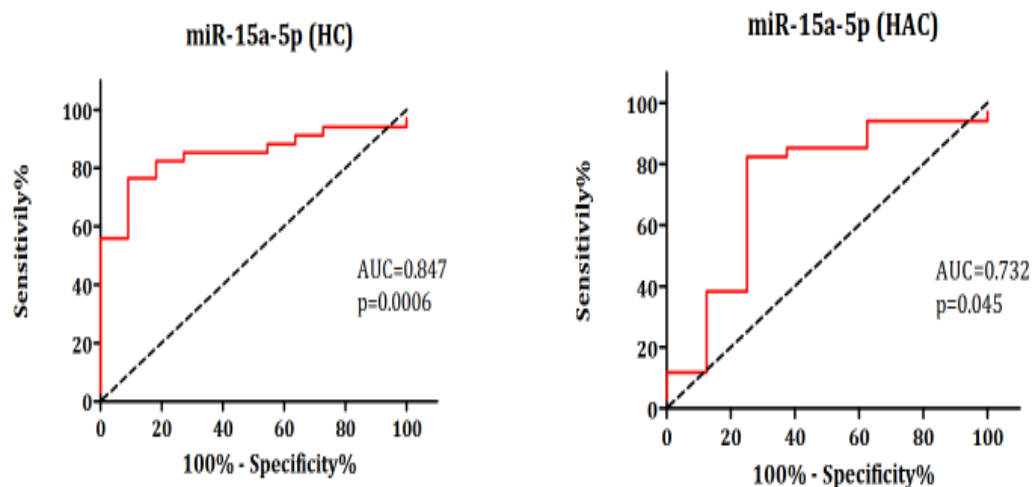
For the remaining two microRNAs, miR-451a and miR-28-5, no statistically significant differences in expression were found among the three study groups (Fig. 13).

Fig. 13. Comparative expression of the two non-significantly different miRNAs in patients with PCOS compared with the control groups



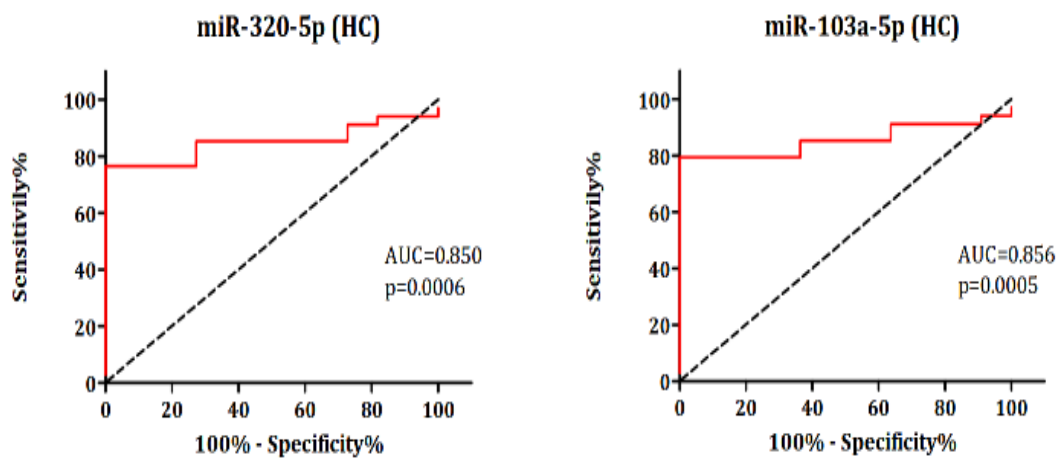
The sensitivity and specificity of each of the four miRNAs as a potential diagnostic biomarker for PCOS were assessed using ROC curve analysis (Fig. 14, 15 and 16, Table 23).

Fig. 14. ROC curve of miR-15a-5p for determining sensitivity and specificity in distinguishing PCOS from HC and HAC



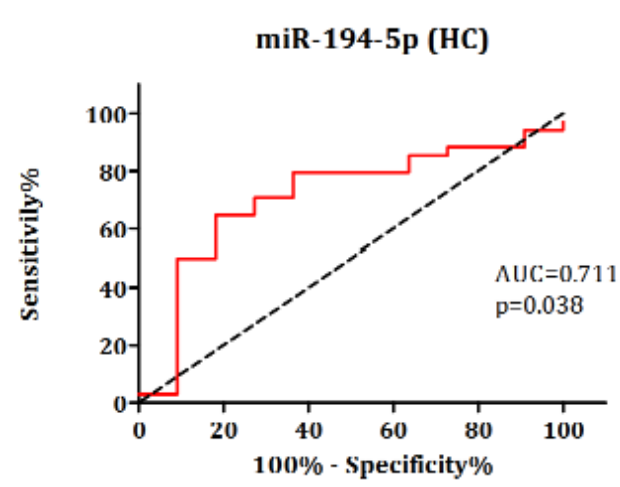
AUC – area under the curve; HC – healthy controls; HAC – hyperandrogenic controls; statistical significance at $p < 0.05$

Fig. 15. ROC curves of miR-320-5p and miR-103a-5p for determining sensitivity and specificity in distinguishing PCOS from HC



AUC – area under the curve; HC – healthy controls; statistical significance at $p < 0.05$

Fig. 16. ROC curve of miR-194-5p for determining sensitivity and specificity in distinguishing PCOS from HC



AUC – area under the curve; HC – healthy controls; statistical significance at $p < 0.05$

Table 23. Sensitivity and specificity of the four miRNAs for distinguishing patients with PCOS from healthy controls

miRNA	Sensitivity	Specificity
miR-320-5p	76%	100%
miR-103a-5p	76%	100%
miR-194-5p	65%	81%
miR-15a-5p	76%	91%

MiR-15a-5p, miR-320-5p, miR-103-5p and miR-194-5p demonstrated statistically significant accuracy as independent diagnostic markers. MiR-15a-5p additionally showed the ability to differentiate PCOS from hyperandrogenic controls. The optimal threshold values for miR-320-5p and miR-103a-5p effectively distinguish patients with PCOS from healthy controls, with 100% specificity and 76% sensitivity. MiR-194-5p showed 65% sensitivity and 81% specificity for diagnosing PCOS. MiR-15a-5p shows higher specificity when distinguishing patients with PCOS from healthy controls (91% specificity, 76% sensitivity) compared with distinguishing girls with PCOS from HAC (75% specificity, 82% sensitivity).

In the PCOS group, correlation analysis was performed to establish correlations between the four altered miRNAs and clinical, metabolic and hormonal parameters associated with PCOS. After adjusting for age and BMI, significant positive correlations were found between miR194-5p and SBP, miR15a-5p and FGS, miR103a-5p with hair loss and SBP, and miR320-5p with FGS and CRP, as well as an inverse correlation of miR320-5p with glucose level (Table 24).

Table 24. Correlation coefficients r between the four miRNAs and clinical and biochemical parameters, and their statistical significance

	miR-194-5p	miR15a-5p	miR-103a-5p	miR320-5p
SBP	$r = 0.462^*$	-	$r = 0.475^*$	-
FGS	-	$r = 0.550^*$	-	$r = 0.513^*$
Hair loss	-	-	$r = 0.508^*$	-
BG	-	-	-	$r = -0.457^*$

	miR-194-5p	miR15a-5p	miR-103a-5p	miR320-5p
CRP	-	-	-	-

*Statistical significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$*

To assess the influence of hyperandrogenism, metabolic and cardiovascular disturbances on the four elevated miRNAs in girls with PCOS, linear regression analysis was performed. The results are presented in Table 25.

Table 25. Results of multiple regression analysis with dependent variable each of the four miRNAs and independent variables CRP and SBP

	miR194-5p	miR15a-5p	miR103a-5p	miR320-5p
CRP	$R^2 = 0.447$, p < 0.001	$R^2 = 0.231$, p = 0.004	$R^2 = 0.243$, p = 0.003	-
SBP	-	$R^2 = 0.159$, p = 0.019	-	$R^2 = 0.188$, p = 0.01

The regression analysis found no influence of hyperandrogenism on the selected miRNAs. An influence of some CV risk markers – CRP and SBP – was found. CRP positively influences the expression of miR194-5p, miR15a-5p and miR103a-5p. SBP has a positive influence on the levels of miR15a-5p and miR320-5p.

PART V. DISCUSSION

1. Characteristics of the participants

A total of 59 girls were included in the study, distributed into three groups matched for age and BMI: a main group of patients with PCOS (n=36), a control group of clinically healthy girls (HC, n=15), and a group of girls with idiopathic hyperandrogenism (HAC, n=8). The formation of this unbalanced design (a ratio of approximately 2.4:1 in favor of PCOS versus HC, and 4.5:1 in favor of PCOS versus HAC) was determined by logistical constraints (the study began during the state of emergency caused by the COVID-19 pandemic) and the strict exclusion criteria used in selecting the healthy controls. To compensate for the smaller size of the control groups and to ensure the validity of the results, non-parametric tests and methods suitable for samples of unequal size were applied in the statistical analysis.

The mean age of all study participants was 15.8 ± 1.3 years (12.9-17.9), with no significant difference between the groups. This is important for the processing and interpretation of the data, since it excludes age as a potential confounding factor and allows for better comparability of the results.

In girls with PCOS, the diagnosis was in practice established upon inclusion in the study. The mean age at diagnosis of PCOS among the participants was 15.9 ± 1.5 years. No large studies on the mean age at diagnosis of PCOS in adolescents are found in the international literature. Özer et al., in a study of 305 girls with menstrual disorders, of whom 36 had PCOS, found a mean age at diagnosis of PCOS of 15.3 ± 1.5 years. Complaints in the girls appeared at a mean age of 13.6 ± 1.6 years (Özer et al., 2025). The International Evidence-Based Guideline for the Assessment and Management of PCOS recommends that assessment for this condition be performed between 13-19 years of age, but only if 2 or more years have passed since menarche (Teede et al., 2023). In a meta-analysis including 20 studies with 14,010 participants aged 10-24 years, most of them (19 studies) with patients aged <19 years, Neven et al. found that the time from menarche to PCOS diagnosis averaged between 2.8-6.9 years (Neven et al., 2024). The girls with PCOS in our study were aged between 12.9 and 17.9 years, and all of them were more than 2 years past menarche, which corresponds to the recommendations of the International Guideline and to the age of diagnostic clarification worldwide.

2. Overweight and obesity

All participants in our study were matched by BMI, which is also of important methodological significance. Thus, in the data analysis, the influence of obesity as a confounding factor is eliminated, since overweight itself causes hormonal and metabolic changes similar to those found in PCOS. In this way, it is possible to distinguish which part of the abnormalities found is due to the syndrome itself.

Overweight (OW) and obesity are common in PCOS, complicating the course of the syndrome. Increased visceral fat tissue in patients with PCOS and obesity increases IR and hyperinsulinism, which stimulates androgen production, increases adipogenesis, and reduces lipolysis (Bila et al., 2025). The frequency of overweight and obesity in girls with PCOS in the present study amounts to 63.9%, with 25% of them overweight and 38.9% obese. This frequency is comparable to that found by other researchers. For example, Elahi et al., in a study of 42 girls with PCOS with a mean age of 15.9 ± 1.6 years, report a frequency of OW and obesity of 61.9%, similar to ours (Elahi et al., 2024). Azargoon et al. found an obesity frequency of 37.9% in 58 girls with PCOS aged 16 ± 0.9 years, which is in full agreement with our finding of 38.9% (Azargoon et al., 2020). A lower frequency is reported by Bhargavi et al. In a study of 100 patients with PCOS aged 15-20 years (54% between 19-20 years), the authors found combined OW and obesity in 46% of participants (Bhargavi et al., 2025). In contrast to these data, and in agreement with ours, in a large-scale study including 137,502 girls aged 15-19 years, of whom 1563 had PCOS, Christensen et al. found obesity in 63.1% of patients with PCOS (Christensen et al., 2014). The data from the cited authors confirm the high frequency of OW and obesity in adolescent PCOS and are fully consistent with our results.

3. Manifestations of clinical hyperandrogenism and menstrual disorders

Manifestations of androgen excess (hirsutism and cystic acne) are a key diagnostic criterion for PCOS. Of the clinical symptoms of hyperandrogenism in girls with PCOS in our study, hirsutism was the most common (88.9%), with significant differences from both control groups. Hirsutism was assessed clinically using the modified Ferriman-Gallwey scale. Girls with PCOS had a significantly higher mean FGS compared with healthy controls (14.9 ± 6.7 versus 6.1 ± 9.4 ; $p < 0.05$). In the literature, the reported frequency of hirsutism in girls with PCOS varies widely. In a meta-analysis of 12 studies, including 149,477 girls with a mean age of 16.99 years, Naz et

al. found an 18% frequency of hirsutism and a 35% frequency of acne (Naz et al., 2019). The authors did not provide FGS values. Our results are similar to those published by Jakubowska-Kowal et al., who found a hirsutism frequency of between 88.1%-91.5% in girls with PCOS, depending on the diagnostic criteria applied (Jakubowska-Kowal et al., 2026). At the same time, the authors found biochemical hyperandrogenism in 54.9%-55.3% of girls with PCOS, a difference that demonstrates the importance of a detailed history regarding the presence of increased hair growth, as well as the importance of a thorough clinical examination (Jakubowska-Kowal et al., 2026). Yetim et al. also found a frequency of hirsutism similar to ours. In their study, including 53 girls with PCOS and 26 healthy controls, the authors found a hirsutism frequency of 87% in the PCOS group (Yetim et al., 2016).

Because of the significant difference in FGS between patients with PCOS and healthy controls, we decided to evaluate the sensitivity and specificity of FGS as a diagnostic marker for PCOS. After ROC curve analysis and determination of the Youden index, a cut-off value for FGS of 6 points was found to distinguish patients from healthy girls with 94% sensitivity and 65% specificity. In our correlation analysis, we found no correlations between FGS, androgen levels and FAI. These results correspond to the data published by Hestiantoro et al. in a study of 76 young women with PCOS (Hestiantoro et al., 2019). In our study, linear regression analysis found that insulin resistance significantly influences FGS and is a predictor of the severity of clinical hirsutism, which confirms the importance of hyperinsulinemia for androgen excess in PCOS.

A number of publications in the scientific literature confirm the validity of FGS for the phenotypic assessment of existing hirsutism and demonstration of hyperandrogenism, not only in PCOS but also in other hyperandrogenic conditions (Azziz et al., 2008, Hagan et al., 2019). In recent years, it has been recommended that the FGS cut-off value for defining hirsutism be adapted to the ethnic characteristics of the population (Spritzer et al., 2022, Teede et al., 2023). Our analysis found that an FGS cut-off of 7.5 points determines the presence of hirsutism with 100% sensitivity and 93% specificity, which corresponds to the value of $FGS \geq 8$ accepted in our country (Orbetsova, 2009). While FGS is a highly sensitive and specific parameter for assessing hirsutism, it has insufficient specificity as a marker for PCOS and does not correlate with

biochemical hyperandrogenism, which is why we do not recommend that this parameter be used alone for diagnosing the disease.

Regarding the remaining symptoms of hyperandrogenism, we found no significant differences between the groups. Acne occurred in 69.4% of our patients with PCOS, 40% of healthy controls, and 62.5% of hyperandrogenic controls. In the above-cited study by Jakubowska-Kowal et al., the frequency of acne in girls with PCOS was between 68.8%-70.2%, similar to that found in our study. In a meta-analysis, Ramezani Tehrani et al. found an acne frequency of 59% in patients with PCOS under 19 years of age, and 39% in healthy girls (Ramezani Tehrani et al., 2021). Despite the lack of statistical significance in the frequency of acne among the three groups in our study, it occurs frequently in patients with PCOS and should not be overlooked as a symptom of hyperandrogenism when the syndrome is suspected.

Oily hair is another pilosebaceous manifestation of hyperandrogenism and occurs in 72.2% of our patients with PCOS. This symptom is rarely described as an independent sign of hyperandrogenism and is usually included among the manifestations of seborrhea, which is why no data on its frequency in girls with PCOS are found in the literature. In a study of 146 women with PCOS, Abusailik et al. found seborrhea in 43.2% of patients and androgenic alopecia in 42.5% (Abusailik et al., 2021). In our patients with PCOS, manifestations of androgenic alopecia occurred in 8.3%, and hair loss in 25%. Alopecia is very rare in girls with PCOS and is not recommended as a diagnostic criterion. There are also no studies on its frequency in adolescent PCOS (Meczekalski et al., 2023; Ramezani Tehrani et al., 2019).

The second mandatory criterion for diagnosing PCOS is the presence of oligomenorrhea (OM) (Ibanez et al., 2017; Teede et al., 2023). For this reason, OM was found in 100% of our patients with PCOS. Since an inclusion criterion for participants in both control groups was the absence of menstrual disorders, OM was not found in any of them. In the international literature, the reported frequency of OM varies depending on the diagnostic criteria used for PCOS. For example, in the study by Yetim et al., who applied the Rotterdam criteria for diagnosing PCOS, the frequency of OM was 83% (Yetim et al., 2016). In their study, Jakubowska-Kowal et al. found an OM frequency similar to ours – 100% when using the ICPE criteria (also applied in our study) and the International Guideline for the diagnosis and management of PCOS, and 91.8% when using the Rotterdam criteria (Jakubowska-Kowal et al., 2026).

4. Hormonal results in the participants

1. Androgens and SHBG

As expected, in the analysis of hormone levels among the participants in our study, significantly higher values of testosterone (T), androstenedione (A4) and free androgen index (FAI) were found in girls with PCOS compared with the healthy control group. Hyperandrogenic controls also had higher T and A4 levels compared with healthy girls, while the difference from the PCOS group was non-significant.

Hyperandrogenism occupies a central place in the pathophysiology of PCOS and is accepted as a mandatory diagnostic criterion in adolescents. From a pathogenetic standpoint, persistently high androgen levels inhibit normal folliculogenesis at the intraovarian level, causing premature atresia of antral follicles, which leads to oligomenorrhea. In addition, hyperandrogenism participates in a “vicious circle” with IR and compensatory hyperinsulinemia. Visceral obesity and IR stimulate ovarian androgen production while simultaneously suppressing hepatic SHBG synthesis, leading to elevated free androgen levels. These worsen insulin sensitivity in peripheral tissues, which deepens the metabolic and hormonal disturbances (Kim et al., 2021).

In the search for reliable biomarkers of biochemical hyperandrogenism (total T, A4, FAI), a number of international studies have attempted to define optimal cut-off values for diagnosing PCOS. However, due to variability in the laboratory methods used and heterogeneity of the cohorts studied, the reported thresholds and their sensitivity and specificity vary considerably. For example, Kumar et al. propose a relatively low T cut-off value of 25.1 ng/dl (0.8 nmol/l). This threshold shows moderate sensitivity of 78.1%, but excellent specificity of 92.6%. However, as the authors themselves note, this lower threshold is suitable for the Asian population, in which T levels are lower (Kumar et al., 2024). Khashchenko et al., studying a cohort of 130 adolescent girls, identified a higher cut-off value ($T > 1.15$ nmol/l). The authors report good sensitivity (75-93%) and high specificity (83-93%) for this cut-off (Khashchenko et al., 2020). The results of our study show partial similarity to the cited literature data regarding the sensitivity of T, but differ significantly in terms of its specificity. In our study, the optimal threshold value of 1.13 nmol/l achieves only 54.5% specificity with a good sensitivity of 80.6%. This discrepancy may be due to the use of standard radioimmunoassay methods (RIA) for T determination in our study and in the

cited studies. Unlike the gold standard LC-MS/MS (liquid chromatography with tandem mass spectrometry), RIA methods exhibit lower accuracy and cross-reactivity at the low testosterone concentrations characteristic of the female sex. Based on the results obtained and the recorded low specificity, we cannot recommend isolated testing of total testosterone as an independent diagnostic marker for PCOS in clinical practice.

In our study, A4 and FAI demonstrated significantly higher diagnostic sensitivity and specificity for distinguishing patients with PCOS from healthy controls, compared with the isolated measurement of T. A4 with a cut-off of 11.6 nmol/l successfully differentiated patients with 69% sensitivity and 100% specificity. Achieving 100% specificity means that values of A4 above this threshold completely exclude false-positive results among healthy controls and serve as a good marker for the presence of PCOS. FAI with a cut-off of 6.2 demonstrated 72% sensitivity and 90% specificity in distinguishing patients from control groups. The higher sensitivity of FAI compared with A4 (72% versus 69%) shows that it succeeds in identifying a larger percentage of truly affected patients, accounting for free androgens, which are the biologically active ones. The advantage of A4 is explained pathophysiologically by the fact that androstenedione is a key precursor in steroidogenesis, reflecting both ovarian and adrenal androgen overproduction simultaneously, the latter of which may be observed in up to 46% of patients with PCOS (Rosenfield, 2020). On the other hand, FAI more accurately reflects actual tissue androgenicity compared with total testosterone.

A number of international studies confirm the importance of FAI as a good diagnostic marker for PCOS, with high sensitivity and specificity. Sağsak et al. found that a FAI cut-off value of 3.45 has 90% sensitivity and 84% specificity for distinguishing girls with PCOS from healthy girls. The authors established a higher threshold of 6.15, which distinguishes patients with PCOS from those with obesity and IR but without PCOS, with 89% sensitivity and 77% specificity (Sağsak et al., 2021). This result is very close to that established in our study, in which the frequency of OW and obesity in the control group was high (53.3%). Khashchenko et al. also determined FAI as a marker with good sensitivity and specificity for diagnosing PCOS. In their study, a threshold value of >2.75 had a sensitivity of 75% and specificity of 93% (Khashchenko et al., 2020). The girls in their control group had a lower BMI than the patients with PCOS, which is the most likely reason for this low cut-off.

Similar to the results of our study, the significance of androstenedione (A4) as a better marker of hyperandrogenism than testosterone in patients with PCOS has been demonstrated in several studies. In a study of 86 women with PCOS and 43 age- and BMI-matched controls, O'Reilly et al. found elevated T in only 65% of patients, while elevated A4 was found in 88%. The authors determined the sensitivity of A4 as a diagnostic marker to be 88.3%, and the specificity 97.7% (O'Reilly et al., 2014). Khashchenko et al. determined a value of A4 of 11.45 ng/ml as suitable for diagnosing PCOS in adolescents, with sensitivity up to 78.2% and specificity reaching up to 93.7% (Khashchenko et al., 2020).

To assess the real diagnostic independence of A4 and FAI, a correlation analysis was carried out in our study, in which the influence of age and BMI was statistically eliminated. For A4, a significant direct correlation with T and FAI was found. This confirms that the increase in A4 goes hand in hand with the increase in total and free testosterone fractions and demonstrates their common pathogenetic source – enzymatic dysregulation in the ovaries. A moderate inverse correlation with SHBG was also found, which demonstrates the influence of hyperandrogenism on its hepatic synthesis. A decrease in SHBG leads to an increase in free testosterone levels and intensification of hyperandrogenic manifestations. For FAI, a significant positive correlation with A4 and a moderate one with T were, as expected, found, as well as a significant negative correlation with SHBG.

To determine the degree of causal relationship between the studied androgen markers, linear regression analysis was applied in the present study. It found no statistically significant influence of FAI on the variability of A4 ($R^2=0.011$, $p=0.544$). The absence of a regression dependence demonstrates that A4 and FAI function as two statistically independent diagnostic biomarkers. They cover different aspects of androgen excess (total precursor pool and free tissue bioavailability), which is an argument in favor of their combined use to achieve better diagnostic accuracy. In contrast to the previous result, linear regression assessing the dependence between serum T and A4 concentrations showed high statistical significance ($R^2=0.336$, $p<0.001$). According to the data obtained, total T levels alone succeed in explaining 33.6% of the total variation in androstenedione in the study group. The direction of this dependence is positive, since A4 is a major precursor for testosterone synthesis. In PCOS there is increased androgen production, resulting in a parallel increase in secretion of both hormones (A4 and T).

In addition, the present study found a significantly lower SHBG level in girls with PCOS compared with those with idiopathic hyperandrogenism ($p < 0.05$), while there was no such difference with the healthy control group. This can be explained by the higher frequency of obesity and OW in girls with PCOS and healthy controls, and the higher (though non-significant) HOMA-IR and insulin values in patients with PCOS compared with the hyperandrogenic control group. It is known that hyperinsulinemia inhibits SHBG synthesis (Ibanez et al., 2027; Nicolaides et al., 2020; Qu et al., 2020).

In summary, our data demonstrate that, unlike total testosterone, testing of A4 and FAI provides significantly higher diagnostic accuracy and reliability in differentiating PCOS. This is also confirmed in the international literature (Khashchenko et al., 2020; O'Reilly et al., 2014; Sağsak et al., 2021). Due to its high specificity, A4 (at a cut-off > 11.6 nmol/l) serves as a good confirmatory marker, while FAI (at a cut-off > 6.2) offers very good sensitivity. These results provide grounds for recommending the inclusion of A4 and FAI as routine practice in the diagnostic algorithm for PCOS in adolescent patients.

2. Anti-Müllerian hormone (AMH)

AMH has long been of scientific interest as a potential diagnostic biomarker for PCOS. Its applicability in diagnostics is directly related to its role in the pathogenesis of the disease. AMH is synthesized by the granulosa cells of preantral and small antral ovarian follicles, modulating the early stages of folliculogenesis. There is evidence that hormone production in PCOS is increased due to intrinsic dysfunction in the granulosa cells themselves, with its high local concentration in the ovary disrupting the selection of the dominant follicle and leading to follicular arrest (Deans, 2019). Thanks to the development of sufficiently sensitive laboratory methods, it has been established that serum AMH levels are between 2 and 4 times higher in patients with PCOS. This increase is partly explained by the increased mass of granulosa cells, resulting from the large number of growing follicles. Due to its close correlation with the total number of ovarian follicles, the increased antral follicle count, and the increase in ovarian volume, AMH has high potential as a diagnostic marker for PCOS, as an alternative to ultrasound examination. In the latest edition of the International Guideline for the diagnosis and management of PCOS from 2023, AMH is included as a diagnostic criterion for PCOS in adults, although it is not recommended as a stand-alone test (Teede et al., 2023). Despite a number of

studies demonstrating its suitability as an appropriate marker in adolescents, AMH is still not accepted as a diagnostic criterion in this age group due to insufficient specificity (Ibanez et al., 2017, Teede et al., 2023).

Among the main objectives of the present study were investigating the potential of AMH as a diagnostic biomarker for PCOS in puberty, determining the optimal threshold value for differentiating between patients and healthy controls, and evaluating the possibilities for its implementation in clinical practice. Analysis of the hormonal profile of the study participants found statistically significantly higher AMH levels in girls with PCOS compared with the healthy control group ($p < 0.05$).

To assess the diagnostic performance (sensitivity and specificity) of the hormone and to define a threshold value distinguishing the two study groups, ROC curve analysis was performed. The results showed good discriminatory ability of the test, with an area under the curve $AUC=0.74$ ($p < 0.01$). By calculating the Youden index, the optimal diagnostic cut-off for PCOS was determined to be 23.6 pmol/l (3.3 ng/ml), characterized by 86% sensitivity and 65% specificity.

The results of the present study correlate to a large extent with the data published by Sopher et al. In a study of 31 non-obese girls ($BMI < 95$ th percentile) – 16 with PCOS and 15 healthy controls, the authors found significantly higher AMH levels in patients, as well as a positive correlation with androgen profile and ovarian volumes. They propose an AMH cut-off value of 3.4 ng/ml, very close to our defined threshold of 3.3 ng/ml (23.6 pmol/l). In the cited study, this value has 40% sensitivity and 93.8% specificity for predicting PCOS (Sopher et al., 2014). In contrast, the threshold established in our study shows significantly higher sensitivity (86%) but lower specificity (65%). This difference is most likely due to the smaller number of participants in Sopher's study ($n = 31$ versus $n = 59$ in our study).

Results close to ours are also reported by Wiweko et al., who define a diagnostic threshold of 4.45 ng/ml with 76.1% sensitivity and 74.6% specificity for PCOS (Wiweko et al., 2014). Higher reference limits are published by Yetim et al. – 6.1 ng/ml (sensitivity: 81.1%; specificity: 92.3%) (Yetim et al., 2016). In a large-scale meta-analysis covering 15 studies, Tsukui et al. recommend an AMH cut-off value for diagnosing PCOS in adolescence of between 6 ng/ml and 7 ng/ml, characterized by 66.3% sensitivity and 81% specificity (Tsukui et al., 2022). Compared with the

cited literature sources, the threshold value established by us of 23.6 pmol/l (3.3 ng/ml) stands out with higher diagnostic sensitivity (86%).

To establish the relationship between anti-Müllerian hormone (AMH) and the clinical manifestations characteristic of PCOS – hyperandrogenism, metabolic disturbances and morphological ovarian changes – partial correlation analysis was applied, controlling for the influence of age and BMI. The analysis registered a statistically significant positive correlation between AMH levels and the volumes of the right ($r=0.519$; $p<0.01$) and left ovary ($r=0.558$; $p<0.01$).

The causal relationships between the volumes of the two ovaries and AMH concentration were examined using linear regression analysis. Assessment of the individual regression coefficients showed that, in isolation, neither the right ovarian volume ($B=1.060$; $p=0.648$) nor the left ($B=5.156$; $p=0.052$) were significant predictors of hormone levels. In contrast, total ovarian volume (TOV) had a statistically significant influence on AMH levels ($R^2=0.304$; $F=12.236$; $p=0.002$), explaining 30.4% of their variability. An increase in TOV of every 1 cm³ is associated with a linear increase in AMH values of approximately 0.73 pmol/l. Consistent with our data, a direct correlation dependence between ovarian volume and AMH levels has been established in a number of international studies, which recommend using AMH as an alternative to ultrasound examination (Pawelczak et al., 2012; Sopher et al., 2014; Wiweko et al., 2014; Yetim et al., 2016).

In conclusion, the diagnostic threshold for AMH established in the present study, of 23.6 pmol/l (3.3 ng/ml), has high diagnostic performance, allowing accurate identification of 86% of girls with PCOS. The demonstrated statistically significant positive correlation with ovarian volume supports the potential application of AMH in clinical practice as an alternative to ultrasound examination. This advantage is particularly important for adolescence, when performing an ultrasound examination is often difficult and inaccurate. Based on the results obtained, we recommend the inclusion of AMH in the standard diagnostic panel for assessing PCOS in adolescents, but given its lower specificity, not as a primary marker, but as one supporting the main diagnostic criteria.

5. Cardio-metabolic disorders in the participants

1. Acanthosis nigricans

Acanthosis nigricans (AN) is a hyperpigmented area of skin with a velvety surface, symmetrically involving mainly the areas of the neck, axillae, popliteal fossae, etc. The etiology of AN is multifactorial, but this dermatological manifestation is most often associated with high serum insulin levels. The pathophysiological mechanism involves binding of insulin to insulin-like growth factor receptors, which triggers proliferation of epidermal cells (Tyebkhan et al., 2024). Patients with PCOS often have a high frequency of AN, since conditions such as obesity, insulin resistance (IR) and hyperinsulinemia are observed in a significant proportion of them. The scientific literature reports an AN frequency between 5.2% and 82%, depending on the presence of concomitant metabolic syndrome, IR, obesity, or type 2 diabetes mellitus (Tyebkhan et al., 2024). In a study covering 270 women with PCOS at a median age of 28 years, Schmidt et al. found AN in 36.9% of cases, demonstrating correlations between AN and BMI, IR, dyslipidemia and free testosterone levels (144). In a study among 175 girls with PCOS from Bangladesh with a mean age of 16.8 ± 1.7 years, Kamrul-Hasan et al. found AN in 77.7% of the individuals studied (Kamrul-Hasan et al., 2021).

In the present study, the frequency of AN is 55.5%, representing a statistically significant increase compared with the control groups ($p = 0.05$). The variation compared with the data of Kamrul-Hasan et al. is probably due to the ethnic characteristics of the populations analyzed, since it is known that AN shows a higher frequency in individuals with darker skin color (Kamrul-Hasan et al., 2021, Tyebkhan et al., 2024). In our study, in the group of patients with PCOS, significant positive correlations with HOMA-IR and insulin levels were registered, as well as moderate positive correlations with clinical manifestations of hyperandrogenism (hirsutism and FGS) and cardio-metabolic disturbances (obesity, elevated blood pressure, dyslipidemia). A moderate negative correlation with SHBG levels was also observed. These results are consistent with the correlations established by Schmidt and colleagues (Schmidt et al., 2015). The data obtained confirm the key role of IR and subsequent hyperinsulinemia as a pathogenetic factor for the development of hyperandrogenism (through stimulation of ovarian androgen synthesis) and as a risk factor for cardiovascular disease (through modulation of the lipid profile and blood pressure).

5.2 Blood pressure

Analysis of systolic and diastolic blood pressure values in the participants of the present study found no significant differences between the mean values of these parameters in the three study groups. Using the definition of elevated blood pressure according to the recommendations of the American Academy of Pediatrics (AAP) for adolescents over 13 years of age (systolic BP ≥ 130 mmHg and/or diastolic BP ≥ 80 mmHg) (Blanchette et al., 2019), elevated blood pressure was found in a total of 44.1% of all participants in the study, with differences between the groups being statistically non-significant. This result is entirely explainable, given that most of the participants in the study were overweight or obese.

Among girls with PCOS, the frequency of elevated BP was 47.2%, with moderate positive correlations found with the presence of obesity, HOMA-IR and insulin levels, as well as a moderate negative correlation with SHBG levels. These results support data on an increased risk of BP dysregulation as a result of the hyperinsulinism characteristic of PCOS, which causes an imbalance in the autonomic nervous system, reduced nitric oxide production, and increased renal sodium reabsorption (Marchesan et al., 2019). The negative relationship between BP and SHBG demonstrates the significance of hyperandrogenism, resulting from elevated free androgen levels, for the increased risk of hypertension as a result of androgen-induced vascular wall remodeling and activation of the renin-angiotensin-aldosterone system (Casella et al., 2006).

The frequency of elevated BP among patients with PCOS established in the present study differs from data published in the literature. Zhang and colleagues found elevated BP in 18.2% of patients with PCOS (Zhang et al., 2018), while Abbas et al. found hypertension in 11.1% of the girls they studied (Abbas et al., 2025). Studies in adult women with PCOS also find a lower frequency of elevated BP compared with our study. For example, in a meta-analysis including 10 studies in women, the established frequency of hypertension in patients with PCOS was 13.1% (Wekker et al., 2020). A slightly higher frequency, 17.6%, was found in one Bulgarian study (Robeva et al., 2025). The reasons for the higher frequency of elevated BP in our patients are probably methodological. BP values are influenced by various factors, such as anxiety or apprehension about upcoming procedures. To avoid this effect, it is recommended that BP be measured several times within a single visit (three times, per the protocol of the present study), which usually leads to a decrease in values, though these changes are not always large enough to

change the classification of BP as elevated (Flynn et al., 2017). In addition, measurements may differ significantly between visits. For example, one study among adolescents found that only 56% of the adolescents studied had elevated BP values at 3 different visits (McNiece et al., 2007). Nevertheless, given the significance of obesity, hyperinsulinemia, dyslipidemia and hyperandrogenism for the development of hypertension, it is advisable that BP be regularly monitored in girls with PCOS, in order to take timely measures for its regulation and to prevent future CV complications.

5.3. Glucose homeostasis

All participants in the present study underwent investigations of glucose homeostasis – fasting BG, fasting insulin and HOMA-IR. Analysis of the data found no statistically significant differences between the values of these parameters in the three study groups. No significant differences were found either with respect to impaired fasting glycemia (defined as fasting BG ≥ 5.6 mmol/l and ≤ 6.9 mmol/l), fasting hyperinsulinemia (defined as fasting insulin ≥ 15 μ IU/ml), or insulin resistance (HOMA-IR ≥ 2.6). In the group of patients with PCOS, impaired fasting glycemia (IFG) was registered in 5.3% of girls, a frequency comparable to that found in other studies. In a study of 66 girls with PCOS with a mean age of 15.8 ± 2.0 years, Flannery et al. found impaired glucose metabolism in 18.2% of patients. IFG was observed in 3%, impaired glucose tolerance (IGT) – in 15.2%, and type 2 diabetes – in 1.5%. The authors found no difference in the frequency of IGT based on the presence or absence of obesity, and recommend screening for glucose metabolism disorders via OGTT in all girls with PCOS, regardless of BMI (Flannery et al., 2013). In a retrospective cohort study covering 98 girls with PCOS and 150 girls with obesity aged between 13 and 18 years, who had 2 or more fasting BG measurements over at least 6 months, Javed et al. found no significantly different change in fasting glycemia levels or increased risk of developing IFG in patients with PCOS compared with controls. The authors conclude that BMI, rather than PCOS, is the strongest predictor of changes in fasting BG levels and the development of IFG. This suggests that, unlike IGT, the isolated risk of IFG in adolescents may be due mainly to obesity, rather than to the pathophysiology of PCOS (Javed et al., 2015). Similar results are reported by Palmert et al. in one of the earliest studies devoted to glucose tolerance disorders in girls with PCOS. In a study of 27 girls, 8 of them (29.6%) had IGT, and 1 was found to have previously undiagnosed type 2 diabetes. The authors note that

fasting BG and the usual surrogate markers of insulin resistance were poor predictors of IGT and diabetes in their study, while the glucose value at the 120th minute of the OGTT proved to be the most reliable screening method – a conclusion later confirmed repeatedly in larger cohorts (Palmert et al., 2002).

Taken together, these data show that, unlike IGT, which occurs regardless of BMI and is considered specifically related to the pathophysiology of PCOS, isolated IFG in adolescents remains a relatively rare finding (in the range of about 2-5% across individual studies) and appears to be determined to a greater extent by concomitant obesity than by the syndrome itself. The results of the present study, in which no significant difference in the frequency of IFG was found between the groups, are consistent with this trend and further support the thesis that testing fasting BG alone is not a sufficiently sensitive method for assessing carbohydrate metabolism disorders in girls with PCOS, and that performing OGTT remains the preferred screening approach, regardless of body weight (Flannery et al., 2013; Palmert et al., 2002).

This difference in the behavior of the two types of carbohydrate metabolism disorders probably reflects different underlying pathophysiological mechanisms. Kim et al. studied 143 women with PCOS and found that isolated IFG and isolated IGT are associated with different patterns of insulin resistance – while IGT is associated mainly with peripheral (muscle) insulin resistance, IFG is determined mainly by hepatic insulin resistance, manifested as an inability to adequately suppress hepatic gluconeogenesis and glucose production. In patients with combined disorder (IFG and IGT simultaneously), both types of insulin resistance were found (Kim et al., 2010).

The substantial differences in the pathophysiology of IFG and IGT also explain why simple fasting glucose measurement is an insufficient screening method in adolescents and adult women with PCOS. Since the predominant metabolic deficit in PCOS affects peripheral insulin sensitivity rather than hepatic gluconeogenesis, most carbohydrate metabolism disorders in this disease manifest as IGT rather than isolated IFG. Hyperandrogenemia and obesity, in turn, appear to contribute further to worsening hepatic insulin sensitivity, which could explain why BMI, rather than the PCOS diagnosis itself, emerges as a stronger predictor of IFG in adolescents in the study by Javed et al. (Javed et al., 2015; Kim et al., 2010).

Based on the data from the studies described above and the insufficient diagnostic value of IFG for detecting disorders of glucose homeostasis, we can recommend including OGTT in the diagnostic algorithm for PCOS in adolescence, regardless of the patients' BMI.

In the present study, after adjusting for BMI and age, positive correlations were found between insulin level and fasting hyperglycemia ($r=0.617$, $p<0.001$), HOMA-IR and FGS ($r=0.436$, $p=0.013$), and HOMA-IR and fasting hyperglycemia ($r=0.709$, $p<0.001$). Linear regression analysis to find a causal relationship between hyperandrogenism, BG, insulin and HOMA-IR found no significant influence of androgens on glucose homeostasis. This result differs from those described in the literature. In a number of studies (e.g., Zhang et al.), FAI is independently associated with the frequency of IGT and with IR markers even after adjusting for age, BMI and waist circumference (Zhang et al., 2018). A possible explanation for this difference is that most studies establishing a significant relationship between androgens and glucose metabolism examine BG values at the 120th minute of the OGTT, whereas when only fasting parameters are examined (as is the case in the present study), this relationship usually weakens or disappears, since hyperandrogenemia mainly affects peripheral insulin sensitivity, which is more clearly manifested after a glucose load (Baculescu et al., 2025; Zhang et al., 2018).

When assessing the influence of glucose homeostasis parameters on androgens and SHBG, a significant influence of HOMA-IR and insulin level on SHBG was found. The linear regression analysis performed showed that HOMA-IR and insulin level have a statistically significant negative influence on SHBG levels. The decrease in SHBG leads to an increase in free testosterone levels and intensification of the manifestations of hyperandrogenism.

These results correspond to the data in the literature. For example, in a study by Biernacka-Bartnik et al., a moderate negative correlation between HOMA-IR and SHBG was established, with patients with low HOMA-IR values having a significantly lower risk of reduced SHBG levels. This relationship is explained by the suppressive effect of compensatory hyperinsulinemia on hepatic SHBG synthesis, since HOMA-IR mainly reflects hepatic rather than muscle insulin resistance (Biernacka-Bartnik et al., 2023).

5.4. Lipid metabolism and CRP

Disorders of lipid metabolism are common in PCOS and are due to the combined effects of obesity, insulin resistance and hyperandrogenism. The most common lipid disorders found in women with PCOS are hypertriglyceridemia, elevated LDL- and VLDL-cholesterol, and decreased HDL-cholesterol (Osibogun et al., 2020). Obesity, through the associated insulin resistance and hyperinsulinemia, is a major factor in the development of dyslipidemia, manifested as increased triglyceride production. On the other hand, hyperandrogenism leads to activation of genes involved in the breakdown of HDL-cholesterol (Diamanti-Kandarakis et al., 2007).

In the present study, all participants were tested for lipid profile (total, LDL- and HDL-cholesterol and triglycerides) and CRP, as markers of CV risk. Analysis of the results found no significant differences in lipid metabolism parameters and CRP levels between the three groups. To determine the frequency of dyslipidemias in all participants, the NHLBI adolescent-age criteria for hypercholesterolemia (total cholesterol ≥ 5.17 mmol/l), hypertriglyceridemia (triglycerides ≥ 1.47 mmol/l), elevated LDL-cholesterol (LDL ≥ 3.36 mmol/l), and low HDL-cholesterol (HDL ≤ 1.03 mmol/l) were used (NHLBI Guidelines, 2011).

In our study, hypertriglyceridemia emerged as the most common lipid metabolism disorder both in girls with PCOS and in healthy controls – $n = 9$ (25%) versus $n = 3$ (20%), without a statistically significant difference between the two groups ($p = 0.283$). Decreased HDL-cholesterol is the second most common lipid homeostasis disorder in the PCOS group, registered in 16.7% of patients ($n = 6$). Among healthy controls, low HDL was found in 6.7% ($n = 1$), again with a non-significant statistical difference between the two groups ($p=0.323$). The difference in the frequency of elevated LDL between the two groups was also non-significant ($p=0.666$) – 1 girl with PCOS (2.8%) and 1 healthy control (6.7%). Elevated total cholesterol was found in 8.3% of girls with PCOS ($n = 3$) and in 6.7% of healthy controls ($n = 1$), with a statistically non-significant difference again ($p=0.698$). In the control group of girls with idiopathic hyperandrogenism, no lipid metabolism disorders were found.

In the PCOS group, after adjusting for the influence of age and BMI, a moderate positive correlation was found between total cholesterol and triglyceride levels ($r=0.434$, $p<0.05$), and a strong positive correlation between total cholesterol and LDL ($r=0.894$, $p<0.001$). TG showed a moderate inverse correlation with HDL ($r=-0.465$, $p<0.05$), while HDL showed a moderate

negative correlation with insulin level ($r=-0.413$, $p<0.05$) and a moderate positive correlation with SHBG level ($r=0.447$, $p<0.05$). Of all the lipid fractions studied, only LDL showed a moderate direct correlation with testosterone ($r=0.472$, $p<0.05$).

In the multiple regression analysis performed in the PCOS group, no influence of androgens on lipid and CRP levels was found. TG levels were significantly and positively influenced by markers of generalized and abdominal obesity (BMI and WC) and insulin level, while HDL levels were positively influenced by SHBG, and negatively influenced by BMI, WC and insulin.

The finding of higher triglyceride and lower HDL levels in the present study partly corresponds to the data published by Fruzzetti et al. In a study of 120 girls with PCOS and 30 healthy controls, the authors found significantly higher triglyceride levels and lower HDL-cholesterol, similar to the results of our study, as well as a positive and negative correlation of these lipids with free testosterone and FAI (Fruzzetti et al., 2009). The lack of statistical differences in lipid levels between the groups in our study, and the lack of correlation with FAI, is most likely due to the smaller number of participants.

In the most recent meta-analysis on the topic, combining 13 studies and 1789 girls aged 10-20 years, diagnosed exclusively according to adolescent-specific PCOS criteria, Tu et al. found that the PCOS group had significantly higher triglyceride values compared with controls, while no statistically significant differences were found regarding HDL-cholesterol (Tu et al., 2026). These results are consistent with the present study, although the difference does not reach statistical significance (probably due to the smaller number of participants). The lack of a significant difference in HDL-cholesterol between the groups is repeated in both analyses, and hypertriglyceridemia stands out as the most sensitive lipid parameter with respect to PCOS.

The established lack of correlation between hyperandrogenism and lipid disorders in the present study is consistent with current understanding that the main factor for dyslipidemia in PCOS is obesity and its associated insulin resistance, while hyperandrogenism has an independent and smaller effect, mainly with respect to HDL (Guo et al., 2022).

In the present study, as the second most common marker of increased CV risk after hypertriglyceridemia in the group of patients with PCOS, elevated CRP ≥ 3.0 mg/l emerged. This was found in 19.4% of girls with PCOS ($n = 7$) and 13.3% of healthy controls ($n = 2$), with a non-

significant statistical difference between the groups ($p=0.373$). No correlations were found between CRP and carbohydrate and lipid metabolism parameters, nor a relationship between CRP and androgens.

The lack of a significant difference in CRP between the groups and the lack of correlation with androgens in the present study coincide with data from a study in Indian adolescent girls, in which CRP showed a higher tendency in patients with PCOS, but without statistical significance compared with controls, with a significant positive correlation found only with BMI (Ganie et al., 2014). The authors conclude that CRP is related more to body weight than to PCOS itself. This contrasts with data in adult women, where CRP is significantly higher in PCOS. In a study including 116 women with PCOS and 94 controls, Boulman et al. found that 36.8% of patients with PCOS had CRP above 5 mg/l, compared with only 9.6% of controls (Boulman et al., 2004). The reason for this difference from the data found in adolescence is probably that the systemic low-grade inflammation associated with chronic insulin resistance has not yet manifested clinically at this earlier stage of the disease. A study in women with PCOS without obesity and IR found that CRP did not differ from controls despite a significantly higher FAI, suggesting that CRP increases mainly with concomitant obesity, rather than directly as a result of hyperandrogenism (Butler et al., 2023).

6. Polycystic ovarian morphology (PCOM)

Ultrasound PCOM is an image of the histopathological changes of the ovaries in PCOS, manifested as the presence of a large number of small antral follicles arrested in development at the preovulatory stage. In diagnostic algorithms for PCOS in adolescents, PCOM is not recommended as a diagnostic criterion for several reasons. In adolescent girls, the use of a transvaginal transducer is not possible, and transabdominal access is not sufficiently informative regarding the number of antral follicles. Ovarian volume in adolescence is larger than in adult women, and a multicystic ovarian appearance is considered a normal variant for up to 8 years after menarche and is not associated with hyperandrogenism. Thus, about 1/3 to 1/2 of healthy adolescent girls meet the criteria for PCOM without having PCOS (Peña et al., 2020; Rosenfield, 2015). Because of these features, it is recommended that PCOM be used only as a supplementary criterion in the diagnosis of PCOS, with ovarian volume being more appropriate than follicle

count. An ovarian volume above 12 ml is considered increased by >2 SD (Ibanez et al., 2017; Rosenfield, 2015).

In the present study, no significant difference in ovarian volumes and the presence of PCOM was found between the groups. In the multiple correlation analysis, adjusting for age, only the already-described positive correlation with AMH was found, but not correlations with clinical and biochemical hyperandrogenism, nor with metabolic parameters.

The lack of a significant difference in ovarian volumes and the presence of PCOM between the groups in the present study is a well-documented and expected finding in the literature. A review article notes that ovarian volume reaches its maximum 1.2-3.8 years after menarche, and during this period follicle count and ovarian volume can significantly overlap between adolescents with PCOS and healthy controls (Welt et al., 2013). The International Guideline for diagnosing PCOS in adolescents recommends avoiding ultrasound examination for up to 8 years after menarche due to this overlap with normal pubertal physiology and the low specificity of PCOM for diagnosing PCOS in this age group (Bahreiny et al., 2023).

7. MicroRNAs (miRNA)

In recent years, a number of studies have drawn attention to the diagnostic potential of various microRNAs (miRNA) as biomarkers for PCOS or its associated metabolic disturbances, but mainly in women of reproductive age. The number of studies concerning adolescents with PCOS is very limited.

In the present study, 6 preselected miRNAs were investigated - miR-451a, miR-15a-5p, miR-320-5p, miR-28-5p, miR-103a-5p and miR-194-5p. Of these, miR-451a, miR194-5p and miR-15a-5p have been studied in girls, while the others had not previously been studied in PCOS.

Analysis of the selected miRNAs found that four of them show different expression in patients with PCOS compared with the control groups. MiR-320a-5p, miR-103a-5p, miR-15a-5p and miR-194-5p are overexpressed in patients with PCOS compared with healthy controls, and miR-15a-5p is also elevated in girls with polycystic ovaries compared with the hyperandrogenic control group. For the remaining two microRNAs, miR-451a and miR-28-5, no statistically significant differences in expression were found among the three study groups.

Of the four overexpressed miRNAs in girls with PCOS, miR-320-5p and miR-103a-5p show the highest specificity and sensitivity for diagnosing the syndrome (76% sensitivity, 100% specificity), followed by miR-15a-5p (76% sensitivity, 91% specificity), and these miRNAs could be used as independent diagnostic markers in patients with the disease. In addition, miR-15a-5p showed a very good sensitivity profile in distinguishing girls with PCOS not only from healthy girls, but also from patients with idiopathic hyperandrogenism (82% sensitivity, 75% specificity).

The first published study of miRNA levels in adolescent patients is by Diaz and colleagues. It includes 31 non-obese girls with PCOS and 13 controls. MiR-451a, miR-652-3p, miR-106b-5p and miR-206 show significantly decreased expression in girls with PCOS compared with controls (Diaz et al., 2020). The authors found that miR-451a was 100% sensitive and 100% specific, miR-652-3p was 81% sensitive and 100% specific, and miR-106b-5p was 100% sensitive and 85% specific for diagnosing PCOS. For miR-451a, miR-652-3p, miR-106b-5p and miR-206, the authors found an inverse correlation with the degree of androgen excess and HOMA-IR, and a positive correlation with SHBG levels (Diaz et al., 2020). In the present study, no significant differences were found in the relative expression levels of miR-451a between patients with PCOS and controls, and the described correlations were not confirmed. Although the number and age of participants in the two studies are similar, in our study the majority of participants are overweight or obese, which probably contributes to the difference in results.

In a study of women with PCOS, Udesen et al. found significantly decreased expression of serum miR-28-3p, miR-103a-3, miR-139-5p and miR-376a-3p. The authors found correlations of miR-28-3p and miR-139-5p with total testosterone and the LH:FSH ratio. MiR-28-3p shows a specificity of 100% and a sensitivity of 59% for diagnosing PCOS, and there are reports that this miRNA is associated with granulosa cell proliferation (Udesen et al., 2023). In the present study, the relative expression of miR-28-5p was assessed, which has also been reported to be associated with granulosa cell proliferation and has been found to be dysregulated in ovarian and other types of carcinoma (Meng et al., 2019), but no significant differences were found in its relative expression between patients and control groups.

MiR-320 participates in glucose and lipid metabolism and is a potential biomarker for related metabolic disorders (Du et al., 2021). In several studies of miR-320 expression in women with

PCOS, mixed results have been found. For example, Krentowska et al. found low serum expression levels of miR-320 in women with PCOS compared with healthy controls, and correlations with fasting BG, insulin levels and HOMA-IR, independent of age, BMI and androgen levels (Krentowska et al., 2024). Rashad and colleagues also found lower serum expression levels of miR-320 in patients with PCOS compared with healthy controls, as well as a negative correlation with fasting serum insulin, HOMA-IR and PCOS phenotype. According to the authors, the sensitivity of serum miR-320 expression levels for diagnosing PCOS is 80%, and the specificity 97.5% (Lewy et al., 2001). On the other hand, Soyman et al. found no significant differences in miR-320 expression levels in 50 women with PCOS and 50 BMI- and age-matched healthy controls (Soyman et al., 2022).

In the present study, levels of miR-320-5p, a member of the miR-320 family not previously studied in PCOS, were assessed. MiR-320-5p (and miR-320) is described as a tumor suppressor, influencing cellular proliferation and migration (Li et al., 2021). In PCOS, there is a significant degree of proliferation and a low degree of apoptosis of granulosa cells, which is associated with abnormal folliculogenesis leading to anovulation (Ruiz-Manriquez et al., 2022). In our study, miR-320-5p is significantly overexpressed in patients with PCOS compared with healthy controls ($p = 0.0006$). It also showed a tendency toward overexpression in the PCOS group compared with the hyperandrogenic control group ($p = 0.079$). In the PCOS group, miR-320-5p correlated positively with FGS and CRP and negatively with glucose level. The regression analysis performed found that SBP has a positive influence on the level of miR-320-5p.

In humans, miR-103 has been found in blood and ovarian granulosa cells and is involved in steroid synthesis, insulin signaling and folliculogenesis (De Nardo Maffiazioli et al., 2022; Sørensen et al., 2014). Murri et al. found decreased expression of miR-103 in women with obesity, but increased expression in patients with PCOS, as well as a positive correlation with testosterone concentrations (Murri et al., 2013). De Nardo Maffiazioli and colleagues reported decreased expression of miR-103a-3p and a positive correlation with androgen levels in 36 Brazilian women with PCOS (De Nardo Maffiazioli et al., 2022). In our study, we investigated the expression of miR-103a-5p, which had not previously been assessed in patients with PCOS. Similar to Murri and colleagues, we found significantly increased expression in the PCOS group compared with healthy controls ($p = 0.0005$), but without significant differences in expression

compared with the hyperandrogenic control group ($p = 0.061$). In the PCOS group, miR-103a-5p showed a positive correlation with SBP and hair loss and, unlike the aforementioned studies, no correlations with other metabolic parameters or androgen levels. The multiple regression analysis performed found a significant positive influence of CRP on the expression of miR-103a-5p. CRP has been validated as a factor for increased cardiovascular risk, together with elevated LDL cholesterol levels (Wong et al., 2022), and has been found elevated in patients with PCOS in a number of studies (Rudnicka et al., 2021).

MiR-194 has been found to be overexpressed in granulosa cells of women with PCOS and in rat models of PCOS, where it induces apoptosis (Wu et al., 2021). Jiang et al., in their study including 65 women with PCOS with impaired glucose metabolism, 65 women with PCOS with normal glucose metabolism, and 45 healthy women, found that miR-194 is overexpressed in women with PCOS and correlates with HOMA-IR (Jiang et al., 2016). Similar to these results, in the present study we found significant overexpression of another miRNA from the miR-194 family. MiR-194-5p is significantly overexpressed in patients with PCOS compared with the healthy control group and shows 65% sensitivity and 81% specificity for diagnosing PCOS. No significant differences were found in its expression compared with the hyperandrogenic control group. In the PCOS group, miR-194-5p correlated positively with SBP, and the regression analysis performed found that its level is significantly influenced by CRP. In the literature, miR-194-5p is reported to have a regulatory role in various types of cancer and has been proposed as a biomarker for breast carcinoma (Yen et al., 2021), but, to our knowledge, has not been evaluated or reported in patients with PCOS to date.

MiR-15a-5p has been found to be significantly overexpressed in the plasma and tumor tissue of patients with endometrial carcinoma (EC) and has been identified as a promising biomarker for its early detection. A relationship has been found between its level and testosterone, which is elevated in PCOS, as well as a correlation with DHEA-S (Du et al., 2021). These findings determined the choice to investigate miR-15a-5p in the present study. It was found to be significantly overexpressed in the PCOS group compared with both control groups ($p = 0.006$ and $p = 0.045$), but showed higher specificity in distinguishing patients with PCOS from healthy controls than in distinguishing patients with PCOS from the hyperandrogenic control group. In addition, miR-15a-5p showed a significant positive correlation with FGS in the PCOS group. The

regression analysis performed found a significant positive influence of some of the CV risk parameters (SBP and CRP) on the expression of this miRNA.

In conclusion, in the present study four significantly overexpressed miRNAs were identified in girls with PCOS. MiR-320-5p and miR-103a-5p showed optimal characteristics as diagnostic markers for PCOS, followed by miR-15a-5p, and these miRNAs could be used as potential independent diagnostic biomarkers for the disease. In addition, miR-15a-5p also showed a very good sensitivity profile in distinguishing patients with PCOS from girls with other hyperandrogenic conditions. The clinical usefulness of these biomarkers must be validated through additional studies including more patients and healthy controls. The precise role of these miRNAs in the etiopathogenesis of PCOS and their correlation with PCOS- associated comorbidities must be further clarified.

PART VI. MAIN CONCLUSIONS

On Objective 1

1.1. Oligomenorrhea is the main clinical manifestation of PCOS in adolescence and is found in all patients.

1.2. Patients with PCOS have pronounced manifestations of clinical hyperandrogenism, the most common being hirsutism, followed by seborrhea and acne. Androgenic alopecia is a very rare hyperandrogenic manifestation in adolescence.

1.3. The Ferriman-Gallwey score is significantly higher in girls with PCOS compared to girls without the syndrome. $FGS \geq 6$ can distinguish patients with PCOS from healthy girls with good sensitivity. FGS correlates positively with hirsutism, HOMA-IR and insulin levels, with HOMA-IR being a statistically significant predictor of the severity of hirsutism.

1.4. Overweight and obesity are found in 2/3 of girls with PCOS.

1.5. Acanthosis nigricans occurs significantly more often in girls with PCOS and correlates positively with obesity, clinical hyperandrogenism, blood pressure and dyslipidemia, and negatively with sex hormone binding globulin.

1.6. Girls with PCOS have significantly higher levels of testosterone and androstenedione, as well as a significantly higher free androgen index (FAI) compared to healthy girls.

On Objective 2

2.1. FAI with a cut-off value of 6.2 is the marker with the highest specificity (90%) and sensitivity (72%) in distinguishing adolescent patients with PCOS from healthy girls,

2.2. A cut-off value of androstenedione of 11.6 nmol/l distinguishes girls with PCOS from healthy controls with high specificity (100%) and good sensitivity (69%).

2.3. AMH with a cut-off of 23.6 pmol/l (3.3 ng/ml) distinguishes adolescent patients from healthy controls with high sensitivity (86%) and good specificity (65%).

2.4. FAI, androstenedione and AMH are suitable for inclusion in the general panel of diagnostic criteria for PCOS in adolescence.

On Objective 3

3.1. Total ovarian volume is a significant predictor of AMH level.

3.2. No correlations are found between AMH level, androgens, metabolic and SS indicators.

3.3. No correlations and causal relationships with metabolic and SS indicators are found for androstenedione and FAI.

On Objective 4

4.1. Increased systolic and/or diastolic BP is found in almost half of the girls with PCOS and correlates positively with the presence of obesity, acanthosis nigricans, HOMA-IR, insulin levels, and negatively with SHBG levels.

4.2. The frequency of glucose homeostasis disorders is high in girls with PCOS, the most common being insulin resistance (IR) and fasting hyperinsulinemia. Increased fasting glycemia is rarely found. In our PCOS group, we did not register patients with type 2 diabetes mellitus.

4.3. Hypertriglyceridemia is the most common lipid metabolism disorder in girls with PCOS and is found in 1/4 of them. The next most common disorder is low HDL-cholesterol, followed by hypercholesterolemia and high LDL-cholesterol.

4.4. TG, HDL and LDL follow the known patterns in obesity and PCOS in adults, including their influence on SHBG.

4.5. No statistically significant influence of androgens on lipid disorders was found.

On Objective 5

5.1. In girls with PCOS, miR-320-5p and miR-103a-5p show the highest specificity (100%) and sensitivity (76%) for diagnosing the disease.

5.2. MiR-15a-5p distinguishes patients from healthy girls with 91% specificity and 76% sensitivity.

5.3. MiR-15a-5p also shows a very good sensitivity profile in distinguishing girls with PCOS from other hyperandrogenic conditions (75% specificity, 82% sensitivity).

5.4. In combination, these miRNAs could be used as independent diagnostic markers in patients with PCOS.

5.5. For the diagnostic for PCOS miRNAs, positive correlations were found with systolic blood pressure (SBP), FGS, CRP.

5.6. No causal relationships were established between miRNA expression and hyperandrogenism, disorders of glucose and lipid metabolism, and ovarian morphology.

PART VII. CONTRIBUTIONS OF THE DISSERTATION

1. The scientific project is the first in the country and the second worldwide to study the expression of selected microRNAs in girls with PCOS and control groups.
2. The present scientific work is the first in the country and worldwide to study the expression of miR-320a-5p, miR-103a-5p, miR-15a-5p and miR-194-5p in girls with PCOS and to determine their sensitivity and specificity as diagnostic markers for PCOS.
3. The present study is the first in the country and one of the few worldwide to determine the most sensitive and specific diagnostic cut-off value of AMH in adolescent girls with PCOS.
4. The present study is the first in the country and one of the few worldwide to determine the most sensitive and specific diagnostic cut-off value of androstenedione and FAI in adolescent girls with PCOS.
5. This is the first study in the country to comprehensively examine PCOS specifically for adolescence, and including a detailed clinical and laboratory evaluation.
6. This study is the first in the country to examine the clinical and laboratory profiles exclusively of patients with PCOS in adolescence.
7. This study is the first in the country to determine the frequency of overweight, obesity, metabolic and cardiovascular disorders in girls with PCOS.
8. This scientific work provides an opportunity to enrich the diagnostic algorithm for PCOS in girls under 18 years of age with new highly specific and sensitive biomarkers.

PART VIII. CONCLUSION

The present dissertation comprehensively and in-depth examines the clinical and laboratory characteristics of PCOS in adolescence, as well as the frequency of associated comorbidities and complications, some of which are already present in this young age group. The data obtained would help to improve the comprehensive care of girls with the disease and reduce or prevent future negative consequences on the overall health of the patients.

The scientific study also enriches the set of markers for earlier and more reliable diagnosis of PCOS in adolescence, when the current valid diagnostic criteria overlap with physiological phenomena characteristic of puberty.

A major limitation of the present scientific work is the relatively small size of the control groups ($n = 15$ and $n = 8$) compared to the patient sample ($n = 36$), which leads to an incompletely balanced design. This limitation was imposed by the strict criteria for selecting clinically healthy girls. To minimize the impact on statistical power, conservative nonparametric analysis methods were applied.

Due to the relatively limited total number of participants ($n = 59$), the results should be interpreted with some caution, as they may not fully reflect the characteristics of the entire population. However, the results obtained coincide with the trends in the international literature and serve as a good basis for future larger-scale studies.

In conclusion, the present dissertation provides valuable data on PCOS in adolescence and enriches the set of diagnostic criteria for the disease. Future multicenter studies with larger and balanced samples are needed to confirm these findings.

PART IX. SCIENTIFIC PUBLICATIONS, PRESENTATIONS AND PARTICIPATIONS RELATED TO THE DISSERTATION

Full-text scientific publications:

1. Mladenov, V.; Radanova, M.; Galcheva, S.; Iotova, V. Selected microRNAs as Potential Diagnostic Biomarkers in Polycystic Ovary Syndrome in Adolescent Girls. *Appl. Sci.* 2025, 15, 2772. <https://doi.org/10.3390/app15052772>
2. Mladenov, V., Radanova, M., & Iotova, V. (2024). Polycystic ovary syndrome in adolescents—controversial diagnostic criteria and potential new biomarkers. *Scripta Scientifica Medica*, 56(2), 7-13.
3. Mladenov, V., S. Galcheva, T. Karamfilova, V. Iotova. Hyperandrogenism in girls of pubertal age – comparison of clinical, biochemical and hormonal characteristics. *Science Endocrinology*, Issue 2 (77) / 2023, p. 73
4. Mladenov, V. “Contemporary aspects of the diagnosis and treatment of polycystic ovary syndrome in adolescents.” *Pediatrics* 62.1 (2022): 14.

International participations:

1. Mladenov V, Galcheva S, Radanova M, Iotova V. Comparison between, clinical, metabolic and hormonal parameters and selected microRNAs in adolescent girls with PCOS and healthy controls. 62nd Annual Meeting of the European Society for Paediatric Endocrinology (ESPE), Liverpool, United Kingdom, November 16–18, 2024.

Participations in Bulgaria:

1. V. Mladenov, M. Radanova, V. Iotova. New biomarkers for the diagnosis of polycystic ovary syndrome in adolescence. Presentation at the XIII National Congress of Endocrinology, 02-05.10.2025, Plovdiv
2. V. Mladenov. Potential new diagnostic biomarkers for polycystic ovary syndrome in adolescence. Presentation at the XVII national conference on clinical laboratory medicine, 18-20.09.2025, Varna

3. V. Mladenov. New biomarkers for polycystic ovaries in adolescence. Presentation at the ninth pediatric meeting “Caring for Children”, 24-27.04.2025, Sveti Vlas

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