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STUDY PROTOCOL

Ovarian Hyperandrogenism in Adolescent Girls and Concomitant Metabolic Changes, Oxidative Stress, Inflammation, Oral Health and Their Relation to Diet

1. Introduction

Hyperandrogenism in women is a state of increased androgen production, manifested by irregular menstrual cycles, a more frequent incidence of infertility, metabolic syndrome, oxidative stress, and inflammation, as well as acne and hirsutism. In addition to ovulation disorders and the multifollicular ovaries structure in an ultrasound examination, hyperandrogenism is a typical clinical feature of the Polycystic Ovary Syndrome (PCOS), which is diagnosed in approximately 8.6% of adolescent girls. Excessive body weight makes it difficult to diagnose at an early stage of the disease and intensifies metabolic and hormonal disorders, as well as those related to oxidative stress and inflammation of the body. Some research studies suggest that PCOS may negatively affect oral health of women, increasing the risk of periodontal problems.

The main purpose of this research was to check whether serum androgen levels and concomitant metabolic changes, oxidative stress, inflammation and oral health in adolescent girls with hyperandrogenism may be related to diet.

The investigators aimed to identify the factors that increase the risk of being overweight and of obesity among adolescents with clinical features of PCOS, related to diet and DEA(Disordered Eating Attitudes).

The objective was also to investigate the relationships between markers of oxidation and markers of Systemic Inflammation and macronutrients intake, such as carbohydrates and fiber, protein (animal, plant), fats and cholesterol in normal and overweight/obese girls with clinical features of PCOS.

Additionally, the investigators would like to evaluate the relation between oral health of girls with PCOS (including condition of periodontium and dental caries) and their dietary habits, hormonal, metabolic and oxidative and inflammatory status.

2. Characteristics of tested population

The research will be carried out with the participation of 200 Caucasian adolescent girls, patients at the Gynecology and Obstetrics Hospital of Poznan University of Medical Sciences and Department of Perinatology and Gynecology, Division of Developmental Gynecology and Sexology, Poznan University of Medical Sciences, aged 13–18 years

Inclusion Criteria:

- Gender: Female
- Minimum Age: 13 Years
- Maximum Age: 18Years
- Written consent for inclusion (girls and their parents)
- clinical and/or biochemical hyperandrogenism (hirsutism with moderate to severe acne, and/or elevation of serum total testosterone or free testosterone,
- oligoovulation (based on oligomenorrhea defined as bleeding episodes occurring less than 8 times per year or secondary amenorrhea),
- polycystic ovarian picture in an ultrasound examination (at least 12 follicles in each ovary each measuring 2-9 mm in diameter and/or ovarian volume >10 mL).

Exclusion Criteria:

- any systemic disease, thyroid dysfunction, diabetes, congenital adrenal hyperplasia, Cushing syndrome, hyperprolactinemia suggestive of pituitary adenoma and androgen-secreting tumors
- medications of continuous use,
- the use of hormonal therapy or antibiotics in the past three months,
- vitamin or supplements use,
- alcohol consumption
- smoking

3. Methods

3.1. Evaluation of anthropometric indices and body composition

3.1.1. Anthropometric Assessment

Body mass index BMI: derived from measured body height (m) and body weight (kg) as body weight / height². Weight will be measured to the nearest 0.1 kg using digital medical scales. Height will be measured with a stadiometer attached to the scales.
Diagnosis and classification of overweight and obesity will base on BMI, according to World Health Organisation (WHO) for children aged 5–19 years overweight and obesity correspond to BMI-for-age greater than 1 standard deviation and 2 standard deviations above the WHO growth reference median, respectively.
Waist circumference (WC) will be measured to the nearest 0.1 mm using Gulick anthropometric tape between the lower border of rib margin and the upper border of iliac crest (WC-mid).

3.1.2. Body Composition Assessment

All measurements will be conducted after a 12 h fast. Body composition will be assessed with a Tanita MC 780 body composition analyzer, which uses bioelectrical impedance analysis (BIA method). In this model, the accuracy of measurement for the individual components, including adipose tissue, is 100 g. The measurements of fat mass (FM) will be expressed as a percentage (%) and kilograms (kg).

3.2. Biochemical parameters

Blood samplings performed in the early follicular phase (days 3–5).
Hormonal and biochemical parameters, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), total testosterone, androstendione, estradiol, dehydroepiandrosterone (DHEA-S), Sex Hormone Binding Globulin (SHBG), fasting glucose and fasting insulin, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG), 17 OH-Progesterone, Prolactin, Cortisol, Thyroid-stimulating hormone (TSH), will be measured in the morning after overnight fasting.

Biochemical analyses will be performed in the central hospital laboratory of the Gynecology and Obstetrics Hospital of Poznan University of Medical Sciences.
Serum concentrations of the inflammation markers: Interleukin 1 (IL-1), Interleukin 6 (IL-1), Tumor Necrosis Factor (TNF-α), C-reactive protein (CRP) and the markers of oxidation: malondiaeldehyde, total antioxidant capacity will be measured with a commercial ELISA assay kits in the Department of Clinical Biochemistry and Laboratory Medicine, Poznan University of Medical Sciences .

3.3. **Saliva assays**
Samples of unstimulated saliva will be taken with a use of a saliva cotton roll commercial collection device at the dental office (University Center of Stomatology and Specialist Medicine). Biochemical analysis of the salivary TNF-alfa, IL-6, IL-1β, and testosterone will be carried out with the use of commercial ELISA assay kits.

3.4. Dental examinations
The state of oral hygiene will be evaluated according to the Silness-Löe plaque index (PI) on the six index teeth: 16, 12, 24, 36, 32, 44. The gingival health will be assessed with the use of the Gingival Index (GI) by Löe and Silness on the same index teeth. The probing depth (PD) measurements will be done using a Williams manual periodontal probe. Bleeding upon probing score (BOP%) will be assessed as the proportion of bleeding sites when stimulated by a periodontal probe (the measurements recorded on all teeth present. Dental caries experience will be presented as a number of teeth with decay (D), missing due to decay (M), and filled due to decay (F) (DMT index).

3.5. Assessment of subgingival microflora
Samples of dental plaque will be collected from each patient by inserting paper points into buccal sites of gingival sulci. A real-time PCR method for the identification of 9 subgingival microorganisms: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, *Prevotella intermedia, Peptostreptococcus micros*, *Eubacterium nodatum*, and *Capnocytophaga gingivalis*, as well as total subgingival bacterial count.

3.6. **Nutrition Evaluation**
Nutrition evaluation will be conducted by continuous recording of all consumed foodstuffs, dishes and beverages, specifying home measures and weight (using "The Album of photographs of products and dishes"). The method used in this study to assess eating habits will be the qualitative-quantitative method of current quotation (3-day food record). Data will be analysed using The Aliant computer program (Cambridge Diagnostics).
In each case, the analyzed daily food intake will be compared with the current, individual daily nutritional and energy requirement in accordance with the Human Nutrition Standards of the Food and Nutrition Institute, taking into account the availability of energy at the level recommended for girls.

3.7. **Disordered Eating Attittudes**

Eating behavior will be assessed with a standard Eating Attitudes Test-26 (EAT-26), a self-administrated questionnaire. The questionnaire consists of 26 questions related to attitudes and beliefs, behavior related to eating, the perception of appearance, and body weight.
The participant replies to a question by selecting one response: always, usually, often, sometimes, rarely, never. In the first 25 questions, the answer 'always' is worth 3 points, 'usually', 2 points, and 'often', 1 point. In question 26, the answer 'never' is worth 3 points, 'rarely', 2 points, and 'sometimes', 1 point. A total score of 20 or more points indicates DEA (Disordered Eating Attittudes).

3.8. The Data From The Child's Health Book

The data were collected from the child's health book regarding newborn body weight and term of delivery. The incidence of macrosomia (body weight over 4000 g) or low birth weight, LBW (body weight less than 2500 g) was assessed on the basis of the newborn's body weight. The full term of

delivery (term births) will be assessed for deliveries that occur between 38–42 weeks of pregnancy, while preterm births will be defined as those deliveries between 23 weeks and 37 weeks.

3.9. Evaluation of physical activity

For this purpose the International Physical Activity Questionnaire will be applied, including records of the type and specific character of the training and activity, its duration, intensity and frequency.

3.10. Statistical analysis

The analyses will be performed in the PQStat v1.8.0 program and the Statistica software version 12 (StatSoft. Inc. 2014, Tulsa, USA) with significance taken as $p < 0.05$. Descriptive statistics of groups will be presented as mean and standard deviation, median and quartiles, as well as numbers and percentages, depending on the data type. The normal distribution of the data will be verified using the Shapiro–Wilk test, and the comparison of the variance of variables between the groups will be performed using the Fisher–Snedecor test. Quantitative data describing two independent groups and meeting the normal distribution will be compared using the Student's t-test or the Student's t-test with the Cochran–Cox correction, when the variances of the groups being compared are significantly different. The Mann–Whitney test will be used when the assumption of normal distribution is not met. The dichotomous data will be compared with the chi-square test or the Fisher's exact test when the Cochran conditions for using the chi-square test is not met. For the ordered category data, the Cochran–Armitage trend test will be used. Pearson correlation coefficient (for normally distributed variables) and Spearman's rank correlation (when the assumption of normality is not met) will be calculated to evaluate association between two quantitative variables.
Logistic regression (univariate and multivariate) will be carried out to identify the independent predictors of dichotomous outcomes. A multiple linear regression analysis model will be built to explain the selected dependent quantitative variables.

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