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Increased levels of serum granzyme-B is associated with insulin resistance and increased cardiovascular risk in adolescent polycystic ovary syndrome patients

Efser Oztas [a,*], Sibel Ozler [a], Aytekin Tokmak [a], Nafiye Yilmaz [a], Huseyin Tugrul Celik [b], Fatmanur Hacıevliyagil Kazancı [b], Nuri Danisman [a], Merve Ergin [c], Halil Ibrahim Yakut [d]

[a] Zekai Tahir Burak Women's Health Education and Research Hospital, Department of Obstetrics and Gynecology, Ankara, Turkey
[b] Turgut Ozal University Faculty of Medicine, Department of Clinical Biochemistry, Ankara, Turkey
[c] Yildirim Beyazit University Faculty of Medicine, Department of Clinical Biochemistry, Ankara, Turkey
[d] Zekai Tahir Burak Women's Health Education and Research Hospital, Department of Pediatrics, Ankara, Turkey

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ABSTRACT

Objectives: Our aim was to determine serum perforin and granzyme-B levels in adolescent PCOS patients, and to investigate whether they are associated with some of the insulin sensitivity, obesity and cardiovascular (CV) risk markers and metabolic syndrome.
Study design: A case–control study was carried out including a total of 172 adolescents (83 PCOS patients and 89 age-matched healthy controls). Participants were recruited consecutively. Homeostasis model assessment (HOMA-IR), lipid parameters, and anthropometric measurements were determined. Serum perforin and granzyme B levels were measured by commercially available ELISA kits. HOMA-IR > 3.16 was considered to indicate the presence of insulin resistance. Logistic regression analysis was applied for the predictive value of granzyme-B for increased CV risk in PCOS patients.
Results: As body mass index (BMI) of the PCOS patients was significantly higher than the controls (median 24.6 kg/m^2 and 21.4 kg/m^2, respectively, $p < 0.001$) all parameters were evaluated after adjustment for BMI. Adolescents with PCOS had significantly higher levels of fasting glucose, insulin, HOMA-IR and granzyme-B when compared with controls. According to the results of logistic regression analysis, granzyme-B levels were found to be significantly associated with increased HOMA-IR (OR = 6.120, 95% CI: 2.352–15.926, $p < 0.001$) in adolescent PCOS patients. Additionally, elevated levels of serum granzyme-B were predictive for increased CV risk in PCOS patients (OR = 0.237, 95% CI: 0.091–0.616, $p = 0.003$).
Conclusions: Increased levels of serum granzyme-B are independently associated with insulin resistance and also with increased CV risk in adolescent polycystic ovary syndrome patients.

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common female endocrinopathy of reproductive age [1]. Since the definition of PCOS was revised by the Rotterdam Consensus in 2003, the prevalence has increased up to 16.6% [2,3]. Previous studies have already shown an increased prevalence of cardiovascular diseases (CVD) and subclinical atherosclerosis in PCOS patients [4]. The increased CV risk in PCOS is attributed mainly to the association of this syndrome with insulin resistance (IR), dyslipidemia, obesity, type 2 diabetes and inflammation, which are already known risk factors for CVD [4–6].

Previously PCOS was recognized as a disorder of adulthood, but now it is accepted as a lifelong condition potentially originated from prenatal period, with a natural course to adolescence and adulthood [7,8]. Since PCOS emerges at puberty and has significant adverse long-term consequences, early intervention is of great importance. Besides, PCOS itself is proposed as a risk factor of atherosclerosis and screening for dyslipidemia is further suggested for the identification of youth at risk for early CVD [9]. So it is evident that the common underlying etiopathogenetic mechanism of PCOS and associated cardiometabolic morbidities should be determined in early stages of life.

* Corresponding author at: Zekai Tahir Burak Women's Health Education and Research Hospital, Talatpasa Street, 06230 Samanpazarı, Ankara, Turkey.
Tel.: +90 0312 3065000; fax: +90 0312 3124931; mobile: +90 0532 6670902.
E-mail address: efseroztas@gmail.com (E. Oztas).

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Recently, two studies demonstrated an expansion of $CD4^+CD28^{null}$ T lymphocytes in all PCOS phenotypes independent from weight [10,11]. The activation of $CD4^+CD28^{null}$ T cells has been already shown to cause the production of inflammatory cytokines and the expression of cytotoxic molecules such as perforin and granzyme-B [12,13]. These cells were also isolated from ruptured atheromas and were suggested to participate in the early stages of atherosclerosis as well [14,15] so it would be proper to propose a causal relation between $CD4^+CD28^{null}$ T cells and CV risk in PCOS patients. Additionally, Giubilato et al. demonstrated the association of $CD4^+CD28^{null}$ T cells with poor glycemic control, the first cardiovascular event and adverse outcomes in type 2 diabetic patients [16]. Furthermore, Yang et al. demonstrated markedly increased granzyme-B^+ adipose-resident T cells in obese mice, suggesting an implication for obesity associated metabolic syndrome, inflammation and insulin resistance [17]. As a result of diabetes, obesity and CVD, the factors associated with PCOS were found to share a common feature: the activation of cytotoxic T cells and the release of cytotoxic enzymes such as perforin and granzyme B.

Perforin, pore-forming protein and granzyme-B, a serin protease are stored in secretory granules inside the cytotoxic T lymphocytes (CTL) and natural killer cells (NK) and together induce the activation of caspase-dependent apoptotic pathways in the target cells [18]. Besides, granzymes were recently recognized to contribute in inflammation and to have additional non-apoptotic, extracellular activities [19]. Previous studies have suggested that perforin and granzyme-B play important roles in CVD and diabetes [20,21].

Based on the results of above-mentioned studies, we hypothesized that serum perforin and granzyme-B levels may be associated with increased CV risk and insulin resistance in PCOS patients. Although CVD are thought to be disorders of older age, it has been proposed that all PCOS patients including adolescents and young adults should be screened for CV risk factors, especially for dyslipidemia, because the probable derangements at a younger age may increase the development of CVD later in life [9,22]. Also, PCOS is identified as an additional risk factor for coronary artery diseases (CAD) and dyslipidemia is proposed to be screened in all patients with CAD risk factors even irrespective of the age. So, we decided to enroll adolescent PCOS patients to see whether they have cardiometabolic profiles leading to increased CV risk later in life or not.

Our aim was to determine serum perforin and granzyme-B levels in adolescent PCOS patients, and to investigate whether they are associated with some of the insulin sensitivity, obesity and CV risk markers, and metabolic syndrome.

2. Materials and methods

2.1. Subjects

Eighty-nine adolescent PCOS patients were recruited consecutively from the outpatient Reproductive Endocrinology Unit of Zekai Tahir Burak Women's Health Education and Research Hospital, between April and August 2014. The diagnosis of PCOS was made due to the recent Amsterdam ESHRE/ASRM proposal and the presence of all three of the following Rotterdam criteria for diagnosing PCOS in adolescents was required: oligo- or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries [2,23]. Ovaries were considered polycystic on transabdominal ultrasound if an ovarian volume is above 10 cm^3 [24].

Exclusion criteria included pregnancy, infectious diseases, use of medications known to alter insulin secretion or action, and lipoprotein metabolism, hypertension, smoking, family history of cardiovascular disease, and endocrinopathies including diabetes, Cushing syndrome, androgen secreting tumors, late-onset 21-hydroxylase deficiency, thyroid dysfunction, current use of oral contraceptives, autoimmune diseases and hyperprolactinemia.

Eighty-three healthy age-matched adolescents were also recruited as control group. All adolescents were further evaluated by an experienced, single pediatrist in order to exclude any possible concomitant endocrinological and other systemic diseases.

All participants provided a written informed consent and in case of participants younger than 18 years old, the parents signed the written informed consent. The study protocol was approved by Instructional Review Board of our hospital.

All participants included in the study were evaluated in the early follicular phase, on the day 3 of a spontaneous or induced menstrual cycle. Clinical examination was performed and anthropometric measurements were recorded. Blood samples were obtained after an overnight fasting at least 12 h, processed within 1 h after withdrawal and were stored at −80 °C. Biochemical evaluation consisted of fasting glucose and insulin, total cholesterol, low-density cholesterol (LDL-C), high-density cholesterol (HDL-C), triglycerides (TG), FSH, LH, estradiol, total and free testosterone (total-T, free-T), 17-hydroxyprogesterone (17OH-P), and dehydroepiandrosterone sulphate (DHEA-S).

2.2. Laboratory assays

Complete blood counting parameters were analyzed with LH780 hematological analyzer (Beckman Coulter, Fullerton, CA, USA). Neutrophil to lymphocyte ratio (NLR) was calculated by proportioning absolute neutrophil count to absolute lymphocyte count. Serum levels of FSH, LH, E2, PRL, total-T, insulin and TSH were measured with UnicelDxI 800 Immunoassay System (Beckman Coulter, Fullerton, CA, USA). Serum levels of 17OH-P, DHEA-S and free-T were measured by radioimmunoassay. Homeostasis model assessment (HOMA-IR) (insulin × glycemia in (μmol/L)/22.5) was estimated. HOMA-IR > 3.16 was considered to indicate the presence of insulin resistance (IR) [25]. The serum levels of glucose, total cholesterol, HDL-C, LDL-C, and TG were determined with the use of AU680 Chemistry System (Beckman Coulter, Fullerton, CA, USA).

Serum perforin concentrations were determined by using human perforin/pore-forming protein (PF/PFP) ELISA Kit (Eastbiopharm Co., Ltd., Hangzhou, China) and the results were expressed as pg/ml. The intra-assay and inter-assay coefficients of variability (CV) for perforin were <10% and <12%, respectively. The sensitivity was 2.41 pg/ml and the assay range was 5–2000 pg/ml.

Serum granzyme-B concentrations were determined by using Human Granzyme-B Platinum ELISA Kit (Ebioscience BMS2027, Vienna, Austria). The intra-assay and inter-assay coefficients of variability (CV) for granzyme-B were 8.5% and 10.4%, respectively. The sensitivity was 0.2 pg/ml and the assay range was 0.66–480 pg/ml.

Dyslipidemia in adolescents is defined as the presence of at least one of the following: LDL-C ≥ 110 mg/dL, HDL-C < 35 mg/dL, TG > 150 mg/dL and total cholesterol > 170 mg/dL. The cutoff values for LDL, HDL and TG were based on American Association of Clinical Endocrinologists' (AACE) Guidelines for Management of Dyslipidemia and Prevention of Atherosclerosis. The cutoff value for total cholesterol in adolescents was chosen according to the report of National Cholesterol Education Program (NCEP) expert panel on blood cholesterol levels in children and adolescents [26,27].

CV risk factors examined in the present study were overweight or obese (BMI >25 kg/m^2), with abdominal obesity [waist circumference (WC) ≥ 80 cm], insulin resistance (defined as a HOMA-IR > 3.16) and dyslipidemia in accordance with serum perforin and granzyme-B levels [25–27]. Increased CV risk is defined as the presence of at least two out of the four mentioned risk factors in the present study because in AACE guidelines the presence of ≥2 risk factors was defined as moderate to high risk for coronary artery disease [26].