Use of Myostatin levels in early detection of PCOS and evaluation of the mechanism

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Abstract
Aim: Polycystic ovary syndrome is a metabolic disease affecting almost 10% of women in the reproductive period. Etiopathology is a debate; obesity, insulin resistance, and genetic inheritance provide the appropriate environment for PCOS. Myostatin is a growth factor in transforming growth factor-beta family which plays a crucial role in suppressing skeletal muscle growth and diminishes insulin sensitivity and increases fat accumulation. Circulating myostatin levels were evaluated to expose the beneficial usage for PCOS diagnosis.

Materials and Methods: A prospective observational case-control study was achieved. Patients diagnosed with PCOS and healthy controls were recruited in the study. Patients’ characteristics, anthropometric measurement, blood lipid parameters, sex hormones, fasting blood glucose, insulin levels, HOMA-IR (homeostasis model assessment insulin resistance index), and serum myostatin levels were reviewed. Correlation analysis, multivariate logistic analysis, and ROC curve were calculated.

Results: Forty patients with PCOS and 38 healthy controls were included in the study. There was a significant difference between the groups across fasting insulin, HOMA-IR, LH levels. Serum androgen levels, lipid profile did not differ significantly. Serum Myostatin levels were significantly higher in patients with PCOS. Logistic regression analysis revealed that Myostatin was significantly associated with a high possibility of having PCOS. The receiver operating characteristic (ROC) curve analysis confirmed that the optimal cut-off value of Myostatin for detecting PCOS was ≥318 ng/L, with a sensitivity of 58.1% and specificity of 62.2%.

Discussion: Our results exposed that myostatin might be used not only for early detection, but also for the treatment follow up of the patients with PCOS. Further contemporary studies with larger participants are essential.

Keywords
MSTN; Myostatin; GDF8; PCOS; PCOS diagnosis
Introduction
Polycystic ovary syndrome (PCOS) is defined as one of the most common diseases among reproductive-aged women. It has appeared in medical literature as a morphologic pathology of the ovaries in the early 18th century and was treated via surgical interventions. To date, PCOS is considered as a metabolic disorder which exposes with menstrual irregularities, hyperandrogenism (clinical or biological) and ovaries with a polycystic view. PCOS patients have an increased risk of infertility, cardiovascular diseases, dyslipidemia, type 2 diabetes, and steatohepatitis [1]. Contemporarily studies have not enlightened the etiology precisely; nonetheless, obesity and insulin resistance have stated as the key contributors to the pathogenesis. Due to the lack of a specific etiological reason, the diagnosis is achieved by the combination of some characters as anovulation disorders, hyperandrogenism, and ultrasound examination of the ovaries. These different characteristics of the PCOS can cause different phenotypes in patients [2]. To date, a diagnostic marker for each PCOS patient is missing. Numerous molecules have been investigated to fill this gap. We hypothesized that, since PCOS is supposed to be a metabolic disorder, a diagnostic tool should be related to metabolism.

Myostatin (MSTN), also known as growth differentiation factor 8 (GDF8), is a growth factor placed in transforming growth factor-beta family [3]. MSTN gene targeting experiments on animals revealed that the deletion of the gene encoding MSTN caused an increase in not only hyperplasia but also hypertrophy [3, 4, 5]. In MSTN-null mice, a dramatic increase in skeletal muscle about twice as much was revealed [6]. Apart from these initial results exposed in MSTN researches, the effect on the skeletal muscle was just like the tip of the iceberg. At the postnatal period, MSTN regulates glucose metabolism and adipogenesis [6]. Of these, myostatin inhibited mice have an evident increase in muscle mass, a decrease in fat mass, and resistance to dietary or genetic dependent obesity [6]. More recently, myostatin has asserted as a fundamental factor in metabolism through improving insulin sensitivity by regulating glucose consumption in muscle and adipose tissue. Considering the possible etiology of PCOS, with the hypothesis that circulating blood MSTN values could be valuable for the diagnosis and treatment follow-up of PCOS, the purpose of the study was to investigate the relationship between PCOS and myostatin, about which there is limited data in the literature.

Material and Methods
A prospective observational study was conducted at a single university-affiliated research and training hospital between January 2019 and January 2020 after approval of the local ethics committee in Bursa Yuksek Ihtisas Training and Research Hospital, University of Health Sciences. The study included participants diagnosed with PCOS and the control group. Forty patients with PCOS and 38 healthy controls were included in the study. All procedures were in agreement with the Helsinki Declaration. Written informed consent was obtained from all the participants. PCOS diagnosis was confirmed based on the minimum two of three 2003 Rotterdam consensus criteria [7]. Control participants were recruited from healthy women who visited the gynecology clinic for a routine annual examination.

A physical examination of each participant was performed and a detailed history was recorded. Age, height (centimeter), and weight (kilogram), and waist circumference were measured. BMI was calculated using the following formula: weight [kg]/square meter of the height [m²]. A score of ≥ 8 was indicated as hirsutism according to the Ferriman-Gallway Hyperandrogenism scoring system.

A biochemical evaluation of sex hormones was determined from blood samples collected from the antecubital veins on the 2-4 day of the menstrual cycle after the onset of early spontaneous menses following at least 12 h of fasting. The samples were centrifuged at 1,000 × g for 15 min at 2–8°C and separated from the serum and stored at −80°C until analysis. The levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), dehydroepiandrosterone sulfate (DHEA-S), total-testosterone, free-testosterone, prolactin, thyroid-stimulating hormone (TSH) were measured with CMIA (Beckman Coulter Inc., Brea, CA, USA). The levels of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, fasting blood glucose (FBG), total cholesterol were measured delineated using an automated analyzer (Abbott Architect C 16000, IL, USA) with its own kits (Abbott Diagnostics, Wiesbaden, Germany). LDL-C was calculated using the Friedewald equation: LDL-C = total cholesterol- (HDL-C) – triglyceride/5. Non-HDL-C was calculated as HDL-C subtracted from total cholesterol. Serum insulin levels were measured by an automated analyzer (Abbott Architect 12000, IL, USA) using a chemiluminescent microparticle immunoassay (CMIA) with its own kit (Abbott Diagnostics, Wiesbaden, Germany). Insulin resistance was calculated with the following formula:

\[ \text{Homoeostasis Model Assessment Of Insulin Resistance (HOMA-IR)} = \frac{\text{fasting insulin [mU/mL] } \times \text{fasting glucose [mg/dL]}}{405} \]

[8].

Serum myostatin concentrations were assessed using an ELISA kit (SUNRED; Shanghai Sunred Biological Technology, Shanghai, China- Catalogue No: 201-12-0404, Human (Myostatin) Elisa Kit, Sensitivity: 5.113ng/L and Assay range: 7ng/L-2000 ng/L). Patients who had any of following properties were excluded from the study: any disease that deteriorates androgenic status or causes for menstrual cycle, congenital adrenal hyperplasia, Cushing’s Syndrome, ovarian or adrenal tumors secreting androgens, primary ovarian insufficiency, acromegaly, hypothalamic amenorrhea, hyperprolactinemia, thyroid disorders, pregnancy or breastfeeding, type 1 or 2 diabetes mellitus, metabolic syndrome or autoimmune diseases, chronic diseases like hypertension, hyperlipidemia, liver disease, muscle diseases or bodybuilders.

Statistical analysis:
Demographic, laboratory characteristics, anthropometric measures, and myostatin levels of the participants were evaluated. Statistical analysis was performed using the SPSS 24.0 software for Windows. The normal distribution of continuous variables was evaluated using the Shapiro-Wilk test. As a consequence of the normality results, categorical variables among the groups were compared with the Pearson chi-square test for normally distributed variables, and the Mann-Whitney U test for not-normally distributed dependent variables which were either nominal or quantitative. The relationship between myostatin and other parameters were
Role of myostatin in PCOS

Calculated by the Pearson's correlation analysis. To examine the independent correlation of myostatin levels in the development of PCOS, odds ratio (OR) with multivariate logistic analysis was calculated. The receiver operating characteristic (ROC) curve was used to achieve the myostatin levels for the prediction of PCOS. The respective areas under the curve, in which sensitivity was plotted as a function of 1-specificity, were evaluated. All reported confidence interval (CI) values were calculated at the 95% level. An overall p-value of less than 0.05 was considered a statistically significant result.

Results

The numbers of patients included in the study with PCOS and controls (without PCOS) were 40 and 38, respectively. Baseline characteristics determined that there was no statistical difference between the groups by age and BMI. Comparing the groups depicted that fasting insulin levels, HOMA-IR, LH, and myostatin levels were statistically different. The groups did not differ statistically in terms of the blood lipid parameters and androgen hormones. All demographic and laboratory comparison parameters are shown in Table 1. The MSTN levels were higher in patients with PCOS, which were statistically significantly different from the control group.

To evaluate the possibility of MSTN in distinguishing PCOS from the healthy controls, correlation analysis, and logistic regression analysis were calculated. Correlation analysis between myostatin and other factors revealed that there was no correlation with MSTN and the parameters. Thus in the present study, it was found that myostatin value is related to PCOS disease. The other parameters, such as age, insulin resistance, androgen status, blood lipid profiles, did not affect MSTN levels. The correlation analysis revealed that MSTN could

Table 1. Comparison of the demographic and laboratory characteristics of the subjects

| Variables         | PCOS group (n=40) | Control group (n=38) | p    |
|-------------------|-------------------|----------------------|------|
| Age, years        | 25.46 ± 4.50      | 26.05 ± 4.63         | 0.567|
| BMI, kg/m²        | 26.41 ± 6.02      | 24.37 ± 3.34         | 0.212|
| FBG, mg/dL        | 90.81 ± 19.01     | 86.67 ± 9.53         | 0.234|
| Insulin, µU/mL    | 17.02 ± 8.10      | 10.21 ± 4.75         | <0.001|
| HOMA-IR           | 3.80 ± 1.94       | 2.17 ± 1.07          | <0.001|
| Total testosterone, ng/ml | 44.14 ± 22.30 | 40.92 ± 14.20        | 0.453|
| Free testosterone, pg/mL | 3.77 ± 9.99     | 2.72 ± 3.88          | 0.304|
| LH (mIU/ml)       | 7.8               | 5.6                  | 0.039|
| DHEAS µg/ml       | 179.60 ± 75.30    | 202.267 ± 01         | 0.163|
| Prolaktin (ng/ml) | 11.8              | 11.0                 | 0.793|
| TSH (µIU/ml)      | 1.77              | 1.71                 | 0.910|
| Total cholesterol, mg/dL | 158.83 ± 26.68 | 155.43 ± 36.66       | 0.633|
| LDL cholesterol, mg/dL | 81.95 ± 24.23 | 77.32 ± 33.90        | 0.482|
| HDL cholesterol, mg/dL | 49 ± 12          | 53 ± 10              | 0.100|
| Triglyceride (mg/dl) | 145.86 ± 78.54   | 122.67 ± 40.63       | 0.110|
| Myostatin (ng/L)  | 686.96 ± 620.29   | 388.59 ± 366.64      | 0.010|

Results are given as mean ± SD.

Table 2. Evaluation of the relationship between myostatin and the other demographic and laboratory variables

| Variables          | PCOS              | Control            | r   | p    | r   | p    |
|--------------------|-------------------|--------------------|-----|------|-----|------|
| Age                | -0.265            | 0.088              | 0.253| 0.131|
| BMI                | -0.088            | 0.576              | -0.122| 0.471|
| Insulin            | 0.023             | 0.884              | -0.027| 0.801|
| FBG                | -0.047            | 0.766              | -0.168| 0.322|
| HOMA-IR            | -0.065            | 0.687              | -0.043| 0.801|
| Total testosterone | -0.065            | 0.686              | -0.160| 0.343|
| Free testosterone  | -0.090            | 0.567              | 0.093| 0.586|
| DHEAS              | 0.148             | 0.343              | -0.233| 0.165|
| Total cholesterol  | -0.149            | 0.341              | -0.126| 0.459|
| LDL cholesterol    | 0.127             | 0.415              | -0.159| 0.346|
| HDL cholesterol    | 0.198             | 0.204              | -0.126| 0.458|
| Triglyceride       | -0.139            | 0.574              | 0.272| 0.103|

Pearson’s correlation analysis was used. r: Pearson’s correlation coefficient. A P- value of <0.05 was considered significant (*). PCOS: Polycystic ovary syndrome; BMI: Body mass index; FBG: Fasting blood glucose; HOMA-IR: Homeostasis model assessment of insulin resistance; DHEAS: dehydroepiandrosterone sulfate; LDL: Low density lipoprotein; HDL: High density lipoprotein.

Table 3. Association of myostatin with PCOS in adjusted models

| Variables         | OR    | 95% CI | P     |
|-------------------|-------|--------|-------|
| Myostatin         | 1.002 | 1.000  | 1.003 | 0.024|
| Age               | 0.878 | 0.765  | 1.006 | 0.062|
| BMI               | 1.179 | 0.995  | 1.398 | 0.058|
| HOMA-IR           | 1.790 | 1.115  | 2.873 | 0.016|
| Total testosterone| 1.006 | 0.974  | 1.039 | 0.712|

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; HOMA-IR: Homeostasis model assessment of insulin resistance.

Figure 1. Receiver operating characteristic (ROC) curve for myostatin for the prediction of PCOS. The estimate of the area under the ROC curve and its 95% confidence interval is shown. The ROC curve is a plot that illustrates the diagnostic ability of a binary classifier system as its discrimination threshold is varied. The area under the curve (AUC) provides a single measure of the overall diagnostic accuracy. A perfect test would have an AUC of 1.0. An AUC of 0.5 indicates no discriminatory ability. AUC value of > 0.5 was considered significant (*).
be a marker for PCOS diagnosis. The statistical evaluation was determined in Table 2. In the multivariate analysis, possible factors identified with univariate analysis were further entered into a logistic regression analysis to determine independent predictors of PCOS diagnosis. This logistic regression analysis exposed that MSTN and HOMA-IR were significant predictors. The ROC curve analysis was evaluated to acquire the favorable MSTN level for PCOS diagnosis. The area under the ROC curves was 0.704 (95% CI 0.589-0.819, p<0.002) for myostatin. The optimal cut-off value of myostatin for detecting PCOS was ≥318 ng/L, at which the sensitivity was 58.1% and specificity was 62.2 %. The ROC curve analysis for myostatin to predict PCOS risk is shown in Figure 1.

Discussion

The prevalence of PCOS varies from 4% to 21% depending on diagnostic criteria and population.[9] Despite this high prevalence, etiology has not been elucidated properly. Most of the patients with PCOS suffer hyperandrogenism, insulin resistance, obesity, and lipid disorders. Consistent with the literature, we detected statistically significant insulin resistance, as well as higher, but not significantly different BMI and blood lipid levels.

One of the main problems is the diagnosis of PCOS. Rotterdam consensus criteria comprise almost all cases however it also lead up to different phenotypes which have different clinical and endocrine characteristics [2]. The novel diagnostic tools should enlighten the therapeutic approach and clinical follow-up. Authors have focused on several markers associated with chronic inflammation, obesity, insulin resistance, pancreatic cell secretion [10, 11, 12]. Besides these all invaluable articles, we aimed to focus on metabolism, thus, any disorder could cause serious diseases such as cardiovascular diseases, metabolic syndrome, obesity, which were supposed to be more important than anovulatory cycle or hyperandrogenism. For that reason, we aimed to investigate MSTN which plays a crucial role in metabolism. MSTN had another positive property, namely, that after treatment, the circulating levels decline significantly, which ensures itself to be used as a treatment follow-up [13, 14].

Myostatin is mainly expressed in skeletal muscle and adipose tissue which constitutes one of the majority of total body volume, thus it has a remarkable role in regulating circulating glucose consumption [15, 16]. In the present study, investigation of the circulating serum myostatin revealed that patients with PCOS had a higher level than healthy controls. There are not many studies in the literature focused on this issue. There has not been a consensus on this issue because of the limited contemporary studies. Only three researches focused on this issue. Two studies have declared increased myostatin levels in contemporary studies. Only three researches focused on this issue. Two studies have declared increased myostatin levels in the literature, we detected statistically significant insulin resistance, as well as higher, but not significantly different BMI and blood lipid levels. These parameters including total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride. Many researches declared that these parameters were affected in patients with PCOS [17, 18, 20]. The incompatibility with literature could pave the way for the early detection of PCOS with MSTN. Patients without elevated serum androgens, lipid, and fasting blood glucose parameters might be detected with MSTN. Logistic regression analysis revealed that MSTN levels were significantly associated with high odds of having PCOS. It was calculated that using the ROC curve, a myostatin value which was ≥ 318 ng/L could detect PCOS with a sensitivity of 58% and specificity of 62%. For the first time in the literature, in the present study, we have demonstrated a proper serum myostatin level to detect PCOS. Early detection and interventions such as lifestyle changes, regulation of dietary supplements, or even medical treatments like insulin-sensitizing agents could ameliorate the MSTN levels and adverse outcomes of PCOS [21, 22, 23]. Although the PCOS name may seem like just a morphologic definition, it is not such an underestimated disease. In 2012, the National Institutes of Health (NIH) Evidence-based Methodology Workshop Panel on Polycystic Ovary Syndrome suggested to rename the syndrome reflecting complex metabolic, hypothalamic, pituitary, ovarian, and adrenal interactions; however, no name could be recommended. Having different phenotypes of PCOS prevents the generalization of the diagnosis and treatment [24]. In the present study, we speculated that myostatin might be a diagnostic or predictive marker in PCOS. Shreds of evidence demonstrated that myostatin had a crucial role in signaling pathways over adipose and skeletal tissues which were both derived from the mesenchymal stem cells [25]. In our opinion, MSTN could be the first exposed marker before deteriorated blood parameters. Although the limitation of the present study was the relatively small study population, the patients had similar characteristics and the number of participants was sufficient for a proper statistical analysis.

In conclusion, myostatin has a crucial role in metabolism especially in the regulation of circulating glucose consumption. It might be used for not only early detection but also treatment follow-up for patients with PCOS providing that our findings were promoted with contemporary studies involving more participants.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.
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