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Serum nonesterified fatty acids, ghrelin, and homocysteine levels in women with polycystic ovary syndrome

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Aim: To investigate the possible relationship of serum levels of nonesterified fatty acids (NEFA), ghrelin, and homocysteine levels to metabolic and hormonal features in women with polycystic ovary syndrome (PCOS).

Materials and methods: Thirty women with PCOS and 30 healthy women with similar age and body mass index (BMI) were recruited. Fasting serum NEFA, ghrelin, homocysteine, growth hormone (GH), cardiac troponin I, glucose, insulin, lipids, and homeostasis model assessment of insulin resistance (HOMA-IR) were measured.

Results: Serum NEFA, homocysteine, insulin, HOMA-IR, cholesterol, and testosterone levels were significantly higher but in contrast ghrelin level was significantly lower in women with PCOS compared to the controls. No significant differences were found in the troponin I and GH levels between the 2 groups. Significant positive correlations between insulin-NEFA and insulin-homocysteine levels were observed. There was no significant correlation between ghrelin-insulin and ghrelin-homocysteine levels.

Conclusion: The decreased ghrelin, elevated NEFA and homocysteine levels, and the correlation of NEFA and homocysteine to fasting insulin level might play an important role in the pathogenesis of the PCOS. These findings need to be confirmed and larger prospective and controlled studies are necessary.

Key words: NEFA, ghrelin, homocysteine, insulin, PCOS

Polikistik over sendromlu kadınlarda serum nonesterifiye yağ asitleri, grelin ve homosistein düzeyleri

Amaç: Nonesterifiye yağ asitleri (NEFA), grelin ve homosistein serum düzeyleri ile polikistik over sendromlu (PCOS) kadınların metabolik ve hormonal özellikleri arasındaki ilişkileri araştırmak.

Yöntem ve gereç: PCOS'lu 30 kadın ile benzer yaş ve vücut kitle indeksi (BMI) ne sahip sağlıklı 30 kadın çalışmaya dahil edildi. Açlık serum NEFA, grelin, homosistein, büyüme hormonu (GH), kardiyak troponin I, glukoz, insülin, lipitler ve homeostasis model assessment-insülin direnci (HOMA-IR) testleri çalışıldı.

Bulgular: Kontrole kıyasla PCOS'lu kadınlarda serum NEFA, homosistein, insulin, HOMA-IR, kolesterol ve testosteron düzeyleri önemli oranda yüksek fakat grelin seviyesi düşük bulundu. Gruplar arasında troponin I ve GH düzeyleri yönünden fark bulunamadı. İnsülin-NEFA ve insülin-homosistein düzeyleri arasında istatistiksel anlamlı ve pozitif korelasyon saptanırken, grelin-insülin ve grelin-homosistein parametreleri arasında anlamlı bir korelasyon saptanmadı.

Sonuç: Azalmış grelin, yüksek NEFA ve homosistein düzeyleri; açlık insülin düzeyleri ile NEFA ve homosistein düzeyleri arasındaki korelasyonlar PCOS patogeneğinde önemli rol oynayabilir. Bu sonuçlar daha büyük prospektif ve kontrollü çalışmalar ile konfirme edilmelidir.

Anahtar sözcükler: NEFA, grelin, homosistein, insülin, PCOS

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine diseases affecting almost 10% of women in the reproductive age (1). Despite the ambiguity of the etiology of PCOS to date, several studies have suggested that insulin resistance (IR) plays an important role in the pathogenesis of the syndrome (2,3). IR is defined as the reduced reaction to normal circulating level of insulin that may be a major risk factor for occurrence of cardiovascular disease (CVD) in women with PCOS (4).

IR in fat cells reduces the effects of insulin and results in elevation of hydrolysis of stored triglycerides (5). The increase of mobility of stored lipids in these cells elevates nonesterified fatty acids (NEFA) in the blood plasma (5,6). Physiological elevations in plasma NEFA concentrations inhibit insulin stimulated peripheral glucose uptake in a dose-dependent manner (6). It is now known that NEFA interfere with insulin signaling in skeletal muscle at the level of IRS-1 serine phosphorylation (7).

Ghrelin, a 28-amino acid peptide recently isolated from rat and human stomach, is the long-sought natural ligand for the GH secretagogue receptor (8). It is associated with energy balance, obesity, IR, and gonadal function. Hypothalamic areas are the major binding sites of ghrelin (9). The available information on ghrelin level in women with PCOS is rather conflicting: some studies reported a reduction (10), some conversely reported elevation (11) of concentrations and some others reported no significant differences between women with PCOS and normal ovulatory women (12).

Homocysteine, a sulfur-containing amino acid formed during the metabolism of methionine, is toxic to vascular endothelium (13). PCOS has been associated with the elevated plasma homocysteine and correlated to IR (14). Higher homocysteine level has been observed in the hyperinsulinemic subjects and so it has been suggested that homocysteine could have a great role in risk factors of CVD in PCOS (13,15).

The available information on serum homocysteine, ghrelin, and NEFA levels in women with PCOS are also conflicting, as well as the number of subjects involved in most of studies is relatively small (5,11,12,14). From this perspective the present

study was designed to assess the possible relationship of NEFA, homocysteine, and ghrelin to metabolic and hormonal features in women with PCOS, employing a substantial number of subjects.

Materials and methods

Thirty women diagnosed with PCOS and 30 healthy female with no known medication or menstrual disorders having similar age and BMI were included in this study. PCOS was documented when at least 2 of the following 3 features were present after the exclusion of other etiologies (Rotterdam criteria): oligo/amenorrhea (fewer than 6 menstrual periods in the preceding year); clinical (Ferriman–Gallwey score > 8) and/or biochemical signs of hyperandrogenism and ultrasonographic findings (16). The ultrasound criteria used for diagnosis of PCOS were the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter, and/or increase of ovarian volume (>10 mL). The PCOS subjects were selected from a group of PCOS patients who were seeking treatment for menstrual irregularity, acne, hirsutism or infertility, and the controls were selected from general community. Clinical hyperandrogenism was quantified by the Ferriman–Gallwey scoring system. Hirsutism scores on each body area were made by 2 experienced physicians.

None of the subjects had medication for at least 6 months prior to the study. None of the studied individuals were undergoing caloric restrictions at the time of the study or doing physical exercise. Their height and weight and waist circumferences were measured. The waist circumference of subjects was measured in standing position by placing a soft tape measure midway between the lowest rib and the iliac crest. All other measurements were performed when the patients were in a standing position with joined feet, relaxed abdomen, and arms at their sides. BMI was measured as the ratio of the weight to the square of the height. Blood pressure was measured on the right arm, with the subjects in a sitting position and relaxed.

The blood of the subjects was sampled in the morning following an overnight fast during early follicular phase (days 2–5) of, spontaneous or progesterone induced, withdrawal bleeding. The blood

samples were centrifuged and then serum or plasma aliquots were frozen at -20 °C until assayed. Patients having type-2 diabetes, thyroid, or renal, liver, or cardiovascular dysfunctions as well as Cushing's syndrome, hyperprolactinemia, androgen-secreting tumors, and late-onset 21-hydroxylase deficiency were excluded from the study. In all patients *a*-basal hormonal evaluation by means of GH, FSH, LH, estradiol, testosterone, dehydroepiandrosterone sulfate (DHEA-S), sex-hormone-binding globulin (SHBG), and *b*-metabolic evaluation by means of concentration of NEFA, ghrelin, homocysteine, cardiac troponin I (cTnI), glucose and insulin, was carried out. The study was performed according to the guidelines of the Helsinki Declaration on human experimentation and was approved by the local ethics committee.

Biochemical studies

In all subjects, fasting serum NEFA concentrations were determined using an enzymatic-colorimetric method with the Wako NEFA C test kit (Wako, Neuss, Germany). Fasting serum total homocysteine levels were determined with an enzyme immunoassay kit (Axis-Shield, Oslo, Norway). Total ghrelin (Linco Research, Missouri, USA) levels were measured by radioimmunoassay with a gamma counter (LKB WALLAC, 1261 Multigamma, Gamma Counter, Turku, Finland). Serum cTnI concentrations were measured by a commercial cTnI kit using a chemiluminescent enzyme immunoassay method (Pathfast™ cTnI, Compact immuno-analyzer, Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). The respective inter- and intra-assay coefficients of variation (CV) of these parameters were 5.1% and 3.8% for NEFA; 4.8% and 3.2% for homocysteine; 14.8% and 10.1% for total ghrelin; 4.1% and 3.7% for cTnI.

Serum FSH, LH, estradiol (E2), total testosterone, SHBG, GH, insulin, and DHEAS levels were measured by competitive chemiluminescent enzyme immunoassay method using the kits with the same brand (Immulite 2000 Analyzer, Diagnostic Products Corporation; DPC, Los Angeles, CA, USA). The respective inter- and intra-assay CV were 7.3% and 5.5% for FSH; 7.6% and 5.0% for LH; 6.6% and 5.1% for E2; 8.3% and 6.2% for total testosterone; 7.0% and 5.2% for SHBG; 6.8% and 5.2% for GH; 5.7% and 4.3% for insulin; 5.3% and 3.9% for DHEAS.

Fasting glucose, triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) concentrations were measured by enzymatic colorimetric assay methods using an Olympus AU 2700 autoanalyser (Olympus Optical Co. Ltd., Japan) and commercially available kits (Olympus Diagnostica GmbH, Wendenstraße, Hamburg, Germany). The respective inter- and intra-assay CV were 3.4% and 3.0% for fasting glucose; 3.2% and 2.7% for TGs; 2.7% and 2.4% for TC; 11.6% and 8.1% for HDL-C. Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using Friedewald formula (17): $LDL-C = TC - HDL-C - TG/5$, and IR was calculated using the homeostasis model assessment insulin resistance index (HOMA-IR) (18), given as: $HOMA-IR = \text{fasting insulin (mU/mL)} \times \text{fasting glucose (mg/dL)} / 405$.

Statistical analysis

The Statistical Package for the Social Sciences, version 11.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The normality of continuous variables in groups was tested by the Shapiro Wilk test. Since the variables did not show a normal distribution Mann-Whitney U-test was used for comparison. Bivariate correlations (computing Pearson's coefficient with their significance levels) between NEFA, ghrelin, homocysteine, GH, cTnI, and other variables in PCOS and control patients were calculated. The data are presented in mean \pm SD. For all comparisons, the statistical significance was defined as $P < 0.05$.

Results

The results manifested through this particular work are shown in Table 1. From basal conditions point of view there were no significant differences between the 2 groups in terms of age and anthropometric parameters, confirming that the 2 cohorts are comparable. However as compared to the healthy control subjects, PCOS patients had significantly higher NEFA, homocysteine, fasting insulin, HOMA-IR, LH and testosterone levels, but lower serum ghrelin, E2, and SHBG levels. In terms of cTnI and GH, the 2 groups were comparable. Also the M-Ferriman-Gallwey score was found as significantly higher in women with PCOS compared

Table 1. Comparison of anthropometric data, and hormonal and biochemical parameters (mean \pm SD) between PCOS and control groups.

| Characteristic | PCOS (n = 30) | Control (n = 30) | P value* |
|-----------------------------|-------------------|---------------------|-------------|
| Age (yr) | 23.13 \pm 4.28 | 23.30 \pm 2.76 | 0.581 |
| Weight (kg) | 58.91 \pm 11.9 | 53.56 \pm 5.63 | 0.076 |
| BMI (kg/m ²) | 22.31 \pm 5.04 | 20.77 \pm 2.08 | 0.505 |
| Waist circumference (cm) | 71.95 \pm 11.7 | 68.40 \pm 5.01 | 0.514 |
| NEFA (mg/dL) | 6.93 \pm 3.51 | 4.36 \pm 2.52 | 0.002 |
| Homocysteine (μ mol/L) | 16.25 \pm 6.76 | 8.91 \pm 6.47 | <0.001 |
| cTnI (ng/mL) | 0.002 \pm 0.004 | 0.002 \pm 0.005 | 0.074 |
| Ghrelin (pg/mL) | 815 \pm 323 | 1459 \pm 306 | <0.001 |
| FSH (mIU/mL) | 5.94 \pm 1.70 | 5.90 \pm 1.63 | 0.802 |
| LH (mIU/mL) | 8.63 \pm 4.47 | 5.63 \pm 3.16 | 0.013 |
| E2 (pg/mL) | 41.85 \pm 16.5 | 112.58 \pm 87.3 | <0.001 |
| T-testosterone (ng/dL) | 62.95 \pm 25.2 | 45.95 \pm 18.0 | 0.005 |
| SHBG (nmol/mL) | 44.53 \pm 42.9 | 54.03 \pm 17.2 | <0.001 |
| GH (ng/mL) | 1.49 \pm 2.24 | 1.83 \pm 3.14 | 0.988 |
| Fasting insulin (mU/mL) | 19.69 \pm 12.64 | 11.99 \pm 5.69 | 0.004 |
| DHEA-S (μ g/dL) | 215.96 \pm 81.4 | 258.16 \pm 98.5 | 0.110 |
| Fasting glucose (mg/dL) | 90.73 \pm 11.0 | 90.93 \pm 14.0 | 0.615 |
| TG (mg/dL) | 99.63 \pm 50.6 | 110.23 \pm 38.7 | 0.117 |
| TC (mg/dL) | 156.86 \pm 35.8 | 168.23 \pm 32.3 | 0.119 |
| HDL-C (mg/dL) | 48.59 \pm 14.8 | 51.43 \pm 12.4 | 0.450 |
| LDL-C (mg/dL) | 96.76 \pm 25.6 | 100.29 \pm 18.3 | 0.222 |
| HOMA-IR | 4.69 \pm 2.87 | 2.57 \pm 1.45 | <0.001 |
| M-Ferriman-Gallwey score | 15.43 \pm 4.98 | 8.53 \pm 2.44 | <0.001 |

*P < 0.05 was considered significant. NEFA: nonesterified fatty acids, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment insulin resistance index, LDL-C: low-density lipoprotein cholesterol, TC: total cholesterol, TG: triglycerides, SHBG: sex-hormone-binding globulin, GH: growth hormone, DHEA-S: dehydroepiandrosterone sulfate, cTnI: cardiac troponin-I

to controls, but there were no distinguishable differences observed between groups in other parameters.

From the results it was observed that NEFA has a positive correlation with GH ($r = 0.256$, $P < 0.05$), fasting serum insulin levels ($r = 0.356$, $P < 0.007$; Figure 1), and HOMA-IR index ($r = 0.294$, $P < 0.05$). Homocysteine level had a positive correlation with

LH ($r = 0.282$, $P < 0.05$) and fasting serum insulin level ($r = 0.545$, $P < 0.001$; Figure 2). Ghrelin level had a positive correlation with HOMA-IR index ($r = 0.364$, $P < 0.004$). Nonetheless, as shown in Figures 3 and 4, there was no significant correlation between ghrelin-insulin ($r = 0.225$, $P = 0.096$) and ghrelin-homocysteine levels ($r = 0.253$, $P = 0.058$). GH was positively correlated with SHBG ($r = 0.342$, $P < 0.01$).

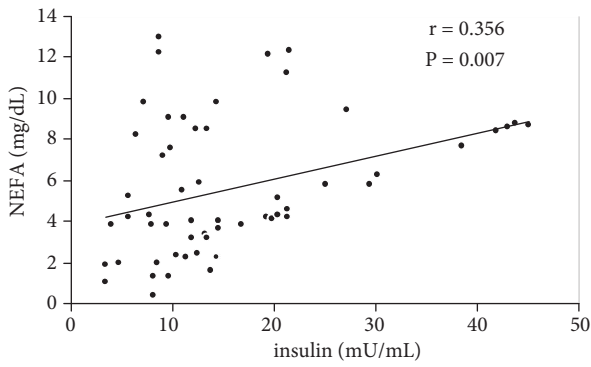


Figure 1. Correlation between serum NEFA and fasting insulin levels in women with PCOS.

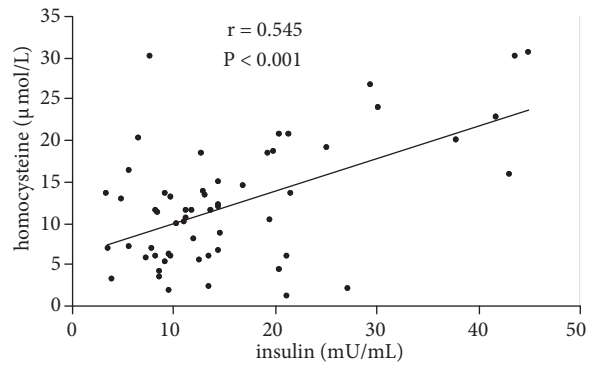


Figure 2. Correlation between serum homocysteine and fasting insulin levels in women with PCOS.

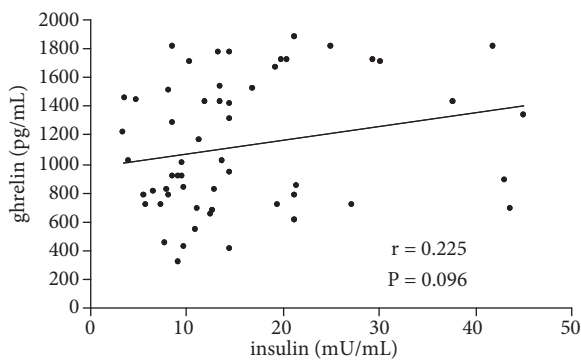


Figure 3. Correlation between serum ghrelin and fasting insulin levels in women with PCOS.

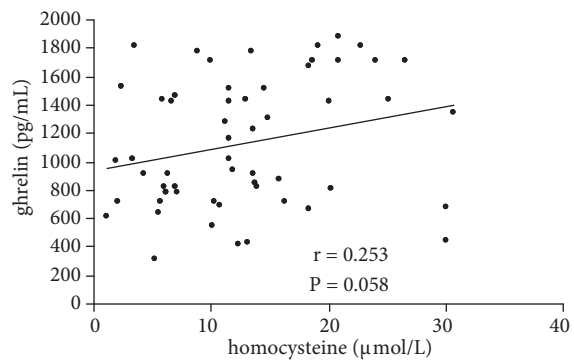


Figure 4. Correlation between serum ghrelin and homocysteine levels in women with PCOS.

Discussion

The obtained results, with reference to basic hormonal profile homocysteine, NEFA and ghrelin levels in women with PCOS, are in accord with well-established evidence on the fundamental characteristics of PCOS (6;14). In most of the previous studies (2,19) a reduction in ghrelin concentration in women with PCOS have been reported, but in some others (11,12), in contrast, such a reduction has not been reported. The present study revealed a lower ghrelin level, which is in line with the majority of the previous reports (2,19), and significantly higher serum NEFA and homocysteine levels but comparable GH and cTnI concentrations in women with PCOS as compared to the controls.

The detected positive correlation of ghrelin level to IR in women with PCOS is also in agreement with

a previous report in which a highly significant positive correlation between ghrelin concentration and the degree of IR in PCOS subjects was shown (2). This interrelationship is in fact not clear enough yet. However, based on currently available information (20), it may be speculated that this relationship could be due to the presence of a moderate to severe IR. Whether ghrelin participates in the mechanisms brings about peripheral IR is also unknown because it is still controversial whether ghrelin stimulates or inhibits insulin secretion (21,22).

From in vivo studies it is known that infusion of ghrelin decreases insulin secretion (23), which could be because of the expression of ghrelin in pancreatic islet-cells, and so exerts a direct inhibitory action on insulin release (21). Other in vivo and in vitro studies, in contrast, suggest a stimulatory action of ghrelin on

insulin release (22). On the other hand, *in vivo* insulin has no impact on circulating ghrelin in humans, which denotes a further complex interplay between both hormones (24). In the PCOS patients involved in the present study, ghrelin did not correlate with insulin and homocysteine, which agrees with a simple mechanism explaining the decrease in serum ghrelin in insulin-resistant PCOS subjects. This could suggest a weak link between ghrelin, fasting insulin, and homocysteine concentrations, and show that the dysregulated ghrelin-homocysteine system in women with insulin-resistant PCOS might be a cause of risk of CVD.

Ghrelin concentration has been reported to have a negative correlation with body mass index (BMI), and in turn with the obesity that increases in response to weight loss (12,20), as well as with indices of IR (11). A negative correlation between circulating ghrelin and androgen levels was also reported (25). In the present study, a fractional relation of ghrelin, NEFA, and homocysteine to BMI was observed but this was not statistically significant. This discrepancy might be due to a relatively narrow BMI range in the subjects involved in the study compared with those reported by others (20).

It remains unclear whether ghrelin is linked to insulin sensitivity via an insulin-mediated process or other mechanisms associated with IR, such as elevated NEFA. When the regulation of energy supply in adipose tissue is impaired, the plasma NEFA levels become elevated due to the increase of mobilization of stored lipids (5). Excessive NEFA causes ectopic fat storage and increase of NEFA metabolites in non-adipose tissues (5), which have lipotoxic effects on skeletal muscle and other tissues (6).

Boden et al. (6) showed an important link in between NEFA, obesity, IR, and type 2 diabetes such that the physiological change caused by elevation of plasma NEFA in most of obese subjects inhibits insulin-stimulated glucose uptake into muscle. It seems that the markedly increase of NEFA levels in women with PCOS manifested in this study closely related to IR, and this relation is supported by previous studies reporting the elevation of serum NEFA levels in PCOS patients, and the suppressive effect of extreme fat diet on fasting ghrelin levels (26,27). Our findings also indicated that ghrelin, apart from its role in the control of appetite and body

weight, could be linked to IR and possibly homocysteine levels. However, whether high NEFA and homocysteine, and low ghrelin levels in PCOS are a cause or the consequence of IR awaits further investigation.

The possible determinants of elevation of homocysteine concentration are still debated among researchers who found significant correlations between homocysteine and IR (14) as well as those who did not observe such correlations (28). Badawy et al. (29) found a significantly higher homocysteine level among PCOS women with IR compared to those without IR. A recent study confirmed the presence of increased serum homocysteine concentration in obese as well as in non-obese PCOS women (28). As the 2 groups involved in our study were compared, the homocysteine level was significantly higher and there were positive correlations among serum homocysteine, insulin, and LH levels in PCOS patients. Although this appears to be a good outcome demonstrating the role of IR, but this is not the sole cause of hyperhomocysteinemia in women with PCOS; this elevation might be due to the influence of LH.

Hyperhomocysteinemia is associated with hyperinsulinemia and partly accounts for increase of risk of CVD associated with IR (30). Meigs et al. (30) proposed a direct association between hyperhomocysteinemia and IR concerning their similar pathogenetic effects on vascular endothelial cells, and a modest association between hyperinsulinemia and fasting levels of plasma homocysteine. Schachter et al. (14) found a significant correlation between IR and homocysteine levels regardless of body weight in women with PCOS. The high rate of homocysteine observed in the PCOS subjects suggests that homocysteine may also play a role in the risks of CVD

In conclusion, significant positive correlations between insulin-NEFA and insulin-homocysteine levels as well as between ghrelin-HOMA-IR index were demonstrated in women with PCOS. As the elevation of NEFA and homocysteine can be linked to IR, they in fact may account for the increased risk of CVD found in insulin-resistant women with PCOS (13). In spite of these profound metabolic aberrations, the GH and cTnI levels were not significantly different between the 2 groups.

Many studies conducted in this direction support existence of interactions between ghrelin and insulin metabolism. The nature of interactions between ghrelin and homocysteine has not been clarified yet which could be due to variations in insulin sensitivity (3). It should be noted that the close relation of insulin to homocysteine and NEFA levels, which is observed here, may indicate some

factors other than simple IR. Also one should bear in mind that PCOS might affect NEFA, homocysteine, and ghrelin concentrations and cause miscellany in clinical and biochemical manifestations. The results from this study suggest that these findings need to be confirmed and a larger prospective and controlled study and long-term follow-up are necessary.

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