Kermanshah University of Medical Sciences

Journal of Kermanshah University of Medical Science

Journal homepage: Htpp://journals.kums.ac.ir/ojs/index.php/jkums

Original Article

The relationship between epicardial fat thickness and insulin resistance in women with polycystic ovary syndrome

Karoon Shahebrahimi[1], Mehrali Rahimi[1], Nahid Salehi[2], Mansoor Rezaei[3], Denial Rezaee[1]*

1. Dept. of Internal Medicine, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.
2. Dept. of Cardiology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.
3. Dept. of Biostatistics&Epidemiology, School of Health, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.

Article Info

Keywords: polycystic ovary syndrome, insulin resistance, epicardial fat thickness

***Corresponding Author**:
Dept. of Internal Medicine, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.
Tel:+989396321617

Email:
dr_danialrezaee@rocketmail.com

Received: 14 February, 2017
Accepted: 30 May, 2017

J Kermanshah Univ Med Sci. 2017; 21(1): 16-22

Abstract

Introduction: Polycystic ovary syndrome is characterized by ovulatory dysfunction and hyperandrogenism. Although insulin resistance is not a diagnostic criterion for polycystic ovary syndrome, it has an important role in the development of the clinical presentation in a majority of patients with this syndrome. Many studies have examined the relationship of epicardial fat thickness with insulin resistance and cardiovascular complications. This study aimed to determine the relationship of epicardial fat thickness with polycystic ovary syndrome and insulin resistance.

Methods: This cross-sectional study recruited women with polycystic ovary syndrome presenting to endocrinology clinic in Kermanshah city. Sixty-four patients with polycystic ovary syndrome, without underlying diseases, were divided into two groups of 32 according to the HOMA-IR index as insulin resistant and insulin sensitive. Their epicardial fat thickness was measured by transtorasic echocardiography.

Results: The results of this study suggest that epicardial fat thickness has a direct and significant relationship with insulin resistance and BMI in patients with polycystic ovary syndrome.

Conclusion: According to the results, the increase in the thickness of epicardial fat is independent of the disease in patients with polycystic ovary syndrome. The cause of the increased thickness is the metabolic syndrome and increased insulin resistance in these patients.

Introduction

Polycystic ovary syndrome (PCOS) is an endocrine disease characterized by irregular menstruation, hyperandrogenism and multiple cysts in the ovaries. PCOS was first introduced in 1935 by Leventhal and Stein in a study on the relationship between multiple cysts, ovarian enlargement, and menstrual problems (1).

The diagnosis of PCOS is based on clinical or biochemical evidence of hyperandrogenism, along with amenorrhea or oligomenorrhea and manifestations of polycystic ovaries confirmed in an ultrasound scan. Clinical manifestations of PCOS encompass a wide range of symptoms. Women with PCOS often complain about menstrual disorders, symptoms of hyperandrogenism and infertility. Menstrual disorders can include oligomenorrhea, amenorrhea and even hypermenorrhea. However, about one-third of patients with PCOS do not have menstrual problems (2).

More than 80% of women who manifest hyperandrogenism symptoms have PCOS and hirsutism is one of the most common clinical findings observed in about 70% of the patients (3).

Infertility is observed in 40% of women with PCOS. The syndrome is the most common cause of infertility and anovulation. Approximately 90%-95% of women presenting to infertility clinics because of infertility and anovulation suffer from PCOS (4).

The prevalence of PCOS can vary based on the diagnostic criteria used. Studies report a prevalence of 4%-8% among women of the reproductive age (5-8).

A history of PCOS in the family is a risk factor for the development of this syndrome and this arises a genetic predisposition for the disease. Furthermore, studies have reported more prevalence of the syndrome in monozygotic twins compared to heterozygotic twins which again suggests a genetic predisposition for the disease (9).

Although insulin resistance is not a diagnostic criterion for PCOS, it plays an important role in its clinical manifestations. The prevalence of insulin resistance in patients with PCOS is 50%-70% (10-13) which is independent of the patients' obesity variables (14).

A total of 30%-40% of patients with PCOS have impaired glucose tolerance test, and 7.5%-10% of them have diabetes type 2 (15, 16). PCOS is associated with an increased risk of dyslipidemia which can be

accompanied by decreased HDL-C, increased triglyceride or decreased LDL-C.

Some studies have reported increased endothelin-1 in patients with PCOS (17) which along with increased concentrations of aldosterone and insulin resistance may underlie cardiovascular problems (e.g. hypertension) in such people. Hyperinsulinism along with insulin resistance was observed in 65% of obese women with PCOS, while insulin resistance is observed in 20% of thin women with PCOS (18-20).

Increased lipolysis leads to increased production of fatty acids and reduced anti-lipolysis effect of insulin in insulin resistance. Increased fatty acids increases access to the substrate and creates resistance to insulin by changing transduction pathways. Fatty acids impair the absorption of glucose by insulin and accumulate in skeletal and vascular muscles in the form of triglyceride, while hepatic glucose production increases and it accumulates in the liver in the form of triglyceride (21).

In addition, PCOS significantly increases the risk of diabetes mellitus type 2, which is apart from the effects of obesity (22).

Furthermore, studies have reported a direct relationship between epicardial fat and the incidence of coronary atherosclerosis and the ability of the tissue in secretion of hormones and cytokines that cause coronary thrombosis. Hence, epicardial fat can be considered a cardiovascular disease risk factor and its assessment by echocardiography is a proper criterion for determining visceral fat, which is the risk factor for cardiovascular diseases and metabolic syndrome in patients (23).

Given the prevalence of insulin resistance in patients with PCOS and the direct relationship between insulin and cardiovascular complications, the need to predict insulin resistance in these patients arises. Several studies have mentioned the relationship between visceral fat and insulin resistance. Moreover, increased epicardial fat is an independent risk factor for coronary artery diseases. Therefore, the present study was conducted to investigate the relationship between epicardial fat as visceral fat and insulin resistance in patients with PCOS in Kermanshah, Iran, so that if this hypothesis is approved, epicardial fat, as a risk factor for insulin resistance and cardiovascular diseases, is measured by transthoracic echocardiography in patients with PCOS to prevent its complications.

Materials and Methods

This analytical cross-sectional study investigated the relationship between epicardial fat thickness and insulin resistance in patients with PCOS. The patients were selected according to the inclusion criteria including an age range of 16-42 years and having Rotterdam criteria including:

1. The presence of ovarian dysfunction, which can include any of the following:
a. Evidence of anovulation
b. Presence of polycystic ovaries in ultrasound
2. Clinical or laboratory evidence of hyperandrogenism which can include:
a. Clinical: Hirsutism, acne or male pattern baldness
b. Laboratory: Increased serum levels of androgens

Patients diagnosed with any of the following were excluded: Cushing's syndrome, congenital adrenal hyperplasia, androgen-secreting tumors, smoking, kidney disease, liver disease, metabolic, cardiovascular disease, diabetes, treated with PCOS drugs. Sixty-four newly diagnosed PCOS patients were divided into two groups of insulin-resistant (case) and insulin-sensitive (control) with 32 patients in each group through fasting blood glucose and insulin level tests and insulin resistance formula (HOMA-IR). At the diagnosis time, a questionnaire was filled for each patient that covered the following information: height, weight, BMI, blood pressure, fasting blood glucose, fasting insulin, transthoracic echocardiography and HOMA-IR indicators. Insulin resistance was calculated using the homeostasis model (HOMA-IR) formula as follows:

Insulin resistance = fasting insulin (microU/L) x fasting glucose (nmol/L)/22.5

By definition, HOMA-IR $\geq$ 3 is considered as resistant to insulin and less than 3 is considered as sensitive to insulin (30).

Insulin serum level was measured by Mono bind kit. The epicardial fat thickness was measured by a vivid 3 expert echocardiography device with a 3S probe by the transthoracic method. The Mmode, long axis and short axis at RVOT site were measured by a cardiologist.

Data from the questionnaires, laboratory results, and echocardiography were entered into the SPSS and interpreted by tests in accordance with the type of variables and their relationship.

First, the Kolmogorov-Smirnov (KS) test was performed for all the three variables (BMI, epicardial fat thickness, and insulin resistance). The Pearson and Spearman correlation coefficients were used to evaluate the relationship for the normal and non-normal data, respectively. After categorizing the subjects as either resistant or sensitive to insulin, Leven test and independent t-test or Mann-Whitney U test were used to compare epicardial fat thickness and BMI in the two groups. ROC curve was used to determine the cutoff point for a maximum sum of sensitivity and specificity. The Error Bar and Box Plot charts were used for comparison of the variables between the two groups of resistant and sensitive to insulin.

Results

Sixty-four newly diagnosed PCOS patients without a history of underlying diseases were divided into two groups of insulin-resistant (case) and insulin-sensitive (control) with 32 patients in each group through fasting blood glucose and insulin level tests and insulin resistance formula (HOMA-IR). Of the 64 subjects in this study, 41 were single and 23 were married. The cumulative percentage was 64.1 indicating the very little difference between the two groups in terms of marital status. Only 6 subjects had children. The mean age of the participants was 27.59 years (range: 16-42 years). The mean weight of the participants was 73.50 kg (range: 49-107 Kg). The mean height of the participants was 163.203 cm (range: 147-80 cm). The mean systolic and diastolic blood pressure of the participants was 118.12 mmHg (range: 90-150 mmHg), and 77.66 mmHg (range: 60-95 mmHg), respectively. The mean HOMA-

IR index among the participants was 2.796 (range: 0.13-5.26). The mean fasting blood sugar among the participants was 87.58 mg/dl (range: 70-200 mg/dl). The mean blood cholesterol level of the participants was 191.79 mg/dl (range: 139-241mg/dl). The mean blood triglyceride level of the participants was 139.16 mg/dl (range: 60-210mg/dl). The mean blood insulin level among the participants was 12.950 mIU/l (range: 2.3-21.1 mIU/l). The mean thickness of epicardial fat (EP) among the participants was 0.466 cm (range: 0.2-0.8 cm).

Table 1. Average quantitative variables in this study (N=64)

Variables	Number	Mean	Standard deviation	Minimum	Maximum
Age (years)	64	27.59	6.276	16	42
Weight (kg)	64	73.05	11.985	49	107
Height (cm)	64	163.203	7.3099	147	180
Systolic blood pressure (mmHg)	64	118.12	12.456	90	150
Diastolic blood pressure (mmHg)	64	77.66	7.917	60	95
HOMA index	64	2.7962	1.18224	0.13	5.26
FBS (mg/dl)	64	86.33	17.177	70	120
LH (IU/l)	41	9.193	15.1611	0.2	96
FSH (IU/l)	41	5.765	3.8417	0.3	24
PRL (ng/ml)	32	121.876	199.0710	5.4	752
Cholesterol (mg/dl)	61	191.79	22.720	139	241
FBS (mg/dl)	61	104.66	18.386	60	158
FBS (mg/dl)	61	51.40	10.484	31	86
Testosterone (ng/dl)	42	1.0074	0.72935	0.05	3.40
17-hydroxiprogesteron (ng/dl)	34	1.607	1.9615	0.2	7.8
Insulin (mIU/l)	64	12.920	5.1075	2.3	21.1
EP (mm)	64	0.446	0.1406	0.2	0.8
FBS (mg/dl)	61	139.16	43.063	60	210

Systolic and diastolic blood pressures were compared in both insulin-resistant and insulin-sensitive groups. The mean systolic and diastolic blood pressure in the insulin-resistant group was more than the insulin-sensitive group (P<0.001) (Figure1).

BMI and HOMA-IR index (Fig. 2 and 3) were compared in both insulin-resistant and insulin-sensitive groups. BMI increase in the insulin-resistant group was more than that in the insulin-sensitive group (P<0.023). HOMA-IR index in the insulin-resistant group was more than that in the insulin-sensitive group (P<0.001).

The cut-off point which indicates the highest sensitivity and specificity of HOMA-IR index was 2.985. Subjects over 2.985 in the HOMA-IR index were in the insulin-resistant group and subjects under 2.985 were in the insulin-sensitive. The area under the curve (AUC) was equal to 1 in the ROC curve (Figure4).

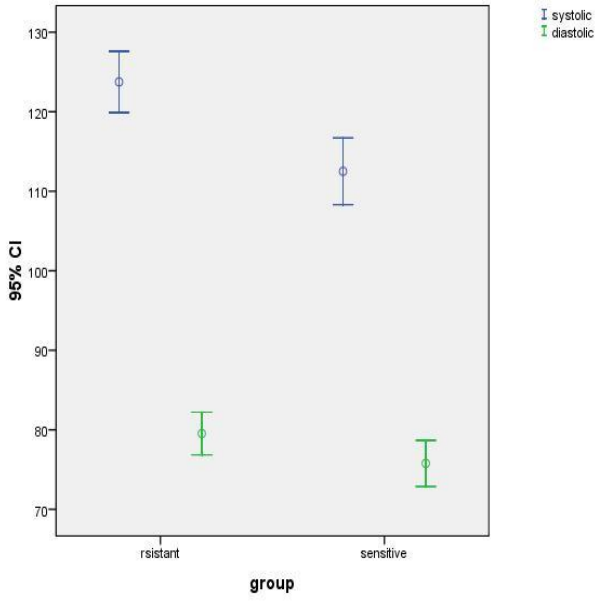

Figure1. The comparison of mean systolic and diastolic blood pressure in the two groups of resistant and sensitive to insulin

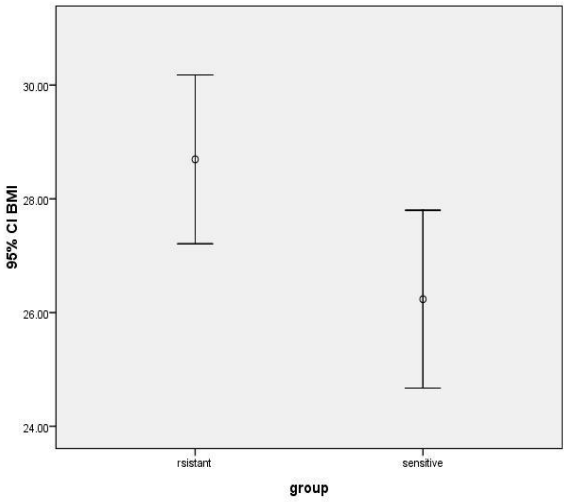

Figure 2. The comparison of mean BMI in the two groups of resistant and sensitive to insulin

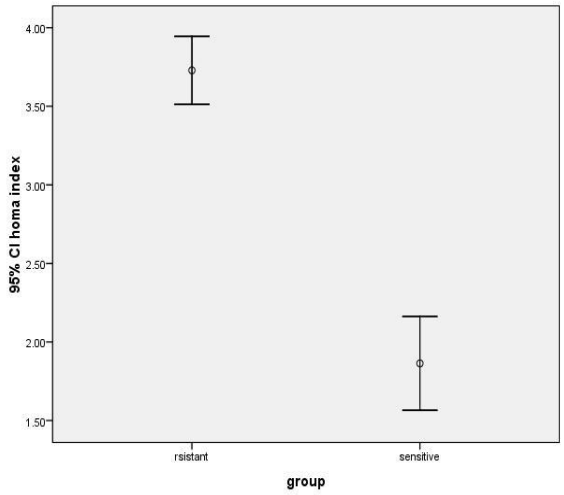

Figure 3. The comparison of mean HOMA-IR index in the two groups of resistant and sensitive to insulin

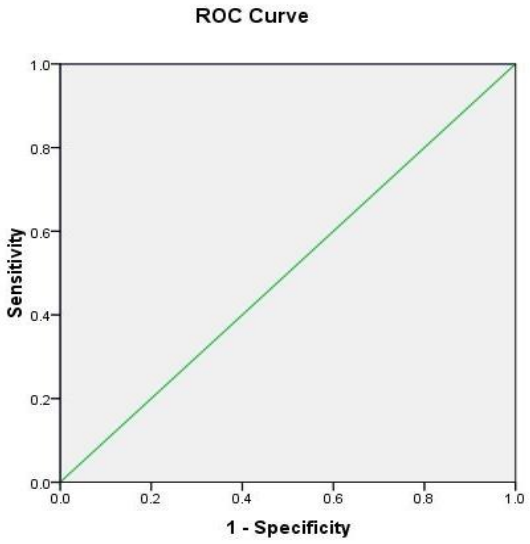

Figure 4. The receiver operating characteristics (ROC) curve, AUC = 1

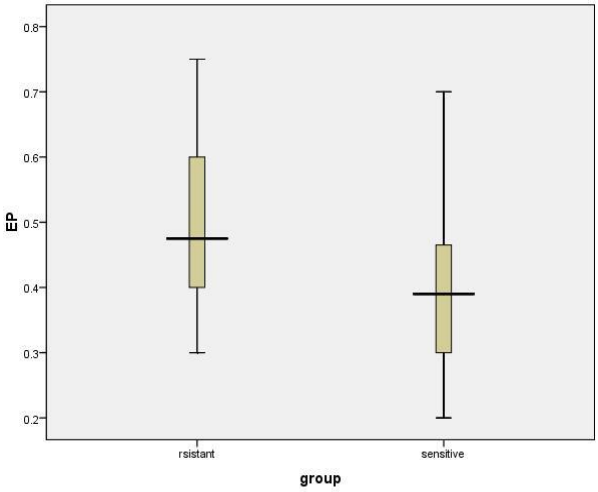

Figure 5. The comparison of mean epicardial fat thickness in the two groups of resistant and sensitive to insulin

There was a significant and direct relationship between the weight of patients with PCOS and epicardial fat thickness, indicating that weight gain will lead to increased epicardial fat thickness (P<0.001).

The relationship between BMI and epicardial fat thickness in patients with PCOS was significant and direct (P<0.023) indicating that an increase in BMI will lead to increased epicardial fat thickness (P<0.001).

There was also a significant relationship between epicardial fat thickness and triglyceride levels in patients with PCOS (P<0.007).

The relationship between HOMA-IR and epicardial fat thickness in patients with PCOS was significant and direct indicating that an increase in HOMA-IR will lead to increased epicardial fat thickness (P<0.001).

The relationship between insulin levels and epicardial fat thickness in patients with PCOS was significant and direct indicating that an increase in fasting insulin levels will lead to increased epicardial fat thickness (P<0.001).

The epicardial fat thickness was compared in both insulin-resistant and insulin-sensitive groups. The epicardial fat thickness in the insulin-resistant group was more than the insulin-sensitive group (Figure 5) (P<0.002).

Discussion

The results of a study by Aydogdu et al. about the relationship between epicardial fat thickness and clinical biochemical manifestations of PCOS, including insulin resistance showed that epicardial fat thickness and insulin resistance in women with PCOS has increased compared to the control group (P=0.0001) (24).

The difference between the study by Aydogdu and the present study was that the controls were healthy subjects matched with the intervention group in terms of MBI and age in the study by Aydogdu, which only measured the epicardial fat thickness and insulin resistance, without any conclusions about the relationship between insulin resistance and increased epicardial fat thickness.

In the present study, the relationship between epicardial fat and insulin resistance was assessed in 64 patients with PCOS in the two insulin-resistant and insulin-sensitive groups who were not matched in terms of age and BMI. The present study showed that HOMA-IR index has a significant and direct relationship with epicardial fat in women with PCOS (P<0.001). The study by Aydogdu et al. compared the amounts of epicardial fat and insulin resistance in healthy subjects and patients with PCOS but did not examine the relationship between epicardial fat thickness and insulin resistance.

In another study by Cakir et al. on subclinical hyperandrogenism and atherosclerosis as independent risk factors for increased epicardial fat thickness in patients with PCOS and idiopathic hirsutism, the epicardial fat thickness and insulin resistance in patients with PCOS were more than those in the controls (P<0.05). Eventually, they suggested using echocardiography to determine the epicardial fat in the early stages of PCOS as a risk factor for cardiovascular diseases (25).

Cakir et al. compared PCOS patients with healthy individuals, too. Both Cakir et al. and Aydogdu et al. showed that epicardial fat and insulin resistance were associated with PCOS, but they did not have any conclusions regarding the relationship between insulin resistance and epicardial fat thickness. That is while the present study clearly showed that increased epicardial fat thickness had a direct relationship with increased weight, BMI, and insulin resistance, and PCOS alone cannot be an independent factor for the increase in epicardial fat thickness. As mentioned in the findings, triglycerides, BMI and blood pressure were clearly more in the insulin-resistant group than in the insulin-sensitive group. Hence, metabolic syndrome was the reason for the increase in the insulin-resistant, that is, the increase in epicardial fat thickness resulted from the metabolic syndrome (abdominal obesity, hypertension, dyslipidemia, insulin resistance) in the insulin-resistant group of PCOS patients. This finding was approved a study by Ranjan Shetty et al. (28). In a study in 2013 by

Huang et al. in Chicago, on increased visceral fat independent of obesity and increased macrophages in adipose tissue as a mechanism for resistance to insulin in patients with PCOS, 14 women with PCOS and 14 healthy women similar in terms of age and BMI underwent MRI of abdominal fat, gluteal fat biopsies, and glucose tolerance test. They reported that increased adipose tissue macrophages and consequently increased insulin resistance (p=0.03), visceral fat (p=0.009) and subcutaneous (p=0.005) fat in women with PCOS. Therefore, it appears that the abdominal fat in patients with PCOS increases independent of the obesity factor, which can be associated with insulin resistance (29).

To determine the relationship between epicardial fat thickness and insulin resistance independent of the metabolic syndrome in patients with PCOS, we need two groups matching in terms of BMI and age. This can be the basis for further studies.

Other studies have suggested that epicardial fat thickness can indicate visceral fat. While MRI is a time consuming and expensive method for the diagnosis of visceral fat, transthoracic echocardiography, as a simple and low-cost method, can measure the epicardial fat thickness and be a good alternative to estimate the amount of visceral fat and cardiometabolic complications of PCOS in its early stages (23, 25, 28).

The present study, which was conducted in collaboration with a cardiologist, demonstrated that transthoracic echocardiography is very quick and much less costly than MRI and the results of epicardial fat thickness estimation by transthoracic echocardiography can be used as a reliable indicator of insulin resistance in patients with PCOS.

Conclusion

The results of the present study suggest that increased thickness of epicardial fat in patients with PCOS is independent of the disease and the cause of the increased thickness is the metabolic syndrome and increased insulin resistance in this group of patients. As a result, epicardial fat can be a predictive factor for insulin resistance and its cardiometabolic complications in high-risk patients such as PCOS patients.

References

1. Stein IF, Leventhal ML. Amenorrhea associated with bilateral polycystic ovaries. Am J Obstet Gynecol. 1935;29(2): 181-91.
2. Balen AH, Conway GS, Kaltsas G, Techatrasak K, Manning PJ, West C, et al. Polycystic ovary syndrome: the spectrum of the disorder in 1741 patient. Hum Reprod. 1995;10(8):2107-11.
3. Unluhizarci K, Kaltsas G, Kelestimur F. Non polycystic ovary syndrome-related endocrine disorders associated with hirsutism. Eur J Clin Invest. 2012;42(1):86-94.
4. Teede H, Deeks A, Moran L. Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. BMC Med.2010;8:41.
5. Azziz R, Wood KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab. 2004;89(6):2745-9.
6. Diamanti-Kandarakis E, Kouli CR, Bergiele AT, Filandra FA, Tsianateli TC, Spina GG, et al. A survey of the polycystic ovary syndrome in the Greek island of Lesbos: hormonal and metabolic profile. J Clin Endocrinol Metab. 1999;84(11):4006-11.
7. Knochenhauer ES, Key TJ, Kahsar-Miller M, Waggoner W, Boots LR, Azziz R. Prevalence of the polycystic ovary syndrome in unselected black and white women of the southeastern United States: a prospective study. J Clin Endocrinol Metab. 1998;83(9):3078-82.
8. Michelmore KF, Balen AH, Dunger DB, Vessey MP. Polycystic ovaries and associated clinical and biochemical feature in young women. Clin Endocrinol (Oxf). 1999;51(6):779-86.
9. Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI. Heritability of polycystic ovary syndrome in a Dutch twin-family study. J Clin Endocrinol Metab. 2006;91(6):2100-4.
10. Dunaif A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocr Rev. 1997;18(6):774-800.
11. Carmina E, Lobo R. Use of fasting blood to assess the prevalence of insulin resistance in women with polycystic ovary syndrome . Fertil Steril. 2004;82(3):661-5.
12. Schachter M, Raziel A, Friedler S, Strassburger D, Bern O, Ron-EI R. Insulin resistance in patient with polycystic ovary syndrome is associated with elevated plasma homocysteine. Hum Reprod. 2003;18(4):721-7.
13. Yildiz BO, Haznedaroglu IC, Kirazli S, Bayraktar M. Global fibrinolytic capacity is decreased in polycystic ovary syndrome, suggesting a prothrombic state. J Clin Endocrinol Metab. 2002;87(8):3871-5.
14. Dunaif A, Sega KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. Diabetes. 1989;38(9):1165-74.
15. Ehrmann DA, Barnes RB, Rosenfield RL, Cavaghan MK, Imperial J. Prevalence of impaired glucose tolerance and diabetes in women with polycystic ovary syndrome. Diabetes Care. 1999;22(1):141-6.
16. Legro RS, Kunselman AR, Dodson WC, Dunaif A. Prevalence and predictors of risk for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome : A prospective, controlled study in 254 affected women. J Clin Endocrinol Metab. 1999;84(1):165-9.
17. Diamanti-Kandarakis E, Spina G, Kouli C, Migdalism I. Increased endothelin-1 levels in women with polycystic ovary syndrome and beneficial effect of metformin therapy. J Clin Endocrinol Metab. 2001;86(10):4666-73.
18. Chang RJ, Nakamora RM, Judd HL, Kaplan SA. Insulin resistance in non-obese patients with polycystic ovary syndrome. J Clin Endocrinol Metab. 1983;57(2):356-9.
19. Duniaf A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocr Rev. 1997;18(6):774-800.
20. Burghen GA, Givens JR, Kitabchi AE. Correlation of hyperandrogenism with hyperinsulinemia in polycystic ovary syndrome. J Clin Endocrinol Metab. 1980;50(1):113-6.
21. Cornier MA, Dabelea D, Hernandez TL, Lindstrom RC, Steig AJ, Stob NR, et al. The metabolic syndrome. Endocr Rev. 2008;29(7):777-822.

22. Azziz R, Carmina E, Chen Z, Dunaif A, Laven JS, Legro RS, et al. Polycystic ovary syndrome. Nat Rev Dis Primers. 2016;2:16057.
23. Rabkin SW. Epicardial fat: properties, function and relationship to obesity. Obes Rev. 2007;8(3):253-61.
24. Aydogdu A, Uckaya G, Tasci I, Baysan O, Tapan S, Bugan B, et al. The relationship of epicardial adipose tissue thickness to clinical and biochemical features in women with polycystic ovary syndrome. Endocr J. 2012;59(6):509-16.
25. Cakir E, Doğan M, Topaloglu O, Ozbek M, Cakal E, Vural MG,et al. Subclinical atherosclerosis and hyperandrogenemia are independent risk factors for increased epicardial fat thickness in patients with PCOS and idiopathic hirsutism. Atherosclerosis. 2013;226(1):291-5.
26. Mannerås-Holm L, Leonhardt H, Kullberg J, Jennische E, Odén A, Holm G, Hellström M, Lönn L, Olivecrona G, Stener-Victorin E, Lönn M. Adipose tissue has aberrant morphology and function in PCOS: enlarged adipocytes and low serum adiponectin, but not circulating sex steroids, are strongly associated with insulin resistance. J Clin Endocrinol Metab. 2011;96(2):E304-11.
27. Iacobellis G, Leonetti F. Epicardial adipose tissue and insulin resistance in obese subjects. J Clin Endocrinol Metab. 2005;90(11):6300-2.
28. Shetty R, Vivek G, Naha K, Nayak K, Goyal A, Dias LS. Correlation of epicardial fat and anthropometric measurements in Asian-Indians: A community based study. Avicenna J Med. 2012;2(4):89-93.
29. Huang ZH, Manickam B, Ryvkin V, Zhou XJ, Fantuzzi G, Mazzone T, et al. PCOS is associated with increased CD11c expression and crown-like structures in adipose tissue and increased central abdominal fat depots independent of obesity. J Clin Endocrinol Metab. 2013;98(1):E17-24.
30. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28(7):412-9.