Serum CCL 18 Levels in women with polycystic ovarian syndrome

ABSTRACT

Background

Polycystic ovarian syndrome (PCOS) is the most common metabolic disorder seen in women of the reproductive age group, with the majority of them having insulin resistance. There is a need to identify sensitive markers of insulin resistance. CC chemokine ligand 18 (CCL 18), secreted from white adipose tissue (WAT) is upregulated in individuals with insulin resistance

Objectives

To study the correlation between serum CCL 18 levels and insulin resistance in PCOS.

Methods

This case-control study included 45 PCOS women and equal number of age and body mass index (BMI) matched controls. Estimation of serum CCL 18, serum testosterone, fasting plasma glucose, fasting insulin and HbA1c and ultrasonography of abdomen and pelvis were done and HOMA IR was calculated.

Results

Serum CCL 18 level was higher in women with PCOS when compared to controls. The mean level of serum CCL 18 (ng/mL) in the PCOS group and control group was 28.32±4.17 and 11.90±4.91 respectively ($p<0.001$). Blood pressure, waist circumference, waist-hip ratio, serum total testosterone, fasting serum insulin and HOMA IR showed a positive correlation with serum CCL 18 levels. High systolic BP, serum CCL 18 and serum total testosterone levels were independent predictors of PCOS ($P < 0.05$). A serum CCL 18 cutoff level of 18.84 ng/mL showed 93.3 % sensitivity and 93.3 % specificity in distinguishing PCOS subjects from healthy individuals.

Conclusion

There is a significant correlation of serum CCL 18 level with insulin resistance in PCOS subjects and serum CCL levels can act as a marker of PCOS.

Keywords: CCL 18, insulin resistance, polycystic ovarian syndrome, HOMA IR.

INTRODUCTION

Globally obesity has tripled between 1975 and 2016, with more than 1.9 billion adults, 18 years and older being overweight and of these over 650 million being obese**.** Concurrently, type 2 diabetes mellitus has reached epidemic proportions with 424.9 million people affected globally and more than 50% being unaware of their disease or undiagnosed[1]. In the face of such overwhelming statistics, insulin resistance as a causative factor for diabetes mellitus is gaining prominence. The inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake in the tissues of the affected individual as much as in a normal person is clinically is defined as insulin resistance. People with insulin resistance syndrome have more than seven times the risk of developing diabetes, compared to those with no cardiometabolic risk factors[2]. It has been estimated that 20%–25% of South Asians have insulin resistance and many more may be prone to it[3] and the prevalence of the insulin resistance syndrome in Asian Indian women is 1.5–2 times higher as compared with men[4].

Polycystic ovarian syndrome (PCOS) is one of the most common metabolic disorders and globally, prevalence estimates of PCOS are highly variable, ranging from as low as 2.2% to as high as 26%[5] and from India ranging from 9.13% to 36%[6]. Severe degree of insulin resistance or impaired glucose tolerance(IGT) is more common in obese PCOS[7]. About two-thirds of females with PCOS are insulin resistant[8] and the degree of insulin resistance exceeds that of women without PCOS matched for BMI or the degree of adiposity. Studies have reported the prevalence of insulin resistance in Indian women with PCOS to be 75%[9] and also more severe than their white counterparts. There is consistent evidence that a large proportion of women with PCOS develop diabetes mellitus later in life[10]. IGT or frank diabetes is evident in approximately 45% of women with PCOS by their fourth decade[11].

PCOS women usually present with abdominal obesity, a marker of insulin resistance, suggesting adipose tissue dysfunction. Both insulin resistance and type 2 diabetes mellitus are linked to

disturbances in white adipose tissue (WAT)[12] with mechanisms involving altered secretion of adipokines, peptides which exert autocrine, paracrine and or endocrine effects on metabolism[13]. The adipose tissue of women with PCOS has hypertrophic adipocytes with reduced lipolysis and insulin action. The expression and secretion of adipokines implicated in insulin resistance suggest that adipose tissue could play a key role in the metabolic abnormalities of PCOS. The major focus of research in adipose tissue has been to identify novel adipokines associated with WAT dysfunction and insulin resistance. In insulin resistance, adipose tissue is characterized by chronic low-grade inflammation and is associated with the infiltration of immune cells like classically activated "M1" macrophages. TNF-α and IL6, cytokines secreted by adipocytes in WAT and these immune cells primarily act as chemoattractants for immune cells, but they also exert direct effects on adipocytes by increasing lipolysis, altering adipokine secretion as well as attenuating insulin signaling and adipogenesis[14]. Fibrotic areas of insulin-resistant obese subjects also have alternatively activated "M2" macrophages.

CC chemokine ligand 18 (CCL18) is a chemokine constitutively expressed in the lung. It is also found in lower levels in lymph nodes, thymus bone marrow and placenta. CCL18 is endowed with chemotactic properties but also acts directly on immunity and participates in fibrotic and cancer processes[15]. The main producers of CCL18 are antigen-presenting cells like alveolar macrophages and follicular dendritic cells[16] in vivo and in vitro, several human cells "spontaneously" secrete CCL18, such as monocyte-derived dendritic cells[17] and alternatively activated macrophages, mast cells and eosinophils. Using gene microarray data from subcutaneous WAT of obese and nonobese women, it was shown that CCL18 expression was significantly increased in obesity and decreased upon weight loss. In a study by Hogling et al[18] in obese women before undergoing bariatric surgery, it has been found that CCL 18 was significantly enriched in macrophages and its secretion reflects adipose gene expression and constitutes a marker of inflammation. CCL 18 was highly expressed by M2 macrophages and showed a correlation with insulin resistance and adiposity[18],[19] and may be an indirect measurement of subcutaneous WAT.

At present, validated risk-assessment tools do not satisfactorily identify the increased risk factors associated with insulin resistance syndrome. Hence there is a need to identify more reliable markers of this syndrome as the currently available markers show conflicting data. Though CCL 18 levels have shown to be correlated to insulin resistance in the Caucasian populations, similar

studies have not been done in the Asian population. We hypothesized that since PCOS patients represent a group that has a high prevalence of insulin resistance, it would be interesting to observe the relationship between CCL 18 levels and insulin resistance in them. The present study was undertaken to study the correlation between insulin resistance and serum CCL 18 levels in PCOS women.

MATERIALS AND METHODS

This study was undertaken at a tertiary care referral hospital, during the period 2017-2018, after due clearance by the institutional ethics committee. Based on a study conducted by Daniel Eriksson Hogling et al in which serum CCL 18 levels in insulin-resistant patients were estimated, a sample size of 45 in each group was required, calculated by N master software developed by the department of biostatistics, CMC Vellore. Forty-five consecutive patients of PCOS in the age group of 18 to 40 years visiting endocrinology outpatient were enrolled in the study and the diagnosis of PCOS was based on Rotterdam criteria. The control group was chosen from the family welfare clinic and included an equal number of age and BMI matched healthy women with regular menstrual cycles and with no clinical evidence of hyperandrogenism, such as hirsutism or acne. Informed consent was taken from all the subjects. The exclusion criteria included other causes of irregular menstrual cycles and or androgen excess such as Cushing's syndrome, hyperprolactinemia, congenital adrenal hyperplasia, other adrenal disorders, thyroid disorders, galactorrhea, lactating women, pregnancy, diabetes mellitus, hypertension, coronary artery disease, acute or chronic infection, known malignancy, on oral contraceptive agents and/or anti-androgen therapy(within the preceding six months), lipid-lowering agents, metformin, pioglitazone or any other oral antidiabetic agent. After a complete clinical examination, 2 mL of a blood sample, after an overnight fast for 8 hours was collected from all the subjects, sera separated and stored at -80 C. Serum testosterone levels, HbA1c and ultrasound examination of abdomen and pelvis for ovaries and adnexa were done as part of routine evaluation. CCL 18 level was estimated by ELISA method (Ray Biotech). Fasting blood glucose level was estimated by the hexokinase method. Serum insulin level was estimated with CLIA technique and serum testosterone was processed with ECLIA. Insulin sensitivity was estimated by HOMA IR model from fasting plasma glucose and fasting insulin levels by using the formula

$$\text{HOMA IR} = [\text{Fasting insulin (miU/mL)} \times \text{Fasting blood glucose (mg/dL)}] \div 405$$

Statistical Methods

The statistical analyses were done using Statistical Package for Social Sciences version 18.0 (SPSS, Inc). All the quantitative parameters such as age, BMI and CCL 18 values are expressed as a mean with standard deviation and median with interquartile range. Differences in the mean values were tested for statistical significance by Student's t-test/ Mann Whitney's test. The association of categorical variables was tested for statistical significance by the Chi-square test of significance. Pearson's correlation analyses were used to examine the relationship between serum CCL 18 levels and other parameters. Logistic regression analysis was done and $P<0.05$ was considered statistically significant.

RESULTS

There were 45 patients each in the PCOS group and age, BMI matched controls. The baseline characteristics are seen in Table 1. The mean age of the subjects was 24.00 (SD 5.240, SEM ± 0.74). The mean BMI in the PCOS and control groups were 28.82 kg/ m^2 (SD 4.78, SEM ± 0.71) and 27.55 kg/ m^2 (SD 2.72, SEM ± 0.40) respectively. The mean HOMA IR in the PCOS group and control group was 3.49 (SD 3.19, SEM ± 0.47) and 2.01 (SD 1.32, SEM ± 0.19) respectively. The mean serum CCL 18 in the PCOS group was 28.32 (SD 4.17, SEM ± 0.62).

In the present study, a cut- off for HOMA IR is 2.31. This is obtained by taking value above the 75^{th} percentile of insulin resistance in controls (Table 2)

The following factors showed a significant positive correlation to serum CCL 18 levels: systolic blood pressure, diastolic blood pressure, waist circumference, waist-hip ratio, serum total testosterone, fasting insulin and HOMA IR (Table 3).

The correlation of insulin resistance with serum CCL 18 level is shown in Fig 1.

In order to identify independent predictors, logistic regression analysis was employed. Those factors which were significant at univariate analysis (systolic blood pressure, diastolic blood, waist circumference, waist-hip ratio, fasting insulin, HOMA IR, serum CCL 18 levels, serum testosterone) were included in the logistic regression analysis to identify the independent predictors for PCOS. It was noted that increased systolic BP (OR 10.58, 95% CI 1.554-72.130) serum CCL 18 levels (OR 82.919, 95% CI 13.658-503.41), serum total testosterone (OR 8.741, 95% CI 1.251-61.06) were found to be the independent factors after

adjusting for other factors. It is shown that patients with higher serum CCL 18 levels have the highest odds ratio of 82.91 in the prediction of PCOS.

A ROC curve was constructed to determine the sensitivity and specificity of serum CCL 18 levels in distinguishing between PCOS subjects and normal individuals. The area under the curve was 0.981. A serum CCL 18 cutoff level of 18.84 ng/mL showed 93.3 % sensitivity and 91.7 % specificity in distinguishing between PCOS subjects and normal individuals.

DISCUSSION

The present study was undertaken, keeping in view the rising prevalence of insulin resistance syndrome and its associated complications affecting the health of the people. There is a renewed interest to identify readily available sensitive markers to detect insulin resistance and the current study was aimed to establish the relevance of serum CCL 18 levels and its association with insulin resistance in women with PCOS, a majority of them having insulin resistance, the major underlying pathophysiological factor.

Studies undertaken previously on serum CCL 18 levels were mainly in inflammatory conditions, and a positive relation has been established between the two. However, recently the role of serum CCL 18 levels as a marker of white adipose tissue inflammation is gaining importance. On reviewing the literature and to our knowledge, the present study is the first to examine the relationship between serum CCL 18 levels and insulin resistance in PCOS.

In the present study, there was a significant correlation with insulin resistance and also a significant difference in serum CCL 18 levels between PCOS subjects and age, BMI matched healthy controls. In addition, there was a significant correlation of serum CCL 18 level with waist circumference, waist-hip ratio, blood pressures, fasting serum insulin level and serum total testosterone level. Multivariate logistic regression analysis proved that serum CCL 18 level could act as an independent predictor of PCOS.

In the present study, the mean age of the controls was 25.18 ± 3.01 was slightly higher than that of the cases, which was 24.00 ± 5.240, though not statistically significant. This was in contrast to the study by Daniel Eriksson Hogling at al[18] where the mean age of the insulin-resistant subjects, was

36.4 ± 6.3. This was higher than insulin-sensitive controls with a mean age of 35.7 ± 5.7. There was no significant correlation noted between serum CCL 18 levels and age in our study and this is in agreement with the study done by Sutter J De et al[20]. However, the study by Sin et al[21] has shown that age was significantly correlated with serum CCL 18 levels and a 10 years increase in age was associated with an 8.7 % increase in serum CCL 18 levels. However, no such relationship was observed in our study. In the present study, there was no correlation of serum CCL 18 levels with the weight although the study by Daniel A Hagg et al[19] has shown a positive correlation with weight.

The finding of the significant difference in the serum CCL 18 levels between PCOS women and normal controls is in agreement with the study done by Daniel Eriksson Hogling et al[18], where it was demonstrated that the mean serum CCL 18 levels were 63.5 ng/ml in insulin-resistant subjects and 41.0 ng/ml in the insulin-sensitive cases, whereas in our study the mean serum CCL 18 levels were 28.32 ng/ml and 11.90 ng/ml in cases and controls respectively. Patients in the primary study were those who were planned for bariatric surgery and their mean BMI was 42.7 kg/m^2 among the insulin resistant and 39.1 kg/m^2 in the insulin sensitive, which was much higher than the mean BMI of 29.29 kg/m^2 in our PCOS subjects and 28.04 kg/m^2 in controls. Though initially it was adjudged that the differences in the levels of serum CCL 18 levels in the two studies could be due to gross difference in the mean BMI between the two sets of patients, this was less likely, as the relationship between BMI and serum CCL 18 levels has been disputed with studies showing conflicting relationships. In a study done by Sin et al[21], it was shown that for every 1 kg/m^2 increase in BMI there was an increase in serum CCL 18 levels by 1.9 %, whereas, in a study done by Sutter J De et al[20], there was no significant correlation between serum CCL 18 levels and BMI. However in another study done by Daniel A Hagg et al[19], though there was no correlation between serum CCL 18 and BMI values, there was a significant correlation of serum CCL 18 levels with waist circumference and waist-hip ratio. In our study too, the relationship between serum CCL 18 and BMI was not significant, whereas a significant relationship was shown between serum CCL 18 and waist circumference and waist-hip ratio. The waist circumference in the primary study done by Daniel Eriksson Hogling et al[18] was 129.8 cm in the insulin-resistant group and 122.3 cm in the insulin-sensitive group when compared with our values of 94.62 cm in the cases and 87.26 cm in the controls, was much higher. We feel that the greater waist circumference, a marker of insulin resistance, seen in the primary study, as well as in our study, could be responsible for the greater

serum CCL 18 levels. The difference could be partly due to the estimation of serum CCL 18 levels by different ELISA kits in the two studies.

In view of higher visceral obesity and increased insulin resistance levels being consistently associated with South East Asian population, we expected higher levels of serum CCL 18 levels in our study group as compared to the Caucasian population. However, the values obtained by us are lower than that in Caucasian population studies. In the only study published from India, which looked at serum CCL 18 levels in patients of Gaucher's disease[22], and controls, an elevation of four times was demonstrated as compared to controls with median and interquartile ranges being 484.1 (334.2–592.2) and 113 (35–185) ng/ml respectively. But the reason behind the high values of serum CCL 18 levels in that study could be Gaucher cells themselves secrete CCL 18[22] and which could have driven the values up. These findings make us think that there could be other factors that could affect the levels of serum CCL 18 in the Indian population.

In comparison with the primary study, wherein BMI matched controls were not included, our study included BMI matched cases and controls and even then, the serum CCL 18 levels are statistically significant between the two groups.

Our study has demonstrated for the first time the significant correlation between serum CCL 18 levels and insulin resistance in PCOS women which was shown to be significant after multivariate regression analysis. In the study done by Daniel Eriksson Hogling at al[18], serum CCL 18 was shown to be a marker of WAT inflammation and is secreted from white adipose tissue and this could account as the possible mechanism for the significant relationship between insulin resistance and serum CCL18 levels. Classically activated M1 macrophages and alternatively activated M2 macrophages are abundant in fibrotic areas of insulin-resistant subjects. It has been previously shown that these alternatively activated M2 macrophages act as a source of CCL 18. As the majority of PCOS subjects are insulin resistant, this could be the underlying reason for the significantly high serum CCL 18 levels and also for its positive correlation.

In our study, there was a positive correlation between serum CCL 18 levels and systolic and diastolic blood pressures. To our knowledge, this is the first time that a positive correlation has been found between blood pressure and serum CCL 18 levels in both PCOS subjects and controls, even after taking into note that none of our cases and controls had ever been diagnosed or treated

for hypertension. This further strengthens the fact of association of high blood pressure with insulin resistance syndrome and hence serum CCL 18 levels in both the conditions. An earlier study done by Daniel A Hagg et al[19] has not shown any positive correlation between CCL 18 levels and blood pressures. Although the study was done in patients with atherosclerotic disease, who had undergone carotid endarterectomy compared with controls, the serum CCL 18 levels were not significantly different and this probably could be the reason for insignificant correlation between blood pressure and serum CCL 18 levels, even though the study had demonstrated as discussed above that serum CCL 18 correlated significantly with weight, waist circumference and waist-hip ratio.

Testosterone is one of the hormones implicated in the pathophysiology of PCOS. In the present study, a very strong correlation was noted between serum total testosterone levels and serum CCL 18 levels and this correlation persists even after adjusting for other confounding variables.

In our study, there was no correlation noted between serum CCL 18 levels and HbA1c or fasting blood glucose. This is in agreement with the study done by Khanian et al[23], who estimated serum CCL 18 levels and ejection fractions in diabetic and non-diabetic subjects with no significant difference between the two groups. Again, in the study done by Daniel A Hagg et al, no similar significant relationship was found. This could reflect that serum CCL 18 levels are elevated in subjects with insulin resistance and not in those with established diabetes mellitus.

Often the diagnosis of PCOS remains inconclusive and solely based on Rotterdam or NIH criteria and also there are no reliable biochemical markers that can be of use in the follow -up of these patients. The outcome of the treatment in PCOS patients is mainly assessed on clinical parameters like regularization of menstrual cycles, decrease in features of hyperandrogenism and regaining of fertility and have limitations being subjective and may not be reproducible. Our study has shown that a serum CCL 18 level of 18.84 ng/ml has 93.3 % sensitivity and 91.7 % specificity in PCOS subjects and this can be of use as a diagnostic marker. Further, it may be an additional tool in follow-up to assess the progress added to clinical parameters.

Our study has a few limitations. Dyslipidemia is a known component of insulin resistance. Firstly, the estimation of lipid profiles of the subjects could have established the relationship between these parameters and serum CCL 18 levels. However, in our study, serum lipid level estimation

could not be done. Secondly, as other inflammatory conditions could influence the serum CCL 18 levels, in our study they have been excluded by thorough clinical examination. However, no investigations have been carried out to detect any asymptomatic, occult conditions which could have affected the serum CCL 18 levels and hence a limitation in the study. However, this is very unlikely. Thirdly, insulin resistance in our study was measured by HOMA IR and was not correlated with the gold standard clamp studies of insulin resistance.

CONCLUSION

There is a significant correlation of serum CCL 18 levels with insulin resistance in PCOS subjects and this can act as a marker of PCOS. There is also a significant difference in serum CCL 18 levels in PCOS subjects with age and BMI matched healthy controls. A significant correlation of serum CCL 18 levels with waist circumference, waist-hip ratio, blood pressure, fasting serum insulin and serum total testosterone has been observed. Multivariate logistic regression analysis proved that serum CCL 18 level could act as an independent predictor of PCOS. Construction of a ROC curve has proved that a serum CCL 18 cutoff level of 18.84 ng/mL showed 93.3 % sensitivity and 93.3 % specificity in distinguishing between PCOS subjects and normal individuals.

Conflicts of interest: None

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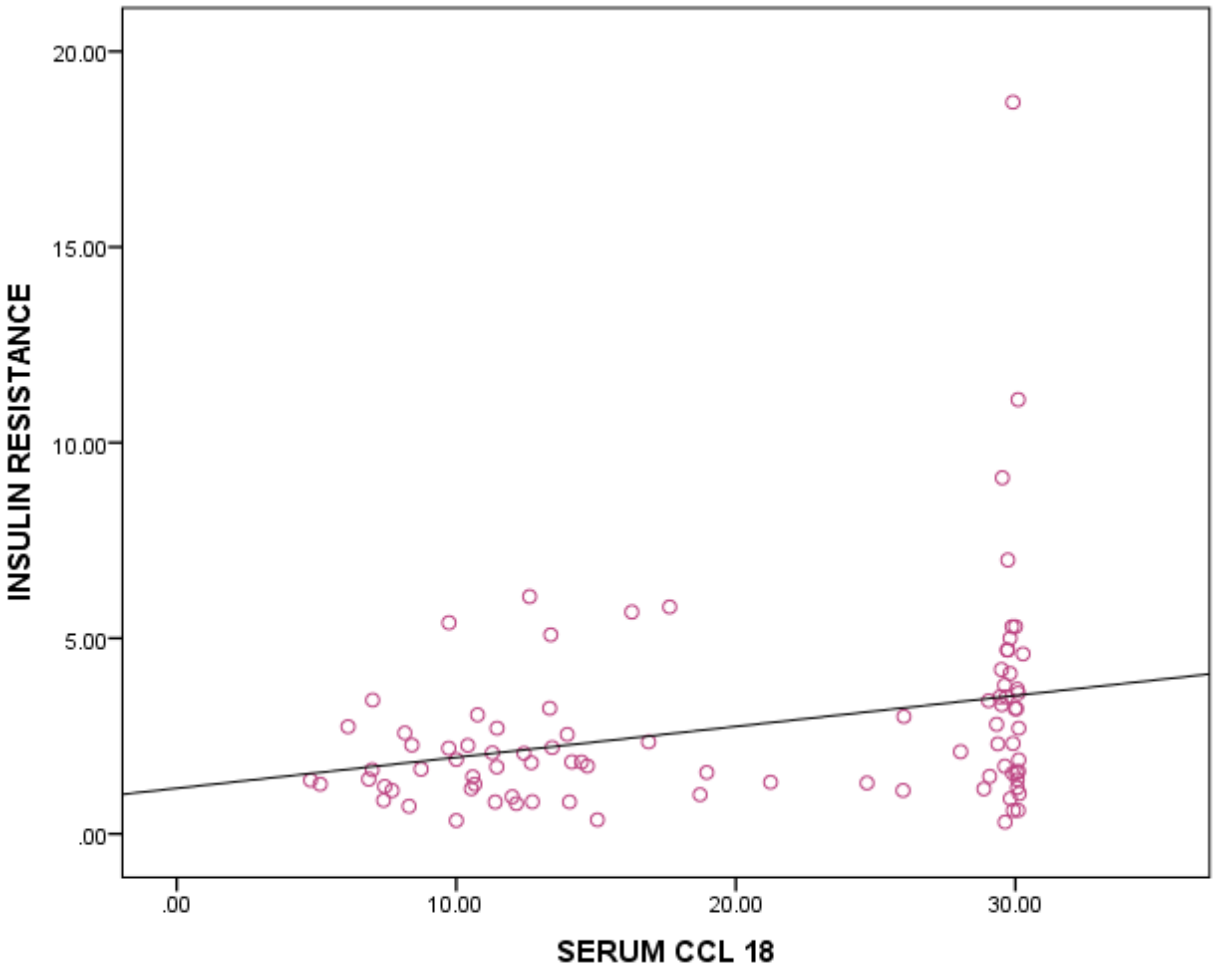

Figure 1: **CORRELATION OF INSULIN RESISTANCE WITH SERUM CCL 18**

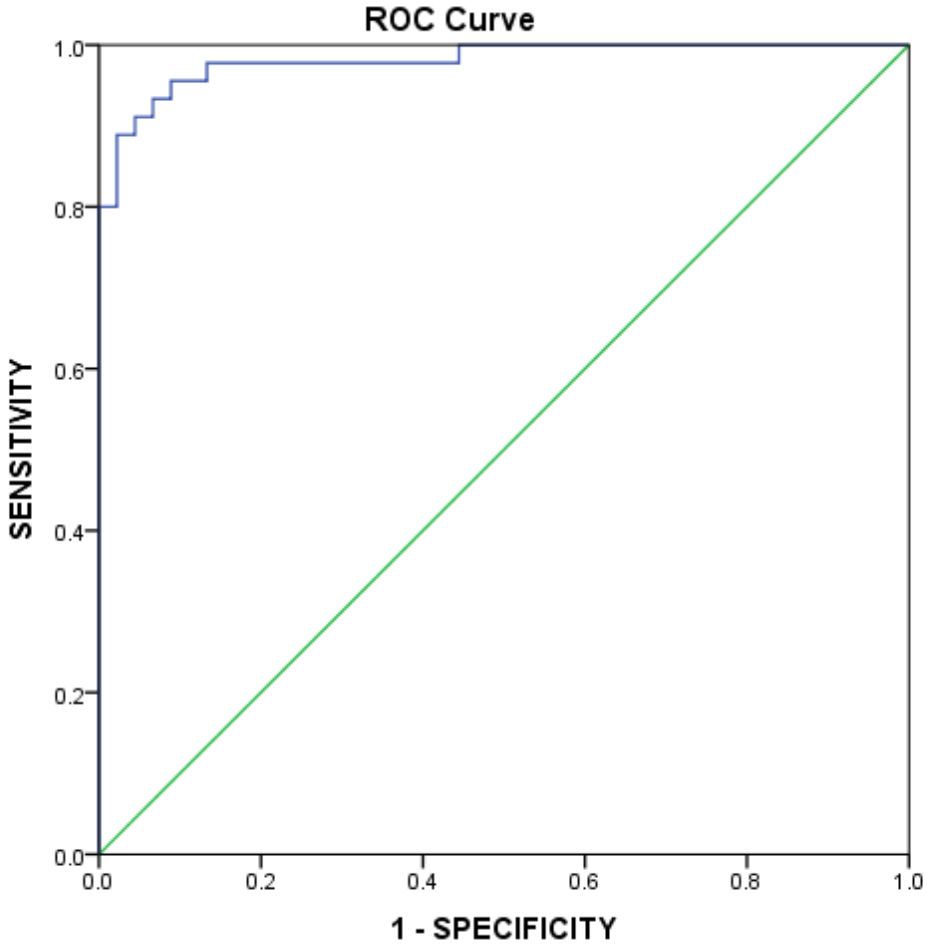

Figure 2: **ROC CURVE FOR SERUM CCL 18 AS A PREDICTOR OF PCOS**

Table 1 : Baseline Characteristics of Cases & Controls			
PARAMETER	**CASES(N=45)**	**CONTROLS(N=45)**	**P VALUE**
AGE (yrs)	24.00 ±5.240	25.18±3.01	0.195
Weight (kg)	74.18±9.52	73.59±8.20	0.875
BMI (kg/m^2)	28.82± 4.78	27.55±2.72	0.77
Waist Circumference(cm)	94.62±9.37	87.26±6.51	**00.03**
Waist Hip Ratio	0.92±0.03	0.90±0.04	**0.004**
SBP (mm of Hg)	120.62±5.78	115.96±6.775	**0.001**
DBP (mm of Hg)	80.44± 3.01	78.53±4.77	**0.026**
Modified FG score	10.09± 4.97	0.56±1.307	**0.000**
HbA1C (%)	5.34±0.33	5.10±0.91	0.100
FBS (mg/dL)	92.5(8.25)*	89.00(10)*	**0.044**
Fasting Insulin (mIU/L)	12.12 (12.45)*	7.72(4.97)*	**0.017**
HOMA IR	3.49±3.19	2.01±1.32	**0.005**
CCL 18 (ng/mL)	28.32±4.17	11.90±4.91	**<0.001**
Serum testosterone (ng/dL)	44(31.07)*	13.5(14.75)*	**<0.001**

*Median(IQR)

TABLE 2: Insulin Resistance by HOMA IR			
HOMA IR	Number (%)	Cases (n, %)	Controls (n, %)
Insulin Resistant (≥2.31)	37 (58.7%)	26 (57.7 %)	11(24.4%)
Insulin Sensitive(<2.31)	53 (41.3%)	19 (42.2 %)	34(75.5%)
Total	90	45	45
p=0.01			

Table 3 : Correlation of CCL 18 with different parameters		
Parameter	Correlation co effecient	P value
HOMA IR	.286	**0.00(<0.01)**
Weight (kg)	0.012	0.914
SBP (mm Hg)	0.338	**0.001 (<0.01)**
DBP (mm Hg)	0.294	**0.005(<0.01)**
BMI (kg/m^2)	0.150	0.158
Waist Circumference (cm)	0.392	**0.00(<0.01)**
Waist Hip Ratio	0.286	**0.006 (<0.05)**
Modified FG score	0.748	**0.00 (<0.01)**
Fasting Insulin (mIU/L)	0.256	**0.015(<0.05)**
FBS (mg/dL)	0.102	0.337
HbA1c (%)	0.151	0.155
S. testosterone (ng/dL)	0.541	**0.00(<0.01)**

Table 4: Logarithmic Regression analysis for independent predictors of PCOS

Factor	Odds Ratio	95 % Confidence Interval		Significance
		Lower	Upper	
SBP	10.588	1.554	72.130	0.016
Serum CCL 18	82.91	13.65	503.41	0.000
Serum Testosterone	8.74	1.251	61.06	0.029