

Reduced Angiotensin-2 as a Predictive Biomarker for Clomiphene Citrate Resistance in Polycystic Ovary Syndrome

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ABSTRACT

Background: Even though angiotensin-2 and interleukin (IL)-23 are known to be altered in polycystic ovary syndrome (PCOS), their association with clomiphene citrate (CC) resistance has not been studied. **Aim:** The objective of the study was to investigate whether angiotensin-2 levels are associated with inflammation and CC resistance in PCOS. **Settings and Design:** This study was conducted in a tertiary care hospital. **Materials and Methods:** Eighty-one women diagnosed with PCOS and on treatment with CC were enrolled in the study. Angiotensin-2 and IL-23 were analyzed in all the subjects. **Results:** Angiotensin-2 was significantly reduced ($P = 0.018$), and body mass index (BMI) ($P = 0.049$) and duration of infertility (0.006) were significantly increased in PCOS women with CC resistance compared to those who are sensitive to CC. In CC resistant PCOS, IL-23 predicts reduction in angiotensin-2 levels ($P = 0.010$). Among angiotensin-2, IL-23, BMI, and duration of infertility, we found that angiotensin-2 ($P = 0.020$) and duration of infertility (0.036) can predict resistance to CC therapy among PCOS subjects. **Conclusion:** We conclude that reduced angiotensin-2 levels predict CC resistance in women with PCOS.

KEYWORDS: Angiotensins, clomiphene citrate, interleukins, polycystic ovary syndrome

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common heterogeneous endocrine and metabolic disorder affecting 6%–8% of women of reproductive years.^[1] Although the etiology of PCOS is still unclear, it is considered as a multifactorial disorder.^[2] One of the first line therapies for PCOS is clomiphene citrate (CC), but around 30% of PCOS patients are resistant to CC treatment.^[3,4] Among several other factors, hyperandrogenism and obesity were found to be commonly associated with CC resistance.^[3] Inflammation and angiogenesis are known to play a role in the pathogenesis of PCOS, but their relation with CC resistance in PCOS has not been explored.

Angiogenesis is necessary for the growth of ovarian follicles and ovulation.^[5] Angiogenesis is regulated

by several proteins and growth factors including angiotensins. Angiotensin-2 is an antagonist of angiotensin-1 and it is responsible for the regulation of angiogenesis.^[6] Angiotensin-2 plays a role in the development of follicles and its levels are increased during follicular enlargement and reduced during maturation of follicles.^[7,8] Studies conducted in animal models of PCOS have demonstrated reduced angiotensin-2 levels, resulting in higher ovarian vascularity and vessel stabilization.^[9] Abnormal expression of angiotensin-2 was found to be associated with defect in the maturation of oocytes.^[10] A previous study has documented elevated follicular fluid angiotensin-2 levels in PCOS subjects during ovarian stimulation.^[11]

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Cytokines have been found to be involved in the maintenance of normal ovarian and menstrual cycles.^[12] Dysregulation of cytokines, a main feature of chronic inflammation in PCOS, is hypothesized to be associated with the blunted ovarian response to CC therapy.^[13] Interleukin (IL)-23 is a proinflammatory cytokine, which belongs to the IL-12 family of cytokines. IL-23 differentiates Th17 cells, which in turn produces other cytokines such as IL-17, IL-22, and tumor necrosis factor alpha.^[14] There are contradictory reports about IL-23 levels in PCOS. An earlier study by Karakose *et al.* have reported elevated serum level of IL-23 in patients with PCOS and concluded that IL-23 can be used as a biomarker to predict the presence of PCOS.^[15] A recent study by Zhang *et al.* has demonstrated reduced IL-23 levels in follicular fluid in PCOS subjects.^[16] Till date, there are no studies in the literature about IL-23 levels in PCOS subjects who sensitive and resistant to CC treatment. The objective of the study was to investigate whether angiopoietin-2 levels are associated with inflammation and CC resistance in PCOS.

MATERIALS AND METHODS

It was a cross-sectional study conducted in a tertiary care hospital from July 2018 to February 2020. Ethical approval was obtained from institute ethics committee (Human Studies) (IEC/2018/0340). Eighty-one women in the age group of 20–40 years, who were diagnosed as PCOS on the basis of revised Rotterdam criteria 2003, and those who were on treatment with CC were included in the study. Rotterdam criteria uses following for diagnosing PCOS: (a) ovulatory dysfunction, (b) clinical and/or biochemical features of hyperandrogenism, and (c) ultrasonographic evidence of polycystic ovarian morphology. At least 2 out of the 3 mentioned criteria are required for the diagnosis of PCOS, after excluding other etiologies with similar manifestations. Patients with other causes of infertility such as tubal factors, documented ovarian failure, associated medical comorbidities, those who are on oral contraceptives, antiandrogens, statins, glucocorticoids, or other infertility medications were excluded from the study. Written informed consent was obtained from all the subjects before participation in the study. The source of funding was from institute intramural fund.

Study procedure

Women who were administered CC (100 mg, OD) as a part of routine treatment once a day for 5 days (day 3–day 7) of each cycle on their cycles 1, 2, and 3 were recruited for the study. The patients who ovulated after three cycles of CC treatment were considered as CC sensitive. Even though International evidence-based

guideline released in October 2018 states that letrozole is the treatment of choice for PCOS, CC was routinely used in our institute for treating PCOS and ethical approval was obtained for the same. Ovulation was confirmed by assessing the ovarian follicle development using transvaginal ultrasonography. Ultrasonography was done to know which PCOS patients were CC resistant (failed to ovulate) and CC sensitive (who ovulated) after ovulation induction with CC. Those who failed to ovulate in response to CC treatment after three cycles were considered as CC resistant.^[17]

Sample collection

Five milliliters of venous blood was collected by peripheral venepuncture from the antecubital vein after in the early follicular phase (day 2–day 5) of menstrual bleeding or after progesterone-induced bleeding in patients in both the groups after the third cycle. Samples were collected in plain tubes without anticoagulant and processed in a centrifuge. The sera obtained were separated and stored at -80°C and were used for the estimation of angiopoietin-2 and IL-23.

Biochemical analysis

Angiopoietin-2 was estimated by ELISA using reagent kits from Elabscience, USA. IL-23 was estimated by ELISA using reagent kits from Fine test, China.

Statistical analysis

Continuous data were represented as mean \pm standard deviation and median (range). Kolmogorov–Smirnov test was used to determine the normality of the data. Independent *t*-test or Mann–Whitney U-test was used to compare the variable differences between CC sensitive and resistant PCOS subjects. Spearman's rank correlation test was used to evaluate the association of angiopoietin-2 with IL-23. Linear regression analysis was used for factors predicting angiopoietin-2 levels in CC-resistant PCOS using interleukin-23, duration of infertility, and body mass index (BMI) as covariates. Logistic regression analysis was used to calculate ODDS ratio and confidence interval to identify the factors which increases the risk of CC resistance in PCOS. Statistical analysis was performed using SPSS software version 19.0 (IBM SPSS Statistics, Armonk, New York, USA), and a $P < 0.05$ was considered statistically significant.

Sample size calculation

The sample size was estimated using the statistical formula for comparing two means with equal variance. The expected mean difference in angiopoietin-2 levels between the clomiphene sensitive and clomiphene resistant PCOS was 138.68 ng/ml with a S.D of 109.37.^[13] The sample size is estimated using this as

Table 1: General and clinical characteristics, hormonal parameters, interleukin-23, and angiopoietin-2 levels in polycystic ovary syndrome

Parameters	PCOS (n=81)
Age (years)	28±4.5
BMI (kg/m ²)	24.2±3.3
Duration of infertility (years)	4.3±3.2
Types of infertility (primary/secondary)	43/38
Menstrual cycle (regular/irregular)	23/58
Hirsutism (presence/absence)	12/69
Acne (presence/absence)	15/66
TSH (mIU/L)	3.11±1.87
LH (IU/L)	5.79±3.54
FSH (IU/L)	6.91±2.64
Testosterone (µg/L)	63.33±26.17
IL-23 (ng/L)	59.84±39.05
Angiopoietin-2 (ng/L)	3908.8±1386.2

PCOS=Polycystic ovary syndrome, BMI=Body mass index, LH=Luteinizing hormone, FSH=Follicle-stimulating hormone, TSH=Thyroid-stimulating hormone, IL-23=Interleukin-23

minimum expected difference at 5% level of significance and 90% power. The sample size estimated was 41 in each group.

RESULTS

Thirty-eight CC-sensitive and 43 CC-resistant PCOS subjects were recruited in the study. Table 1 shows the general and clinical characteristics, hormonal parameters, IL-23, and angiopoietin-2 levels in PCOS. The mean age group of the participants in this study was around 28 years with a BMI of 24.2 ± 3.3 kg/m² and the mean duration of infertility was 4.3 ± 3.2 years. Among the 81 participants, 43 (53%) women presented with primary infertility and 38 (47%) had secondary infertility. Majority of the participants (72%) presented with irregular menstrual cycle. Hirsutism was present in 12 (15%) women and acne was present in 15 (19%) women. The mean thyroid-stimulating hormone, luteinizing hormone, and follicle-stimulating hormone levels were within normal range. The mean testosterone levels were around 63 µg/L indicating the presence of hyperandrogenemia in PCOS subjects. The mean IL-23 was around 60 ng/L and angiopoietin-2 was around 3909 ng/L.

The clinical characteristics, IL-23, and angiopoietin-2 levels in CC-sensitive and CC-resistant PCOS subjects were shown in Table 2. BMI ($P = 0.049$) and duration of infertility (0.006) were significantly increased and angiopoietin-2 levels were significantly reduced ($P = 0.018$) in PCOS women with CC resistance compared to those who are sensitive to CC. There was no significant difference in age and IL-23 levels between the two groups.

When correlation analysis was done, angiopoietin-2 was negatively associated with IL-23 in PCOS subjects ($P = 0.034$) and those who are resistant to CC ($P = 0.003$). In PCOS subjects who are sensitive to CC, we did not observe any association between angiopoietin-2 and IL-23 levels. Angiopoietin-2 was not associated with age, BMI, and duration of infertility in women with PCOS.

Linear regression analysis was done on factors predicting angiopoietin-2 levels in CC-resistant PCOS using interleukin-23, duration of infertility, and BMI as covariates. Among various factors, we found that only IL-23 predicts reduction in angiopoietin-2 levels ($P = 0.010$) in PCOS women who resistant to CC.

Table 3 shows univariate logistic regression analysis for the factors predicting CC resistance in PCOS. Among angiopoietin-2, IL-23, BMI, and duration of infertility, we found that angiopoietin-2 ($P = 0.020$) and duration of infertility (0.036) can predict resistance to CC therapy among PCOS subjects.

DISCUSSION

In PCOS, ovulation induction is usually done with CC owing to low cost, ease of administration and minimal side effects.^[18] The factors which predict the responsiveness to CC includes age, BMI, testosterone levels, ovarian volume and menstrual status of the women.^[18] Earlier studies have reported that obesity is one of the factor which increases the risk of CC resistance.^[3] In our study, we observe that BMI and duration of infertility are increased in CC resistance group compared to CC-sensitive group suggesting that PCOS women with obesity and increased duration of infertility may not respond to treatment with CC. Since angiogenesis is involved in the pathogenesis of PCOS,^[19] we wanted to investigate whether reduced angiopoietin-2 levels increases the risk of CC resistance in women with PCOS.

Angiopoietins are known to be altered in PCOS. Earlier investigators have reported elevated angiopoietin-1 levels in PCOS, but they did not observe any significant difference in serum angiopoietin-2 levels between PCOS and controls.^[11,20] In the present study, angiopoietin-2 levels were significantly reduced in PCOS women with CC resistance compared to those who are sensitive to the CC treatment. These findings were in agreement with an earlier study from Wang *et al.* who reported reduced angiopoietin-2 levels in CC-resistant PCOS subjects.^[13]

PCOS is considered as a state of chronic inflammation and alteration in proinflammatory and anti-inflammatory cytokines have been reported. IL-23 is a proinflammatory

Table 2: General and clinical characteristics, interleukin-23 and angiopoietin-2 levels in clomiphene citrate sensitive and clomiphene citrate resistant polycystic ovary syndrome subjects

Parameters	PCOS		P
	Clomiphene citrate sensitive (n=38)	Clomiphene citrate resistant (n=43)	
Age (years)	27.47±4.3	28.47±4.569	0.322
BMI (kg/m ²)	23.37±3.41	24.92±3.51	0.049
Duration of infertility (years)	2 (1-12)	5 (1-16)	0.006
IL-23 (ng/L)	52.3 (42.6-357.5)	50.4 (27.1-137.3)	0.296
Angiopoietin-2 (ng/L)	4481.1 (1544-6591)	3289 (1561-6643)	0.018

BMI=Body mass index, PCOS=Polycystic ovary syndrome, IL-23=Interleukin-23

Table 3: Univariate logistic regression analysis to predict clomiphene resistance in polycystic ovary syndrome

Parameters	OR	CI	P
Angiopoietin 2	0.340	0.137-0.842	0.020
IL-23	0.641	0.266-1.542	0.321
BMI	0.636	0.511-2.999	0.636
Duration of infertility	2.622	1.067-6.442	0.036

BMI=Body mass index, IL-23=Interleukin-23, OR=Odds ratio, CI=Confidence Interval

cytokine, known to be elevated in PCOS.^[15] However, to the best of our knowledge, there are no studies about IL-23 levels in CC-sensitive and CC-resistant PCOS subjects. In the current study, we did not find any significant difference in IL-23 levels between two groups indicating inflammation may not be controlled in PCOS women who are treated with CC.

Since inflammation influences angiogenesis, we did correlation between IL-23 and angiopoietin-2 in PCOS and those who are sensitive and resistant to CC treatment. In all PCOS cases and PCOS women who are clomiphene resistant, IL-23 was negatively associated with angiopoietin-2 levels. Among several factors which can influence angiopoietin-2 levels in clomiphene resistant PCOS group, we observe that only IL-23 was found to be responsible for reduction in angiopoietin-2 levels in these patients. Based on our findings, we suggest that IL-23 reduces angiopoietin-2 levels which in turn increases angiogenesis and disrupts maturation of follicles, leading to reduction ovulation rates and increase in the formation of cysts.^[19] We speculate that all these processes may cause resistance to CC treatment in PCOS women.

Since age, inflammation, and angiogenesis can cause CC resistance, we did logistic regression analysis to predict the factors associated with clomiphene resistance. Among age, duration of infertility, IL-23, and angiopoietin-2, we observe that angiopoietin-2 and duration of infertility were associated with increased risk of CC resistance in PCOS women.

Noninclusion of control group is one of the limitations of the study. We did not follow-up the subjects to

study the maternal and fetal outcome. Furthermore, other cytokines and angiopoietins were not analyzed due to financial constraints. In the present study, the sample size was less in CC resistance PCOS ($n = 43$) and most of the patients belong to phenotype A having all the features of Rotterdam criteria. Hence, we did not correlate resistance PCOS with phenotypic classification. Since the present study is a short-term student project, we recruited consecutive PCOS cases who are sensitive and resistant to CC. Hence, we were unable to control the confounders such as BMI and duration of infertility.

CONCLUSION

We conclude that angiopoietin-2 levels are reduced and associated with IL-23 and CC resistance in women with PCOS. Angiopoietin-2 can act as predictor of CC resistance in PCOS subjects. Hence, we suggest that angiopoietin-2 levels should be estimated before starting treatment with CC and if levels are reduced alternative therapeutic strategies such as laparoscopic ovarian drilling should be considered in women with PCOS.

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Conflicts of interest

There are no conflicts of interest.

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