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ORIGINAL ARTICLE

Genetic Variants of *CYP11B2* and *CYP1A1* Among the North-Indian Punjabi Females with Polycystic Ovary Syndrome

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ABSTRACT

Polycystic ovary syndrome (PCOS) is a complex endocrinopathy in women of reproductive age. The genetics of PCOS is heterogeneous with the involvement of number of genes in the steroid synthesis pathway. The *CYP11B2* encodes aldosterone synthase and the genetic variants might increase aldosterone secretion in PCOS cases. *CYP1A1* is known to enhance the intraovarian catechol estrogen production and thus the propensity for PCOS. The present case-control study analyzed a total of 619 females for *CYP11B2* (rs1799998) and *CYP1A1* (rs4646903) polymorphisms. Obesity was examined according to body mass index (BMI) and waist hip ratio (WHR) categorization. Biochemical (lipid profile) analysis was performed in PCOS females. BMI (P=0.0001) and WHR (P=0.0001) revealed a statistically significant difference between PCOS cases and controls. The overall levels of triglycerides were higher in PCOS females. The genotype frequency distribution of *CYP11B2* (rs1799998) polymorphism revealed statistically significant difference between PCOS cases and controls (P=0.017). However, *CYP1A1* (rs4646903) polymorphism did not showed any association with PCOS. The present case-control association analysis is first from our region for *CYP1A1* and *CYP11B2* polymorphisms and is suggestive of genetic predisposition of steroidogenic genes among PCOS patients in the North-Indian Punjabi females.

INTRODUCTION

The frequently occurring disorder in the women of reproductive age is polycystic ovary syndrome (PCOS) which was for the first time reported by Stein and Leventhal [1, 2]. It is a complex endocrinopathy, with the presence of oligo/anovulatory cycles, hyperandrogenemic features (hirsutism, acne), insulin resistance and polycystic ovaries. About 9.3% of the Indian and 5~10% worldwide females [3, 4] are affected with PCOS. Different criteria have been proposed for the diagnosis of PCOS: Rotterdam 2003 criteria being the most widely applied [5]. The genetics of PCOS is heterogenous with the involvement of number of genes reported to be associated with the etiology and pathophysiology of PCOS. Majority of them are known to be involved in steroid synthesis pathway.

The *CYP11B2* is a member of cytochrome P450 superfamily that encodes a mitochondrial enzyme: aldosterone synthase, which is involved in the final step of aldosterone biosynthesis from, 11-deoxycorticosterone in adrenal cortex [6]. The gene is located at 8q22 and consists of 9 exons [7]. Genetic variations found in *CYP11B2* may increase the aldosterone secretion, raising the normal values of aldosterone-to-renin ratio (ARR) in plasma. The polymorphism -344 T>C (rs1799998) is known to be responsible for increasing the levels of

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aldosterone involved in a putative binding site for steroidogenic factor-1 (SF1) in the 5' regulatory promoter region of *CYP11B2* [8]. There is suppression in insulin signaling due to decreased expression of insulin receptor substrate-1 (IRS-1), as a result of increased aldosterone levels [9, 10]. The increased levels of aldosterone and testosterone have been observed in the cases who were carriers of mutant genotype, thus suggesting the effect of rs1799998 polymorphism on rennin-angiotensin system of the ovaries [9, 10].

CYP1A1 is localized on 15q24.1 with 7 exons and spanning 6 kb [11]. It encodes a phase I detoxifying enzyme involved in the activation of procarcinogens and affecting the metabolism and transportation of estrogens. The transcription of *CYP1A1* is upregulated by Aryl hydrocarbon receptor (AhR) [12]. The *CYP1A1* rs4646903 polymorphism is a T to G substitution at nucleotide 3801 in the 3' noncoding region. This leads to increased risk of PCOS by oxidative metabolizing the estradiol to catechol estrogen, 2-hydroyestradiol (2-OHE2) to inhibit the granulose cell DNA replication and folliculogeneis [11]. Several studies have reported the increased *CYP1A1* activity, which further enhancing the intraovarian catechol estrogen production and so the chances of PCOS [13].

The present study evaluated the association of two genetic variants, *CYP11B2* polymorphism rs1799998 and *CYP1A1* polymorphism rs4646903 with the PCOS. To the best of our knowledge no study has been done in the North Indian region for the explanatory roles of above genetic variants in PCOS.

MATERIALS AND METHODS

1. Study design and subjects selection

The sample size incorporated in the present case-control study was 619 females, including PCOS cases (N=311) and age-matched healthy controls (N=308) from Punjab, India. The samples were collected from June 2017 to March 2020. All the PCOS samples were collected from Hartej Hospital. In the present study the CaTs-power calculator was used to calculate sample size to attain minimum 90% power of study with 95% confidence interval [14].

2. Ethics statement

The study was permitted by ethical committee of Guru Nanak Dev University, Amritsar, India with provisions of declaration of Helsinki (reference number: 96/HG, dated: 09/01/2015).

3. Clinical assessment

The cases fulfilling the Rotterdam 2003 criteria were selected for the study [5]. The consent was obtained from all the study participants. The information of the subjects was collected on a predesigned proforma. Detailed demographic information, menstrual and reproductive history, family history and pedigree were obtained from all the included subjects.

The venous blood sample (5~6 mL) was withdrawn using sterile disposable syringes by venipuncture by trained professional. The blood was divided into two parts, 3~4 mL was stored in vacutainers containing 0.5 M EDTA (act as an anticoagulant agent) for molecular approach and 2 mL blood was stored in clot activator for serum (used for biochemical analysis). The blood and serum were stored in −20℃ and −80℃ till further use.

4. Anthropometric assessment

Anthropometric measurements such as height, weight, waist ratio and hip ratio were recorded. Obesity has inference on reproductive complications and elevates hirsutism, hyperandrogenism, insulin resistance in women suffering with PCOS [15, 16]. The obesity was examined according to body mass index (BMI) and waist hip ratio (WHR) categorization. The WHO 2004 criteria were followed for categorization [17].

5. Biochemical assessment (lipid profile)

Biochemical analysis for lipid profile including cholesterol, triglyceride, high density lipoprotein

(HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) was done in PCOS cases using Erba Mannheim kits (Erba Mannheim, Germany) on Erba Mannheim biochemical analyzer (Erba Mannheim).

6. Genotype analysis

DNA isolation was done from 1 mL blood using organic method given by Adeli and Ogbonna (1990), with slight modifications. Quantification of DNA was done using nanodrop and agarose gel electrophoresis.

After DNA quantification, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for genotypic analysis of *CYP11B2* (rs1799998) and *CYP1A1* (rs4646903) polymorphisms. The amplification conditions for *CYP11B2* (rs1799998) were denaturation at 94℃ for 1 minute, annealing at 64℃ for 45 seconds and extension at 72℃ for 1 minute and were repeated for 35 cycles. The PCR product for *CYP1A1* (rs4646903) was amplified by these conditions denaturation at 72℃ for 1 minute, annealing at 57℃ for 45 seconds and extension at 72℃ for 1 minute (repeated for 35 cycles). The selected primers along with restriction enzymes of rs1799998 and rs4646903 polymorphisms are illustrated in Table 1.

7. Statistical analysis

The statistical analysis was done using SPSS (version21, IBM SPSS, NY, USA). Student t-test and Fisher's exact test was used to calculate the genotypic and allelic frequency distribution between PCOS cases and healthy controls. The risk association was calculated using Pearson's chi square (χ^2) test. The genetic models were constructed by binary logistic regression and the relative risk was calculated by adjusting other co-variates specifically body mass index (BMI), waist hip ratio (WHR) and age at menarche (AAM).

The distribution of all the biochemical parameters including cholesterol, triglyceride, HDL, LDL and VLDL were analysed by one-way analysis of variance (ANOVA).

RESULTS

1. Demographic and clinical features

The mean age females with PCOS was 24±4.80 and 23±4.17 in healthy females (P=0.08). The mean of age at menarche was 13.20±1.31 and 13.26±1.33 in PCOS cases and controls. The clinical presentation in PCOS females showed hirsutism (N=123), obesity (N=145), acne (N=41) and sleep apnea (N=10). Control females enrolled did not had hirsutism, acne and sleep apnea. However, 25% (N=77) of the control females were obese.

BMI (P=0.0001) and WHR (P=0.0001) revealed a statistically significant difference between PCOS cases and controls (Table 2). There were more obese PCOS females (53.70%) than non-obese PCOS females (46.29%) as compared to control females where non-obese were higher (71.11%) as compared to obese females (28.51%).

Univariate and multivariate regression analysis was done to determine the most significant demographic as well as risk factors associated with PCOS. After application of multivariate logistic regression BMI denoted significance of $P=4.3\times10^{-7}$, in addition WHR also revealed significant association towards PCOS ($P=2.1\times10^{-12}$). Family history of PCOS and diabetes as well as lifestyle conferred a significant risk towards progression of PCOS (Table 3).

The comparison of the biochemical variables between TT, TC and CC genotypes of rs1799998 polymorphism revealed no significant association of any of the observed variables. The mean values for cholesterol and LDL were

Table 1. Representation of selected primer pairs and fragments generated after digestion with specific restriction enzymes

Genes	SNPs	Primer pairs	Size	Enzyme	Fragmented size
CYP11B2	rs1799998	5'-GACTCCAGGACCCTGGTTGATA-3' 5'-CAGCCAAAGGTAGATGAAGGAG-3'	390	SFoI	390, 204, 186
CYP1A1	rs4646903	5'-CAGTGAAAGAGGTGTAGCCGCT-3' 5'-TAGGGAGTCTTGTCTCATGCCT-3'	340	MSPI	340, 205, 135

observed to be higher in PCOS cases with TC genotype as compared to other two genotypes. Triglycerides have higher levels of mean with respect to CC genotype. There was a significant difference observed in BMI distribution with high mean value (28.4±6.2) in CC genotype (P=0.003) (Table 4).

Table 2. Presentation of clinical parameters in cases and controls

Clinical parameter		PCOS (N=311)	Control (N=308)	P-value
Age (years)		24±4.80	23±4.17	0.088
Age at menarche (years)		13.20±1.31	13.26±1.33	0.761
Height (cm)		160±0.5	163±0.6	0.006
Weight (kg)		65.5±7.2	58.5±6.1	0.04
Waist circumference (cm)		93.4±13.9	79.2±7.8	0.000
Hip circumference (cm)		103.6±10.6	93.9±7.3	0.000
BMI	<18.5	19	22	1.0×10^{-9}*
	18.5~22.9	106	171	
	>23	145	77	
WHR	<0.81	39	170	1.0×10^{-11}*
	>0.81	231	100	

*P<0.05 statistically significant.
Abbreviations: BMI, body mass index; WHR, waist hip ratio.

Table 3. Representation of univariate and multivariate logistic regression

Co-variates	Univariate regression			Multivariate regression		
	OR	95% CI	P-value	OR	95% CI	P-value
Age of females	0.99	0.96~1.03	0.893	1.02	0.98~1.06	0.243
Age at menarche	1.03	0.91~1.17	0.582	1.02	0.90~1.17	0.689
BMI	0.88	0.84~0.92	1.0×10^{-11}*	0.87	0.83~0.92	4.3×10^{-7}*
WHR	0.10	0.06~0.15	1.0×10^{-10}*	0.11	0.07~0.19	2.1×10^{-12}*
Diet intake	1.01	0.70~1.46	0.927	0.88	0.55~1.41	0.622
Family history of PCOS	18.91	7.49~47.70	4.7×10^{-9}*	16.12	5.76~45.10	1.2×10^{-6}*
Family history of diabetes	7.23	4.57~11.44	1.0×10^{-12}*	6.56	3.79~11.36	2.0×10^{-10}*
Family history of other complex disorders	2.48	1.59~3.88	6.0×10^{-5}*	1.01	0.56~1.41	0.953
Lifestyle (active/sedentary)	1.68	0.91~3.11	0.096	2.24	1.02~4.88	0.043*

*P<0.05 statistically significant.
Abbreviations: See Table 1; PCOS, polycystic ovary syndrome; OR, oddi ratio; CI, coefficient index.

Table 4. Comparison of mean values for baseline characteristics with respect to genotypes among PCOS cases for *CYP11B2* (rs1799998) polymorphism

Characteristics	PCOS cases (N=311, mean±SD)							
	rs1799998				rs4646903			
	TT (N=253)	TC (N=47)	CC (N=11)	P-value	TT (N=253)	TC (N=47)	CC (N=11)	P-value
Age (years)	24.0±4.9	24.4±4.2	26.4±4.6	0.245	23.8±5.0	24.2±4.0	26.0±5.0	0.07
Age at menarche (years)	13.1±1.3	13.0±1.2	13.6±0.9	0.447	13.1±1.6	13.0±1.3	13.0±1.2	0.803
Age of onset of PCOS (years)	20.0±5.4	19.5±5.2	18.6±6.3	0.591	20.2±5.3	19.2±5.2	19.8±4.5	0.366
BMI (kg/m^2)	23.8±4.39	23.6±4.0	28.4±6.2	0.003*	23.6±4.3	24.1±4.5	25.5±5.3	0.088
WHR	0.8±0.06	0.8±0.06	0.8±0.05	0.526	0.8±0.06	0.8±0.07	0.8±0.06	0.524
Cholesterol (mg/dL)	168.8±49.9	174.3±50.2	151.0±51.8	0.381	168.4±47.3	175.0±58.9	156.6±41.7	0.241
Triglycerides (mg/dL)	168.5±102.5	157.5±95.7	207.8±108.7	0.338	172.8±106.9	155.3±94.3	81.0±15.3	0.441
HDL (mg/dL)	44.8±14.4	44.7±13.8	44.8±11.4	1.00	46.1±14.4	42.0±14.7	43.0±9.7	0.079
LDL (mg/dL)	90.2±57.6	98.0±59.3	65.1±54.1	0.237	87.7±55.7	101.9±66.3	79.5±44.6	0.109
VLDL (mg/dL)	32.9±20.1	31.4±19.1	41.0±21.8	0.369	33.6±21.0	31.0±18.8	34.0±16.2	0.615

*P<0.05 statistically significant by one way ANOVA.
Abbreviations: See Table 1, 2; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

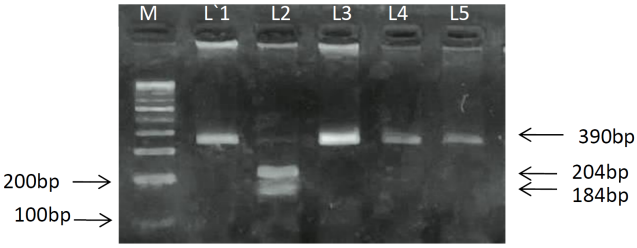

Figure 1. Picture RFLP products of *CYP11B2* (rs1799998) polymorphism. Lane M represents 100 bp ladder. Lane L1, L3, L4 and L5 represent homozygous wild genotype (TT). Lane L2 represents homozygous mutant genotype (CC).

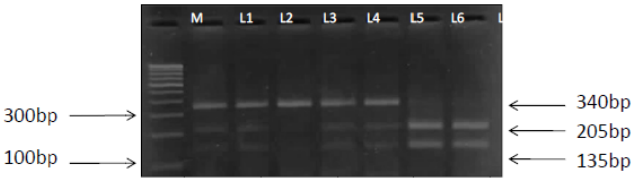

Figure 2. RFLP products of *CYP1A1* (rs4646903) polymorphism. Lane M represents 100 bp ladder. Lane L3 represents homozygous wild genotype (TT). Lanes L1, L2, L4 and L5 represents heterozygous genotype (TC). Lanes L6 and L7 display homozygous mutant genotype (CC).

Table 5. Frequency distribution of genotypes and alleles among cases and controls with relative risk towards PCOS

SNP	Genotypes/ allele value	Case (N)	Control (N)	χ^2-*P* value	OR (95% CI)	*P*-value
CYP11B2 (rs1799998)	TT	253	235		reference	-
	TC	47	69	0.017*	0.6 (0.4~0.9)	0.02
	CC	11	4		2.5 (0.8~8.1)	0.11
	T	553	539		reference	-
	C	69	77	0.44	0.8 (0.6~1.2)	0.44
CYP1A1 (rs4646903)	TT	207	228		reference	-
	TC	76	60	0.121	1.3 (0.9~2.0)	0.09
	CC	28	20		1.5 (0.8~2.8)	0.15
	T	490	516		reference	-
	C	132	100	0.02*	1.3 (1.0~1.8)	0.02

*$P<0.05$ statistically significant, OR after adjustment for BMI, WHR, AAM.

No significant difference was shown by any parameter with respect to three genotypes TT, TC and CC of rs46464903 polymorphism. There was increase in mean values of triglyceride with TT genotype (Table 4).

2. Genotype analysis

After amplification of promoter region of *CYP11B2* including -344T>C (rs1799998) variant, PCR product of 390 bp was generated. Restriction digestion was done by *SFoI* enzyme (Figure 1). The genotype frequency distribution of *CYP11B2* (rs1799998) polymorphism revealed statistically significant difference between PCOS cases and controls (P=0.017) (Table 5). On comparing the TT genotype of rs1799998 polymorphism with TC genotype, a statistically significant protection was shown against development of PCOS (Table 5). This risk was calculated after application of adjustments of other confounding factors such as BMI, WHR and age at menarche.

The *CYP1A1* (rs4646903) PCR fragment of 340bp was digested with restriction enzyme *MSPI* (Figure 2). The genotype frequency did not demonstrate statistically significant difference between PCOS cases and controls of *CYP1A1* rs4646903 polymorphism (P=0.121) (Table 5).

DISCUSSION

The polycystic ovary syndrome is one of the reasons that cause infertility issues among young females. The anovulation (menstrual disturbance) in PCOS females causes difficulty in conception and leads to dilemma of infertility. Abnormal levels of androgens in PCOS are result of defect in functioning of various enzymes and genes in steroid synthesis pathway [18]. Ovarian hyperandrogenism gets affected by overexpression of steroidogenic enzymes.

The cases and controls selected in the present study were age matched and no significant difference was found between the two studied groups (P=0.088).

However, in relation to age a study by Zhang and colleagues found significant difference between PCOS cases and controls (P<0.05) [19]. A study on South Indian females did not reveal difference in age [20].

The age at menarche (AAM) calculated for PCOS cases and controls in the present study did not reveal any comparable difference (P=0.761). The mean age at menarche in PCOS cases was 13.20±1.31 and 13.26±1.33 in controls. This indicated that average age of menarche in females of our study to be around 13 years. Dasgupta and Reddy did not find any significant difference of AAM between cases and controls (P=0.852) [21]. However, a significant difference for AAM was found in study by Sadrzadeh and colleagues [22]. Analogous to present investigation, Okamura and colleagues observed no comparable difference for AAM between cases and controls [23]. Age at onset of PCOS has been undertaken in many studies to find its role in progression and complications of PCOS. In the present work the age of onset of PCOS cases was found to be 20.94±5.59, whereas study on South Indian cohort revealed age of onset to be 16.66±4.93 [20]. A study done by Bronstein and colleagues suggested that age of onset of PCOS may be earlier in case of females who have early menarche and thelarche [24].

It is well documented that PCOS women are more vulnerable to obesity related health problems like diabetes, hypertension, cardiovascular disorders, anovulation, infertility, difficulties in conception and adverse pregnancy outcomes [25]. BMI provides the measure of obesity which throws light on associated problems with it. It has been established that Asian population has higher fat deposition at a lower BMI. A significant difference was observed in BMI between PCOS cases and controls (P=1.0×10^{-9}) in the present study (Table 3). A significant difference in BMI of PCOS cases and controls was observed in Pakistani females where PCOS females faced more pregnancy related issues as compared to non-PCOS females [26]. Studies on South Indian cohort have mentioned differences in BMI in PCOS females and controls as well its related conditions including high glucose level, LH and LH/FSH ratio, dyslipidimia and fasting insulin [27, 28]. Similar findings were reported by Dasgupta and Reddy in South Indian PCOS females (P<0.001) [13]. In our previous studies also BMI was found to be positively related with PCOS [29, 30].

WHR is known to provide estimation of abdominal obesity. Abdominal obesity is known to be related with disorders of reproductive system. In the present study a significant difference was observed for WHR in case of PCOS as compared to controls (P=1.0×10^{-11}). In a study on lean PCOS cases and controls, higher fat body mass as well as android obesity was observed as compared to lean controls, suggesting obesity trend in even in lean PCOS females [31].

The most common metabolic abnormality observed in PCOS is the additive role of dyslipidemic profile (high triglycerides and low high density lipoprotein-cholesterol [HCL-C]) with insulin resistance [29, 32]. In the present study lipid profile analysis was done in PCOS cases and the overall mean calculated for cholesterol was 168.99±50.09, triglyceride (168.30±101.81), HDL (44.82±14.24), LDL (90.53±57.91) and for very low density lipoprotein 33.14±20.03. The overall mean of triglycerides was higher and the levels of HDL were lower than required. About 23.33% PCOS females had cholesterol above the threshold value of 200 mg/dL, 21.11% PCOS females had LDL above the threshold of 130 mg/dL, 74.44% females had HDL lower than the threshold of 50 mg/dL, 26.29% cases had higher VLDL levels than threshold of 40 mg/dL and 48.51% cases had higher triglycerides than threshold value of 150 mg/dL (Table 4).

The *CYP11B2* is known to affect the ovarian renninangiotensin system. Aldosterone synthase (*CYP11B2*) is one of the enzymes that plays essential role in aldosterone synthesis and is expressed only in adrenal cortex specifically in the zona glomerulosa cells. The minor allele of −344T>C (rs1799998) is thought to enhance the aldosterone-to-renin ratio [8] and a difference in the expression level of rs1799998 of *CYP11B2* in the

Table 6. Comparison of genotype distribution of *CYP11B2* (rs1799998) and *CYP1A1* (rs4646903) polymorphisms in the present study with other studies

Type	Country (etnicity)	Cases/ controls	Cases			Controls			*P*-value	Refs
			TT	TC	CC	TT	TC	CC		
rs1799998	Chinese	92/92	40	52		58	34		<0.001	[34]*
	Japanese	50/100	22	18	10	49	44	7	0.022	[33]*
	North India	311/308	253	47	11	235	69	4	0.017	Present study*
rs4646903	South India	184/72	77	74	29	40	28	4	0.013	[37]*
	Turkish	54/50	26	18	0	36	14	0	0.188	[35]*
	Indian	100/100	50	43	7	59	38	3	0.32	[36]*
	Egyptian	120/120	60	49	11	72	45	3	0.054	[38]
	North India	311/308	207	76	28	228	60	20	0.121	Present study*

*PCOS cases were recruited according to Rotterdam 2003 criteria.

adrenal gland has been reported in the GTex portal. In the present study a significant difference was observed between PCOS cases and controls (*P*=0.017) (Table 5). Many experimental studies have shown that the mutant allele of rs1799998 genetic variant elevates the expression of the gene and therefore increases the enzymatic activity [7]. Only a few studies have been conducted till date on *CYP11B2* rs1799998 polymorphism in association with PCOS [33]. However, rs1799998 polymorphism has been widely studied in other disorders.

CYP1A1 plays an important task in bioactivation of procarcinogens and proteratogens and finally binds to placental or fetal DNA in form of DNA-adducts. It has been reported by different studies that *CYP1A1* activity may enhance production of intra-ovarian catechol estrogens and thus increased chances of developing PCOS. The genotypes and allele distribution was not statistically significantly different between PCOS cases and controls in the present study (*P*=0.121) (Table 5). *CYP1A1* T6235C genetic variant has been reported to have role in hypertension and endometrial cancer and obesity is one of the major risk factor for both the conditions as well as for PCOS. They focused on individuals who were having TC and CC genotypes of rs4646903 polymorphism and observed the significant association of the genotypes with PCOS. In a meta-analysis conclusion was drawn in favor of rs4646903 polymorphism of *CYP1A1*, which after critical analysis provided a strong evidence of the genetic variant with susceptibility to PCOS [11]. The comparison with other studies is illustrated in Table 6 [33-38].

In conclusion, *CYP11B2* and *CYP1A1* play a crucial role in conversion of enzymes in steroidogenic pathway and disturbances in this pathway can lead to hyperandrogenism in females, which is a hallmark feature of PCOS. The case-control association analysis is suggestive of genetic predisposition of steroidogenic genes among PCOS patients in the North-Indian Punjabi females. However, a discrepant association of *CYP11B2* and *CYP1A1* is also noted in this study which implies the need of further studies focusing on genes in the steroidogenic pathway to validate their therapeutic potential in pathophysiology of PCOS. Also androgens may influence insulin sensitivity indirectly via effects on lipid metabolism, adiposity/body fat distribution and cytokine secretion causing hyperinsulinemia. The present study also brings an insight on other risk factors in PCOS (family history, dietary habits, BMI, WHR), identification of these factors can lead to early prognosis and timely management of cases with PCOS. This study gives a way to spread out this research to a larger population to attain more encouraging results for the women undergoing fertility problems.

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