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REPRODUCTIVE GENETICS: ORIGINAL ARTICLE

Analyzing the Impact of *FSHR* Variants on Polycystic Ovary Syndrome—a Case-Control Study in Punjab

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Abstract

Polycystic ovary syndrome (PCOS) is an endocrine-metabolic syndrome that involves hyperandrogenism, menstrual irregularities, and/or small cysts in one or both ovaries which might lead to infertility in women. The genetics of PCOS is heterogenous with the involvement of several genes reported in the hypothalamic-pituitary-gonadal axis. Follicular growth and steroidogenesis regulation are both critically dependent on follicle-stimulating hormone (FSH). The variants of *FSHR* cause abnormal folliculogenesis, steroidogenesis, and oocyte maturation at various stages of growth and may render women more susceptible to PCOS development. The present case-control study evaluated the association of *FSHR* rs6165 and rs6166 variants with PCOS. A total of 743 females were recruited. PCR-RFLP method was used for the genotypic analysis of *FSHR* polymorphisms. Obesity was examined according to the categorization of body mass index (BMI) and waist-hip ratio (WHR). Biochemical analysis, including a lipid profile, LH, FSH, and testosterone levels, was done in both PCOS women and controls. BMI and WHR revealed a statistically significant difference between PCOS cases and controls. Overall, levels of HDL were significantly lower, whereas cholesterol, triglycerides, LDL, and VLDL levels were higher in PCOS women ($p < 0.05$). The genotypic and allelic frequencies of rs6165 and rs6166 did not demonstrate significant differences when PCOS women were compared with the control group. However, clinical features of PCOS including gonadotropic hormone (FSH), hyperandrogenism, and dyslipidemia were significantly correlated with variants of *FSHR*. The present study concludes that rs6165 and rs6166 were significantly related to clinical features of PCOS, regardless of providing direct disease risk.

Keywords Polycystic ovary syndrome · FSHR variants · FSH · LH

Introduction

Polycystic ovary syndrome (PCOS) is an endocrine-metabolic disorder characterized by multiple hormonal imbalances that affect women at their childbearing age [1]. It is a physiological disorder that has detrimental effects on the endocrine, metabolic, psychological, and reproductive systems [2]. Classical features of PCOS include hyperandrogenism, menstrual irregularity, infertility, and polycystic ovarian (PCO) morphology [3]. However, PCOS women also experience hirsutism, acne, alopecia, and psychological issues [4]. It is estimated that 2-26% of women at their reproductive age suffer from PCOS worldwide and in India prevalence is claimed to be 11.96% [5, 6]. The most common diagnostic criteria for PCOS are the Rotterdam criteria (2003), which states that 2 out of 3 conditions should be present: (1) hyperandrogenism, (2) oligo-ovulation or anovulation (3), 12 or more cysts in one ovary, and/or ovarian volume >10 mL [7]. It was also reported

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that women with PCOS have a higher risk of endometrial cancer, cardiovascular disease, dyslipidemia, and type 2 diabetes mellitus [2].

PCOS was first studied by two American gynecologists in 1935 after they observed the irregularities in women with amenorrhea, hirsutism, obesity, and histological evidence of polycystic ovaries; hence, the condition was named after them as Stein-Leventhal syndrome [8]. Family studies suggested that PCOS is inherited in a pseudo-autosomal inheritance pattern with variable penetrance [9]. Studies in identical twin sisters proposed the heritability to be over 70% in women with PCOS [10, 11]. However, PCOS is a multifaceted disorder that occurs due to the interactions among multiple proteins and genes of different pathways such as steroid hormone synthesis [12], insulin-signaling pathway [13], and gonadotrophin hormone action [14] influenced by epigenetic and environmental factors [15].

Follicle-stimulating hormone (FSH) is a pituitary glycoprotein that plays a pivotal role in follicle development and regulation of steroidogenesis and triggers the oocyte maturation by binding to its receptor, FSH receptor (FSHR). FSHR belongs to the G protein coupled receptor family and is present in the granulosa cells of ovaries [16]. The biological signals of FSH are transferred to the downstream cascade through FSHR. Due to the significant role in the signal transmission of FSH, *FSHR* might be a candidate gene for PCOS pathogenesis. *FSHR* is located on the short arm of chromosome #2 (2p21) and consists of 10 exons and 9 introns [17]. The extracellular domain of the receptor is encoded by the first nine exons, while the C-terminal end of the extracellular domain, the transmembrane domain, and the intracellular domain of the FSHR are all encoded by exon 10. The C-terminal of the intracellular domain is enriched in Ser/Thr residues which can be phosphorylated by intracellular kinases and mediate the transduction of signal originating from FSH/FSHR interaction [18, 19]. *FSHR* is highly polymorphic and mutations in this gene can result in aberrant folliculogenesis, steroidogenesis, and oocyte maturation at several phases of follicular growth [20]. Exon 10 of the *FSHR* harbors two variants (rs6165 and rs6166) that alter amino acids at locations A307T and N680S respectively. The extracellular domain of FSHR is responsible for hormone binding (FSH) and it has been noted that A307T change in this domain affects hormone trafficking and signal transduction. Within the intracellular regions of FSHR, phosphorylation of the Ser and Thr residues may have an impact on protein uncoupling from adenylyl cyclase. Therefore, amino acid changes associated with the corresponding SNPs could affect the receptor's function, including the FSH's efficacy [21].

Thus, the present study was conducted to investigate the genetic association between the genetic variants of *FSHR* rs6165 and rs6166 with clinical features of PCOS in Punjab.

Materials and Methods

Selection Criteria

In the present case-control study, out of 743 subjects, 421 women with PCOS were recruited from Beri Maternity Hospital, Amritsar, Punjab and 322 age-matched women with regular menstrual cycles and no sign of PCOS were enrolled as the control group. Sample collection was done from November, 2016 to March, 2021. A Rotterdam criterion 2003 was used to diagnose PCOS women. Participants exhibiting the signs of Cushing syndrome, congenital hyperplasia, androgen secreting tumors, and thyroid dysfunction were excluded from the study. The study satisfied the guidelines of the Ethics Review Board of Guru Nanak Dev University and each participant provided written informed consent before sample collection. All the information like reproductive background, anthropometric measurements, demographic information, family history, lifestyle habits, and pedigree were taken from each individual and recorded on the predesigned questionnaire at the time of sample collection. After taking the informed consent, 5 mL of intravenous blood was withdrawn from each case and control subject. For biochemical analysis, 2 mL of blood was transferred to the vacutainer blood collection tube containing a clot activator. The remaining 3 mL of blood was poured into 0.5 M EDTA vacutainer for molecular genetic analysis. The samples were transported to the laboratory in an ice box and were kept at −20°C until further analysis.

Biochemical Measurements

Serum was isolated from 2 mL of blood by centrifuging the vial at 2000-2500 rpm for 10 min and stored at −20°C until further analysis. The hormone levels including follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) were measured by Calbiotech's ELISA kits. Serum cholesterol, high-density lipoprotein (HDL), and triglycerides were also measured using specific Erba kits on Erba Mannheim biochemical analyzer. Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were calculated using Friedwald's formula [22].

Genotype Analysis

Genomic DNA was isolated using the standard phenol-chloroform method [23]. The polymerase chain reaction was carried out in a 15 µl reaction cocktail containing 50 ng of DNA, 0.2 mM dNTP, 10 pmol of each primer, and 0.25 U Taq DNA polymerase. The primer sequences and cyclic conditions were adopted from Kambalachenu et al.

[24]. Restriction fragment length polymorphism (RFLP) analysis was performed for genotyping the genetic variants of the *FSHR*. For the digestion of polymorphisms, rs6165 and rs6166 restriction enzymes *AhdI* and *BseNl* were used, respectively. For rs6165, products of 403 bp and 175 bp represented homozygous wild genotype (GG), a digested product of 403 bp, 175 bp, 144 bp, and 31 bp signified heterozygous genotype (AG) and bands of 403 bp, 175 bp, and 31 bp represented homozygous mutant genotype (AA). Digestion of rs6166 variant generated a product of 384 bp and 114 bp which represented homozygous GG genotype, a digested product of 498 bp and 384 bp and 114 bp signified heterozygous genotype (AG) and a band of 498 bp represented homozygous AA genotype.

Statistics

The statistical analysis was performed using SPSS (version 21, IBM SPSS, NY, USA). Chi-square (χ^2) test was carried out to compare the genotypic and allelic frequencies of the PCOS cases and healthy controls. To estimate the effects of alleles, odds ratios (ORs) were calculated at a 95% confidence interval (CI) level. The student's *t*-test was used to compare the clinical features of both groups. The distribution of biochemical parameters including cholesterol, triglycerides, HDL, LDL, VLDL, FSH, LH, and total testosterone levels among rs6165 and rs6166 was evaluated by one-way analysis of variance (ANOVA) test. A *p*-value of <0.05 was considered statistically significant. CaTS power calculator was used to determine the power of the study and sample size, which was >90% at CIs of 95% [25]. Haplotype combinations and blocks were constructed by Haploview 4.2 [26]. Linkage disequilibrium (LD) was examined, and all the haplotypes were assessed for association with PCOS.

Results

This study included 421 PCOS cases with a mean age of 24.3 ± 4.89 and 322 healthy women with a mean age of 24.6 ± 4.88 (Table 1). Early menarche was observed in PCOS cases (12.88 ± 1.33) in comparison with control women (13.09 ± 1.34). A significant difference in BMI and WHR was observed between PCOS women and controls ($p = 0.00001$ and $p = 0.00001$ respectively). Among PCOS cases, 36.5% were obese and 63.5% were non-obese; however, in controls, 20.4% were obese and 79.6% non-obese women. In our study, 58% of cases had hirsutism and 65% of them also suffered from cystic acne. Cholesterol, triglycerides, LDL, and VLDL levels were significantly higher ($p = 0.00001$, $p = 0.00002$, $p = 0.009$, and $p = 0.00001$ respectively), whereas HDL levels were lower in cases than in controls ($p = 0.0001$). It was seen

Table 1 Socio-demographic and clinical features of PCOS cases and controls

Variables	Cases ($n = 421$)	Controls ($n = 322$)	*p*-value
Age	24.3 ± 4.89	24.6 ± 4.88	0.45
Age at menarche	12.88 ± 1.33	13.09 ± 1.34	0.001*
Lifestyle pattern			
Sedentary	315	187	0.00001*
Active	106	135	
Obesity-related parameters			
BMI	23.9 ± 4.6	22.1 ± 3.39	0.0001*
WHR	0.89 ± 0.06	0.83 ± 0.08	0.001*
Lipid profile			
Cholesterol (mg/dL)	175.62 ± 49.94	161.17 ± 40.61	0.00002*
Triglycerides (mg/dL)	161.44 ± 90.50	106.06 ± 63.51	0.00001*
HDL-C (mg/dL)	44.85 ± 13.3	51.14 ± 25.9	0.0001*
LDL-C (mg/dL)	98.8 ± 54.1	88.8 ± 47.4	0.009*
VLDL-C (mg/dL)	31.9 ± 17.7	21.1 ± 12.7	0.00001*
Hormonal profile			
LH (mIU/mL)	7.9 ± 4.4	5.2 ± 2.7	0.006*
FSH (mIU/mL)	6.2 ± 2.9	6.1 ± 3.4	0.49
LH/FSH	2.6 ± 1.79	0.89 ± 0.68	0.11
Total testosterone (ng/mL)	0.90 ± 0.33	0.58 ± 0.31	0.00001*

BMI body mass index, *WHR* waist hip ratio, *HDL-C* high-density lipoprotein-cholesterol, *LDL-C* low-density lipoprotein-cholesterol, *VLDL-C* very low-density lipoprotein-cholesterol, *LH* luteinizing hormone, *FSH* follicle-stimulating hormone

*$p < 0.05$ significant

that a sedentary lifestyle additionally increments the risk of PCOS ($p = 0.00001$) (Table 1).

Association Studies

The difference in allelic and genotypic frequencies of rs6165 and rs6166 was not significant between PCOS cases and controls (shown in Table 2). Further biochemical parameters were analyzed for rs6165 and rs6166 variants (Table 3). For rs6166, PCOS cases with AA genotype had significantly higher levels of cholesterol and LDL, whereas VLDL levels were significantly high in cases with the GG genotype (Table 4). HDL levels showed a significant difference in distribution with genotypes of rs6165. FSH level distribution was significantly different for rs6165 genotypes; however, total testosterone level was significantly different between genotypes of rs6166, despite non-significant association for *FHSR* variants (rs6165 and rs6166) and LH levels (Table 3).

Table 2 Distributions of genotypic, allelic, and genetic models of the rs6165 and rs6166 polymorphisms in *FSHR* in PCOS cases and controls

SNP	Genotype/allele	Cases (%)	Controls (%)	HWE *p*-value	χ^2 *p*-value	OR (95% CI)	*p*-value
rs6165	GG	153 (36.4)	100 (31)			Reference	
	GA	175 (41.6)	146 (45.3)	0.11	0.31	0.78 (0.56-1.09)	0.15
	AA	93 (22)	76 (23.6)			0.79 (0.53-1.18)	0.22
	G	481 (57.13)	346 (53.7)		0.19	Reference	
	A	361 (42.9)	298 (46.8)			0.87 (0.7-1.07)	0.19
Dominant model	G/G G/A+A/A	153 268	100 222		0.31	0.78 (0.57-1.07)	0.13
Recessive model	G/G+G/A A/A	328 93	246 76		0.63	0.92 (0.64-1.2)	0.6
Heterozygous model	G/G+A/A G/A	246 175	176 146		0.3	0.85 (0.64-1.4)	0.3
rs6166	GG	104 (24.7)	74			Reference	
	AG	198 (47.03)	156	0.61	0.85	0.95 (0.63-1.42)	0.82
	AA	119 (28.27)	92			0.81 (0.43-1.53)	0.52
	G	406	304		0.69	Reference	
	A	436	340			0.89 (0.65-1.21)	0.46
Dominant model	G/G G/A+A/A	104 317	74 248		0.58	0.9 (0.64-1.27)	0.5
Recessive model	G/G+G/A A/A	302 119	230 92		0.92	0.98 (0.7-1.3)	0.7
Heterozygous model	G/G+A/A G/A	223 198	166 156		0.7	0.98 (0.7-1.3)	0.9

HWE Hardy Weinberg equilibrium, χ^2 chi-square, *OR* odds ratio, *CI* confidence interval

*$p < 0.05$ significant

Table 3 The relationship between the rs6165 and 6166 of *FSHR* and hormone levels in the PCOS group

Parameters	rs6165				rs6166			
	GG	AG	AA	*p*-value	GG	AG	AA	*p*-value
FSH (mIU/mL)	5.6 ± 3.03	5.6 ± 1.81	10 ± 2.87	0.0001*	6.87 ± 3.9	5.99 ± 2.3	7.7 ± 3.7	0.401
LH (mIU/mL)	6.67 ± 4.3	7.97 ± 4.9	9.18 ± 3.7	0.438	6.94 ± 5.5	8.1 ± 4.5	7.5 ± 2.2	0.76
LH/FSH	1.64 ± 1.6	1.76 ± 1.8	0.94 ± 0.383	0.475	1.24 ± 1.01	1.84 ± 1.96	1.14 ± 0.54	0.447
Total testosterone (ng/mL)	0.74 ± 0.28	0.88 ± 0.31	0.75 ± 0.34	0.41	0.60 ± 0.21	0.88 ± 0.32	0.86 ± 0.31	0.03*

One-way ANOVA, *$p < 0.05$ significant

Table 4 Distribution of biochemical parameters concerning genotypes in PCOS cases

Variant	Genotype	Cholesterol		Triglycerides		HDL		LDL		VLDL	
		Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
rs6165	GG	170.5 ± 46.4	162.7 ± 43.8	168.5 ± 100.6	100.3 ± 58.1	45.5 ± 13.1	53.63 ± 14	91.8 ± 49.4	89.1 ± 59.7	33.2 ± 19.7	21.8 ± 13.4
	AG	176.1 ± 50.7	157.7 ± 37.5	156.5 ± 80	111.9 ± 68	42.6 ± 11.4	50.51 ± 15	102.2 ± 55.1	84.8 ± 40.2	31.2 ± 16	22.39 ± 13.75
	AA	183.1 ± 53.4	165.3 ± 41.5	158.9 ± 90.3	102.8 ± 60	48.1 ± 16	49.02 ± 12	104.1 ± 58.6	95.8 ± 40.6	30.9 ± 17.2	20.56 ± 12
	p-value	0.157	0.376	0.46	0.331	0.004*	0.471	0.126	0.271	0.516	0.331
rs6166	GG	173 ± 42.5	166.44 ± 50.56	165.1 ± 100	105.75 ± 60.3	46.5 ± 15.3	56.7 ± 13.2	95 ± 48.5	88.5 ± 68.91	31.9 ± 17.7	19.5 ± 12.3
	AG	171.2 ± 51.8	160.12 ± 34.32	169.3 ± 85.4	111.32 ± 66.04	43. ± 12.2	49.7 ± 14.67	94 ± 54.5	88.08 ± 35.6	28.6 ± 16	22.26 ± 13
	AA	185.9 ± 52.2	158.64 ± 41.14	146.4 ± 88	97.7 ± 61.8	42.5 ± 12.5	48.8 ± 12.6	110.7 ± 56.8	90.2 ± 43.3	33.8 ± 17	21.1 ± 12
	p-value	0.04*	0.428	0.102	0.276	0.085	0.09	0.02*	0.9	0.04*	0.27`

One-way ANOVA, *$p < 0.05$ significant

Table 5 Haplotype analysis between the variants (rs6165 and rs6166) of the *FSHR*

Haplotypes	Case frequency	Control frequency	OR	*p*-value
GG	0.408	0.388	1.05 (0.88-1.25)	0.57
AA	0.367	0.333	1.1 (0.9-1.35)	0.31
AG	0.150	0.177	0.84 (0.61-1.2)	0.31
GA	0.074	0.101	0.71 (0.44-1.14)	0.16

OR odds ratio, *CI* confidence interval

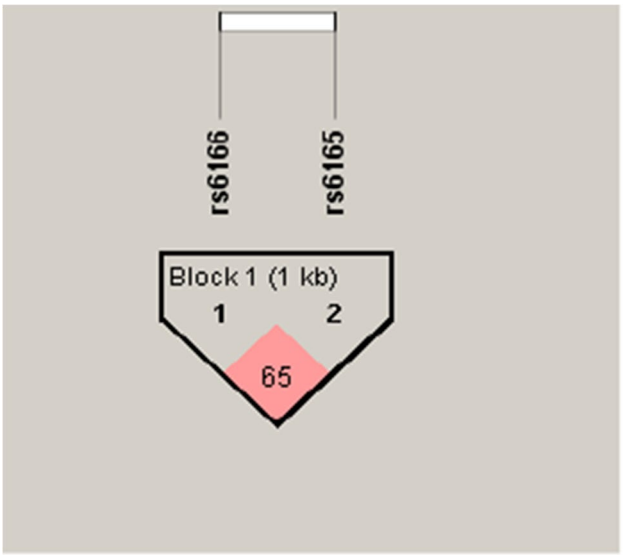

Figure 1 LD plot for *FSHR* variants

Haplotype Analysis

Haplotype analysis for *FSHR* rs6165 and rs6166 was done using the Haploview software ver 4.2. The haplotype frequency comparison is illustrated in Table 5. Two SNPs were revealed to be in linkage disequilibrium among PCOS cases and controls ($D' = 0.652$, $LOD = 63.25$, $r^2 = 0.313$) (Figure 1). None of the haplotypes showed a significant difference in their frequency distribution among PCOS cases and controls (Table 5).

Discussion

The present study was designed to investigate the relation of *FSHR*, exon 10 (rs6165 and rs6166) variants with PCOS. These two variants are crucial as protein structure analysis has shown that rs6165 affects the binding of a ligand (FSH) and rs6166 is related to the coupling of G protein for downstream signaling [20]. Besides, GWAS on Han Chinese and

European populations recognized the 2p16.3 region (containing *FSHR* loci) to be associated with PCOS, with notable differences according to racial background [27]. In previously reported studies, these two polymorphisms have been widely studied to evaluate the potential association with PCOS and the results were inconsistent.

In the present study, age at menarche (AAM) was found to be lesser in PCOS women as compared to controls (Table 1). BMI and WHR of PCOS women were significantly higher than controls (Table 1). Levels of serum cholesterol, triglycerides, LDL-C, and VLDL-C were found to be significantly higher among PCOS women (Table 1). LH and total testosterone levels were also statistically significantly different between PCOS women and controls. According to our study, the genotypic distribution of rs6165 and rs6166 was not statistically significantly different between PCOS cases and controls (with *p* values 0.31 and 0.61 respectively) (Table 2). It was observed that PCOS women carrying the AA genotype of rs6166 showed elevated levels of serum cholesterol, LDL, and VLDL, and the HDL levels were also found to be significantly distributed among the rs6165 genotypes (Table 4). In the present study, LH, FSH, and total testosterone levels were also analyzed in relation to genotypes of *FSHR* polymorphisms and a significant increase in FSH levels in relation to the mutant genotype (AA) of rs6165 was found. In addition, it was also noted that distributions of total testosterone levels were significantly correlated with the rs6166 variant (Table 3).

Obesity leads to a hyperandrogenic state by increasing insulin resistance in the liver and boosting androgen synthesis in the ovaries [28]. To ascertain obesity, BMI is generally used as a measuring factor that helps to anticipate the risk for metabolic disorders [29]. In the current study, the BMI of PCOS women was significantly higher than controls which is known to increase the risk of diabetes mellitus, cardiovascular morbidities, and other metabolic syndromes in PCOS females (Table 1). Our results are in agreement with studies on North Indian [30], South Indian [31, 32], Chinese women [33, 34], and Turkish [21]. A study conducted on the Sardinian population disagrees with our findings [35].

The waist-to-hip ratio (WHR) is a tool that aids in assessing the probable health risks linked to obesity and being overweight. The likelihood of developing metabolic diseases like type 2 diabetes and heart disease is increased when the majority of fat accumulation occurs around the waist rather than the hips. In our study, WHR was revealed to be significantly different between both groups (Table 1). This finding is in disagreement with a study done on Iranian [27] and Polish women [36].

For all women with PCOS, lifestyle management is the first line of treatment. Physical activity is an important lifestyle management strategy that promotes better reproductive, metabolic, and mental health. The prevalence of metabolic diseases, anxiety, and mortality are greatly influenced by a sedentary lifestyle [37]. In our study, a significant correlation between a sedentary lifestyle and the development of PCOS was observed (Table 1). A study on Greek women that found that PCOS girls were physically inactive than controls confirmed our findings [38]. Lin et al. [39], in contrast to the present study, showed no statistically significant correlation between PCOS cases and physical activity in the USA.

Dyslipidemia is common in PCOS which leads to metabolic disturbances. It was reported PCOS women with higher cholesterol, triglycerides, and LDL-C levels undergoing unstimulated natural cycles were correlated with the increased number of oocytes retrieved that are of poor quality, hence resulting in less live birth outcomes [40]. Likewise, elevated cholesterol was related to the conceptive failure of PCOS women that were undergoing assisted reproduction [41]. Therefore, evaluation for dyslipidemia may not only assist with assessing cardiometabolic health but also be useful to predict reproductive outcomes after fertility treatment for women with PCOS [42]. In the present study, overall cholesterol levels, triglycerides, LDL-C, and VLDL-C levels were higher among PCOS women (Table 1). Subsequent studies in distinctive regions have stated comparable results to the current finding [43, 44]. Wild et al. did a meta-analysis and concluded that the levels of triglycerides and LDL-C were higher and HDL-C concentrations were lower in women with PCOS than in controls [45].

Exon 10 of FSHR contains rs6165 and rs6166 SNPs that lead to changes in amino acids at positions 307 and 680, respectively. These SNPs may have subtle effects on the FSHR's sensitivity to FSH. In the present study, allelic and genotypic frequencies of rs6165 and rs6166 were compared between both groups. The genotypes and allele distribution of rs6165 were not statistically significantly different between PCOS cases and controls in our study (Table 2). This study is in line with studies on South Indian [24], Sri Lankan [46], Chinese [47], Turkish [21], Polish [48], and Korean women [16]. However, studies on Japanese [20], Italian [49], Singapore [50], and Iranian women [51] showed that rs6165 was significantly associated with PCOS development. In South Korean women, the results were contradictory; a study by Gu et al. [16] postulated that the distribution of rs6165 genotypes did not differ significantly while another study by Kim et al. [52] showed a significant difference in genotypic frequency between PCOS cases and controls.

In the current study, a non-significant association was observed for the genotypic and allelic frequency of rs6166 (Table 2). Our results were supported by studies conducted on Sri Lankan [46], Iranian [51], Chinese [47], Turkish [21], Polish [48], Dutch [53], and UK [54]. Contrary to our results, studies on South Indian [18] and Korean women [16, 52]. Kambalachenu et al. [24] conducted a case-control study and demonstrated the non-significant difference

in the distribution of allele or genotypes between PCOS and controls, and found a significant association of rs6166 (Ser680Ser) genotype with PCOS in the recessive model in South Indian women. A pilot study was performed in North India, on 30 PCOS cases and 30 controls, which did not show any association between rs6166 and PCOS [55]. The conflicting results for rs6165 and rs6166 are likely due to ethnic/racial variations and the small sample sizes in some studies.

FSHR polymorphisms (rs6165 and rs6165) were revealed to be in moderate linkage disequilibrium among PCOS cases and controls ($D' = 0.652$ and $r^2 = 0.31$) as their frequency of coinherited together was 65.2% (Table 5). Also, none of the haplotypes was provided risk towards to development of PCOS in our group. Gu et al. [16] and Kim et al. [52] also revealed that both polymorphisms were in linkage disequilibrium. They showed that the haplotype having a homozygous variant combination was significantly associated with PCOS. In South India, these two polymorphisms did not show to be linked together (D': 0.09 and r^2: 0.008) which is in disagreement with our study [24].

We also assessed the possibility that *FSHR* polymorphisms affect clinical characteristics including lipid and hormonal profile (LH, FSH, and total testosterone). Our study showed higher levels of cholesterol, LDL, VLDL, and testosterone in association with the mutant allele of rs6166 (Table 4). A study carried out on South Indian women revealed that Ser680 was associated with high levels of FSH in PCOS [18]. Studies on the Dutch population illustrated that levels of FSH and LH were high with Ser680 polymorphism and they also concluded the mutant genotype of rs6166 was also associated with testosterone levels [53, 56]. The present study also demonstrated that higher FSH and HDL levels were also significantly associated with the mutant genotype of rs6165 (Table 3). It was observed that the level of FSH was approximately 2 times higher in the PCOS women with mutant genotypes than with wild type or heterozygote genotypes. This was in agreement with the study performed by Dolfin et al. [49] which showed that Ala to Thr polymorphism was associated with higher responsiveness to exogenous FSH in Italian women. Women with PCOS have FSH levels that are within normal ranges [53]. So, it does not appear likely that altered FSH sensitivity is a key in the ovulatory dysfunction that is typically seen in PCOS. It might be because carriers of the rs6165 mutant genotype, which had a higher FSH threshold, can increase FSH levels in a compensatory manner. Increased FSHR resistance results in lower production of E2 and inhibin B that exert an inhibitory feedback action at the level of the pituitary gland and further increases ovarian androgens and may hinder folliculogenesis [53]. We have observed a significant relationship between *FSHR* variants and higher levels of FSH and testosterone (Table 3).

Exon 10 polymorphisms of *FSHR* are known to cause FSH resistance to FSHR and that could result in hyperandrogenemia and hyperinsulinemia. In the current study, we also demonstrated that there is an interrelation between higher FSH levels and HDL levels with the AA genotype of rs6165 polymorphism of *FSHR*, which is unique in our study. However, a study conducted on Iraqi women demonstrated higher FSH levels with GG genotype and lower HDL levels with AG genotype of rs6165 [57]. HDL level was less strongly associated with FSH in post-menopausal women by Serviente et al. [58]. Also in our study, it is evident that women having the mutant genotypes of both polymorphisms (rs6165 and rs6166) will have higher levels of FSH, total testosterone, and dyslipidemia. Higher FSH levels in the blood may bind to the FSHR in the liver in PCOS women with the mutant genotype rs6165, which reduces LDLR expression. Low levels of LDLR have decreased LDL metabolism which may result in higher levels of LDL in circulation.

Our study has several strengths, such as the power of the study is >90%, and ethnicity of cases and controls were matched (only from the Punjab region), which reduces type I errors, common to genetic association studies and hence supports the findings. Additionally, the analysis of genetic association was done at the allele, genotype, genetic models, and haplotype levels and genotypes were also correlated with clinical features of PCOS. There are some limitations of our study. Only two polymorphisms of *FSHR* were evaluated and hospital-based sampling prevented the results from generalizing the entire community.

This is the first study from Punjab carried out to investigate the possible association of exon 10 variants of the *FSHR* gene for the development of polycystic ovary syndrome. Though many studies have been carried out on these variants, their association with the North Indian population and especially the Punjabi population was not conducted. The present study postulated that *FSHR* variants did not provide a significant association with PCOS, but significantly impact the clinical features of PCOS such as dyslipidemia and endocrine hormones. Thus, these polymorphisms did not directly contribute to disease progression but were linked to PCOS severity. The current study also sheds light on other PCOS risk factors (lifestyle pattern, BMI, and WHR), and identifying these factors can help with early diagnosis. A cohort study with a greater number of participants is necessary to further understand the relationship between *FSHR* polymorphisms and PCOS, and to produce more promising outcomes for women experiencing fertility issues.

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Availability of Data and Material The data will be available from the corresponding author on request.

Code Availability Not applicable.

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Data Availability The data will be available from the corresponding author on request.

Declarations

Ethics Approval The study was approved by the ethical committee of Guru Nanak Dev University, consistent with provisions of the Declaration of Helsinki.

Consent to Participate The informed consent was taken from all the study participants.

Consent for Publication All the authors have given their consent for publication.

Conflict of Interest The authors declare no competing interests.

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