Original Article

Luteinizing Hormone β-subunit Gene Polymorphisms and Androgen Levels are Less Predictive of Ovarian Response in Polycystic Ovary Syndrome *In vitro* Fertilisation Women: A Nested Case–Control Study

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Abstract

Background: Genetic polymorphisms in the luteinizing hormone (LH) β-subunit gene have been associated with responses to controlled ovarian hyperstimulation (COH) in *in vitro* fertilisation (IVF) patients. Variants in rs1800447 and rs34349826 may increase androgen production, potentially impairing folliculogenesis and ovarian response. **Aim:** The aim of this study was to investigate the clinical significance of LH β-subunit single-nucleotide polymorphism (SNP) (rs1800447 and rs34349826), levels of testosterone, sex hormone-binding globulin (SHBG) and free testosterone index in predicting COH response in polycystic ovary syndrome (PCOS) women. **Settings and Design:** This nested case–control study enlisted 122 women with PCOS in the Morula IVF Jakarta Clinic, Jakarta, Indonesia. **Materials and Methods:** The selection of cases and controls was in the ratio of 1:2. Blood samples were taken on day 2 or 3 of menstrual cycle. Sanger sequencing was utilised to genotype the LH β-subunit genes (rs1800447 and rs34349826). Levels of testosterone and SHBG were measured to calculate the free testosterone index. Women were retrospectively grouped as hyporesponders (<8 oocytes) or normo/hyperresponders (≥8 oocytes) according to the number of retrieved oocytes. **Statistical Analysis Used:** SPSS Software version 21.0 (IBM Corp., USA). Independent *t*-test or Mann–Whitney test for numerical variables and Chi-square test for categorical variables were employed. **Results:** A unique automatic transmission (AT) variant in both rs1800447 and rs34349826 LH β-subunit was discovered, which has yet been reported previously. Notably, the proportion of heterozygous LH β genotypes (AT and AG) in both rs1800447 and rs34349826 was similar between hyporesponder and normo/hyperresponder groups ($P > 0.05$). All participants were normoandrogenic PCOS women, indicated by the normal level of testosterone (0.84 ± 0.35 nmol/L) and SHBG (57.87 ± 29.20 nmol/L) as well as free testosterone index (1.88 ± 1.34). No difference in testosterone levels, SHBG and free testosterone index was observed among the studied

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groups ($P > 0.05$). **Conclusion:** LH β gene SNPs (rs1800447 and rs34349826), testosterone level, SHBG level and free testosterone index were not significantly correlated and less effective clinical indicators for COH response in normoandrogenic PCOS women.

Keywords: *Androgen, clinical relevance, in vitro fertilisation, luteinizing hormone β-subunit, polycystic ovary syndrome, polymorphisms*

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder affecting women of childbearing age, with a 5% to 10% prevalence.[1,2] Three diagnostic criteria for diagnosing PCOS are available, including the National Institutes of Health (1999), Rotterdam criteria (European Society of Human Reproduction and Embryology and American Society for Reproductive Medicine) in 2003 and Androgen Excess and PCOS Society in 2006. Of all the guidelines, the Rotterdam criteria are the most commonly utilised for PCOS classification in clinical practice worldwide. According to the Rotterdam consensus, PCOS is diagnosed by the presence of two out of three following criteria: (i) ovulatory dysfunction, (ii) clinical or biochemical hyperandrogenism and (iii) polycystic ovaries (PCOs) represented by the presence of ≥12 follicles measuring 2–9 mm in diameter on the ultrasound scan.[3] Although some women with PCOS have regular periods, PCOS has been associated with impaired fertility, as 80% of PCOS women exhibit irregular/abnormal ovulation and consequently would require hormonal therapy to conceive.[4-6]

In vitro fertilisation (IVF) is the third-line treatment for PCOS-associated infertility after unsuccessful treatment with ovulation induction agents.[7] Through controlled ovarian hyperstimulation (COH), an optimum number of mature oocytes can be retrieved for insemination *in vitro*. However, ovarian response to COH varies substantially between patients and is not always as expected.[8] Women with PCOS frequently hyperrespond to gonadotropin stimulation, often yielding >15 oocytes. However, PCOS women who are hyporesponsive are also found in clinical practice. A hyporesponse is defined as an unexpectedly poor ovarian response to gonadotropin therapy despite adequate prestimulation ovarian reserve parameters, resulting in suboptimal IVF outcomes.[9] Genetic polymorphisms in gonadotropins and their receptors have been associated with varying COH outcomes in IVF patients.[10,11] In particular, follicle-stimulating hormone receptor (FSHR) polymorphisms have been implicated as a cause of unexpected poor ovarian response in PCOS women.[12] Single-nucleotide polymorphisms (SNPs) in follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and/or their receptors could affect proper folliculogenesis.[11,13] Alviggi *et al*. reported a high frequency of LH β-subunit gene polymorphism in normogonadotropic women, linked to menstrual disturbances, recurrent miscarriages and a higher exogenous FSH consumption during COH.[14] To date, no study has evaluated the effect of LH and LH-receptor polymorphism on IVF outcomes in PCOS women.

Physiologically, the LH β-subunit gene variant is known to increase androgen synthesis, causing atresia or apoptosis. Hence, elucidating the association between LH β-subunit gene polymorphism and androgen levels (testosterone, sex hormone-binding globulin [SHBG] and free testosterone index) with ovarian response. In addition, LH β-subunit polymorphism Trp8Arg in rs1800447 and Ile15Thr in rs34349826 were found to affect androgen production. Considering the crucial role of LH in stimulating follicular growth and maturation during folliculogenesis and also to fill the gap, this study aimed to evaluate the clinical significance of LH β-subunit polymorphisms, especially in rs1800447 and rs34349826, testosterone level, SHBG and free testosterone index as predictors for ovarian response to COH in PCOS Indonesian population.

Materials and Methods

This was a nested case–control study conducted at Morula IVF Jakarta Clinic from April 2020 until November 2021. Women with PCOS were diagnosed with the presence of two out of three Rotterdam criteria as follows: presence of oligo-anovulation, PCOs confirmation through ultrasound or biochemical sign of hyperandrogenism. Exclusion criteria were women with PCOS, a history of endometriosis and/or a laparoscopic ovarian drilling operation. A total of 131 PCOS patients who underwent an IVF program were eligible as study participants. Eight eligible subjects were excluded since those women failed to continue the IVF program due to failing to properly respond to ovarian stimulation [Figure 1]. As these patients could not be categorised into either high or hyporesponse groups, sequencing was not performed. Eventually, 122 women were enrolled for further analysis. All participants underwent the antagonist protocol. Of these, 88% (107/122) received FSH-LH regimen (Pergoveris, Merck or Menopur, Ferring), while the remaining participants received FSH regimen (Gonal-F, Merck). Briefly, gonadotropin injection was initiated on day 2 or 3 of menstrual cycle, with a starting dose

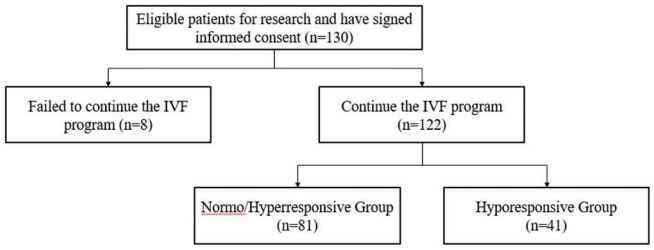

Figure 1: Sample recruitment flow

ranging between 150 and 300 IU. Gonadotropin-releasing hormone antagonist (0.25 mg Cetrotide, Merck) was administered on day 5 or 6 of ovarian stimulation. When at least 2 or 3 follicles have reached 18 mm, the ovulation trigger was given (6500 IU recombinant-human chorionic gonadotropin (Ovidrel®; Merck Serono). Ovum pick-up (OPU) was conducted 36 h after final maturation trigger administration. Mild sedation was administered before follicle aspiration. Follicular fluid was aspirated and evaluated under a microscope by an experienced embryologist in a petri dish. The embryologist then collected the cumulus–oocyte complex. Blood samples for androgen analysis were collected before the commencement of ovarian stimulation, while blood sample for genotyping was collected on the trigger day. All samples were stored until the completion of the OPU procedure was completed. The subjects were then divided into two groups based on the number of mature oocytes retrieved. In the present study, subjects with <8 mature oocytes retrieved were grouped as hyporesponders. This criterion was based on presumption that after undergoing a proper controlled ovarian stimulation protocol, a successful pregnancy can be attained if patients receive at least 8 mature oocytes.[15,16] Therefore, we predefined it *a priori* as <8 mature (MII) oocytes retrieved after standardised COS in this study. Women meeting this criterion were classified as hyporesponders. Eventually, 41 cases were compared with 81 controls (1:2 ratio).

This study was conducted adhering to all regulations and the Helsinki Declaration and received ethical approval from the ethics committee of the Faculty of Medicine, Universitas Indonesia, approved the study protocol on December 5, 2019, with approval number KET-139/UN2.FI? ETIK/PPM.00.02/2019. Signed informed consent was obtained from all studied participants.

Sequencing

As the present study targeted LH β-subunit gene polymorphisms, Sanger sequencing was used as it is more cost-efficient and accurate to detect targeted polymorphisms instead of next-generation sequencing. Sanger sequencing uses chain-terminating dideoxynucleotides (ddNTPs) to generate fragments of gene targets, with each fragment's end in a labelled base. These fragments are separated by capillary electrophoresis. Eventually, the fluorescent signal of terminal bases is read to determine the DNA sequence. QIAmp DNA Blood Mini Kit (Qiagen) was used for DNA extraction. Polymerase chain reaction (PCR) for amplification of SNP rs180447 gene was done utilising "CCA GCA TCC TAT CAC CTC CT" as the forward primer and "TGG TGT TGA CGG TGA TG" as the reverse primer, while PCR for SNP rs34349826 was done using "CGT CAA CAC CAC CAT CTG T" and "GCG GAT TGA GAA GCC TTT ATT G" as the forward and reverse primers, respectively. Sanger sequencing proceeded using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), that widely utilised for high-quality, capillary electrophoresis-based sequencing and the same primer sets used in PCR. Sequencing outputs for the rs1800447 and rs34349826 were then aligned with the Information Disclosure Statement reference sequence (NC_000019.10) using BioEdit® software (Tom Hall, North Carolina State University, United States). The software is commonly used for visualising, editing and comparing nucleotide sequences, thus facilitating the identification of point polymorphism in sequencing targets and ensuring accurate alignment and interpretation of target sequences relevant to the reference genome.

Electrochemiluminescence immunoassay

Electrochemiluminescence immunoassay was used to analyse the testosterone and SHBG levels in the blood samples. Briefly, 4.5 ml of blood contained in a clot activator tube was used. The blood was then centrifuged at 3500 rpm for 15 min to collect the serum for measurement of SHBG concentration (Cobas 6000-e601, Roche Diagnostics GmbH, Germany) and testosterone level (Cobas 6000-e411, Roche Diagnostics GmbH, Germany).

Statistical analysis

OpenEpi software (Bill and Melinda Gates Foundation, Emory University, Atlanta, United States) was used to calculate the requirement of sample size. At least 122 PCOS women were required to detect differences between the case and control groups (hyporesponse vs. normo-hyperresponse group). SPSS software version 20 was used for statistical analysis (IBM SPSS Statistics, New York, United States). The Kolmogorov–Smirnov test was carried out to test for the normality of data distribution. Normally distributed variables were analysed using an independent *t*-test. Nonnormal data distributions were transformed through either a log or square root method and subsequently analysed using an independent *t*-test. Variables that remained nonnormally distributed were analysed using the Mann–Whitney test. Pearson's Chi-square test was used to measure the proportion difference between the studied groups. Both SNPs were tested for departure from Hardy–Weinberg

equilibrium (HWE). Genotype distributions and linkage disequilibrium (LD) were calculated using r^2 coefficient equation as previously proposed.[17]

$$r^2 = \frac{D^2}{f(A)f(a)f(B)f(b)}$$

in which D is calculated as $D = f(AB) - f(A)\ f(B)$ and $f(AB)$, $f(A)$, $f(a)$, $f(B)$ and $f(b)$ are observed frequencies of haplotypes AB and alleles A, a, B and b, respectively. The higher the r^2, the stronger the LD.

Results

Baseline and clinical characteristics of polycystic ovary syndrome women

Overall, the median age of women who participated in this study was 31 years old. Body mass index, the proportion of obesity, levels of basal hormones including LH and estradiol, the type of gonadotropin and total gonadotropin dose did not differ between the two groups. In contrast, basal FSH levels and number of mature oocytes retrieved differed significantly between the hyporesponders and normo/hyperresponders ($P < 0.05$) [Table 1].

Polymorphisms of luteinizing hormone β-subunit gene

The present study observed two reported polymorphisms of LH β-subunit genes rs1800447 and rs34349826. Both SNPs present base conversion of adenine to guanine (A → G) and adenine to thymine (A-T), resulting in amino acid modifications (rs1800447>Trp8Arg and rs34349826>Ile15Thr). As a consequence, three genotypes of each rs1800447 and rs34349826 were obtained (AA, AG and AT). In addition, one woman carried a homozygote TT rs34349826 variant. Moreover, the genotype frequency of AT was higher than that of AA and AG variants.

In AG and AT genotypes of rs1800447, the translation of tryptophan is modified to arginine, while AG and AT genotypes of rs34349826 shifted the translation of isoleucine to threonine and isoleucine to asparagine, respectively. Conversion from isoleucine to asparagine was suspected to have an impact on the conformation

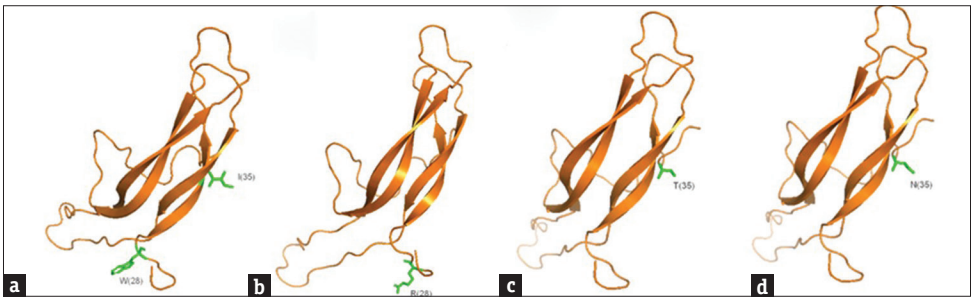

Figure 2: The 3D protein comparison of LH β-subunit gene. (a) wildtype protein. (b) the 3D protein of AG and AT vairants in rs1800447. (c) the 3D protein of AG variant in rs34349826. (d) the 3D protein of AT and TT variants in rs34349826

Table 1: Baseline and clinical characteristics of studied subjects

Baseline and clinical characteristics	Overall value (*n*=122)	Response groups		*P*
		Normo/hyperresponder (*n*=81)	Hyporesponder (*n*=41)	
Age (years)	31 (22–40)	31 (22–40)	32 (23–38)	0.528
BMI (kg/m^2)	23.66±3.54	23.57±3.43	23.86±3.78	0.704
Nonobese	20.78±1.59	39 (48.1)	19 (46.3)	0.520
Obesity	26.28±2.68	42 (52.9)	22 (53.7)	
Basal FSH levels (mIU/mL)	6.11±1.50	5.79±1.39	6.75±1.51	0.001
LH levels (mIU/mL)	6.27±3.80	6.58±4.24	5.66±2.66	0.330
Basal E2 (pg/mL)	37.03±12.02	35.86±11.73	39.33±12.38	0.123
Type of gonadotropin				
FSH	15 (12.3)	10 (66.7)	5 (33.3)	1
FSH + LH regimen	107 (87.7)	71 (66.4)	36 (33.6)	
Starting dose (IU)	225 (150–300)	225 (225–300)	225 (150–300)	0.745
Total dose of gonadotropin (IU)				
rFSH only	2100 (1200–3000)	19,125 (1200–3000)	2700 (2100–3000)	0.098
rFSH + LH regimen	1967 (600–3600)	1800 (600–3600)	1875 (1050–3375)	0.575
Number of mature oocytes	10 (2–55)	12 (8–55)	6 (2–7)	<0.001

Data was presented in the mean±SD, median (minimum–maximum), categorical variables were presented in *n* (%). SD=Standard deviation, BMI=Body mass index, FSH=Follicle-stimulating hormone, LH=Luteinizing hormone, rFSH=Recombinant FSH

of LH β protein due to the different chemical properties between the two amino acids. Therefore, a three-dimensional (3D) protein model using Pymol software (Schrödinger, New York, United States) was constructed [Figure 2].

Table 2 shows genotype distribution and allele frequency at two SNP points. Distribution of AA, AT and AG genotypes of both rs1800447 and rs34349826 revealed no significant difference between normo/hyperresponders and hyporesponders (P = 0.271 and P = 0.835, respectively). Similarly, the frequency of alleles including A, T and G in rs1800447 and rs34349826 was observed to be similar between the two study groups (P = 0.889 and 0.429, respectively).

Table 2: Distribution of rs1800447 and rs34349826 luteinizing hormone subunit beta gene polymorphisms

Polymorphism variants	Response groups		*P**
	Normo/hyperresponse (*n*=81), *n* (%)	Hyporesponse (*n*=41), *n* (%)	
rs1800447			
Wild type (AA)	20 (24.7)	5 (12.2)	0.154
Heterozygote (AG/AT)	61 (75.3)	36 (87.8)	
Genotype			
AA	20 (24.7)	5 (12.2)	0.271
AT	46 (56.8)	27 (65.9)	
AG	15 (18.5)	9 (22.0)	
Allel frequency			
A	62.3	56	0.889
T	28.4	33	
G	9.3	11	
rs34349826			
Wild type (AA)	13 (16.0)	8 (19.5)	0.777
Heterozygote (AG/AT)	67 (82.7)	32 (78.0)	
Homozygote (TT)	1 (1.2)	1 (2.4)	
Genotype			
AA	13 (16.0)	8 (19.5)	0.835
AT	63 (77.8)	31 (75.6)	
AG	4 (4.9)	1 (2.4)	
TT	1 (1.2)	1 (2.4)	
Allel frequency			
A	58	59	0.429
T	40	40	
G	2	1	

*Pearson Chi-square was used

LD was then calculated to determine the probability of the occurrence of mutations at these two SNPs, rs1800447 and rs34349826. The square root coefficient of correlation of the LD calculation was 729. Both rs1800447 and rs34349826 did not depart from HWE.

Testosterone, sex hormone-binding globulin levels and free testosterone index

Table 3 presents the androgen measurements. Levels of testosterone, SHBG and free testosterone index in overall participants were comparable to those of a normal healthy woman (0.84 ± 0.35 nmol/L, 57.87 ± 29.20 nmol/L and 1.88 ± 1.34, respectively). No difference was also observed between normo/hyperresponse and hyporesponse groups in the testosterone level (0.84 ± 0.35 nmol/L vs. 0.84 ± 0.34 nmol/L, P = 0.991), SHBG levels (55.60 ± 30.05 nmol/L vs. 62.34 ± 27.26, respectively) and free testosterone index (2.02 ± 1.50 vs. 1.59 ± 0.91, respectively).

Discussion

This present study demonstrated that LH β-subunit gene SNPs, rs1800447 and rs34349826, in PCOS patients had no association with ovarian response during COH. The LH β SNPs, rs1800447 and rs34349826, were selected for analysis in this study due to their known roles in the pathogenesis of PCOS. Furthermore, previous studies suggested that both SNPs were rarely found in the Asian women population,[18] which has been proven otherwise in this study. A higher distribution of the AT and AG genotypes was observed compared to the AA wild type. However, no correlation was observed between the presence of polymorphism in either SNP and response to gonadotropin stimulation. Notably, a systematic review and meta-analysis highlights that specific polymorphisms may influence ovarian response in mixed COS populations, particularly in FSHR (rs6165, rs6166 and rs1394205), showing associations with oocyte yield, stimulation duration and FSH consumption, while polymorphisms in LH and its receptor remain underpowered for quantitative analysis due to limited or small data reported.[13] LH/Lueteinizing hormone/choriogonadotropin receptor (LHCGR) polymorphism may modestly influence ovarian response in terms of dosage and oocyte yield; however, its robust predictive value remains to be further validated.[19]

Table 3: Evaluation of androgens levels

Androgens	Overall value	Response groups		*P*
		Normo/hyperresponder (*n*=81)	Hyporesponder (*n*=41)	
Testosterone levels (nmol/L)	0.84±0.35	0.84±0.35	0.84±0.34	0.991
SHBG levels (nmol/L)	57.87±29.20	55.60±30.05	62.34±27.26	0.337
Free testosterone index*	1.88±1.34	2.02±1.50	1.59±0.91	0.253

*Data was presented in the mean±SD, Free testosterone index was calculated as follow=testosterone levels ×100 /SHBG levels.
SD=Standard deviation, SHBG=Sex hormone-binding globulin

Interestingly, while the adenine to guanine (AG) conversion in both rs1800447 and rs34349826 has been recorded in the National Center for Biotechnology Information, a new variant of adenine (A) conversion to thymine (T) was discovered in this study. Of utmost importance is the identification of the AT genotype of both rs1800447 and rs34349826, which up to now has not yet been reported in studies of other ethnicities. Furthermore, the distribution of the heterozygous AT genotype in Indonesian PCOS women was found to be more prominent than the AG genotype. AG variant of rs1800447 has been previously reported in a different population. The mutation causes the change of amino acid translation from tryptophan to arginine, consequently compromising hormone immunoreactivity.[20] Similarly, AG genotype of rs34349826 renders the amino acid modification from isoleucine to threonine, which translates into a protein with an added glycosylation chain and thus, a shorter half-life than the wild-type form.[20-22]

While similar amino acid modifications are noted in the heterozygous AT and AG genotype of rs1800447, AT variant of rs34349826 results in a change of amino acid from isoleucine to asparagine. Isoleucine is a nonpolar, aliphatic essential amino acid, while asparagine is a polar, nonessential amino acid. As such, a 3D model predicted a change in LH protein conformation due to the polarising properties of the different amino acids [Figure 2]. Although we cannot directly describe the clear-cut effect of the deviations in protein conformation, these variants were not correlated with ovarian response to gonadotropin stimulation in our cohort. Correspondingly, Manna *et al*. reported that polymorphisms of rs1800447 and rs34349826 did not affect the binding affinity of LH and its receptor.[22]

In terms of androgen levels, this study also found no difference in androgen levels, specifically testosterone, SHBG and free testosterone index, before COH between the studied groups and ovarian response to gonadotropin, suggesting that androgen levels may have limited predictive value. Comparable levels of testosterone, SHBG and free testosterone index were observed between the hyporesponder group and normo-highresponder groups.

In general, women with PCOS exhibit high levels of circulating androgens. Batista *et al*. investigated the same SNPs but utilised a different gene reference (NCB9:NG_011464.1) and noticed that more than 80% of the studied participants carried the AG variant linked to hyperandrogenemia.[23] In comparison to our findings, the PCOS participants in this study were normoandrogenic, which is defined by PCOS with oligo-anovulation, PCO and normal androgen levels. We presumed that the dominant AT genotype polymorphism may have an influence on the androgen level and manifested as the normoandrogenic phenotype. A 2014 study conducted by Wiweko *et al*.[24] demonstrated that approximately 63% of Indonesian PCOS women were normoandrogenic, also known as the Rotterdam criteria Group D. In addition, the meta-analysis conducted by Mumusoglu and Yildiz also indicated that 14%–25% of PCOS phenotype worldwide was categorised as normoandrogenic PCOS.[25] This present study, therefore, corroborated the previous findings of a high prevalence of normoandrogenic PCOS women, particularly in the Indonesian population. Another possible explanation for the lack of association of ovarian response with both polymorphism and androgen levels is that the participants in the present study were predominantly normoandrogenic/euandrogenic PCOS women, as indicated by testosterone, SHBG and free testosterone index that fell within the normal range.

This study has its limitations. First, the nature of the nested case–control study did not allow homogenisation in the type of gonadotropin used for COH. Notably, recombinant FSH (rFSH) only as or rFSH supplemented with rLH or LH-like activity were utilised for ovarian stimulation using the antagonist stimulation protocols, which could have affected the study outcomes. Second, the relatively small sample size may not be statistically adequate in detecting the association between LH β-subunit SNPs and androgen levels with ovarian response to COH with gonadotrophin. A larger sample size with a clinical trial study design would be more appropriate to correlate the effects of LH β-subunit polymorphisms and androgen levels with ovarian response to gonadotropin. Establishing a correlation between genetic variations, such as polymorphisms and clinical phenotypes is inherently complex and requires large datasets to achieve statistical significance. Third, our hyporesponse group was predefined with a threshold of <8 mature (MII) oocytes after standardised COS. This differs from the POSEIDON framework, which classifies ≤9 total oocytes as a suboptimal response. This yielded minor differences in classification but produced similar results. This study serves as proof of concept, demonstrating that such polymorphisms do exist. It also highlights the need for larger-scale studies with broader SNP analysis to uncover meaningful clinical associations. Thirdly, further investigation on the effect of LH protein modifications (due to genetic polymorphisms) in its function is required to elucidate its potential clinical implications in PCOS women.

Conclusion

Findings in this study suggest that the LH β-subunit gene polymorphisms (rs1800447 and rs34349826) androgen

levels including testosterone, SHBG and free testosterone index, are not significant predictors for ovarian stimulation response in PCOS women undergoing an IVF program. We propose further validation of the present findings in a larger cohort of Indonesian women with PCOS, utilising whole-gene sequencing to obtain a more comprehensive understanding of the potential correlation.

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Author contributions

IS designed, coordinated this study and drafted the manuscript. BW, RM, AS, WI, SGM, AB and TF critically reviewed and revised the content. IRS and RT carried out the study and data analysis, and the statistical analysis was reviewed by WI. IS and BS conceived of the study and participated in study coordination. All authors contributed to the article and approved the submitted version.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement

Data related to this study are available from the corresponding author (IS) upon reasonable request.

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