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Submission date: 14-Mar-2024 02:59PM (UTC+0700)

Submission ID: 2320051897

File name: ojbsci.2024.154.169.pdf (761.79K)

Word count: 12489

Character count: 67123

Original Research Paper

Associations of HSD17B1 Gene Expression with its DNA Methylation and Estradiol Level in Polycystic Ovary Syndrome Indonesian Patients

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Article history

Received: 07-08-2023

Revised: 06-10-2023

Accepted: 01-11-2023

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Abstract: PCOS's origin and mechanism are still unknown. Epigenetics has been linked to PCOS in an increasing number of studies in recent years. The most extensively researched epigenetic alteration is DNA methylation. During organismal development, DNA methylation can control gene expression by altering transcription factor binding. The alterations in DNA methylation are directly associated with follicular development in PCOS. Studies show that increased levels of pregnenolone and estrogen in the follicular fluid may affect follicle formation in PCOS patients; the process is largely associated with the expression of HSD17B1. There is evidence to suggest that these levels may have an impact on follicle development in PCOS patients. The mechanism for this effect is partially linked to HSD17B1 expression, which catalyzes the final step in estrogen biosynthesis, 17 β -estradiol (E2). We speculated that defects in DNA methylation increase gene dysregulation, resulting in decreased mRNA expression of HSD17B1, which eventually generates insufficient E2 in PCOS patients. The objective of this study is to investigate DNA methylation, mRNA expression, and E2 level in PCOS patients and healthy women groups; the correlation between DNA methylation and mRNA expression in PCOS patients; and the correlation between mRNA expression and E2 serum level in PCOS patients. We provided informed consent to participants; we studied 60 female patients, 30 PCOS patients and 30 healthy women served as the control group, we used the Methyl-Specific PCR (MSP) method and quantitative PCR (qPCR) for DNA methylation and mRNA expression analyses, respectively; and we examined E2 serum levels and hormonal levels. The methylation of the HSD17B1 gene in PCOS women was 42.64% and a healthy group showed 53.80% ($p = 0.160$). The two groups' differences were not statistically significant. The relative expression value of the HSD17B1 gene was 0.70-fold lower compared with the healthy women ($p = 0.003$) group. Significant variances were between the two groups. The average E2 serum level in the PCOS group is 25.78 pg/mL and in the healthy women group, it is 36.74 pg/mL. Compared to the group of healthy women, the PCOS group had a decreased E2 serum level. The correlation of DNA methylation level versus mRNA

expression in PCOS patients is not significant. ($p = 0.076$). A significant negative association has been seen between the mRNA. There is a significant negative correlation between the mRNA expression of the HSD17B1 gene and serum E2 levels. ($p = 0.020$). "The more down-regulated mRNA expression of the HSD17B1 gene, the lower serum E2 levels." The integrated analysis in this study was hypomethylated DNA and down-regulated mRNA expression of HSD17B1 genes. The hypomethylated DNA was not involved in down-regulating mRNA expression. Therefore, down-regulated mRNA expression of the HSD17B1 gene in PCOS patients can cause lower E2 levels in PCOS, preventing cell growth and potentially contributing to the cause of PCOS pathogenesis.

Keywords: HSD17B1 Gene, DNA Methylation, mRNA Expression, Estradiol, Polycystic Ovary Syndrome

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Introduction

The most prevalent metabolic and endocrine condition affecting women of reproductive age is called Polycystic Ovary Syndrome, (PCOS). However, with the scientific community's growing interest neither the pharmaceutical industry nor international health authorities have made comparable advances in PCOS. 1935 saw the first description of PCOS as a combination of obesity, enlarged cystic ovaries, amenorrhea, persistent anovulation infertility, and hirsutism (Escobar-Morreale, 2018).

Clinical trial registration in PCOS is clearly seen at this time. Although there are similar global prevalences for both PCOS and diabetes mellitus, there were only 28 commercial research on PCOS registered at ClinicalTrials.gov in 2017 compared to 4,632 studies on the latter condition. PCOS is not well understood by patients, doctors, or scientists and there is a widespread misconception about its long-term effects. This could be the reason for the lack of interest in the disease. Some explanations could be that it is too diverse to be adequately named, that its definition is debatable, that its etiology and pathophysiology are unknown, or that it is too general to be defined (Escobar-Morreale, 2018).

The world health organization estimates that 8-13% of women who are of reproductive age have PCOS; globally, up to 70% of afflicted women do not receive a diagnosis (Abbott *et al.*, 2019; Bellver *et al.*, 2018; Carbone *et al.*, 2019). In Indonesia, there is a lack of information about the number of PCOS cases. As a result, determining which city has the highest frequency of occurrence is challenging. The information on PCOS cases is based on medical records from hospitals in each province.

However, the researcher's interest in PCOS keeps growing, both genetic and epigenetic. Epigenetic variables have been linked to PCOS in an increasing number of studies in recent years (Dyke *et al.*, 2019). The most extensively researched epigenetic alteration is DNA methylation. Liu *et al.* (2022) DNA methylation in PCOS

has been considered a fresh biological target for creating effective diagnostic markers for predicting PCOS risk or its progression Smirnov *et al.* (2023); Sarkies (2020). In order to adapt to changes in the environment and in lifestyle, DNA methylation has the ability to alter the way genes are expressed without altering the sequence of DNA (Li *et al.*, 2020; Hosseini *et al.*, 2019; Concha *et al.*, 2017). Gene silencing and activation are linked to hypermethylation and hypomethylation in DNA, respectively (Guéant *et al.*, 2020; Rotondo *et al.*, 2018). Promoter regions are where methylation patterns are found. Through its impact on transcription factor binding during organismal development, DNA methylation has the ability to influence gene expression (Liu *et al.*, 2020).

Studies reveal that high levels of pregnenolone and estrogen in follicular fluid may affect follicle formation in PCOS patients; the process is largely associated with the expression of HSD17B1 (Yu *et al.*, 2021). Investigate expression changes of the steroidogenic enzyme PCOS. The results of the PCOS rat model showed that, in comparison to the normal control group, the PCOS group exhibited elevated levels of 3 β -HSD and 17 β -HSD mRNA and protein expressions. According to these findings, the current rat model of PCOS may include 3 β -HSD and 17 β -HSD in the control of ovarian hormones (Lin *et al.*, 2013). The hydroxysteroid 17-beta dehydrogenase-1 (HSD17B1) gene, chromosome 17: 42,549,214-42,555,213 is home to the hydroxysteroid 17-beta dehydrogenase-1 (HSD17B1) gene, type I marker locus (D17S934). Homer *et al.* (2017).

Due to its ability to selectively reduce the weak estrogen Estrone (E1) to produce the powerful estrogen 17 β -Estradiol (E2), the HSD17B1 gene is known as the "estrogenic" 17 β -HSD. During reproduction, the majority of E2 in females is generated by ovarian granulosa cells through the aromatization of androstenedione, which is generated in theca follicular cells, to E1. Subsequently, 17 β -HSD transforms E1 into E2 (Homer *et al.*, 2017; Konings *et al.*, 2018). Because of the strong correlation between the HSD17 β gene and E2, this gene is one of the best candidates to be investigated in PCOS's pathogenesis.

Individuals with PCOS have ovarian defects like impaired folliculogenesis, poor oocyte quality, and anovulation that lead to infertility (Budihastuti *et al.*, 2019; Amiri *et al.*, 2020; Zhang *et al.*, 2019; Sagvekar *et al.*, 2019). Granulosa cells were gathered as samples because they help the oocyte and theca cells communicate. Theca cells are controlled by the hormones FSH and LH, which stimulate the production of estrogen and "support" the oocyte (Ai *et al.*, 2019). Lack of E2 may indicate insufficient granulosa cell development in this study since granulosa cells release E2 mostly in follicles. Anovulation may result from low E2 levels, which have been proposed as a predictor of follicle development (Huang *et al.*, 2018).

Numerous research studies have linked HSD17B1 to PCOS, including the association between SNP-71G in type 5 of the 17βHSD polymorphism and androgen excess in some PCOS patients. The HSD17B6 gene's SNP rs898611 has been linked to PCOS's metabolic phenotype. In Chinese women, ovarian steroidogenesis has been linked to elevated expression of HSD17B6 in theca cells of follicles with PCOS; SNP rs1937845 of HSD17B5 is strongly connected with PCOS (Shaaban *et al.*, 2019).

We speculated that defects in DNA methylation increase gene dysregulation, resulting in decreased mRNA expression of HSD17B1, which eventually generates insufficient E2 in PCOS patients. We aim to investigate DNA methylation, mRNA expression, and E2 level in PCOS patients and healthy women groups; the relationship between PCOS patients' DNA methylation and mRNA expression; and the relationship between PCOS patients' mRNA expression and E2 serum level.

The only research by Wang *et al.* (2014) found a correlation between hypomethylated DNA and up-regulated mRNA expression of the HSD17B1 gene in PCOS using fresh ovarian tissue in the Chinese population (Wang *et al.*, 2014). The research on the associations of HSD17B1 gene expression with its DNA methylation and estradiol level in PCOS Indonesian patients has never been explored. This is the first study to examine DNA methylation and mRNA expression of the HSD17B1 gene in granulosa cells along with E2 serum levels in Indonesian patients.

Materials and Methods

Ethical Statements

The Helsinki declaration of 1975 and ethical guidelines were followed in the conduct of the inquiry.

Sample Size Calculation

The Lemeshow (1990) calculation formula was used to determine the sample size for this study. The prevalence of PCOS is 45.7% among women within the age of reproduction at Dr. Cipto Mangunkusumo hospital

Indonesia's national reference center hospital a leader in services, education, and research.

The lame show's formula:

$$N = \frac{(Z\alpha)^2 p(1-p)}{d^2}$$
$$= \frac{(1.96)^2 0.457(1-0.457)}{(0.2)^2} = 23.83$$

Description:

N = Total sample number

$Z\alpha$ = Error rate (1, 96)

p = PCOS ratio (10%)

d = Precision (20%)

The total number of samples used in this calculation was 60 people divided into two subjects; we looked at 30 PCOS patients, 30 healthy women, and 60 female patients overall.

Respondent Criteria

1. Healthy women as the control group:

a. Inclusion criteria

- Women who underwent ovum pick-up as part of the IVF procedure had normal ovarian function and no signs of PCOS symptoms
- Male factors cause infertility
- Women with non-patent tubes or tube diseases
- Women who agree to participate in the study sign a consent form after being informed about it

b. Exclusion criteria

- Women with endometritis
- Endometriosis-affected females
- Endometrial cancer sufferers
- Women who have ovarian cancer

2. PCOS group:

a. Inclusion criteria

- Women who had PCOS based on Rotterdam criteria identified through laboratory and ultrasound testing and were undergoing IVF
- Women who agree to participate in the study sign a consent form after being informed about it

b. Exclusion criteria

- Cushing's syndrome in females
- Women with endometritis
- Endometriosis-affected females
- Endometrial cancer sufferers
- Women who have ovarian cancer

Research Strategy

The research strategy carried out is as follows:

1. Sample collection
2. Isolate DNA samples using the Qiagen kit
3. Measurement of DNA concentration and purity using a nanodrop (Maestronano)
4. Bisulfite conversion using the EpiTect Bisulfite Kit, Qiagen 59104
5. Design methylation and unmethylation primers using the MethPrimer program
6. The primers are used for amplification using the IDT website's Primer Quest software (<https://www.idtdna.com/PrimerQuest>)
7. Amplification of target DNA (in the promoter region of the HSD17B1 gene) using Methylation-Specific Polymerase Chain Reaction (MSP) using EpiTect MSP Kit Qiagen 59304
8. The analysis of the methylation status of the HSD17B1 gene promoter regions by visualization electrophoresis Using Ultraviolet (UV) light was calculated using ImageJ software
9. Isolate the RNA sample using the High Pure RNA Isolation Kit for tissue. Quick-RNA MiniPrep Plus 200 preps, R1058 (ZYMO Research)
10. RNA concentration and purity were measured using Nanodrop (Maestronano)
11. Synthesis of RNA into cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover 200 reactions FSQ-301. (Toyobo)
12. Analysis of mRNA expression of target gene (HSD17B1) and reference gene (ACTB) in granulosa cells using the relative quantitative real-time polymerase chain reaction (qPCR) method with the Thunderbird® SYBR® qPCR Mix (3x1.67 ml) QPS kit (Toyobo)
13. Analysis of E2, Anti-Mullerian Hormone (AMH), Luteinizing Hormone (LH), Follicle-stimulating Hormone (FSH) and LH/FSH ratio
14. Statistical Analysis

The Research Strategy Carried Out is as Follows

Sample Collection

We studied 60 female patients who were being seen for *In Vitro* Fertilization (IVF) at the Dr. Cipto Mangunkusumo hospital at Yasmin clinic and the IVF Morula Bunda Jakarta Indonesian reproductive science institute Bunda Medik. These patients, who were aged 25-40 years, were divided into two groups (30 PCOS patients and 30 healthy women as the control group).

The blood serum of a patient following an IVF program is taken after the patient signs an informed consent. In patients undergoing IVF programs, granulocyte samples are taken after the patient gets

hormonal stimulation first. Granulosa cells play a crucial role in the synthesis of steroid hormones by supplying the oocyte with nutrients and other growth factors that may interact with it as it develops in the ovarian follicle.

An ovum pick-up is planned after a transvaginal ultrasound reveals the dominant follicle. In addition to flushing fluid, follicular fluid is acquired through piercing during ovum pick-up. The follicular fluid is then kept for a maximum of 6 h at room temperature or a maximum of 2×24 h in the refrigerator.

Isolate DNA Samples Using the Qiagen Kit

A 1.5 mL microtube was filled with up to 500 L of granulosa cells that had been kept in RNA later. The microtube was then centrifuged for 5 min at a speed of 300 g to separate the pellet from the supernatant and the supernatant was discarded. The pellet was mixed with 200 L of 1% Phosphate-Buffered Saline (PBS) to resuspend the cells, after which 20 mL of Proteinase K was added and vortexed for 10 sec at 60°C. Add 200 L of Gel Sample Buffer (GSB) to the cells, vortex for 10 sec and then incubate for 5 min with inversions every 2 min to lyse the cells. For DNA binding, add 200 L of 100% ethanol and vortex for 10 sec. Place the sample in the granulosa cell column tube, which has been positioned above the collecting tube. Centrifuge the sample for 1 min at a speed of 14000 g, then discard the supernatant. The sample is washed by adding 400 L of W1 buffer and the supernatant is then discarded after centrifuging the sample at a speed of 14000 g for 30 sec. Centrifuge the GS column with 600 mL of wash buffer at a speed of 14000 g for 30 sec, then discard the supernatant. Repeated centrifugation at a speed of 14000 g for 3 min and incubation at 60°C for 5 min were used to dry the material. Add 80 L of 60°C-warmed elution buffer to the DNA mixture for optimum absorption, then let it sit at room temperature for 3 min. Centrifuge at 14000× g speed for 30 sec. The DNA-containing supernatant should be kept at -20°C if it is not going to be used.

Measurement of DNA Concentration and Purity Using a Nanodrop (Maestronano)

After isolation, measure the concentration and purity of DNA from the DNA isolation to determine its purity and concentration. DNA concentration values are needed to calculate the next stage of the reaction, bisulfite conversion. This instrument uses nano drops with the principle of a spectroscopic photometer on the sample (2 µL). Once the inspection is complete, the indirectly used DNA can be stored at a temperature of -20°C for a relatively long period of time. The average gDNA yield was 4 µg (per 10⁶ cells).

Bisulfite Conversion Using the EpiTect Bisulfite Kit, Qiagen 59104

Bisulfite conversion is a technique used to study DNA methylation using sodium bisulfite that converts non-methyl cytosine to uracil while methylcytosine remains unchanged. As the 'gold standard' used to assess the DNA methylation status, these bisulfite conversions can be followed with applications such as PCR or sequencing so that it can be distinguished between methylcytosine residues and nonmethylations. This is probably because DNA that has been transformed into bisulfite can be used directly as a template on PCR and during this PCR, all uracil and thymine residues will be reinforced as thymine and only the 5-methylcytosine residual will be enhanced as cytosine. Implementation of bisulfite conversion using the EpiTect Bisulfite Kit, Qiagen 59104 DNA isolates are prepared according to the volume that has been determined based on a DNA concentration of 50 ng/mL. An aliquot of bisulfite mixture that is still in powder condition was added to 800 μ L of RNA-free water to dissolve. The mixture is homogenized with the vortex until mixed or heated at 60°C and prepared for a bisulfite reaction in a 140 μ L PCR tube. The solution of the mixture of bisulfites in the PCR tubes will turn blue after adding a DNA protection buffer since the solution has been mixed well and the pH is correct. A total of 20 μ L was used for the bisulfite reaction, 85 μ L for the bisulfite mix, 35 μ L for the DNA protect buffer, and 140 μ L for the combined volume of DNA solution and Rnase-free water. Denaturation 5 min 95°C, incubation 25 min 60°C, incubation 85 min 60°C, denaturation 5 min 95°C, incubation 175 min 60°C and hold indefinitely at 20°C are all achieved with the bisulfite conversion thermal cycler. The temperature adjustment is adapted to the protocol using the thermal cycler T100TM from Biorad, USA. The sample PCR tube was inserted into the thermal cycler block.

The washing for DNA purification is carried out by means of a PCR tube of the thermal cycler at the spindown and transferred into the 1.5 mL ephendorf tube, then added 540 μ L BL buffer and divortex until all is mixed and in the spindown. The DNA solution is transferred to the spin column that has been placed on the collection tube, centrifuged at a speed of 13,000 rpm for 1 min, the rest of the centrifuge removed and then put back on the spin column in the collect tube. 500 μ L of BW buffer is added to the spin column, which is then withdrawn and centrifuged in the collection tube before being reattached to the collecting tub.

The spin column is then sealed and left to stand at room temperature for 15 min. After centrifuging the previously incubated solution for 1 min at 13,000 rpm, the spinocolumn is transferred to the new collecting tube, and the remaining centrifugation in the collector tube is released. After transferring the spin column to a

fresh 1.5 mL Ephendorf tube, 20 μ L of EB buffer is immediately added to the spin column. In the next step, it's centrifuged at a speed of 13,000 rpm for 1 min to circle DNA. The result is re-inserted into the spin column and centrifuged at a velocity of 12,000 rpm for a minute. After all the stages are completed, the DNA that has been purified is stored at a temperature of 2-8°C for 24 h and for a long time at -20°C.

Design Methylation and Unmethylation Primers Using the MethPrimer Program

The MSP operation uses kits specially designed for MSP processes and uses primers in the categories of Methylated (M) and Unmethylated (U) DNA. Primers M is used for modified and methelilated DNA, while primers U are used to modify and non-methelilate DNA. Two primary sets are amplified in two different reaction tubes. Amplification with primary M indicates methylation at CpG sites, while U indicates unmethylated sites. The primer methylation design was made with the Li-Lab Methprimer program (<http://www.urogene.org/methprimer/>) for HSD17B1 genes.

The primary sequence used for this research is the HSD17B1 gene, taken from the NCBI genebank (<https://www.ncbi.nlm.nih.gov>) with the reference sequence: NC_000017.11, located on chromosome 17p21.2 with a sequence length of 2657 base pairs. The primary methylation result used is found in the first order. The forward primary is at base position 1473 and the reverse starts from base position 1596. The number of cytosin-guanin dinucleotides in the area amplification product of the promoter gene HSD17B1 is 11.

The primer Methylated (M) and Unmethylated (U) promotor gen HSD17B1 Forward-M, 126 bp, annealing 65.6°C, GGGTTAGTAGCGTGAGTTTGATC. Reverse-M, 126 bp, annealing 65.6°C, ATTAATACAAAAACGAAAAATCCG. Forward-U, 124 bp, annealing 64.3°C, GGGTTAGTAGTGTGAGTTTGATTGA. Reverse-U, 124 bp, annealing 64.3°C, TAATACAAAAACAAAAATCCAAA.

The Primers are Used for Amplification Using the IDT Website's Primerquest Software (<https://www.idtdna.com/PrimerQuest>)

The primer pair sequences for the HSD17B1 gene amplification were as follows: Forward CCAGGACAGTTTGTGTGCCA (Sense), annealing 60°C and reverse CCCACGCAATCTCAAGGATAAG (Anti-sense), annealing 60°C; and the ACTB gene was employed as a housekeeping gene, using the reverse primer AGA GAA GTG GGG TGG CTT TT (Anti-sense) and the forward primer AAA CTG GAA CGG TGA AGG TG (sense).

Amplification of Target DNA (in the Promoter Region of the HSD17B1 Gene) Using Methylation-Specific Polymerase Chain Reaction (MSP) Using EpiTect MSP Kit Qiagen 59304

PCR amplification was done using the Hotstat method using a standard protocol and a prepared PCR tube containing components with a DNA concentration of 10 ng/mL and a volume of 5 μ L. Reaction components for MSP, PCR-grade water up to 50 μ L, 2 \times KAPA Hifi hotstat uracil + ready mix 25 μ L, forward primer 0,5 μ L and reverse primer 0,5 μ L, DNA template as required. The PCR pipe that contains the above components is inserted into the thermal cyclor machine. The methylation positive control used is Qiagen's epitect-methylated DNA with an amplicon size of 132 bp, the purpose of which is to assess the conversion of bisulfite and the quantity of DNA metylation.

The MSP-PCR cycle protocol is: Initial denaturation 2-5 min 95°C, 1 cycle; denaturation 20 sec 98°C; annealing 15 sec 45-55°C, 15-40 cycle; extension 30-60 sec/kb 75°C; final extension 1 min/kb 75°C, 1 cycle.

The Analysis of the Methylation Status of the HSD17B1 Gene Promoter Regions by Visualization Electrophoresis Using Ultraviolet (UV) Light Was Calculated Using Imagej Software

The MSP amplification product was observed with an electrophoresis gel using 2.4% agarose (Promega) in a 1% TAE (Tris Acid EDTA) buffer that was given a red gel of 0.1 μ g/mL. The volume of the MSP product from the sample as well as a positive control of 7 μ L were run inside the gel with a strong current of 100 volts for \pm 45 min. The result is then visualized using UV rays on the transluminator and documented with the camera. One primary set contains DNA cytosin to amplify methylated DNA and another set contains thymine for amplifying unmethylated DNA.

Amplification with MSP will produce three different alternative tape patterns when running on electrophoresis agarose. When only one tape appears in M, it indicates the methylated CpG area; when one tape appears in U, it indicates promoters that have not experienced methylation; and when a tape appears in both M and U, it indicates partial methylation in the promoter area. Generally speaking, the differences in the results of electrophoresis can be seen by direct visualization. But to obtain the value of the DNA thickness, the electrophoresis result is photographed using a camera under ultraviolet rays and then the result is processed using ImageJ software. The principle of reading using image J is based on the result of the band/band DNA of the sample in the form of a densitogram so that it can be known the width of the peak area of each DNA band; the thicker the tape or band, the greater the image value. To evaluate the level

of methylation, large-scale measurements of the area and the intensity of the target gene tape methylated compared to the total area and intensities of the methylated and unmethylated tape were multiplied by one hundred percent, or the unmethylation rate was looked for by comparing the width of the non-methylated tape compared with the total methylation and unmethylated tape width multiplied by one hundred percent. The results of the data were subsequently tested using statistical tests using SPSS software.

Isolate the RNA Sample Using the High Pure RNA Isolation Kit for Tissue. Quick-RNA Mini Prep Plus 200 Preps, R1058 (ZYMO Research)

Centrifuging at 3000 g for 5 min will first separate the granulosa cells from the RNA; the supernatant should be discarded. Granulosa cells in a volume of 300 liters were taken, placed in a 1.5 mL microtube with a 300 mL DNA/RNA shield (2 \times), homogenized with a mixer until crushed, and then added together with 30 mL of PK digestion buffer and 15 mL of proteinase K. Take the homogenate tube next. A thermal block was used to incubate the sample for 30 min at a temperature of 55°C. The mixture was mixed with 400 L of RNA prep buffer and the column was centrifuged at 13,000 g, discarding the supernatant. After that, 700 L of wash buffer I was added, and 13,000 g was centrifuged for 30 sec. After pouring 400 L of wash buffer II onto the column and centrifuging it at 13,000 g for 2 min, each sample was combined with 100 L of elution buffer, and it was put onto the Zymo III CG column matrix. To extract the data for total RNA, the sample was centrifuged at 16,000 g for 30 sec.

RNA Concentration and Purity were Measured Using Nanodrop (Maestronano)

After the isolation, measure the concentration and purity of the RNA isolated with the aim of knowing its qualities of purity and concentration. The value of the RNA concentration is required for the calculation of the next phase of the reaction, cDNA. The measuring instrument uses the nanodrop with the principle of spectroscopic photometry on the sample (2 μ L). Once the inspection has been completed, indirectly used RNA isolates can be stored at a temperature of -80°C for a relatively long period of time. The average RNA yield was 10 μ g (per 10⁶ cells).

Viruses, bacteria, fungi, and parasites are all efficiently rendered inactive by the DNA/RNA shield, which is utilized to preserve the genetic integrity and expression patterns of materials at room temperature. Freeze-thaw cycles and freezer malfunctions are avoided, preventing deterioration. It has been rigorously tested for its ability to deactivate various pathogens without additional steps or the need for heat treatment, homogenization, or alcohol sterilization.

Synthesis of RNA into cDNA Using ReverTra Ace® qPCR RT Master Mix with gDNA Remover 200 Reactions FSQ-301. (Toyobo)

All the mixing activities in this cDNA synthesis are done on the ice shredder (0-4°C). The first step is to make a mix consisting of 4× DN Master mix and gDNA remover with a ratio of 5:1, considering making the mix adjusted to many samples to work with because the mix only lasts for 3 months when mixed. Before entering the next step, the RNA concentration is equated first with a concentration of 25 ng/μL, then enter the Dnase I treatment phase to remove DNA. At this stage, the mix is made consisting of 4× DN master mix (2 μL) plus 6 μL of RNA mix and RNA-free water to obtain a total volume of 8 μL. The solution is diverted and spindown, then incubated at a temperature of 37°C for 5 min using a thermal block and then the solution is placed on the ice. The next stage is the synthesis of cDNA by means of a mix DnASE I treatment with a volume of 7 μL added with 5× RT master mix II of 2 μL, then homogenized so that a total of 10 μL volume is obtained. This step is done on ice. This cDNA solution has an extended shelf life at -20°C.

Analysis of mRNA Expression of the F Target Gene (HSD17B1) and Reference Gene (ACTB) in Granulosa Cells Using the Relative Quantitative Real-Time Polymerase Chain Reaction (qPCR) Method with the Thunderbird® SYBR® qPCR Mix (3×1.67 mL) QPS kit (Toyobo)

At this stage, each reaction has a volume of 20 μL with component details, namely: 10 μL cyber mix, 0.04 μL ROX cyber mix, 1 μL primary forward and primary reverse, 6.96 μL ddH₂O and 1 μL cDNA template. After inserting and mixing in a 0.2 mL PCR tube, the solution is inserted into the PCR machine following the cycle protocol.

The process of the lightcycler cycle is 40 cycles of amplification, 1 cycle of cooling and 1 cycle of pre-incubation. The ideal temperatures (in degrees Celsius) are pre-incubation at 95°C for 1 min, amplification at 95°C for 15 sec, cooling at 40°C for 30 sec, and pre-incubation at 72°C for 1 min.

The mRNA expression analysis of the HSD17B1 genes is performed using the Livak method or delta threshold comparison ($\Delta\Delta Ct$), which is a method of quantification relative to one of the samples specified as a calibrator and target compared to a calibrator to show a relative expression level. The reason this method is used is because the standard value is unknown, so the target is compared to the reference value of the selected gene. In order for the Ct value to be valid, the efficiency at the time of amplification of the target gene must be the same as the reference gene amplification. The value obtained is a relative value to the control:

- ΔCt gene sample = Ct gene target minus Ct gene reference
- CT gene control = CT gene target-CT gene reference
- $\Delta\Delta Ct = \Delta Ct$ Gen sample- ΔCt gen control
- Gene expression comparison = $2^{-\Delta\Delta Ct}$

Statistical Analysis

The numbers, frequencies, and percentages or ratios are used to represent categorical data for each category. The normal data distribution is provided as the mean. The normal data distribution, the T-independent test, is used to examine hormonal characteristics such as AMH, LH, FSH, LH/FSH ratio, and serum E2 level. The nonparametric test, Mann-Whitney to examine methylation and expression of the HSD17B1 gene, the person correlation test to examine the relationship between methylation and expression of the HSD17B1 gene, using Spearman's Rho correlation to look at the connection between blood E2 levels and the HSD17B1 gene.

Results

Hormonal Characteristics

Independent T-Test

The independent t-test is an inferential statistical test that may be used to determine the mean difference between two unrelated groups. For the test to be successful, the dependent variable has to be approximately normally distributed within each group. This may be confirmed using tests like the Shapiro-Wilks test of normality or a Q-Q plot. Modify the data or do the Mann-Whitney test if there are substantial differences between the groups and the data is not nearly normally distributed. Assuming that the variances of the two groups in the population are equal, the independent t-test may be performed using Levene's test of equality of variances.

Since the independent t-test was used for all hormonal investigations during menstruation in this study, all results were homogeneous across the two unpaired groups and had a normal distribution. The AMH and FSH levels of healthy women and those with PCOS differed significantly, according to the hormonal data of the female research participants. Data on average AMH levels in healthy women subjects was 3.98 ng/mL and in PCOS subjects it was 6.49 ng/mL. The average LH level in healthy female subjects was 6.04 and 6.38 mIU/mL in PCOS patients. The average FSH level in healthy female subjects was 7.36 and 6.18 mIU/mL in PCOS patients. The average ratio LH/FSH of healthy women subjects was 0.8517 and 1.073 in PCOS subjects.

DNA Methylation Level of the HSD17B1 Gene

Electrophoresis MSP results (Fig. 1) are representative of 5 samples from the PCOS group and 5 samples from the healthy women group. The methylated HSD17B1 gene in granulosa cells from the 30 PCOS patients was 42.64% and from 30 healthy women (control) it was 53.80% (Fig. 2). The Mann-Whitney test statistical analysis of nonparametric data revealed no significant difference between the two groups ($p = 0.160$) at $p < 0.05$.

DNA methylation is attracting a lot of attention in studies, which raises a lot of issues like, "How does a transcription pattern become stable?" If specific genes are not active, how can a lens cell remain a lens cell? How do cells go through several cycles of mitosis while remaining differentiated? It's called DNA methylation. Inactive gene promoters are methylated at certain cytosine residues, resulting in the formation of methylcytosine, which stabilizes nucleosomes while blocking transcription factor binding. The lack of methylation of the HSD17B1 gene in PCOS demonstrates that there is no methylation activity inhibiting HSD17B1 expression. The DNA methylation of HSD17B1 on the cell granulosa in PCOS is rarely discussed in the literature, making it difficult to compare. The only one the literature demonstrates and has the same outcome: Wang *et al.* (2014), DNA in ovarian tissue is hypomethylated.

All processes are carried out in accordance with the drawing instructions. Design methylation and unmethylation primers using the MethPrimer program; the biggest island is CpG 874 bp, starting from 1306-2179 bp. The amplification product region promoter gene HSD17B1 has 11 cytosine-guanin dinucleotides.

mRNA Expression Level of the HSD17B1 Gene

The qPCR study showed that PCOS patients had lower levels of HSD17B1 mRNA expression than did healthy women. By applying the Livak approach, we discovered that PCOS patients had a 0.70-fold lower relative expression value of the HSD17B1 gene than did healthy women (Fig. 3). The difference was statistically significant ($p = 0.003$), according to the Mann-Whitney test statistical analysis of nonparametric data. At $p < 0.05$.

Human granulosa cells in the ovaries and human placenta syncytiotrophoblasts both use the human HSD17B1 enzyme in glandular E2 production. The enzyme's enzymatic properties and expression in these cells indicate a central role in E2 production. High expression in the human placenta is also indicative of its association with E2 production. HSD17B1 is expressed in various peripheral sex steroid target tissues. It supplies extremely potent ligands for estrogen receptors. Human disorders that are endocrine-dependent have been linked to elevated HSD17B1 expression (Hakkarainen *et al.*, 2015).

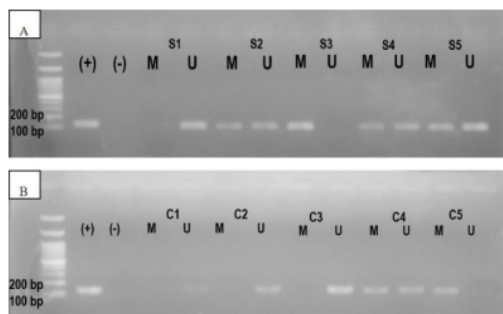


Fig. 1: Electrophoresis HSD17B1 gene

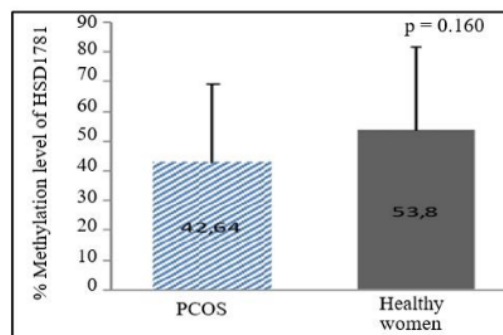


Fig. 2: Methylation percentage of the HSD17B1 gene

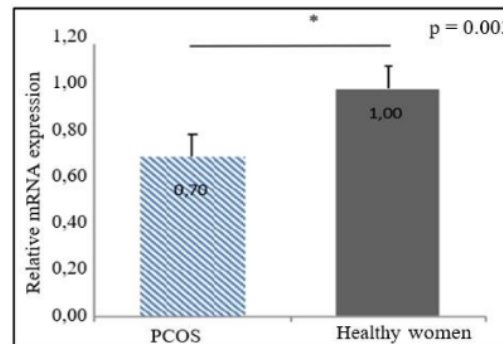


Fig. 3: mRNA expression HSD17B1 gene

According to the research, HSD17B1 controls the availability of estrogen ligands for estrogen receptors in theca cells, which is essential for balanced steroidogenesis in the ovaries. The function of HSD17B1 in luteinization and the onset of pregnancy (Hakkarainen *et al.*, 2015) As a result, HSD17Bs are thought to be interesting targets for medications designed to reduce the amount of estrogen present in peripheral estrogen target tissues (Hakkarainen *et al.*, 2015).

Serum E2 Levels in PCOS Patients and Healthy Women

Compared to women in good health, the average E2 serum level in PCOS patients was 25.78 pg/mL. Using an independent t-test, the difference in E2 serum levels was shown to be statistically significant ($p = 0.009$) $p < 0.05$.

Clinical laboratories test serum estradiol in women to assess ovarian activity. Blood tests for estradiol quantify the level of the hormone. It is used to assess the placenta, adrenal glands and ovaries' overall health. It can identify estrogen in women going through menopause, hypoestrogenicity, amenorrhea, or other menstrual disorders. Estradiol levels rise with follicular development (peak: 200 pg/mL) and are typically less than 50 pg/mL during menstruation in a normal menstrual cycle. In PCOS patients, low estradiol levels were found when compared to healthy women due to the disruption of the work of the genes involved in steroidogenesis, so conversion into estradiols is disrupted, which ends with little or inadequate estradiol circulating in the blood (Homer *et al.*, 2017).

Correlation Between DNA Methylation and mRNA Expression of the HSD17B1 Gene

DNA methylation levels and mRNA expression in PCOS patients did not significantly correlate, according to the correlation analysis utilizing Pearson's correlation test ($p = 0.076$) ($p < 0.01$) (2-tailed).

The most significant epigenetic fingerprint seen in the genomes of higher eukaryotes is the methylation of DNA at the cytosine ring's position (C-5). Depending on where DNA methylation occurs in an area, it might operate as a limiting or activating mark for gene expression. DNA methylation can thus, through interaction with histone modifiers, either prevent the transcription apparatus from binding or create an environment that is favorable to transcription.

The methylation of DNA at the location of the cytosine ring (C-5) is the most notable epigenetic imprint observed in the genomes of higher eukaryotes. DNA methylation may function as a limiting or activating mark for gene expression, depending on where it happens in the region. Therefore, DNA methylation may either inhibit the transcription apparatus from binding or promote transcription by interacting with histone modifiers.

Studies reveal that favorable correlations between DNA methylation and gene transcription are rare. A study of DNA methylation and gene expression revealed that certain genes were hypomethylated and up-regulated, hypermethylated and down-regulated, hypomethylated and up-regulated, and hypermethylated and down-regulated. A few of these differences were connected to the promoter regions' methylation patterns. may impact methylation changes and transcription factor

binding in gene coding regions, which can modulate gene expression through alternative splicing mechanisms even if they do not directly control gene transcription (Hakkarainen *et al.*, 2015).

In contrast to our findings, mRNA expression was down-regulated. Wang *et al.* (2014), on the other hand, utilized ovarian tissues as a sample, whereas our investigations employed granulosa cells. The change in the same gene may be caused by the various environmental and lifestyle variables that we know have an impact on PCOS etiology.

Correlation Between the mRNA Expression of the HSD17B1 Gene and Serum E2 Levels

The substantial ($p = 0.020$) association between the blood E2 levels and the mRNA expression of the HSD17B1 gene was demonstrated using the Spearman's Rho correlation test at $p < 0.05$.

HSD17B1 genes that are missing or deficient cause downregulation in mRNA genes, which then create E2 that is insufficient for the formation of the estrogen enzyme in the ovaries; this occurrence was demonstrated in this study. An indicator of ovarian activity is serum E2. It is used to assess the placenta, adrenal glands, and ovaries in order to identify hypoestrogenicity and menopause, as well as to detect estrogen in women who have amenorrhea or menstrual disorder. When compared to women in good health, PCOS patients have lower E2 levels because the normal actions of genes involved in steroidogenesis are disturbed, which leads to insufficient or inadequate amounts of E2 circulating in the blood.

Electrophoresis of the MSP product of the HSD17B1 gene in granulosa cells (Fig. 1A) 5 PCOS patients (Fig. 1B) and 5 healthy women as controls (+) = positive control; (-) = negative control; M = methylated; U = unmethylated; S1-S5 = sample 1-5 (sample = PCOS group); C1-C5 = control 1-5 (control = healthy women group). The methylated product size was 126 bp, whereas the unmethylated product size was 124 bp.

Methylation percentage of the HSD17B1 gene in granulosa cells from PCOS patients and healthy women. Methylation of the HSD17B1 gene in PCOS patients was 42.64% and in healthy women, it was 53.80%. and the statistical analysis of parametric data by the Mann-Whitney test, no significance was found between the two groups ($p = 0.160$).

Through the Livak method, the relative expression value of the HSD17B1 gene in PCOS patients was down-regulated by 0.70-fold compared with that in healthy women (1.00-fold) ($n = 30$ in PCOS patients; $n = 30$ in healthy women). According to the statistical analysis of nonparametric data by the Mann-Whitney test, the result was significant ($p = 0.003$).

Discussion

Correlation Between DNA Methylation and mRNA Expression of the HSD17B1 Gene

Electrophoresis MSP results (Fig. 1) are representative of 5 samples from the PCOS group and 5 samples from the healthy women group. The methylated HSD17B1 gene in granulosa cells from the 30 PCOS patients was 42.64% and from 30 healthy women (control) it was 53.80% (Fig. 2). In the statistical analysis of nonparametric data by the Mann-Whitney test, no significance was found between the two groups ($p = 0.160$). As revealed in the qPCR analysis, the HSD17B1 mRNA expression compared to women in good health, and PCOS patients had a lower level.

According to qPCR results using the Livak technique, PCOS patients had a 0.70-fold lower relative expression value of the HSD17B1 gene than did healthy women (Fig. 3). According to the statistical analysis of nonparametric data by the Mann-Whitney test, the statistically significant change was observed ($p = 0.003$). HSD17B1's mRNA expression level was significantly decreased in PCOS. Patients than in healthy women, according to the qRT-PCR study. As a result, the amount of HSD17B1 expression was reduced in this research.

The hypomethylated DNA and mRNA expression linked to PCOS are analyzed together with down-regulated mRNA expression of HSD17B1 genes. DNA methylation levels and mRNA expression in PCOS patients did not significantly correlate, according to the correlation analysis utilizing Pearson's correlation test ($p = 0.076$).

Lerner *et al.* (2019) carried out a study along these lines. Granulosa cell dysfunction has been connected to the pathogenesis of PCOS. Ovarian dysfunction in PCOS may be influenced by abnormal regulation of steroid metabolism or activity, according to granulosa cells from women with ovulatory and anovulatory PCOS. Gene expression in control and PCOS-afflicted women was compared using RT-qPCR on cells obtained after oocyte extraction for IVF/ICSI. When treated with 10 nm dihydrotestosterone, the expression of HSD17B1 was decreased, according to the results. Compared to healthy women, PCOS women's granulosa cells expressed HSD17B1 1.8 times less frequently ($p = 0.02$). These results imply that ovarian dysfunction in PCOS may be related to abnormal steroid metabolism or action (Lerner *et al.*, 2019).

According to Al-Omar *et al.* (2020), the oocytes' HSD17B1 expression level varied significantly ($p 0.05$).

Thirteen Meiosis II (MII) stage oocytes were collected from IVF patients. Seven oocytes were extracted from oocyte donor patients who had polycystic ovaries and six oocytes were retrieved from female oocyte donors who did not exhibit any indications of polycystic ovaries (control group). Human MII-stage

oocytes from donors between the ages of 18 and 30 showed HSD17B1 expression levels. Patients with polycystic ovaries showed slightly greater follicle and oocyte numbers compared to the control group. HSD17B1 expression in oocytes was statistically different, indicating that polycystic ovaries had changed steroidogenesis pathways, potentially playing a role in their development (Al-Omar *et al.*, 2020).

There is evidence that there are few instances in which gene transcription and DNA methylation are positively correlated. Wang *et al.* (2014) used newly harvested ovarian tissue from three cervical cancer patients (controls 1-3) who were less than 40 years old, in the follicular phase, had undergone pelvic lymphadenectomy and radical hysterectomy for cervical cancer, and had regular menstrual cycles (25-35 days). Three PCOS patients (PCOS 1-3) with an irregular menstrual cycle, had laparoscopic wedge resection during the follicular phase and were less than 40 years old and had their fresh ovarian tissue harvested. Wang *et al.* (2014) found a connection between up-regulated mRNA expression and hypomethylated DNA (Hakkarainen *et al.*, 2015).

In contrast to our findings, hypomethylated DNA and mRNA expression were down-regulated. Wang *et al.* (2014), on the other hand, utilized ovarian tissues as a sample, whereas our investigations employed granulosa cells. The change in the same gene may be caused by the various environmental and lifestyle variables that we know contribute to the pathogenesis of PCOS (Pan *et al.*, 2018).

In European and Chinese women, there have been detected variants of the LH Receptor (LHR) and certain allelic variations of Fibrillin-3 (FBN3), which are PCOS susceptibility genes. An altered metabolic profile, including insulin resistance, is seen in the FBN3, A8 allele linked to PCOS. Transformation-promoting Factor (TGF) signaling is regulated by the extracellular matrix protein FBN3. Variations in LHR can impact the function of the corpus luteum, development of ovarian follicles, ovulation caused by LH surge, and generation of stroma cell T in ovarian theca (De Leo *et al.*, 2016).

Asian women are more likely to have PCOS in developed nations than Caucasian women, who have varying rates of the condition. Lifestyle, physical activity, and diet are examples of environmental variables that might affect how the condition presents phenotypically. Adult-onset disorders like PCOS are largely influenced by environmental variables and epigenetic pathways. In fetal reproductive tissue, exposure to hyperandrogenism during pregnancy may cause aberrant epigenetic reprogramming, which translates into the adult PCOS phenotype. The PCOS phenotype may also arise as a result of postnatal environmental variables including nutrition and genetics. There appears to be a correlation between cardiovascular illness, prenatal growth restriction, and the risk of type 2

Diabetic Mellitus (DM). Genetic polymorphisms may alter an individual's insulin resistance characteristics, which helps to explain why persons exposed to unfavorable in-utero environments have varying degrees of insulin resistance. These fetuses' overnutrition has long-term consequences for insulin resistance, obesity, and susceptibility to glycemic management issues. New avenues for preventative care during the crucial prenatal stage will become available with more studies. If this idea is confirmed by more studies, new opportunities for preventative therapy during the crucial prenatal time will need to be considered (De Leo *et al.*, 2016).

Correlation Between the mRNA Expression of the HSD17B1 Gene and Serum E2 Levels

As revealed by Spearman's Rho correlation test, the correlation between the mRNA expression of the HSD17B1 gene and the serum E2 levels was significant ($p = 0.020$) $p < 0.05$. There is a significant negative correlation between the mRNA expression of the HSD17B1 gene and serum E2 levels. "The more down-regulated the mRNA expression of the HSD17B1 gene, the lower the serum E2 levels."

In this investigation, compared to healthy women, PCOS patients had decreased average serum E2 levels. Reduced E2 levels in the PCOS group, according to our findings, were associated with reduced mRNA expression. Because of the reduced mRNA expression, the HSD17B1 gene was unable to catalyze the conversion of E1 from its inactive form to its active E2 form; hence, E2 remained in an inactive state, resulting in low serum E2 levels in PCOS patients, leading to infertility.

The three main forms of physiological estrogen in women are Estrone (E1), Estradiol (E2), and Estriol (E3). E2 is the most powerful estrogen during the premenopausal period. Its deactivation is regulated by estrogen metabolism, which converts it to less active forms and sulfates it to 17-beta-estra-1,3, 5-trien-3, and 17-diol 3-sulfate. The ratio of circulating estrogens in the blood indicates dynamic metabolism and a balance between estrogen synthesis and deactivation. E2 is primarily produced in ovarian follicles through cholesterol conversion processes and chemicals. It is converted into estrone and E2 by aromatase or testosterone. HSD17B1 and HSD17B2 are regulated by E2, according to Hilborn *et al.* (2017), who reveal that estrogen regulates HSD17B1 and HSD17B2 (Hilborn *et al.*, 2017).

The lower E2 serum level is intriguing because PCOS patients often develop endometrial hyperplasia as a result of high levels of circulating estrogen. High estrogen levels induce endometrial hyperplasia. Endometrial hyperplasia is a histological diagnosis defined by endometrial gland growth to the point where the gland-stromal ratio exceeds that of normal endometrium. Endometrial hyperplasia can be caused by chronic estrogen exposure without progesterone exposure. The researchers hypothesized that

estrogen generated in the peripheral blood would not penetrate the granulosa cells in this investigation, but estrogen circulating in the periphery would trigger ovarian receptors. When estrogen binds to its receptors in the ovary, it induces estrogen in the endometrium, resulting in endometrial hyperplasia. Estrone (E1) is the kind of estrogen that causes endometrial hyperplasia.

AMH Analysis

It is reasonable to assume that women with PCOS have lower E2 levels, greater AMH levels, and an inverse connection between FSH and AMH levels (Table 1).

The AMH levels of the two patients were significantly different from one another. In women without PCOS, the average AMH level was 6.49 ng/mL, whereas in healthy women it was 3.98 ng/mL. According to this study, female PCOS patients had greater AMH levels than healthy persons; in PCOS patients, AMH levels increased by twice as much as in non-PCOS patients.

Numerous studies have also found higher AMH levels in PCOS individuals (Wiweko *et al.*, 2014; Sova *et al.*, 2019). This might be because preantral and small antral follicles produce and secrete more AMH (Tal *et al.*, 2020). The Antral Follicle Count (AFC) is correlated with a steady rise in AMH of 0.2 ng/ml per follicle. Additionally, it has been shown that granulosa cells in PCOS patients' follicles produce 75 times more AMH than normal cells (Muharam *et al.*, 2022). PCOS may be suggested by elevated AMH levels brought on by a high number of follicles in the early stages of development (Mohammed and Qasim, 2021).

AMH exposure during pregnancy has been demonstrated to have a probable causative role in the development of PCOS in animal models. AMH may inhibit the release of Follicle-Stimulating Hormone (FSH) and decrease the recruitment of primordial follicles from women's resting oocyte pool, worsening ovulatory problems. Compared to women who are ordinarily ovulatory, individuals with PCOS typically have serum AMH levels that are much higher. Based on these findings, AMH may be a helpful surrogate marker or a substitute for the FNPO count on ultrasonography in the diagnosis of PCOS as a whole or in the detection of PCOM (Teede *et al.*, 2019).

Table 1: Hormonal characteristics in the form of Anti-Mullerian Hormone (AMH), Luteinizing Hormone (LH), Follicle-stimulating Hormone (FSH) and LH/FSH ratio

Variable	Healthy women	PCOS	p-value
	n = 30 mean ± SD	n = 30 mean ± SD	
AMH (ng/mL)	3.9800±2.79000	6.490±2.940	0.000 ^(*)
LH (mIU/mL)	6.0400±2.86000	6.380±3.690	0.91 ^(*)
FSH (mIU/mL)	7.3600±2.01000	6.180±1.000	0.011 ^(*)
Rasio LH/FSH	0.8517±0.41600	1.073±0.700	0.233 ^(*)

* $p < 0.05$

The reason for such AMH overproduction is unknown; however, evidence suggests that androgens play a role. A favorable relationship was discovered between serum androgens and AMH levels (Dewailly *et al.*, 2016). It is also related to intrinsic granulosa cell dysregulation, which results in an increase in AMH. It is also caused by intrinsic granulosa cell dysregulation, which has been linked to an increase in AMH receptor type II (Pierre *et al.*, 2017; Alebić *et al.*, 2015). Certain papers state that gonadotropins, especially FSH, decrease the production of AMH in the blood. Conversely, it explained how FSH stimulates the expression of AMH in both normal and polycystic ovaries (Alebić *et al.*, 2015). The recent revelation that E2 inhibits the generation of AMH through the estrogen receptor β may provide a resolution to this controversial subject. AMH in small antral follicles may be directly increased by FSH.

FSH, on the other hand, can suppress AMH expression by boosting E2 synthesis in bigger follicles due to the negative feedback of E2 (Grynberg *et al.*, 2012). In PCOS women, this process is disrupted, since they do not have a corresponding increase in E2 levels. AMH expression and levels in follicular fluid are lower in gonadotropin-dependent follicles in women who are normally ovulatory, but not in PCOS patients (Kristensen *et al.*, 2019).

The ovarian reserve may be predicted using the serum AMH value, which represents the quantity and quality of follicular deposits in the ovary. Antral follicle count on ultrasonography, testosterone levels, and ovarian volume are all connected to serum AMH levels. Serum Patients with PCOS have serum AMH levels that are 2-3 times higher than those of healthy women. Study revealed that AMH levels may be utilized as a predictor in the diagnosis of PCOS as an alternative to analyzing polycystic ovary pictures and the incidence of PCOS increases as AMH levels rise (Harzif *et al.*, 2023).

Wiweko *et al.* (2014) discovered that people with high AMH levels were 9 times more likely to have PCOS, with the AMH cutoff value as a predictor of PCOS being 4.45 ng/mL. The AMH concentration (6.49 ng/mL) in PCOS participants was above the AMH cut threshold in this investigation (Wiweko *et al.*, 2014).

LH Analysis

Studies have shown that blood levels of Luteinizing Hormone (LH) were greater in PCOS patients than in the general population. Despite the lack of a statistically significant difference in this study, LH levels were greater in PCOS individuals. Serum LH levels have been shown to be high in 40-60% of PCOS patients (Yu *et al.*, 2015).

LH production is frequently elevated in PCOS patients. High testosterone levels, together with low FSH levels, contribute to poor ovum formation and anovulation. When the menstrual cycle begins, LH levels

in PCOS patients are frequently elevated. LH is also more abundant than FSH. Ovulation does not occur without the LH surge and menstruation is irregular. LH hypersecretion is a defining feature of PCOS. This increase in LH production is assumed to be caused by an increase in hypothalamic GnRH pulse frequency. Increased androgen production by theca cells in the ovaries is facilitated by a surge in LH. Excess LH is induced by a fast pulse of release (GnRH) in the hypothalamus, which leads to a dominance of LH hormone output in the pituitary gland. Ovulation abnormalities in PCOS are hypothesized to be caused by elevated LH levels and reduced FSH levels. Normal folliculogenesis is aided by the hormone FSH, which controls follicular development and generates a dominant follicle ready for ovulation. Insulin indirectly increases inhibitory secretion, which suppresses FSH.

Folliculogenesis is significantly impacted by this. In PCOS patients, hypersecretion of LH and insulin results in early luteinization and stops the maturation process in ovarian follicles. Ovarian follicles have polycystic ovarian morphology when their maturation process is interrupted during development, leading to an increase in follicle count. PCOS patients have an increase in theca cells lining their ovaries, which produces androgens (Yu *et al.*, 2015).

This leads to premature luteinization of granulosa cells and hyperstimulation of theca cells when paired with the elevated LH levels associated with PCOS. Furthermore, after LH stimulation, PCOS women's granulosa cells expressed more AMH than did those of normal ovulatory women (Alebić *et al.*, 2015; 2018; Pierre *et al.*, 2017).

FSH Analysis

The average FSH level in patients without PCOS was 7.36 mIU/mL, whereas it was 6.18 mIU/mL in PCOS participants. Despite the fact that the FSH levels were statistically different, both healthy women and PCOS women had normal levels. FSH levels between 3 and 10 mIU/mL are considered normal, whereas values greater than 12 mIU/mL suggest decreased ovarian reserves. More than 25 mIU/mL is associated with ovarian failure and is seen in postmenopausal women (Harzif *et al.*, 2023).

The pituitary gland synthesizes 55-57% more LH than FSH due to abnormal control of hypothalamic GnRH production. The serum LH concentration rises, the FSH level falls and the LH/FSH ratio rises. The abnormal dynamic LH expenditure, which is indicated by an increase in the frequency and amplitude of LH pulses, is what is responsible for this elevation in blood LH levels. An increase in GnRH pulse frequency, a persistent negative feedback effect of estrone concentration, and a little increase in inhibin cause FSH levels to decline (Yu *et al.*, 2015). Rather than fluctuating cyclically as it does in ovulating women,

the LH pulse frequency in PCOS women is very constant at around one beat per hour. The same rise in hypothalamic GnRH pulse frequency that encourages the production of LH rather than FSH is most likely the cause of this trend (Harzif *et al.*, 2023).

LH/FSH Ratio Analysis

The LH/FSH ratios of the two groups, which were found to be 0.857:1.073 for PCOS patients, did not significantly vary from one another. The ratio typically has a value of 1. The LH/FSH ratio between the two groups in this investigation was discovered to be 1:1. There was no difference in the LH/FSH ratio between the two groups. This ratio, which is around 1:1, indicates that blood levels of FSH and LH are similar and that neither group's ovarian stereogenesis changes are influenced centrally.

Abnormalities in the adrenal or hypothalamic-pituitary-ovarian axis have been linked to the etiology of polycystic ovarian disease. An increase in the ratio of LH to FSH release is caused by a change in the Gonadotrophin-Releasing Hormone (GnRH) secretion rhythm. The abnormal feedback mechanism responsible for the rise in LH production is caused by ovarian estrogen. Because of a high LH/FSH ratio, individuals with polycystic ovarian disease do not ovulate (Saadia, 2020).

Conclusion

Gene expression can be either activated or inhibited by DNA methylation. DNA methylation can either facilitate or hinder the transcription machinery's ability to bind when combined with histone modifiers. This study found that HSD17B1 does not hinder transcription machinery binding but rather creates a transcription-friendly landscape. Reduced mRNA expression of the HSD17B1 gene in PCOS patients can result in reduced E2 levels, thereby contributing to the etiology of PCOS. HSD17B1 is thought to be an interesting target for medicines aimed at decreasing estrogenic burden in peripheral estrogen targets. In clinical laboratories, serum E2 is tested in women to determine ovarian activity. E2 blood tests determine the quantity of E2 present in the body.

Limitations

There is a lack of knowledge on genetic deterioration factors in the family as well as a history of PCOS in the family.

Since pyrosequencing can detect DNA methylation and allele frequency as well as reveal the genetic code of DNA and identify single nucleotide polymorphisms, insertion-deletions, and other sequence variations, it should be used.

We should examine the FSHR gene's DNA methylation because damage methylated or not expressed in this gene can affect the receptor's ability to bind FSH or activate the signal

transduction pathway, inhibit the work of estrogen enzymes, and interfere with ovarian and folliculogenesis, all of which contribute to PCOS in women.

We should examine the DNA methylation of the estrogen receptor gene, even if the low level of E2 in PCOS due to down-regulated HSD17B1 expression is already proven, but we also anticipate that the receptor estrogen's potential may be impaired by DNA methylation.

Acknowledgment

We are grateful to every woman who consented to take part in our study. We are appreciative of all the doctors who consented to their patients to participate in the study, the IVF Morula Bunda Jakarta Indonesian reproductive science institute Bunda Medik, the nurses who take blood samples for hormonal analysis at the Yasmin clinic at Dr. Cipto Mangunkusumo hospital and the embryologists who gathered the granulosa cells.

Funding Information

The doctoral final assignment fund 2018 from the research council and Indonesia University's community services provided funding for this study.

Author's Contributions

10 Rina Puspita and Asmarinah: Concept designed, the definition of intellectual content, literature search, clinical studies experiments studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript edited manuscript reviewed, and guarantor.

Andon Hestiantoro: Concept designed, the definition of intellectual content, clinical studies, experiments studies, statistical analysis, manuscript preparation and guarantor.

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Batara Immanuel Sirait: Concept designed, experiments studies, data acquisition, data analysis, manuscript edited, manuscript reviewed, and guarantor.

74 Arie Adrianus Polim: Concept designed, data acquisition, statistical analysis, manuscript editing, and guarantor.

Ethics

The author provided informed consent to participants, minimizing the risk of harm, protecting anonymity and confidentiality and exercising the right to withdraw. The Indonesia university health research ethics committee granted ethical permission. Central Jakarta, Indonesia. ethics approval number: 18-03-0233.

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