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Genetic Polymorphisms of the Vitamin D Receptor *ApaI* Gene and Physiological Parameters Diversity of Polycystic Ovary Syndrome in Iraqi Women

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Abstract

Polycystic ovary syndrome (PCOS) is a prevalent hormonal disorder among women of reproductive age, requiring diverse management strategies. This study, conducted at Kamal Al-Samarrai Hospital Fertility Center/Baghdad from January to March 2023, included 80 Iraqi women aged 18-45, comprising 40 with PCOS and 40 healthy controls. Hormonal markers (FSH, LH, T, PRL, Ca2+, and Vitamin D3) were systematically assessed, along with Apa1 gene polymorphisms in whole blood genomic DNA using PCR-RFLP. PCOS patients showed significantly elevated LH, LH/FSH, T, and PRL levels ($p < 0.01$) and lower Vitamin D and calcium levels ($p < 0.05$) compared to controls. The scrutiny of VDR gene Apa1 polymorphism uncovered a notable prevalence of "Aa" and "aa" genotypes among PCOS patients, constituting 65.00% and 25.00%, respectively, in contrast to the control group's 17.50% and 0.00% in controls. The "aa" allele, more frequent in PCOS, emerged as a potential risk factor for the condition. This study establishes a strong association between VDR gene (Apa1) polymorphism and PCOS, highlighting the significance of the "aa" allele. It emphasizes the homozygous variant genotype "aa" as a substantial risk factor for PCOS, providing insights into its genetic basis.

1. Introduction

Polycystic ovary syndrome (PCOS), the most common gynaecological heterogenetic condition during childbirth, is caused by pituitary gland, ovarian, and testosterone problems. Female infertility is often caused by PCOS [1]. PCOS is caused by insulin resistance (IR) and/or hyperandrogenism (HA)-induced hormonal imbalance. Obesity, ovarian dysfunction (OD), and hypothalamic abnormalities combine with genetic, environmental, and hormonal abnormalities to create PCOS [2]. PCOS is distinguished by irregular menstrual cycles, increased androgen (male hormone) levels, and an abnormal number of follicles in the ovaries [3]. The genetics of PCOS

have been studied using candidate gene approach, connection analysis, family studies, genome wide association studies, and other methods [4].

Vitamin D, a fat-soluble vitamin, is considered a prohormone because the body can make it from its precursor (7-dehydrocholesterol) when exposed to ultraviolet (UV) radiation at 290–315 nm. Everyone needs good circulation. 25-hydroxyvitamin D for metabolic, immunological, muscular, skeletal, cutaneous, and respiratory systems [5]. Vitamin D is vital for reproductive biology in rats, but humans have just recently discovered that vitamin D signalling may be important for reproductive health[6][7].

Vitamin D receptors are proteins produced by the vitamin D receptor gene. Food or sunlight can provide the vitamin. Vitamin D balances minerals like calcium and phosphate, which are necessary for strong bones and teeth. Vitamin D regulates intestine-to-blood calcium and phosphate absorption. Vitamin D also contributes to bone and teeth development cycles [8]. VDR gene is located on chromosome 12q13, it contains 14 exons and covers over 75 kb of genomic DNA [9]. Eight protein-coding exons (2-9) and six untranslated exons, along with two promoter regions, make up this gene (1a–1 f) [10].

The aim of this study is to investigate the association between the (Apa1) variant of the vitamin D receptor gene and polycystic ovary syndrome (PCOS), as well as to examine the relationship between (VDR) polymorphism genes (Apa1) and various physiological parameters in individuals with PCOS.

2. Experimental Procedure

The samples for this study were from the Fertility Centre of Kamal Al-Samarrai Hospital in Iraq/ Baghdad, and the research was conducted at the Research and Industrial Development /Al-Razi Centre for Research between January and March of 2023. Two groups of Iraqi women were studied: (40) with polycystic ovary syndrome and (40) without as controls. The Patients were identified if they are between the ages of (18 – 40) and have at least two of the following characteristics: they have a medical history consistent with PCOS, PCOS was first detected by ultrasound scan, and they experienced oligomenorrhea, amenorrhea, or highly irregular menses. Ultrasounds can detect PCOS. On follicular phase days 3, 4, and 5, patients and healthy controls gave eight ml venous blood samples. Five ml of whole blood was coagulated at 37°C for 30 minutes in a vacuum sterile glassware gel tube before centrifugation.

DNA was extracted from the all blood samples (PCOS cases and controls) by taking 5ml whole blood that placed in EDTA tubes for molecular studies using DNA purification kit (Genaid/Korea). Detection of concentration and purity of DNA by measuring using a Nano-Drop spectrophotometer. By Polymerase chain reaction (PCR), amplification were detecting in a total volume of 25µl involved (50ng/µl Template DNA, 9.5µl Deionized water, 12.5µl Go Taq G2 promeg , 1µl Forward primer (10 pmol/µl) and 1µl Reverses primer (10 pmol/µl). The Apa1 gene repeat regions were amplified using the forward primer (5´-GGATCCTAAATGCACGGAGA-3´) and the reverse primer (5´-ACGTCTGCAGTGTGTGTTGGAC-3´)[10]. Below the optimal temperature profile for an *Apa1* program, which begins with a 5-minute denaturation at (96°C) and continues with (35 cycles) of denaturation at (94°C) for 1 minute, annealing at (55 °C) for 48 seconds, and elongation at (72°C) for 1 minute. The PCR result was detected by electrophoresis on a 1.5% agarose gel, where the DNA band (265 bp) was visualized and documented using a gel documentation system. Following PCR amplification, PCR products could be consumed using Apa1 restriction enzymes from thermo scientific , Lithuania, and enzyme buffer (RFLP), the Apa1 PCR were incubate at room temperature for 24 hours before being electrophoretic ally separated on a 2% agarose gel for use in determining Apa1 polymorphisms.

The Statistical Analysis System [11] program was used to detect the effect of difference factors in study parameters. T-test and Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). Estimate of Odd ratio and CI in this study.

3. Results and Discussion

3.1. Distribution of Study Samples According to Age

Table (1) showed that the highest number and percentage of PCOS-infected patients and controls 26 (65.00%) and 29 (72.50%) were in the age group (25-40) years, followed by 13 (32.50%) and 10 (25.00%) in the age group (<25) years, and 1 (2.50%) in the age group (>40) years, with non-significant differences between the groups (p>0.05). Within the same group, there is a significant difference (P < 0.01).

Table (1): Distribution of study samples according to age.

Factor		Patients (No=40)	Controls (No= 40)	P-value
Age group: No (%)	<25 yr.	13 (32.50%)	10 (25.00%)	0.531 NS
	25-40 yr.	26 (65.00%)	29 (72.50%)	0.686 NS
	>40 yr.	1 (2.50%)	1 (2.50%)	1.00 NS
	P-value	0.0001 **	0.0001 **	---
** (P≤0.01), NS: Non-Significant.				

3.2. Hormonal Profile

Data in table (2) demonstrated that the Mean ± SE of FSH, LH, LH/FSH, Testosterone and Prolactin hormones in the patient group were (4.40±0.32), (8.05±0.31), (2.44±0.40), (1.17±0.20) and (28.06±1.52) respectively compared to their mean and SE in the control group (5.81 ±0.24), (4.83±0.15), (0.871±0.03), (0.391±0.03) and (14.13 ±1.01) respectively with highly significant differences (P≤0.01).

Table (2): Distribution of hormones among the study groups.

Group	Mean ± SE				
	FSH (mIU/mL)	LH (mIU/mL)	LH/FSH	Testosterone (ng/mL)	Prolactin (ng/mL)
Patients (N=40)	4.40 ±0.32	8.05 ±0.31	2.44 ±0.40	1.17 ±0.20	28.06 ±1.52
Control (N= 40)	5.81 ±0.24	4.83 ±0.15	0.871 ±0.03	0.391 ±0.03	14.13 ±1.01
T-test	0.801 **	0.688 **	0.802 **	0.399 **	3.632 **
P-value	0.0008	0.0001	0.0002	0.0002	0.0001
** (P≤0.01).					

3.3. Distribution of Vitamin D and Calcium Levels among the Studied Groups

Table (3) shows Vitamin D and Calcium findings. It was found a significantly significant drop in vitamin D levels in the PCOS group (18.38 ±1.49) ng/dL compared to the control group (62.21±4.53) (P < 0.01). Calcium Mean ± SE values were significantly lower in PCOS patients (9.55 ±0.17) than in the control group (10.54 ±0.31) (P < 0.01).

3.4. Molecular Analysis

This study analyzed vitamin D receptor gene variants in the *Apa1* (rs7975232; intron 8) genes and inflammatory PCOS prevalence. Polymerase chain reaction amplifies the vitamin D receptor gene in 80 samples, 40 from PCOS women and 40 from healthy controls.

3.5. PCOS and *Apa1* Polymorphism

PCR product of (*ApaI*) Polymorphic variant (VDR) gene at molecular weight (265bp) is shown in Figure (1).

Table (3): Distribution of Vitamin D and Calcium levels among the study groups.

Group	Mean ± SE	
	Ca (mg/dL)	D3 (ng/dL)
Patients (N=40)	9.55 ±0.17	18.38 ±1.49
Control(N= 40)	10.54 ±0.31	62.21 ±4.53
T-test	0.709 **	9.513 **
P-value	0.0069	0.0001
** (P≤0.01).		

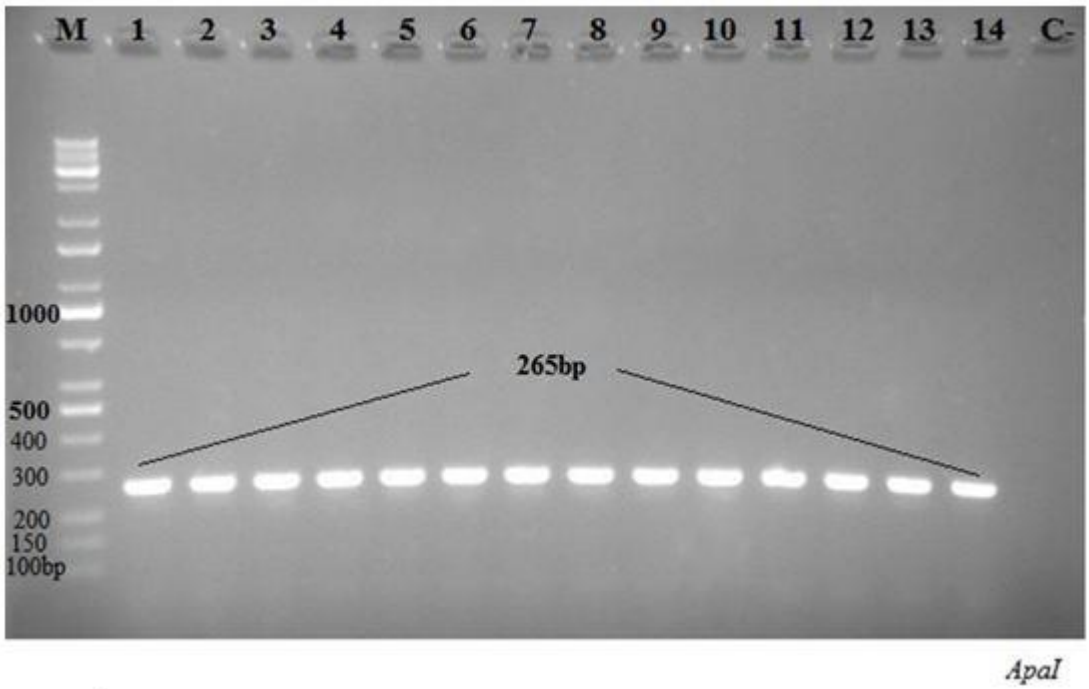

Figure (1): Gel electrophoresis of the PCR product (*Apa1* gene/265bp) by (1.5%) (1h/90v). Lane M: DNA marker (100bp), Lane C-, negative control. Lanes (1-14) are samples.

Figure (2) shows the (*APa1*) Polymorphic variant (VDR) gene PCR product with 265bp. Digestion of (*Apa1* gene) Gene. *Apa1* enzymes (Thermo Scientific, USA) digested VDR-*Apa1* gene PCR products. *Apa1* PCR product was digested overnight at room temperature and electrophoresed on a 2% agarose gel, yielding bands of homozygous wild type (AA) (265bp), heterozygous variant type (Aa) (265bp and 146bp, 119bp), and homozygous variant genotype (aa) (146bp, 119bp) .

3.6. Genotype and Allele Association of the VDR (*Apa1*) Polymorphism in PCOS Patients and Controls

Table (4) shows the association between PCOS patients and healthy control; found that PCOS patients had 4 (10%) AA/AA alleles of the *Apa1* gene, while the control group had 33 (82.5%). This was a highly significant difference ($p<0.01$). The same data indicated that PCOS patients had 26 (65.00%) AC/Aa alleles compared to 7 (17.50%) controls, a highly significant difference ($p<0.001$). PCOS patients had 10 (25.00%) CC/aa alleles, compared to 0 (0.00%) in controls, a highly significant difference ($p<0.001$).

Moreover, it was shown in table (4) that the A allele frequency and percentage among the PCOS patient group was 34 (0.425%)compared with its frequency and percentage among the controls 73 (0.91%), with a highly significant difference (p<0.001), and the a allele frequency and percentage among the PCOS patient group was 46 (0.575%) compared with its frequency and percentage among the controls 7 (0.09%), with a highly significant difference (p<0.001). The allelic frequency of a allele of the gene *Apa1* is considered a risk factor for the disease.

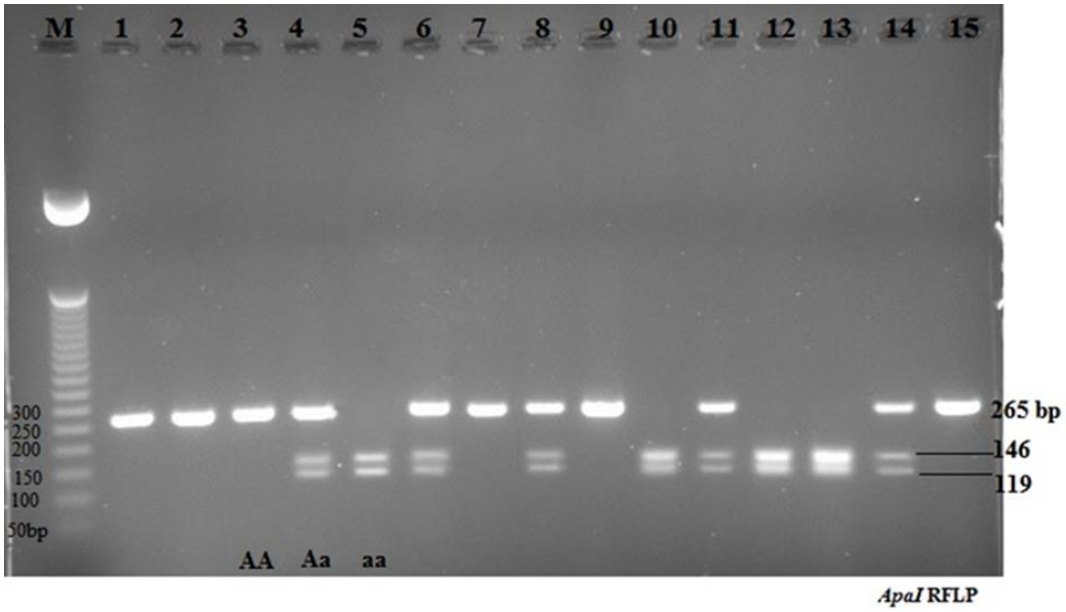

Figure (2): The PCR products of the (*Apa1*) gene after enzyme digestion *Apa1* and electrophoresis on (2%) agarose gel (1h/90vol). Lane M (100bp; DNA marker, lane (4,6,8,11,13) heterozygous variant type (Aa) (265 bp and 146bp,119bp), lane (1,2,3,7,9,15) homozygous wild type (AA) (265bp) and lane (5,10,12,13) homozygous variant genotype (aa) (146 bp,119bp).

Table (4): Genotype and allele frequency of *Apa1* gene in patients and control groups.

Genotype/ *ApaI* gene	Patients No. (%)	Control No. (%)	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
AA/AA	4 (10.00%)	33 (82.50%)	22.729 **	0.0001	Ref. =1
AC/Aa	26 (65.00%)	7 (17.50%)	10.939 **	0.0009	1.74 (0.92-3.02)
CC/aa	10 (25.00%)	0 (0.00%)	8.415 **	0.0022	1.25 (0.74-2.24)
Total	40	40	---		
P-value	0.0001 **	0.0001 **	---		
Allele	Frequency				
A	34 (0.425%)	73 (0.91%)	0.0001 **		
a	46 (0.575%)	7 (0.09%)	0.0001 **		
** (P≤0.01), NS: Non-Significant.					

The Mean ± SE of (FSH), (LH), (Testosterone and Prolactin) hormones in the AA/AA allele was 4.72 ±0.73, 8.17 ±0.82, 0.367 ±0.12 b, and 32.10 ±4.42, as shown in table (5) . The AC/Aa allele's mean ± SE of (FSH), (LH), (Testosterone and Prolactin) hormones was 4.47 ±0.44, 7.99 ±0.42, 1.09 ±0.23 ab, and 28.01±2.08 table (5). In the same table, *Apa1* gene polymorphism and mean ± SE of FSH, LH, Testosterone, and Prolactin hormones in the CC/aa allele were 4.09±0.51, 8.15±0.51, 1.69±0.45, and 26.57±2.25 correspondingly. Comparing genotypes, the Apa1 gene therapy increases testosterone hormone by a substantial proportion $P < 0.05$.

Table (5): Relationship between *Apa1* gene polymorphism and patients group according to the selected Hormonal profile.

Genotype/ *Apa1* gene	**Mean ± SE**			
	FSH (mIU/mL)	**LH (mIU/mL)**	**Testosterone (ng/mL)**	**Prolactin (ng/mL)**
AA/AA	4.72 ±0.73	8.17 ±0.82	0.367 ±0.12	32.10 ±4.42
AC/Aa	4.47 ±0.44	7.99 ±0.42	1.09 ±0.23	28.01 ±2.08
CC/aa	4.09 ±0.51	8.15 ±0.51	1.69 ±0.45	26.57 ±2.25
LSD value	2.151 NS	2.017 NS	1.099 *	10.131 NS
P-value	0.841	0.967	0.0490	0.638
Means having with the different letters in same column differed significantly. * (P≤0.05), NS: Non-Significant.				

4. Discussion

The *Apa1* polymorphism (rs7975232) in the VDR gene's intron 8 is 3'-end. The 3′-UTR (untranslated region) aids mRNA stability and post-transcriptional processing [12]. The *Apa1* polymorphism regulates gene expression, not amino acid sequence, therefore the results showing a high significant difference was recorded homozygous variant genotype "aa" are discordant [13]. A previous study with meta-analysis confirmed *Apa1*'s association with polycystic ovarian syndrome (PCOS) [14]. The *Apa1* "a" and "aa +Aa" haplotypes increase PCOS risk in Caucasian women, while the variant "aa" increases PCOS risk in Asian women ; This was also proven in the results of this study [15]. And it was noted that Carriers of the "aa" genotype had a greater risk, whereas carriers of the "Aa" genotype had a reduced risk. Another research in Indian women indicated that the Apa1 "AA" genotype was related with greater androstenedione levels than the "aa" genotype, but lower estradiol levels [16]. The PCOS group had higher insulin, dehydroepiandrosterone sulphate, 17-hydroxyprogesterone, and overweight/obesity values, but lower vitamin D mean serum levels [17]. Researchers investigated metabolic and endocrine issues in a Rotterdam group. The subjects had higher total testosterone and free androgen index than the control group, but lower sex hormone binding globulin [18].

5. Conclusions

Apa1 gene polymorphism are linked to PCOS, The VDR gene (*Apa1*) polymorphism is high significantly associated with PCOS in "aa" allele, This study showed that there is a high significant relationship between homozygous variant genotype "aa" with PCOS and that "a" is a risk factor for this diseases. It is found that PCOS patients have increase serum levels of LH, LH/ FSH, PRL as well as testosterone and decrease serum levels of FSH compared with healthy control.

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References

[1] K. M. Hoeger, A. Dokras, and T. Piltonen, "Update on PCOS: consequences, challenges, and guiding treatment," *J Clin Endocrinol Metab*, vol. 106, no. 3, pp. e1071-e1083, 2021.

[2] J. Wang, W. Daichao, G. Hui, and Meixiang, Li. Meixiang, "Hyperandrogenemia and insulin resistance: The chief culprit of polycystic ovary syndrome," *Life Sciences* vol. 236, pp. 116940, 2019.

[3] L.H. Zeng, S. Rana, L. Hussain, M. Asif, M.H. and et al., "Polycystic Ovary Syndrome: A Disorder of Reproductive Age, Its Pathogenesis, and a Discussion on the Emerging Role of Herbal Remedies," *Frontiers in Pharmacology*, vol. 13, pp. 874914, 2022.

[4] D. Hiam , A. Moreno-Asso, HJ .Teede, Laven, J. Stepto NK, L. Moran and et al., "The Genetics of Polycystic Ovary Syndrome: An Overview of Candidate Gene Systematic Reviews and Genome-Wide Association Studies," *J Clin Med*, vol. 8, no. 10, pp. 1606-22, 2019.

[5] B. Al-Zohily, A. Al-Menhali, S. Gariballa, A. Haq, and I. Shah, "Epimers of Vitamin D: A Review," *Int. J. Mol. Sci*, vol. 21, no. 2, pp. 470, 2020.

[6] J. Luk, S.Torrealday, G. Neal Perry, and L. Pal, "Relevance of Vitamin D in reproduction," *Hum Reprod*, vol. 27, no. 10, pp. 3015-27, 2012.

[7] M. Grzesiak, M. Tchurzyk, M. Socha, A. Sechman and A. Hrabia, "An Overview of the Current Known and Unknown Roles of Vitamin D3 in the Female Reproductive System: Lessons from Farm Animals, Birds, and Fish," *Mol. Sci*., vol. 23, no. 22, pp. 14137, 2022.

[8] S. Bazal-Bonelli, L.Sánchez-Labrador, J. C. Brinkmann, C .Cobo-Vázquez, N .Martínez-Rodríguez and et al., "Influence of Serum Vitamin D Levels on Survival Rate and Marginal Bone Loss in Dental Implants: A Systematic Review," *Int. J. Environ. Res. Public Health*, vol. 19, no. 16, pp. 10120, 2022.

[9] X. Yuan Shi, H. A. Ping, X. D. Wen and Y. X. Long, "Association of vitamin D receptor gene variants with polycystic ovary syndrome: a meta-analysis," *BMC Medical Genetics*, vol. 20, no. 32, 2019.

[10] A. Hajj, R. Chedid, E. Chouery, A. Megarbane, and M. Helen , "Relationship between vitamin D receptor gene polymorphisms, cardiovascular risk factors and adiponectin in a healthy young population," *PHARMACOGENOMICS*, vol. 17, no. 15, 2016.

[11] SAS, Statistical Analysis System, User's Guide. Statistical. Version 9.6th ed. SAS. Inst. Inc. Cary. N.C. USA. 2018.

[12] S. Siddamalla, TV. Reddy, S. Govatati, N. Erram, M. Deenadayal, S. Shivaji, M. Bhanoori, "Vitamin D receptor gene polymorphisms and risk of polycystic ovary syndrome in South Indian women," *Gynecol Endocrinal*, vol. 34, pp. 161–165, 2018.

[13] B. Luana, G.Natália , R.Kathryna, C.Ana, D. Fernando, "Polymorphisms in vitamin D receptor gene, but not vitamin D levels, are associated with polycystic ovary syndrome in Brazilian women," *Gynecol Endocrinol* , vol. 35 pp. 146–149, 2018.

[14] D.Flavia, C. Antonino, B. Federica, M. Gabriella and B. Salvatore, "Vitamin D, Bone Metabolism, and Fracture Risk in Polycystic Ovary Syndrome, Metabolites," vol. 11, no. 2, pp. 116, 2021.

[15] F. Liang, N. Ren, H.Zhang, Z. Jian, W. Qingguo, S. Rui, S. Zhenfeng , Z. Zhanxiu, and W. Kuixiang, "A meta-analysis of the relationship between vitamin D receptor gene *ApaI* polymorphisms and polycystic ovary syndrome," *Clinical and Experimental Medicine*, vol. 28, pp. 255–262, 2019.

[16] T. Mahmoudi, "Genetic variation in the vitamin D receptor and polycystic ovary syndrome risk," *Elsevier Fertility and Sterility*, vol. 92, no. 4, pp. 1381–3, 2009.

[17] S. Dipanshu and R. Chakravorty, "Genetic polymorphism in the vitamin D receptor gene and 25-hydroxyvitamin D serum levels in East Indian women with polycystic ovary syndrome," *J Mol Biomark Diagn*., vol. 6, pp. 24, .2015.

[18] B. Santos, S. Lecke, and P. Spritzer, "*Apa-I* polymorphism in VDR gene is related to metabolic syndrome in polycystic ovary syndrome: A cross-sectional study," *Reprod Biol Endocrinol*, vol. 16, no. 38, pp. 2060, 2018.