

RESEARCH ARTICLE



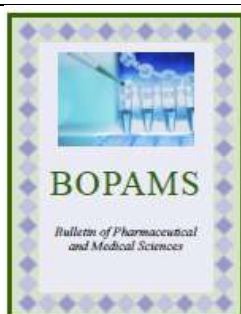
THE ESTROGEN RECEPTOR BETA POLYMORPHISMS ASSOCIATION WITH OVARIAN DISORDERS IN WOMEN OF SALADIN GOVERNORATE IN NORTH OF IRAQ

ANAS Y. AL HAYAWI¹, AYAT S. ABBAS

¹Biology Department (Assist. Prof. Dr., genetic), College of Education for Pure Science, Tikrit University, Iraq

Corresponding authors.

E-mail addresses: dr.anas77@tu.edu.iq (Dr. A.Y. Al-Hayawi), www.tu.edu.iq/ tu@mohehsr.gov.iq



ABSTRACT

Ovarian disorders (OD) is an An ovulation in women during fertilization age that reflects changing clinical symptoms. The genetic concept of OD is unclear and no significant genetic association with an ovulation has been established. In humans, Estrogen Receptor Beta (*ESR2* or *ER-β*) is encoded by the *ESR2* gene and the level of Estradiol is encoded by receptor (*ESR2*) and abnormal *ESR2* affects abnormal menstrual cycle and an ovulation and consist of 1 introns, 8 exons, and the region of chromosome promoter at 14q23.2. Sample of 60 OD (Polycystic ovary syndrome PCOS and abnormal menstrual cycle) patients and 30 controls were collected from Province of Saladin in north of Iraq. Genomic DNA was extracted from the blood and genotype dissected was improved for the two population of study using PCR-RFLP with the restriction enzyme *AluI*. The genotype distributions and allele frequency of *ESR2* polymorphisms were not statistically various between the controls and the patients. Significant elevation of body mass index with all genotype of patients was found when compared with controls. There were statistical differences in the BMI and most of the serum hormone and lipid profile parameters including Estrogen, Testosterone, Cholesterol and HDL, there were no significant various in FSH, LH and Progesterone levels of hormones and Triglyceride, LDL and VLDL with patients group conveying different genotypes of *ESR2* polymorphisms. The variant of *ESR2* was not associated with OD patients in Saladin women; there was no relationship between the OD and gene of *ESR2* polymorphism at codons 307. There was a significant difference in Estrogen, Testosterone, Progesterone, and HDL levels with OD patients conveying different genotypes of *ESR2* polymorphism.

KEYWORDS: Ovarian disorders, *ESR2*, rs4986938, Polymorphism, PCR-RFLP

I. Introduction

Ovarian disorders (OD) and menstrual disorder is common, with approximately 9-30 % of Reproductive aged women [1]. The Causes of menstrual disorder include obesity, polycystic ovarian syndrome, uterine fibroids, hormonal imbalance, and other causes. Menstrual disorder is defined as an abnormal cycle from where duration bleeding and time of occurrence. It can also include heavy menstrual bleeding, oligomenorrhoea, and lack of amenorrhea [2]. Estrogen plays important roles in many tissues in human body [3]. May be involved in the pathogenesis of many endocrine related diseases, such as uterine cancer, breast cancer, autoimmune diseases, prostate cancer osteoporosis and metabolic disorders. The action of estrogen is through Mediated estrogen receptor (*ESR2*) [4].

There are two types of estrogen receptors, alpha and beta, estrogen receptors belonging to nuclear receptor (NR) superfamily that includes receptors for mineral corticoids, glucocorticoids, estrogens, progesterone, androgens, thyroid hormones, retinoic acid and vitamin D. The estrogen receptor alpha was discovered for the first time in 1986 and after of this history beta-receptor were discovered [5]. Both *ERα* and *ERβ* have separate coded genes located on different chromosomes, But similar in structure to some extent. *ERα* is located on the long arm of chromosome 6, while *ERβ* is located on the long arm of the chromosome 14 [6].

The gene of *ESR2* located on the chromosome 14 is located in the q22-24 region of the arm Long. Estrogen receptors have four functional regions, A / B, C, D and E / F. Rate Compatibility Between *ERα* and *ERβ* regions vary by region where they are in Region A / B 17%, Region C 97%, Region D 36%, Region E 56% and Region F 18%. *ESR2* Gene consists of 8 exons and 530 amino acids with a molecule (MM) weighing 60 kDa [7]. Exon 1 encodes to A / B region and exon 2 and 3 encodes to C region and there is one intron, Exon 4 encodes part of C region, D region and part of E region, while exons of 4-8 encode part of E region and F region. Estrogen receptors are present in the nucleus or in the cytoplasm cell thus, these receptors have a direct effect on the process of DNA cloning, when estrogen enters the cell and binds to the estrogen receptor, in this case receptors are released from the inhibitory protein [8]. The present study aim was to evaluate the association of rs4986938 polymorphism of *ESR2* gene with Ovarian disorders (OD) and with clinical features of OD patients in Tikrit city, women in north of Iraq.

II. Materials and methods

A. Sampling

1. OD sampling and controls women

In the present study included two groups of women. The first group was the OD group, which included Sixty infertile women with mean age of 27.65 ± 5.46 . and the second group was the control group, which included 30 healthy women with mean age of 27.65 ± 6.67 , with regular menstrual cycles, Samples were recruited randomly from Centers of Obstetrics and Gynecology in the city of Tikrit, within the period from "November 2016 to February 2017" OD patients were chosen according to the presence of polycystic ovary morphology and Oligomenorrhea or an ovulation or amenorrhea for at least 6 months OD patients were chosen according to the presence of polycystic ovary morphology and Oligomenorrhea or an ovulation or amenorrhea for at least 6 months[9]. Biochemical and clinical signs of hyperandrogenism, PCOS on ultrasound [10].while healthy women were chosen according to normal ultrasonography, regular menstrual cycle, normal endocrinal hormones and number of pregnancies and children

2. Biochemical and hormonal findings

Five milliliters from blood was collected from each subject after 12-h fast at two to three days after the menstruation start. 3 ml of each blood sample was used to obtain serum samples and the separated serum was stored at -80°C for further measurement of the biochemical parameters, the levels of hormones, which measured FSH, LH progesterone and Testosterone, They were measured by I-CHROMA, (Boditech Belyium and Korea). Estrogen were measured by ELISA (Biotek, USA) and Lipid profile, were measured and investigated to determine the contrasts between the samples acquired from the patients and the controls and the result shown in Table 1.

3. Blood Sampling and DNA Extraction

Two milliliters from blood were collected in tubes containing anticoagulant EDTA and were stored at 4°C for genomic DNA extraction and performing genotyping working. Genomic DNA was extracted using the manual method described by Saremi et al [11] with some modifications to obtain the best results in terms of concentration and purity. The DNA was stored at -20°C until processing

B. Genotype Analysis

1. Polymerase Chain Reaction Analysis

Genotyping for the *ESR2* gene polymorphism (rs4986938) was performed by polymerase chain reaction (PCR), PCR was interacted with the use of PCR_Primax kit (Bioneer, USA) and the use of the specialized prefix with the sequence primer listed below:

F 5"TTTTGTCCCCATAGTAACA 3"

R 5"AATGAGGGACCACAGCA3"

Components of PCR Interaction which was achieved in a 20 µl mixture includes: 2.5 µl 1x PCR buffer, 1.5mM MgCl₂, 250 µM dNTP mixture, 1 µl of each primer, 1Utaq DNA polymerase, 15 µl double distilled H₂O, and 1 µl genomic DNA. All reactions had an initial denaturation step of 7 min at 95 °C followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 51 °C for 1 min and 72 °C for 1 min, and a final elongation step at 72 °C for 7 min.

2. Restriction Fragment Length Polymorphism (RFLP)

The rs4986938 of *ESR2* was detected by restriction fragment length polymorphism (RFLP) analysis [12]. The *ESR2* fragment were digested with enzyme *AluI* (Thermo Fisher Scientific Inc., USA). Digestion of the T allele delivered one section with lengths 307 bp, assimilation of the A allele created three parts with lengths 307, 240 and 67 bp. The digestion products were settled after electrophoresis in 3% agarose gel (see Figure 1).

C. Statistical Analysis

Statistical analysis was performed using SPSS Statistics Version 22.0 and Minitab Version 17.0 program on windows 10. To analyze the variance between two continuous variables, a T test was used. Genotype frequencies were measured for Hardy–Weinberg equilibrium. The test of Chi squared was used Hardy–Weinberg test and measured the variables in allele frequencies and genotype distribution between patients and controls by contrasting the observed genotype frequencies with the expected ones, odds ratio (OR), and 95% confidence interval (CI) were done to assess the association between the groups. The results obtained were expressed as mean ± standard deviation (SD) and/or percentage. A p-value <0.05 was expressed statistically significant.

A number of children in the two groups were adopted as a measure of women's fertility with regard to the age of marriage, and were considered the first year of marriage outside of this test. The results in Figure 2, showed that the rate of fertile women in the control group were 1.9 ± 1.53 compared to the OD groups of 0.8 ± 1.10 . This indicates that the number of women with children is significantly lower than who do not have; this is a diagnostic phenotypic measure for women with ovulation disorders. Figure 3 showed that the genotype distribution of *ESR2* polymorphism (observed number), percentage, mean and standard deviations (number of children) of patient group.

B. Hormone

In this study, 60 samples of women with ovulation disorders and 30 women healthy were collected from Tikrit city. The statistical results in Table 1 showed no significant differences in age between patients and control. There were difference of statistically significant in BMI women with patients when compared with healthy group, p-value (0.000). There were a significant increase in the level of Estrogen and Testosterone in OD women compared to the control, p-value (0.001) and (0.010) respectively. Whereas Luteinizing hormone (LH), Follicular stimulating hormone (FSH) and Progesterone were no significant reduction in mean of their level were found in OD women when compared with control.

B. lipid profile

Lipid profile were measured for patient and healthy women. There were significant elevation in cholesterol and HDL-C in OD women when compared with control with p-value 0.017 and 0.002 respectively. There was no significant difference in the mean of Triglycerides, LDL and VLDL as shown in Table 1

C. Genotype and Allele Frequencies

Three patterns of genotype were obtained: TT, TA and AA, of genotyping distributions of SNP rs4986938 T/A of *ESR2* gene in women with menstrual cycle disorder and 30 healthy women. The *ESR2*

polymorphism were analyzed by PCR-RFLP, A 307bp of PCR yield were obtained, and for which the absorption with restriction enzyme *AluI* exhibited 3 fragment 307bp., 240bp. and 64bp. distinctive patterns to 1730T→A replacement. Fragment 307 bp. band demonstrates TT genotype; 307bp. 240bp. 67 bp (heterozygote), and bands indicate TA genotype; and 403 bp, 240bp. and 67bp bands indicate AA genotype (mutant homozygous).

The genotype distribution and allele frequencies of *ESR2* in OD group and control were shown in Table 2. This table indicates the relative genotypes (n), allele frequencies, genotype percentage (%), confidence interval (CI), evaluated odd ratios (OR) at 95% and heterozygosity alongside the P values. There were no statistically significant differences in the distribution of (homozygote TT, AA and heterozygote TA) genotypes between the OD and Control groups. No statistically significant differences was observed in T and A allele frequencies between the two groups (0.375% in patients vs. 0.350% in controls) with A allele and (0.625% in patients vs. 0.650% in controls) with T allele (OR = 0.89, 95% CI = 0.47-1.71, $P = 0.742$).

Hormones, lipid profile and anthropometric values of OD women group within their particular rs4986938 genotypes were shown in Table 3 and there were no significant differences of the age and BMI, endocrine and the lipid profile values.

III. Results

A.Fertility

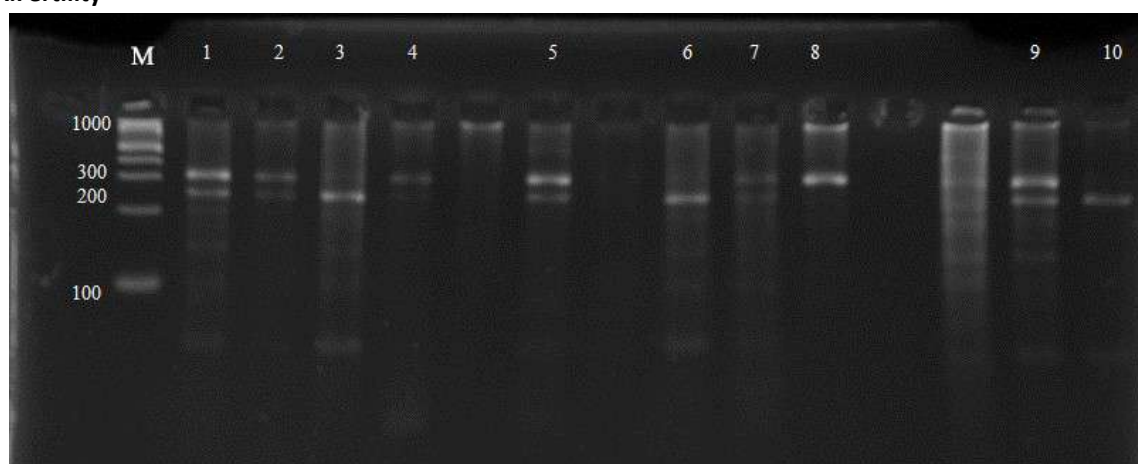


Figure 1: Electrogram of DNA Restriction fragment length polymorphism (RFLP) analysis of the *ESR2* receptor variant of rs4986938. Agarose gel (3%) electrophoresis after *AluI* digestion of the PCR. DNA ladder (100-1000) lane M. Homozygote T/T was shown by the band of 307 bp.. lane (8) samples. Heterozygote T/A was shown by the bands of 307 bp., 240 bp. and 67 bp. lane (1,2,4,5,7,9) samples. Homozygote A/A was shown by the bands of 240 bp. and 67 bp. lane (3,6,10) sample.

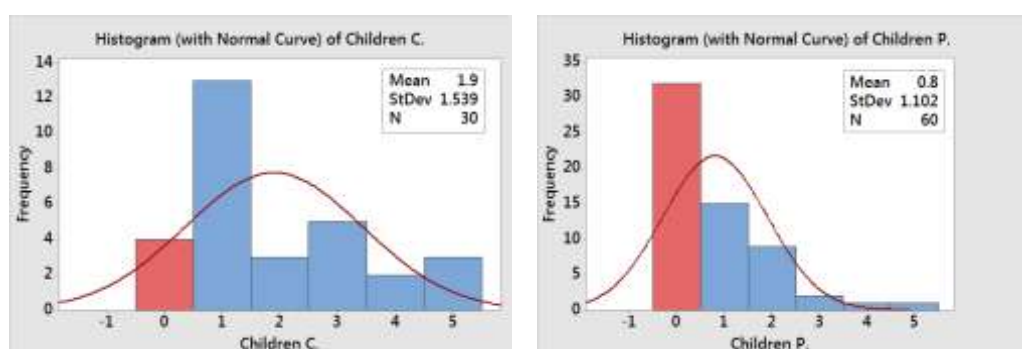


Figure 2: Histogram (with normal curve) of fertile (Children C: control) and infertile (Children P: Patient) women with *ESR2* polymorphism (Red column: no children, StDev: Standard Deviations)

Table 1: Anthropometric Characteristics and biochemical characteristics of *ESR2* patients and controls.

Characteristics	<i>ESR2</i> (n = 60) Mean + SD	Controls (n = 30) Mean + SD	p value
Age (year)	27.65 ± 5.46	27.65 ± 6.67	0.997
BMI (kg/m ²)	29.68 ± 3.06	25.09 ± 4.13	*0.000
<u>Serum hormone</u>			
Estro.(pg/ml)	21.79 ± 10.30	31.60 ± 12.52	0.001*
LH (mIU/ml)	5.37 ± 2.87	4.77 ± 2.54	0.324
FSH (mIU/ml)	6.65 ± 3.26	7.51 ± 3.72	0.295
Pro. (mmol/l)	3.57 ± 1.79	4.03 ± 2.77	0.420
Testosterone (ng/mL)	2.55 ± 1.49	1.28 ± 0.94	0.010*
<u>Lipid Profile</u>			
Cholesterol (mg/dl)	145.51 ± 35.36	128.41 ± 28.67	*0.017
Triglycerides (mg/dl)	138.45 ± 57.77	123.79 ± 45.35	0.197
HDL (mg/dl)	47.65 ± 6.61	41.48 ± 8.74	0.002*
LDL (mg/dl)	74.45 ± 35.04	60.48 ± 30.17	0.057
VLDL (mg/dl)	27.60 ± 11.55	24.44 ± 8.66	0.155

BMI: body mass index; Esrto: Estrogen hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; Pro: Prolactin hormone; HDL: High-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; SD: standard deviations; *Significant values (p < 0.05).

Table 2: Genotypes distribution and allele frequencies of *ESR2* polymorphism among women with *ESR2* and healthy control

rs4986938	Genotype			p value	H-W test
	TT	TA	AA		
<i>ESR2</i> (n=60)	20 (34%)	35 (58%)	5 (8%)	0.166	3.585
Control (n=30)	12 (40%)	15 (50%)	3 (10%)	0.863	0.293
<u>Allele</u>					
	T	A		Or (95 % CI)	
<i>ESR2</i> (n=60)	0.625	0.375		0.742	0.89 (0.47 ~1.71)
Control (n=30)	0.650	0.350			

ESR2: Estrogen receptor beta; Or: Odds ratio; CI: confidence interval; H-W test: Hardy-Weinberg test.

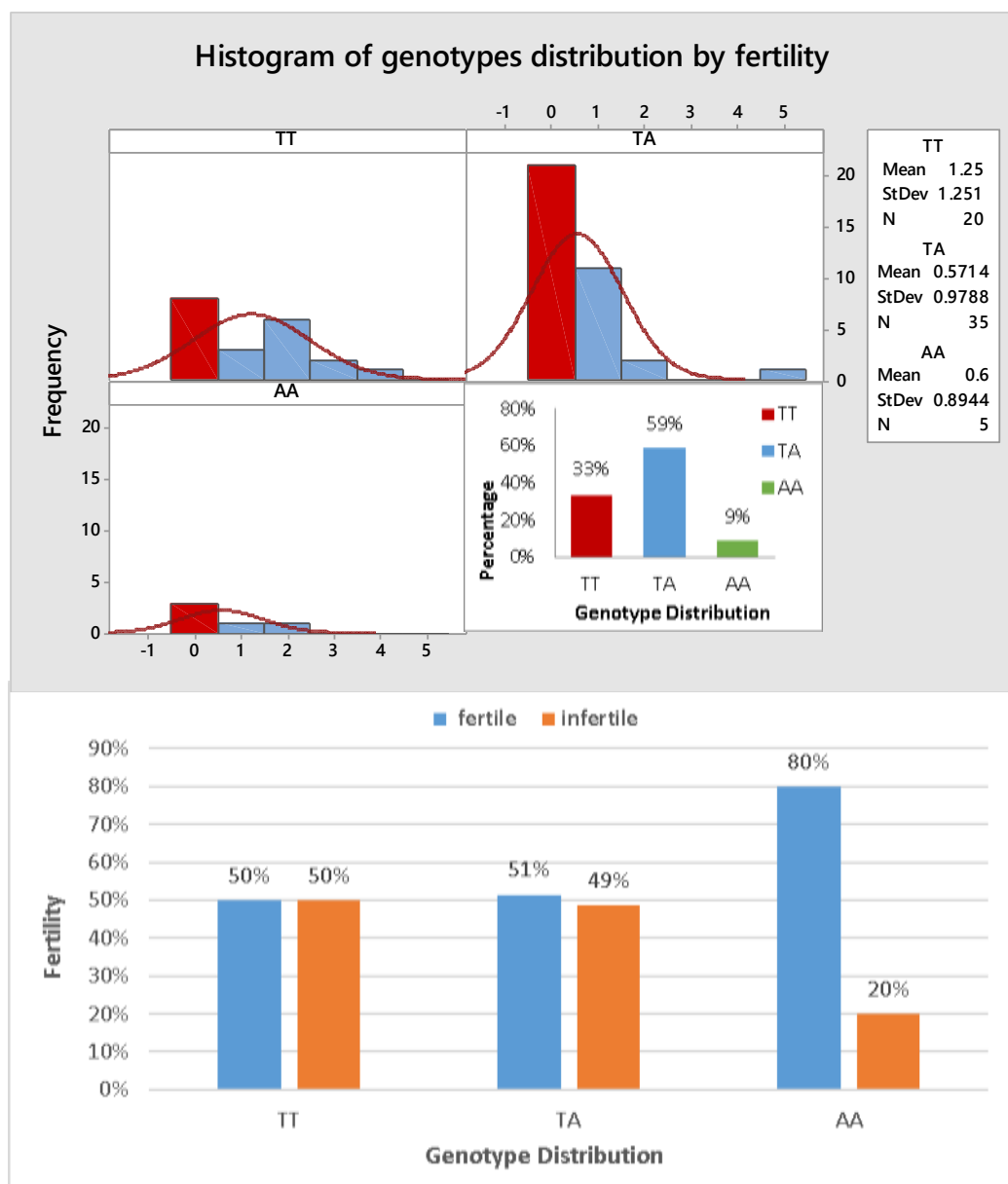


Figure 3: Histogram of genotype distribution and percentage of fertile (control) and infertile (patient) women with ESR2 polymorphism

Table 3: Distribution of clinical Characteristics of each rs4986938 genotype in patients

Characteristics	ESR2 (n = 93) Mean + SD			p value
	TT (n= 20)	TA (n= 35)	AA (n= 5)	
Age (year)	29.25 ± 5.97	26.51 ± 4.84	29.20 ± 6.96	0.164
BMI (kg/m ²)	28.93 ± 2.48	29.88 ± 3.32	31.27 ± 2.93	0.264
Hormone				
Estro.(pg/ml)	23.74 ± 8.44	20.10 ± 0.70	25.92 ± 13.72	0.296
LH (mIU/ml)	5.98 ± 3.79	5.24 ± 2.31	3.85 ± 1.78	0.313
FSH (mIU/ml)	6.51 ± 2.08	7.16 ± 3.75	3.61 ± 1.50	0.071
Pro. (mmol/l)	3.03 ± 1.65	3.91 ± 1.94	3.31 ± 0.50	0.210
Testosterone (ng/mL)	2.19 ± 0.49	2.56 ± 0.31	4.03 ± 1.99	
Lipid Profile				

Cholesterol (mg/dl)	149.05± 37.46	145.66± 31.28	130.4 ± 55.8	0.581
Triglycerides (mg/dl)	146.9± 66.2	135.03± 51.22	128.6± 74.4	0.713
HDL (mg/dl)	49.55± 6.07	46.60± 7.03	47.40± 4.72	0.286
LDL (mg/dl)	75.00± 33.86	74.37± 31.60	72.80± 64.6	0.992
VLDL (mg/dl)	29.60 ± 13.29	26.69± 10.19	26.00 ± 14.78	0.641

p values from one-way ANOVA test and data are shown as mean + SD,* Significant values (*p* is <0.05), BMI body mass index, Esrto: Estrogen hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; Pro: Prolactin hormone; HDL: high density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein.

IV. Discussion

In this study, there were no significant differences in age-specific values as observed in the table(1). According to the observed values and the studied ratio, it is clear that the collection of the samples was carefully conducted and made sure to approximate the ages between the healthy and the patient to achieve a balance of body activity and to avoid hormonal differences as a result of aging and reaching menopause. It is, therefore, judged that the apparent differences in the taken and studied measurements are due to a pathos or not. It is found that obese women have a higher frequency of disorders in ovulation than healthy women, as overweight works to increase the formation of fat around the ovary, which leads to the lack of access to ovulation as a result of polycystic of ovums inside the ovary and thus disturbance in the menstrual cycle and in the case of continued lack of access, ovulation prolonged uterine hyperplasia and uterine cancer occur [13].

This indicates that the number of women who have children is significantly lower than the number of women who do not have children and this is considered a diagnostic measure for women with ovulation disorders. The weakness of fertility increases according to many factors in women, the most important is polycystic ovaries, which has increased very much lately and is one of the most dangerous diseases accompanying disorders of ovulation in women in adulthood; and weakness in fertility of women increases with polycystic ovaries as a result of increase of body mass (weight) and the result as observed in the Fig. 2 and 3. Obesity causes disorders in the menstrual cycle, which can cause infertility among women[14].

The large overlap of the work of hormones with each other and their side effects lead to the occurrence of such disorders. As there is an inverse relationship between testosterone hormone and estrogen hormone, since when the testosterone hormone increases then the estrogen hormone decreases and this is consistent with this study[15]. Desoto *et al.* (2003)[16] in a study conducted on a group of women with ovarian disorders referred to the fact that estrogen hormone is affected by the acute psychological state of the patients when there is delay or lack of menstrual cycle in addition to taking contraceptive drugs at the beginning of the first year of marriage, especially those taken orally which leads to a decrease in the hormone's concentration in the period of menstruation and the first week following the menstrual cycle.

This is due to the relationship of estrogen hormone with serotonin given with contraceptive drugs or its secretion during the women's acute psychological state [16]. This hormone is one of the factors causing ovarian disorders as well as cancer tumors, especially cancer of the uterus and ovaries. Fatty tissue has the ability to manufacture androgens and thus to increase the fat tissue (obesity). The more obesity increased, the more increase of the free androgen in the body and thus increase of the testosterone hormone, and the transformation of cholesterol by a series of metabolic processes to testosterone hormone. The increase in testicular fat concentration is one of the ways of diagnosing polycystic ovary syndrome, and the decrease in the concentration of this hormone indicates the absence of polycystic ovary syndrome [17, 18]. There is a positive relationship between LH hormone and testosterone hormone since they both rise together and this is consistent with our results [19].

The rise in the level of cholesterol confirms the presence of accumulated fats in the women patients' serums and these fats cause obesity, as well as high blood pressure or suffering from heart diseases. These high concentrations are mainly related to nutrition patterns, where nutrition is one of the most important

factors causing high fat because it contains saturated fats in its composition, which raises the level of cholesterol [20]. The high level of cholesterol concentration is due to the lack of estrogen hormone and the first substance forming estrogen is cholesterol. This condition occurs when the ovary stops and ovarian failure occurs early, where the formation of estrogen hormone is an important factor in reducing cholesterol and this is in agreement with the researcher [21].

The level of high-density lipoproteins was limited to normal values and was no longer considered as a pathosis. Herrington (2002) [22] mentioned that the presence of estrogen hormone in blood plasma works on raising the levels of high - density lipoproteins HDL-C and this explains the low percentage of women with heart diseases before reaching the age of menopause and after the age of menopause. We are likely to have a cause of varying concentrations of high-density lipoproteins due to unknown genetic factors, which is consistent with the results of our study but contradicts with the study by Conway *et al.* (1992) [23]. As the concentration of high-density proteins decrease with the increase of the triglycerides as a result of an increase in the concentration of fatty acids and their lack of esters, this increases the concentration of triglycerides. Van Deelin *et al.*, (1997) [24] mentioned that women with low concentrations (HDL-C) are more likely to develop atherosclerosis due to lack of transport as well as reduced oxidation of lipoproteins. Arora *et al.* (2010) [25] stated the presence of a positive relationship between progesterone and HDL-C and this is consistent with the results of our present study.

The gene *ESR2* is located on the chromosome 14q22-24 and the most common mutations in the cryptographic regions with part of the most common mutations 30 and part of the 50 region of the gene *ESR2*, which is the singular polymorphism of region 30, where there is conversion of G to A in the nuclei 1730 in the exon 8; and the transient mutation in 1082 in which G is converted to A in the exon 5 which is silent. Although the functional significance of polymorphism 1730 does not alter the amino acids in the protein of the product, but it is possible that this polymorphism has a link or correlation to imbalances in different regulatory sequences that may affect gene expression or function. In addition, it has been reported that genes in which polymorphism occurs can lead to different folds in the synthesis of mRNA and these changes in mRNA may have different biological functions interacting with other cellular components. The *AluI* enzyme in the gene *ESR2* is associated with increased risk of endometriosis in Japanese women, but no effect was found in Italian and Korean women [26].

The results obtained in this study with the results of Sun *et al* 2005[27]. showed that the estrogen receptor genes *ESR2* were associated with neither cervical cancer nor breast cancer in more than 800 Chinese women. All of these factors lead to ovarian disorders and in agreement with the study by Chen *et al.* (2007)[28] and Cox *et al.*, 2008[29] who confirmed the lack of association of the gene of *ESR2*, despite the large size of the sample and attributed the affect of women with ovulation disorder to the health status of each patient and the nature of food and race.

The different genotype and genotype of mutants are more likely to have ovarian disorders in the genotype TT when studying the gene *ESR2*, and this is what is obtained in this study. A study by de Mattos *et al.* (2014)[30] conducted on 136 Brazilian women suffering from ovarian disorders confirmed via the study of the polymorphism of the genes of *ESR2* that the most genotypes affected by infertility are: mutant pattern, which is consistent with this study. It is not easy to understand the mechanism by which each genotype is affected in this study, because it is governed by a number of mechanisms.

In this study, the future receptors of estrogen gene (*ESR2*) are studied and this does not confirm the disruption of ovulation, i.e. it is not easy to understand the effect of each genetic model alone if the significant values did not refer to the affecting of one genotype rather than the other, contrary to what appeared in the study of estrogen hormone and the male hormone Testosterone and its high level of patient compared with healthy women. This indicates that all genotypes in patient women were affected by this increase compared to the control. The hormonal imbalance experienced by women at childbearing age was not shown in this study but as being the result of having specific polycystic ovaries genes or genes with genetic predisposition to cancer and may be due to cases of excessive drug use or psychological condition of patients who are affected

by the delay of childbearing after the first year of marriage. This is always what happens in our Eastern societies, as the study included a sample of the stratum within the areas governed by customs and traditions in the subject of reproduction, which reflects negatively on the psychological state of married women[31,32].

Most studies agree with the results of the current study on the effect of fat on estrogen receptors in beta, especially in relation to race, as race plays an important role in women being affected by the level of lipids and its relationship with these receptors [33]. In his study of the Caucasian group, their effect was not found in the genes of the estrogen receptor beta. The current study differed with the study that found the mutant lipid effect of this future [34]. The table(3) showed no significant differences in fat levels. This is due to the non-influence of women in SNPs, especially those who did not show a clear effect. The difference may be due to other nucleotides in this gene.

V. Conclusion

The polymorphism of *ESR2* is associated with Ovarian disorders (OD); we found no association between *ER-β* gene polymorphisms at positions 307 and OD in the studied Iraqi women. *ESR2* showed non-significantly difference was observed among the studied females groups. Prolactin, testosterone, and were significantly higher in *ESR2* group in comparison to control group. In Lipid profile there were statistically significant in the concentration of Cholesterol and HDL in *ESR2* groups compared to control group. The efficiency of the PCR-RFLP technique in the creation of the multiple nucleotide polymorphisms (SNPs) of the co dominant genes and in the development of the SNP mutation located within a small piece of DNA.

VI. References

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