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Genetic Polymorphism in MTHFR and PCO genes associated with the incidence of Polycystic Ovary Syndrome in A sample of Iraqi Women

A Thesis

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By

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بسم الله الرحمن الرحيم

يَا أَيُّهَا النَّاسُ إِنْ كُنْتُمْ فِي رَيْبٍ مِنَ الْبَعْثِ فَإِنَّا خَلَقْنَاكُمْ مِنْ تُرَابٍ ثُمَّ مِنْ نُطْفَةٍ ثُمَّ مِنْ عَلَقَةٍ ثُمَّ مِنْ مُضْغَةٍ مُخَلَّقَةٍ وَغَيْرِ مُخَلَّقَةٍ لِنُبَيِّنَ لَكُمْ ۚ وَنُقِرُّ فِي الْأَرْحَامِ مَا نَشَاءُ إِلَىٰ أَجَلٍ مُسَمًّى ثُمَّ نُخْرِجُكُمْ طِفْلًا ثُمَّ لِتَبْلُغُوا أَشُدَّكُمْ ۖ وَمِنْكُمْ مَنْ يُتَوَفَّىٰ وَمِنْكُمْ مَنْ يُرَدُّ إِلَىٰ أَرْذَلِ الْعُمُرِ لِكَيْلَا يَعْلَمَ مِنْ بَعْدِ عِلْمٍ شَيْئًا ۚ وَتَرَى الْأَرْضَ هَامِدَةً فَإِذَا أَنْزَلْنَا عَلَيْهَا الْمَاءَ اهْتَزَّتْ وَرَبَتْ وَأَنْبَتَتْ مِنْ كُلِّ زَوْجٍ بَهِيجٍ (٥)

سورة الحج (الآية ٥)

صدق الله العظيم

Dedication

This research is dedicated to

To my father

Thank you for always keeping me in your prayers, believing

in me and supporting my decisions .

To my mother

The light that illuminates my life and spring, which saturate from him

love and affection.

To my sister Rania

For taking me in and providing the love, support and comfort

That can only come from family

To my fiancé Mahmood (my other half)

You are more than a dream for me and deeper than the homeland

Reem

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Reem Jaafar

Summary

This study was constructed to investigate Polycystic ovary syndrome (PCOS) related infertility through a biochemical and molecular base associated with single nucleotide polymorphism (SNP) at methylene tetrahydrofolate reductase (MTHFR) gene and Poly cystic ovary (PCO) gene in Polycystic ovary syndrome (PCOS) Patients.

The study included fifty blood samples of patients women suffering from Polycystic ovary syndrome (PCOS) during the period from November 2015 to January 2016, collected from Kamal al-Samarrai Hospital in Baghdad governorate. Twenty tissue samples were collected during period January 2016 till February 2016 from Madinat al-Amamin al-Kazimin al-Ttbbia Hospital. Fifty blood samples from healthy women were collected serving as the control group.

The average ages of patients and control group were 20-50 years.

The biochemical study include the fifty patients and fifty healthy samples (control group).Then, the subjects were divided in to three age groups (20-30), (31-40) and (41-50), years old. Serum samples from all Polycystic ovary syndrome (PCOS) patients were analyzed to detect the fertility hormones Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and Testosterone hormone.

It was found that there is a significant ($P<0.05$) difference in hormone concentration in serum patients when compared with normal. Hormone Follicle Stimulating Hormone (FSH) recorded a significant decreased, while hormones Luteinizing (LH) and Testosterone recorded a significant increase when compared to the normal. The greatest decreased of the fertility hormone Follicle Stimulating Hormone (FSH) and the greatest increased of Luteinizing Hormone (LH) were in the age group (41-50)years old, while increased in Testosterone

hormone was in the age group (31-40) years old. The study confirmed the incidence of SNPs detected in Polycystic ovary (PCO) gene of Polycystic ovary syndrome (PCOS) patients. Polymerase chain reaction (PCR) was done using a specific set of primers. One primer was selected to amplify the exon (2) of the methylene tetrahydrofolate reductase (MTHFR) gene, another three primers were designed to amplify the exons region of the Polycystic ovary syndrome (PCOS) gene (5-10).After optimization of the amplification condition, the product of (198,278,278 and 284 bp), was sent for DNA sequencing which was the tool for the detection of variation within patients. It was found that the percentage of substitution mutation was 88%, while the deletion mutation percent was 12%. The SNPs in the exons of the methylene tetrahydrofolate reductase (MTHFR) gene and Polycystic ovary (PCO) were detected and these polymorphism alteration in the expression attributed to altered transcription factor gene binding.

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List of Abbreviation

Abbreviation	Meaning
AES	Androgen Excess and PCOS Society
ASRM	the American Society for Reproductive Medicine
ACTH	Adrenocorticotrophic hormone
Bp	base pair
CVD	Cardiovascular disease.
CYP17	17α-hydroxylase/17,20-lyase cytochrome P450
D.W	Distal water
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxy Ribonucleic Acid
EC	Endometrial Cancer
EDTA	Ethylene Diamine Tetra Acetic Acid
ESHRE	European Society for Human Reproduction and Embryology
FSH	Follicle stimulating hormone
FDA	Food and Drug Administration
GnRH	Gonadotropin-releasing hormone
HGDP	Human Genome Diversity Project
HMG	human menopausal gonadotropin
Hcy	Homocysteine
HCG	Human chorionic gonadotropin
IGFBP-1	Insulin-Like Growth Factor Binding Protein-1
IGF1	Insulin Like Growth Factor 1
IVF	In vitro fertilization
IR	Insulin Resistance
LSD	Least Significant Differences
LH	Luteinizing Hormone
MAPK	Mitogen-Activated-Protein Kinase
MTHFR	methylenetetrahydrofolate reductase
M	Micro ($\times 10^{-6}$)
M±SD	Mean ± Standard Deviation
mL	Milliliter
µL	Microliter
NCBI	National Center for Biotechnology Information
NIH	National Institutes of Health
PE	Paraffin embedded tissue
PI3K	Phosphatidylinositide-3-Kinases

List of Abbreviation

PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
SAS	The Statistical Analysis System
SHBG	Sex hormone-binding globulin
SNP	Single nucleotide polymorphism
T2DM	Type 2 Diabetes Mellitus
TBE	Tris Borate EDTA
TAF-1	Transcriptional activation function-1
U.V.	Ultra Violate

Chapter One

Introduction

and

Literatures Review

1. Introduction and Literature Review.

1.1.Introduction.

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age, with a prevalence of 6.5–6.7% among premenopausal women (Diamanti-Kandarakis *et al.,*1999 ; Escobar-Morealle *et al*., 2000). PCOS was defined as the combination of chronic an ovulation or oligomenorrhoea and clinical or biochemical hyperandrogenism (Zawadski and Dunaif, 1992). Consequently, PCOS has significant implications for the health and quality of life of these patients. PCOS was firstly reported as Stein-Leventhal syndrome in (1935), and since then has attracted more and more attention due to its genetic heterogeneity and divers clinical manifestations. It has been used an important clinical model to investigate the relationships among endocrine functions, reproductive activity and energy metabolism (Diao *et al*., 2004). The methylenetetrahydrofolate reductase (MTHFR) enzyme plays important roles in metabolism of folates, remethylation of homocysteine to methionine and reduces 5, 10-methylenetetrahydrofolate to 5-methyltetrahydro folate (Jacques *et al.,* 1996). It has been established that MTHFR enzyme activity is associated with mutations within the MTHFR gene. The two most defined mutations of the MTHFR gene are missense mutations that include substitution of cytosine to thymine at nucleotide 677 which results in the conversion of alanine to valine. Another mutation is the transfusion of adenosine to cytosine at nucleotide 1298 which results in the conversion of glutamate to alanine (van derPut *et al.,* 1998). The influence of theses mutations varies in degree from mild to severe regarding the deficiency of MTHFR enzyme activity, folate as a universal methyl donor, contributes to the synthesis of nucleic acids, repair and methylation, and gene expression (Friso *et al.,* 2002). This function implies that gene-nutrient interactions mainly influence the

pattern of DNA polymorphisms (Stern *et al*., 2000). This study was suggested to fulfill the aims of:

- Investigation the hormonal factors causing polycystic ovary syndrome (PCOS).
- Detection the single nucleotide polymorphism in MTHFR gene associated with the incidence of Polycystic Ovary Syndrome.
- Measurement of fertility hormone change in the body of female patients as an indicator for the disease.

1.2 Literature Review.

1.2.1. Ovaries.

The ovary controls the growth, maturation and release of the oocyte in preparation for fertilization. It produces various steroids required for maintaining regular menstrual cycles and supporting pregnancy (Leung and Adashi, 2004). The follicle is one of the functional units in the ovary and consists of an innermost oocyte, surrounding granulosa cells and an outer layer of theca cells. Under the precise control of endocrine and intraovarian regulators, the follicles develop through primordial, primary and preantral stages before forming an antrum as show in Figure (1-1). At the antral stages, the predominant follicle is selected. Most developing follicles undergo atresia, and only a few continue to develop to pre-ovulatory stages and eventually ovulate in response to gonadotropins (McGee , 2000).

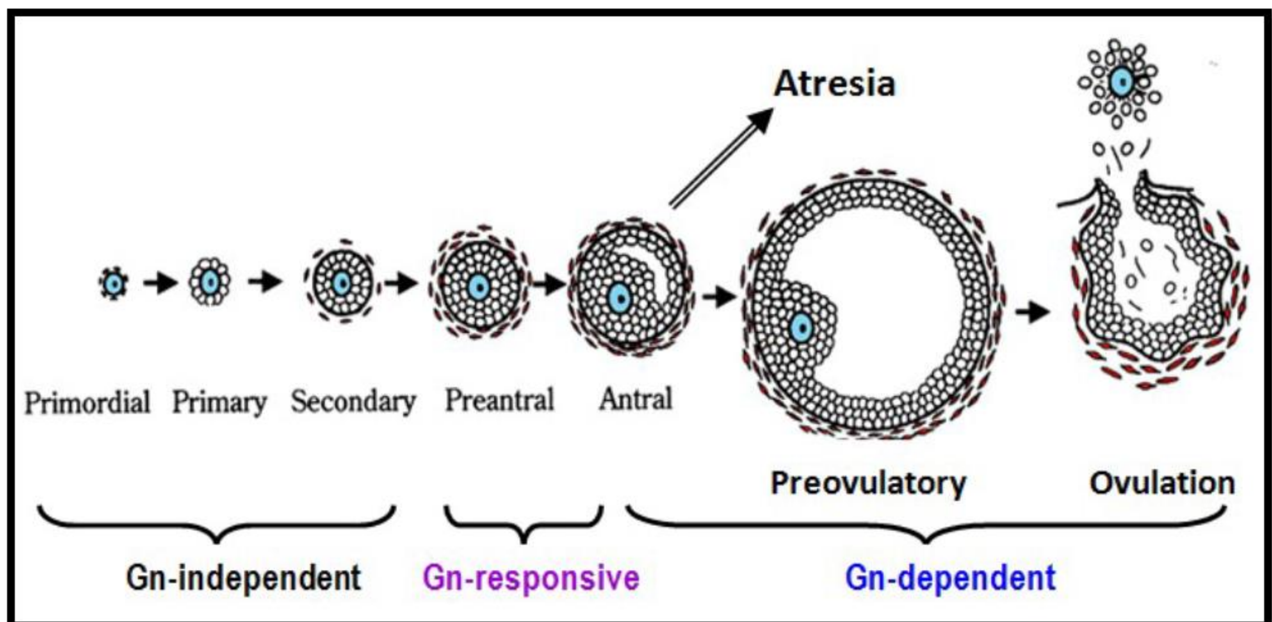


Figure (1-1): A schematic representation of ovarian follicular development Follicles in different stages (primordial, primary, seconday, preantral, antral, preovulatory and ovulation) are shown. According to the responsiveness of follicles, the follicle development stages could be categorized as gonadotropin (Gn)-independent, Gn-responsive and Gn-dependent stages (McGee, 2000 ; Gougeon, 2004).

1.2.2. The Ovulation Cycle.

Early in a woman's cycle the fertility clock stimulates the pituitary gland to secrete a large amount of follicle stimulating hormone (FSH). FSH stimulates growth of the Ova and the cells lining the follicle, the tiny bubble that holds the egg, so that the follicle enlarges and moves out towards the surface of the ovaries. At this stage, the follicle does not respond to stimulation by luteinizing hormones (LH) (Palomba *et al*., 2004). Around days 10, 11 or 12 of the cycle, the fertility clock stimulates the pituitary gland to make a very large amount of LH. By this time the follicle is 9.5 millimeters in diameter and has become sensitive to LH stimulation. The surge in LH from the pituitary gland always stimulates the final step of maturation of the follicle after which no further growth is possible. At this stage the follicle and egg are ripe or mature and the follicle will rupture or ovulate, releasing the Ova (Azziz *et al*., 2004).

Early in the cycle, the ovary and the developing follicle produce a female hormone called estradiol or estrogen. Estrogen stimulates the lining of the womb to grow and thicken. After the follicle ruptures and releases the egg in the middle of the cycle, the ruptured follicle (or egg shell) changes its function and produces the second female hormone, progesterone. Progesterone changes the lining of the womb so that it no longer grows thicker but becomes receptive to the implantation of a fertilized egg. This change also allows the lining of the womb to separate from the womb promptly and evenly after blood estrogen levels fall if fertilization and implantation have not occurred that cycle. This will result in a normal menstrual period that lasts from four to six days (Gnoth *et al.*, 2005) .

1.2.3. The Polycystic Ovary Syndrome.

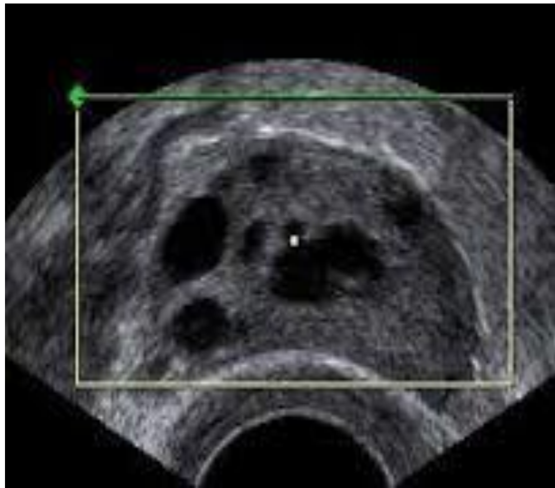
The polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women worldwide , PCOS is a complex, heterogeneous disorder of

uncertain etiology, but there is strong evidence that it can to a large degree be classified as a genetic disease (Fauser *et al.,* 2012).

PCOS produces symptoms in approximately 5% to 10% of women of reproductive age (12–45 years old). It is thought to be one of the leading causes of female subfertility and the most frequent endocrine problem in women of reproductive age (Aittomaki *et al.,* 1995).

The definition of the PCOS morphology has also varied over the years. The first definition was the one by Stein and Leventhal and they described the macroscopic appearance of PCOS ovaries as usually bilateral, enlarged, tense ovaries that were often distinctly globular in shape, the histological description was that of the presence of multiple cysts, rarely larger than 15 mm and these cysts were lined by ahypertrophic theca cell layer, it was also noted that the tunica albuginea, which is the collagen-rich stroma immediately below the ovarian surface epithelium, was much wider than in normal ovaries and that the ovaries were devoid of corpora lutea (Stein and Leventhal , 1935).

At the introduction of gynecological transvaginal ultrasound in the 1970s and as the ultrasound technology improved, the PCOS morphology diagnosis was made using ultrasound instead of ocular inspection or histology. The definition used today is the presence of twelve or more follicles measuring 2-9 mm in diameter, and/or at least one enlarged (>10 cm3) ovary. If a follicle is >10 mm in diameter, the scan should be repeated(Rotterdam ESHRE/ASRM, 2003). Images of typical ultrasound scan of a normal ovary and of a PCOS ovary as show in Figure (1-2):



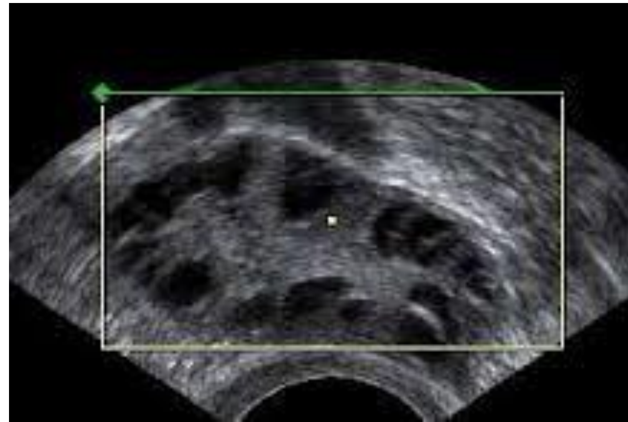


Fig.3a **Fig.3b**

Figure (1-2): typical image by transvaginal ultrasound of a) normal ovary and b) PCOS ovary (Rotterdam ESHRE/ASRM, 2003).

1.2.4. Etiology of Polycystic Ovary Syndrome.

Nobody is suring why these hormonal problems emerge, Some say that the problem could originate in the ovary itself, part of the brain that controls hormonal production, or in other glands in the body. It is also possible that insulin resistance triggers these changes (Palomba *et al.*, 2004).

The symptoms are related to abnormal hormone levels:

- High luteinizing hormone (LH) - LH stimulates ovulation, but if levels are excessively high, the proper functioning of the ovaries may be disrupted (Nugente *et al*., 2005).
- Insulin is a hormone that controls the level of glucose (a type of sugar) in the blood. If you have PCOS, your body may not respond to insulin (this is known as insulin resistance), so the level of glucose is higher. To try to prevent the glucose levels becoming higher, your body produces even more insulin. High levels of insulin can lead to weight gain, irregular periods,

fertility problems and higher levels of testosterone with glucose intolerance and hyperandrogenism (Charnvises *et al*., 2005).

1.2.5. Genetics of polycystic ovary syndrome.

Previous studies suggest that genetic factors play a major role in the etiology of PCOS (Legro *et al*., 1998 ; Govind *et al*., 1999). However, the mode of inheritance of PCOS remains unknown, and recent studies indicate that this disorder could be a complex trait (Crosignani and Nicolosi, 2001). This means that several genes are interacting with environmental factors to provoke the phenotype. In contrast, biochemical parameters, including fasting insulin levels or hyperandrogenism, seem to be highly heritable parameters, suggesting that some clinical signs, symptoms, or biochemical parameters of PCOS could be transmitted as mendelian autosomal dominant (Carey *et al*., 1993) or X-linked traits (Legro and Strauss, 2002), but the genetic studies have not as yet concluded the pattern of heredity (Diamanti-Kandarakis and Piperi, 2005)Because the heritability of PCOS is probably more complex, similar to that of type 2 Diabetes Mellitus or cardiovascular disease(CVD). However, a positive family history appears to be the most informative risk factor for the development of PCOS. The heterogeneity and lack of universally acceptable clinical or biochemical diagnostic criteria make genetic studies of PCOS difficult to perform. PCOS is a disorder which primarily affects women of reproductive age and it is therefore difficult to study in more than one generation. There is no commonly accepted male phenotype. Male pattern premature balding has been demonstrated in male relatives in familial PCOS studies (Legro and Strauss, 2002).

1.2.5.1. Chromosomal abnormalities.

A relation between PCOS with the X chromosome aneuploidies and polyploidies in addition to other cytogenetic abnormalities has been confirmed. Some of the cases of PCOS may represent an intermediate condition in a spectrum that extends from the streak gonad of Turners syndrome to the normal ovary. The concept is that at least some cases of PCOS may be due to X chromosomal factors causing an abnormal follicular apparatus (Hickey *et al*., 2002). In addition, large deletion of the long arm of chromosome 11 was seen in some of the PCOS cases (Meyer *et al*., 2000).

1.2.5.2. Adrenal androgen production.

The adrenal cortex synthesizes all the three major androgens; dehydroepiandrosterone sulfate (DHEAS), androstenedione and testosterone, and this is the other major site of female androgen production, besides the ovaries. DHEAS is almost exclusively (97-99%) produced by the adrenal cortex and androstenedione is produced in both the adrenal gland and the ovaries whereas 25% of testosterone is synthesized by the adrenal gland,25% in the ovary and the remaining part being produced through peripheral conversion from androstenedione in liver, adipose tissue and skin (Burger , 2002). Around 60-80% of PCOS women have high concentrations of circulating testosterone (Chang *et al*., 2005).In PCOS women, the prevalence of DHEAS excess is 20-30%, depending on ethnicity and DHEAS levels decline up to the age of ~ 45 years (Kumar *et al*., 2005). The increased DHEAS levels in PCOS women compared with controls is verified up to the perimenopausal ages (Dahlgren *et al*., 1992).However, the mechanisms of the adrenal androgen excess in PCOS is still unclear, although it has been proposed that it may result from increased metabolism of cortisol, which could lead to decreased negative feedback on adrenocorticotrophic hormone (ACTH) secretion (Tsilchorozidou *et al*., 2003).

1.2.6. Diagnostic Criteria for Polycystic Ovary Syndrome.

Three different sets of standard diagnostic criteria have been proposed, reflecting the heterogeneity of the syndrome, (Table 1-1). The first attempt to define PCOS was made during an expert conference held at the National Institutes of Health (NIH) in 1990, and this included both hyperandrogenism and ovulatory dysfunction (Zawadzki and Dunaif, 1992).

In 2003, the Rotterdam conference, sponsored by the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), broadened the definition of PCOS by including PCOS morphology, and the requirement for at least two of the three diagnostic features (Louise, 2010).

Finally, the Androgen Excess and PCOS Society (AES) proposed new diagnostic criteria in 2006, which made hyperandrogenism fundamental and excluded the phenotype of the non-hyperandrogenic woman with ovulatory dysfunction which is included by the Rotterdam criteria (Azziz *et al.,* 2006). All three sets of PCOS diagnostic criteria require the exclusion of other disorders that cause hyperandrogenism and ovulatory dysfunction, Table (1-1) (Zawadzki and Dunaif, 1992) .

Table (1-1): Polycystic Ovary Syndrome Diagnostic Criteria (Louise, 2010).

Definition	**Diagnostic criteria** A
NIH (1990)	Requires the presence of: 1) Hyperandrogenism B 2) Ovulatory dysfunction C
Rotterdam (2003)	Requires the presence of at least two of: 1) Hyperandrogenism B 2) Ovulatory dysfunction C 3) PCO morphology D
AES (2006)	Requires the presence of: 1) Hyperandrogenism B 2)Ovarian dysfunction (ovulatory dysfunction C or PCO morphology D)

A: Exclusion of other disorders causing hyperandrogenism and ovulatory dysfunction is a criterion of all three definitions.

B: Clinical and/or biochemical signs of hyperandrogenism.

C: Oligo menorrhea, amenorrhea, oligo ovulation, and anovulation.

D: Twelve or more 2-9 mm follicles and/or at least one enlarged ovary (>10ml).

NIH : National Institutes of Health

AES : Androgen Excess and PCOS Society.

Estimates of the prevalence of PCOS depend on the definition used. According to the NIH criteria, 6-8% of women in the general population have PCOS (Azziz *et al.,* 2004). A recent Australian study, including primarily Caucasians, found that the prevalence of PCOS under the Rotterdam and AES criteria was almost twice that produced by the NIH criteria (March *et al.,* 2010).

1.2.7. Other Conditions Associated With Polycystic Ovary Syndrome.

1.2.7.1. Obesity.

Compared with women of similar age who don't have polycystic ovary syndrome ; women with PCOS are significantly more likely to be overweight or obese (Martínez-Bermejo *et al.,* 2007). Furthermore, about half of all women with PCOS manifest central obesity, in which there is a greater deposition of visceral fat around internal organs in the abdominal region, as opposed to the fat being located on the thighs and hips. Abdominal fat distribution is associated with increased risk of hypertension, diabetes and lipid abnormalities (Faloia *et al*., 2004).

1.2.7.2. Insulin Resistance.

Insulin resistance, i.e., impaired stimulation of glycogen formation in all major target tissues (skeletal muscle, adipose tissue, liver, kidney), is a pathogenic characteristic feature of PCOS, particularly among obese subjects (Dunaif *et al*., 1989). The molecular mechanisms of insulin resistance involve defects in the insulin-receptor signalling pathway in both adipocytes and in skeletal muscle (Dunaif , 1997).Insulin resistance causes compensatory hyperinsulinemia and might contribute to hyperandrogenism and gonadotropin aberrations through several mechanisms. Insulin may act directly in the

hypothalamus, the pituitary or both and thereby contribute to abnormal gonadotropin levels (Adashi *et al*., 1981). High insulin can also serve as a co-factor to stimulate ACTH-mediated androgen production in the adrenal glands (Moghetti *et al*., 1996). the stimulation of the ovaries is exerted by a synergistic effect of insulin upon LH stimulation of the theca cells(Nestler ,1997 ; Diamanti-Kandarakis *et al*., 2008) and insulin may also directly stimulate theca cell proliferation(Duleba *et al*., 1998). In addition, high insulin concentrations also cause decreased circulating SHBG, thereby increasing the levels of free bioavailable testosterone (Nestler *et al*., 1991 ; Yki-Jarvinen *et al*., 1995). Administration of insulin in young non-PCOS women resulted in increased LH-puls frequency, thereby implying an association between insulin and hypothalamus-pituitary-ovarian-axis-activity(Moret *et al*., 2009). In conclusion, excess of androgens in PCOS of ovarian and/or adrenal origin initiates or maintains a vicious circle, where hyperandrogenism leads to hypothalamus/pituitary abnormalities, ovarian dysfunction, insulin resistance and abdominal obesity, which in turn stimulates further androgen production (Escobar-Morreale and San Millan 2007).

1.2.7.3. Infertility.

Polycystic Ovary Syndrome is the most common cause of female infertility. Many women with polycystic ovary syndrome experience infrequent ovulation or lack of ovulation altogether and may have difficulty in becoming pregnant; PCOS is also associated with spontaneous abortion and preeclampsia (Van der Spuy and Dyer , 2004).

1.2.8. Hormone Fertility.

1.2.8.1. Luteinizing Hormone (LH) .

The level of LH is important in ovulation process not only releasing the egg, but also initiating the conversion of the residual follicle into a corpus luteum that, in turn produces progesterone to prepare the endometrium for a possible implantation, LH is necessary to maintain luteal function for the first two weeks. LH supports theca cells in the ovary that provide Androgens and hormonal precursors for Estradiol production (Mason, 2004). It was Connected to hormonal stimulation for IVF it has been found that both too high as well as too low levels of LH exert detrimental effect on the developing conceptus (Westergaard *et al*., 2000). Luteinizing Hormone is constituted of two glycoprotein molecules α- and β-subunits, which together constitute a heterodimer forming a functional protein. Luteinizing Hormone has a common α-subunit containing 92 amino acids while the β-unit is different. The β-subunit of LH is built of 120 amino acids and share for 81% the same amino acids and stimulate the same receptor (Kessler *et al*., 1979). The difference in structure of the subunits, decide both bioactivity as well as half-life, which is approximately 20 minutes for LH shorter than that of FSH (3-4 hours).The secretion of LH from the anterior pituitary lobe is controlled by pulsatile waves of Gonadotropin-releasing hormone (GnRh) from the hypothalamus(Damewood *et al*., 1989).

1.2.8.2. Follicle Stimulating Hormone (FSH).

Follicle stimulating hormone (FSH) is a glycoprotein that is synthesized and secreted by the basophilic cells gonadotropes of the anterior pituitary gland, FSH regulates the development, growth, pubertal maturation and reproductive processes of the human body. (Negi , 2009) .

FSH has a beta subunit of (118) amino acid, which confers its specific biologic action and is responsible for interaction with the FSH receptors**,** (Murray *et al*., 2000) . In the female FSH is necessary for the selection and growth of ovarian gonadotrophic effects of FSH may be subserved by a number of intermediaries (Richards , 1994). That form part of the cellular and tissue (Christenson and Stouffer , 1997) which is response to FSH stimulation culminating in ovulation, Such cellular responses illustrate the complex nature of FSH since they indicate that FSH activity has many components, *i.e.* FSH is a growth factor or tropic hormone and a modulator of cellular development (Li *et al*., 1997). It is generally thought that FSH exerts most of intracellular actions via the cAMP-mediated signaling pathway, although FSH may also utilize other signal transduction pathways such as Ca (Sharma *et al*., 1994). The biological activity of FSH is the sum of a complex combination of processes. Release from the pituitary, survival in the circulation, transport to the site of action (*i.e*., the gonad), binding to the receptor, and activation of signal transduction pathways. These processes may be modified by other factors that may affect release (pulse frequency or amplitude), clearance from the circulation, receptor binding and desensitization, cellular responsiveness, and modifications to the hormone during circulation such as neuraminidase action (Lapthorn *et al*., 1994). According to the two cell–two gonadotropin hypothesis as show in Figure (1-3), LH stimulates theca cells to produce androstenedione from cholesterol. This is then converted to estrogens in the granulosa cells by the action of FSH-dependent aromatase (CYP19 gene). In fact, every molecule of estrogen is derived from a molecule of androgen, The two principal factors influencing the total amount of androgen secreted by the ovary are the total number of theca cells and their steroidogenic capacity, both of which are disturbed in women with PCOS (Magoffin, 2005).

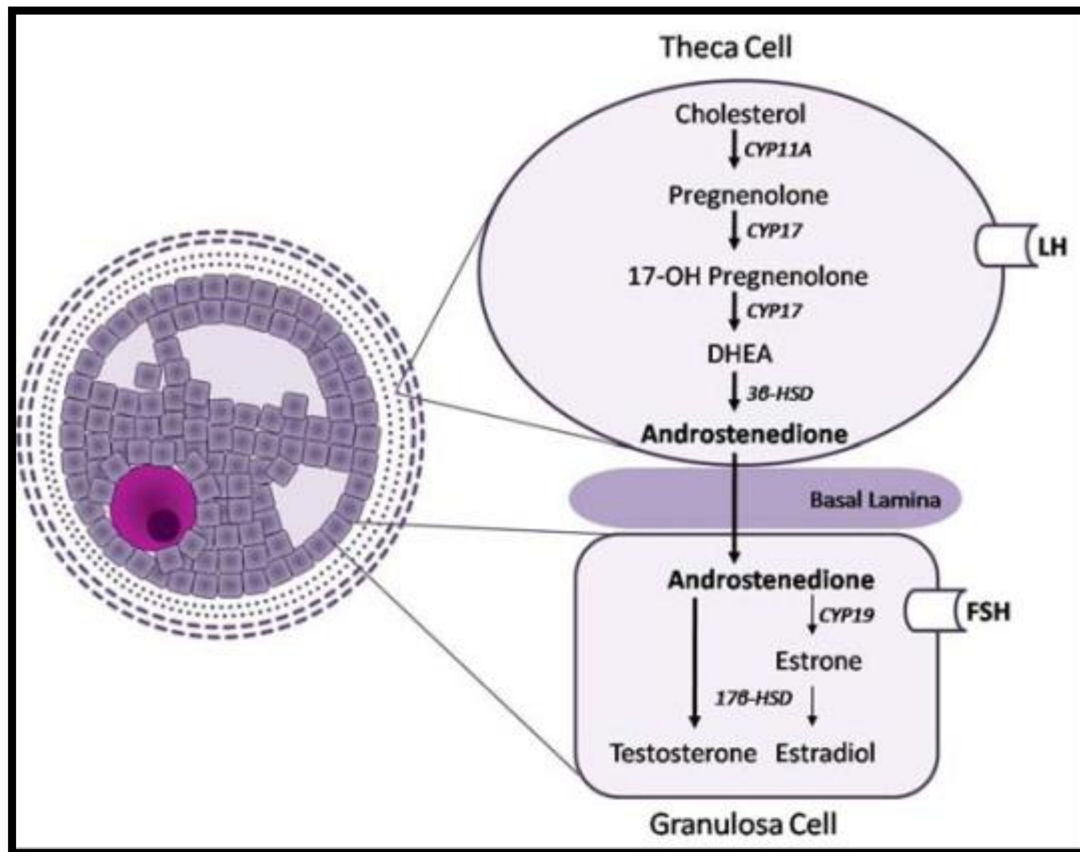


Figure (1-3): The "Two-cell Two Gonadotropin Concept" For Sex Steroid Production (Hanley, 2000).

1.2.8.3. LH and FSH in Polycystic Ovary Syndrome (PCOS).

An appropriate gonadotropin secretion is associating with the classic form of PCOS, Compared with the follicular phase of the normal menstrual cycle, women with PCOS exhibit an unreasonably high LH secretion with comparatively constant low FSH secretion. Therefore, an elevated LH/FSH-ratio of 2-3/1 is commonly used to indicate abnormal gonadotropin secretion. The prevalence of increased serum LH in PCOS ranges from 30% to 90 % (Franks , 1989 ; Conway *et al*., 1989).

1.2.8.4. Testosterone Hormone.

The ovarian and adrenal glands of women with PCOS are usually the sites of production of elevated androgens, It is postulated that these women have a hyperactive production of 17α-hydroxylase/17,20-lyase cytochrome P450 (CYP17) enzyme, which is responsible for forming androgens in the ovaries and adrenals from DHEA-S(Hopkinson *et al*., 1998) Elevated total and free Testosterone correlate with the typically elevated LH levels. Serum total Testosterone is usually up to twice the normal range (20 to80 ng/dL). High androgen levels in the ovary inhibit FSH, there by inhibiting development and maturation of the follicles (Hopkinson *et al*., 1998 ; D'Hooghe *et al*., 2002) DHEA is found to be elevated in 50% of women with PCOS, The elevated DHEA is due to stimulation by ACTH, produced by the pituitary in response to stress. The excess DHEA then converts to androgens via adrenal metabolism, which in turn contributes to the typical elevated androgen levels in PCOS (Hopkinson *et al*., 1998).

1.2.9. Polycystic Ovary Syndrome and Risk of Endometrial Cancer.

Risk factors for endometrial cancer are excessive weight, hyperinsulinemia, nulliparity, and a longer time of exposure of estrogens and these factors are also associated with PCOS. In addition, increasing age and a sedentary lifestyle add to the already mentioned risk factors . The mechanism behind endometrial cancer in PCOS that has been discussed is the unopposed stimulation by estrogens of the endometrium, which can cause endometrial hyperplasia with increased risk of atypia and eventually endometrial cancer (Amant *et al*., 2005). No large prospective studies regarding PCOS and endometrial cancer exist and the results of studies are conflicting, however a recent meta-analysis showed an almost three times higher risk of developing

endometrial cancer for PCOS women compared to women without PCOS (Chittenden *et al*., 2009). Regarding breast cancer, the discussed cause of an association with PCOS, is that of obesity, hyperandrogenism, the longer time-periods of unopposed estrogen and of infertility (Balen , 2001). Most studies of PCOS and breast cancer show no increased risk for women with PCOS, as supported by two review articles on the subject (Balen , 2001; Chittenden *et al*., 2009) and the meta-analysis by(Chittenden *et al*., 2009).The risk of ovarian cancer seems to be increased in women with multiple ovulations late menopause and early menarche, Many of these factors are present in PCOS women and, theoretically, these women could have an increased risk of ovarian cancer, although this is contradicted by the fact that a large percent of women with PCOS are oligo/anovulatoric, As for endometrial cancer and breast cancer, data of an association between PCOS and ovarian cancer are conflicting, studies are small and prospective studies are lacking. Most studies seem to show no association between ovarian cancer and PCOS (Pierpoint *et al*., 1998 ; Balen , 2001).

1.2.9.1. Polycystic Ovary Syndrome and Hyperandrogenism.

Hyperandrogenism is the essential characteristic of PCOS and is the most consistent biochemical finding in all women with PCOS (Franks , 1989).Familial aggregation of reproductive endocrine biochemical abnormalities in PCOS relatives suggests that these traits have a genetic basis(Legro *et al*., 1998). Clinically, the most common sign of hyperandrogenism in PCOS women is hirsutism. The range of the prevalence of hirsutism in PCOS women varies between 17% and 83%. Another common sign of hyperandrogenism is acne. Thus, an adolescent female with moderate to severe acne should be investigated for PCOS, Furthermore, the progress or persistence of acne into adulthood is unusual and should raise awareness. However, it has also been shown that hyperandrogenism may be related to overt

signs of virilization, i.e. male pattern balding, alopecia, increased muscle mass, a deepening voice or clitoromegaly but these signs partly resolves before menopause in women with PCOS. These women tend to gain more regular menstrual cycles with increasing age (40 years and more) (Elting *et al*., 2000).

1.2.9.2 Polycystic Ovary Syndrome, Hyperestrogenism and Progesterone Deficiency.

The majority of Endometrial Cancer (EC) occur due to the unopposed estrogen environment. In this situation, that the excess of estrogen stimulation is not sufficiently counterbalanced by progesterone results in promoted mitogenesis, atypical hyperplasia, and the transition to malignant adenocarcinoma (Wang *et al*., 2014). The presence of increased endometrial proliferation rates at follicular phase of menstrual cycle, during which progestin levels are low, whereas estradiol levels at normal premenopausal concentrations and increased endometrial cancer risk among women using exogenous estrogens without progestins are two important signs that verify this relation (Kaaks *et al*, 2002). Progesterone reduces estrogenic activity in the endometrium by enhancing the local synthesis of 17β-hydroxysteroid dehydrogenase and estrogen sulfo-transferase. These enzymes play a significant role in the conversion of estradiol into the less potent estrogen estrone, and into estrogen sulfates that are rapidly excreted from cells and from the body (Sinreih *et al.,* 2013). Mostly, the proliferative effects of estradiol on endometrial tissue are mediated by an increase in the local production, especially by stromal tissue, of insulin-like growth factor-1 (IGF-I) (Eritja *et al.,* 2013). Progesterone provides the key stimulus for endometrial gene expression and synthesis of insulin-like growth factor binding protein-1 (IGFBP-1), which inhibits IGF-1 action in endometrial tissue (Yang *et al.,* 2011). In patients with PCOS the increased estrogen levels and reported resistance of endometrium to

progesterone stimulate the hyperplasic effects of estrogen to endometrium and the risk of EC (Shen *et al.,* 2008).

1.2.9.3. Polycystic Ovary Syndrome, Insulin Resistance and Obesity.

Although the demonstration of Insulin Resistance (IR) is not necessary to diagnose PCOS, it is well established that hyperinsulinemic (IR) has a great importance in PCOS, the prevalence of IR in PCOS ranges from 50%-70% and occurs independently of obesity. However, obesity has an additive effect on IR in women with PCOS (Sirmans and Pate , 2013). IR is a condition in which target tissues become less sensitivity to insulin, and results in elevated blood insulin levels, to overcome this β cells produce more insulin, and compensatory hyperinsulinemia occurs. Consequently, increased insulin levels may induce cancer formation including breast cancer, colorectal cancer, prostate, pancreatic cancer and EC by several direct and indirect pathways (Mu *et al*., 2012).

Insulin directly promotes endometrial cell proliferation and activates protein-kinase enzyme systems phosphatidylinositide-3-kinases (PI3K) and mitogen-activated-protein kinase (MAPK) which have mitogenic and anti-apoptotic effects that contribute significantly to cellular transformation and development of EC (Subramaniam *et al*., 2013). Moreover, the interaction between insulin, estrogen and IGF-1 also contributes to the development of EC. Since the insulin receptors and IGF-1 receptors are partially homologues, insulin can bind to IGF-1 receptor to activate protein-kinase signaling pathways (Janssen and Varewijck , 2014). As these pathways activated, they can stimulate transcriptional activation function-1 (TAF-1) of estrogen receptor, which regulates cell growth and division (Kato *et al.,* 2000). Increased insulin levels can also inhibit IGFBP-1 mRNA and protein expression in endometrial stromal cells (Lathi *et al*., 2005) which leads elevated levels of free IGF-1. IGF-1 is a potent mitogen and survival factor that can promote the development of EC (Mu

et al., 2012). In the indirect mechanism, hyperinsulinemia can stimulate theca cell androgen activity, elevate serum free testosterone levels through decreased hepatic sex hormone-binding globulin (SHBG) production, amplify LH- and IGF-I-stimulated androgen production, and enhance serum IGF-I bioactivity through suppressed IGF-binding protein production which in turn promote the development of EC (Goodarzi *et al.*, 2011). Obesity is common in PCOS, which is a strong risk factor for EC (Crosbie *et al.*, 2012). Insulin can behave as a growth factor in adipose tissue, it enlarges adipocytes as well as increases the number of adipocytes that causes obesity. In adipose tissue, estrogens can be produced by stromal cells or aromatization of adrenal androgens, in addition, the level of sex hormone binding globulin is decreased in obese women, these hormonal changes in obese women with PCOS contribute to increased activity of free estrogens that amplifies estrogenic activity in the endometrium and act in an additive manner to promote the development of EC, as the obesity and overweight are clearly correlated to insulin resistance and hyperinsulinemia, briefly the increased risk of EC in obese women is likely to be associated with steroid hormone regulation during the insulin-resistant state (Li and Shao, 2014).

1.2.10. Genetic Basis of Polycystic Ovary Syndrome.

Familial clustering of PCOS has been consistently reported suggestion that genetic factors play a role in the development of this syndrome, it is now established that PCOS represents a complex trait similar to type2 Diabetes and obesity. and that both genetic and environmental factors contribute to the PCOS pathogenesis, overall PCOS can be viewed as a heterogeneous androgen excess disorder with varying degrees of gonadotropic and metabolic abnormalities (Unluturk *et* al., 2004).However, there is strong evidence for a major genetic component in the etiology of PCOS, in families with PCOS cases, there is evidence of heritability of both hyperandrogenaemia and hyperinsulinaemia in

affected siblings (Legro *et al*., 1998 ; Franks and McCarthy,2004), Numerous genetic mechanisms, including autosomal dominant, X-linked dominant, modified autosomal dominant and multifactorial, have been proposed, still, the precise mode of inhertince of PCOS has not been established (Franks *et al*., 2001). It has been suggested that multiple loci and epigenetic modifications may play a role in the phynotype (Menk and Strauss, 2007). Family history, as a reflection of genetic risk, can also be considered as a risk factor and, therefore it is important for determining an individual's risk of developing PCOS (Kahsar-Miller and Azziz , 1998).

1.2.11. Genetic Models of Polycystic Ovary Syndrome.

While most studies have focused on defining the characteristics of this disorder, few have attempted to elucidate the genetic mechanisms behind the development of PCOS. This depend on whether the disorder is viewed as the combination of defect unique to this syndrome or as a defect already present in the general population. Three general genetic models of PCOS have been proposed (Kahsar-Miller and Azziz , 1998). Which are following :

1.2.11.1. The first model.

Termed as the "**single – gene Mendelian**" model, considers that the majority of the defects, that are present in PCOS, is unique. This model would suggest that the inheritance of PCOS should demonstrate a recessive or dominant pattern, indicative of a single– gene defect, if a dominant mode of transmission is assumed, all women who inherit the defect would develop clinically evident PCOS (Urbanek *et al.,* 2007).

1.2.11.2. The second model.

Termed "**multifactorial**", suggests that the defects present in PCOS are not unique to it, and this disorder simply represents the conglomeration of

abnormalities already present in the general population, under this concept, PCOS would be considered as a multifactorial genetic disorder such as type 2 DM and CVD. Hence, women carrying multiple defects (both via inheritance and via environmental influence) would be at increased risk of developing clinical PCOS (Franks and Mccarthy , 2004).

1.2.11.3. The third model.

It is called the "**variable expression – single gene**" model, is a modified combination version of the above two models, under this model, PCOS is caused by a major gene defect, which is transmitted to 50% of offspring. However, the expression of PCOS would then be modified by additional factors, both environmental and/or genetic (i.e. "genetic background"), so that the actual observed segregation ratio could be less than expected for an autosomal dominant disorder. women who possess the mutation would be at almost 100% risk of developing some degree of PCOS, although additional factors would determine the clinical severity of the disorder. Genetically predisposed women not exposed to these other influences might develop only subclinical forms of PCOS (Crosignani and Nicolosi , 2001).

1.2.12. Methylenetetrahydrofolate reductase (MTHFR).

Methylenetetrahydrofolate reductase (MTHFR) is a rare genetic defect that leads to complications in pregnancy (Ivy *et al*., 2007). MTHFR gene produces an enzyme called methylenetetrahydrofolate reductase and mutation in the gene inhibits the production of this enzyme, result in hyperhomocystinemia, which is an elevated level of homocysteine found in blood plasma. When the body is deficient in methylenetetrahydrofolate reductase, its ability to absorb folate, such as folic acid, is inhibited. Folic acid and B9 are both essential to the development and health of the fetus. Because of a mother with MTHFR’s inability to efficiently metabolize folic acid and vitamin B9, the disorder has

been linked to a variety of pregnancy complications such as congenital malformations. Elevated levels of homocysteine have been associated with placental disease (Foka *et al.* , 2000).

1.2.13. . Methylenetetrahydrofolate reductase (MTHFR) Gene mutation.

(MTHFR) is a key enzyme in one-carbon metabolism, the enzyme catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the predominating circulating form of folate. 5-methyltetrahydrofolate participates in the vitamin B12-dependent remethylation of homocysteine to methionine that is converted to S-adenosylmethionine that serves as a methyl group donor in the methylation of DNA, proteins, neurotransmitters and phospholipids (Mattson and Shea , 2003). MTHFR gene polymorphisms are commonly associated with hyperhomocysteinemia (van der Put *et al*., 1998). and recurrent embryo loss (Nelen *et al*., 2000 ; Gris *et al.*, 2003). Also reported an association between increased levels of homocysteine and a first early pregnancy loss. The best characterized MTHFR genetic polymorphism consists of a 677C>T transition which results in an alanine to valine substitution in the predicted catalytic domain of MTHFR (Frosst *et al*.,1995). This substitution renders the enzyme thermolabile, and homozygotes andheterozygotes have about a 70% and 35% reduced MTHFR activity *in vitro*, respectively. Homozygosity for the 677T allele is associated with elevated homocysteine levels, predominantly in individuals who have a low plasma folate level (Jacques *et al*., 1996). Furthermore, the level of plasma homocysteine can be lowered in homozygous individuals by folate supplementation (Malinow *et al*., 1997). About half the general population carries at least one mutated allele and the frequency of the homozygous mutated genotype (677TT) ranges from 1 to 20% depending on the population (Botto and Yang, 2000). A second common polymorphism in the MTHFR gene is a

1298A>C transition which results in a glutamate to alanine substitution within a presumed regulatory domain of MTHFR (van der Put *et al*., 1998 ; Weisberg *et al*.,1998). The 1298C allele has been reported to lead to decreased enzyme activity, although not to the same extent as the 677T allele (van der Put *et al*., 1998 ; Weisberg *et al*.,1998 ; Chango *et al*., 2000). Individuals who are compound heterozygous for the 677T and 1298C alleles, which produces a 677CT/1298AC genotype, have according to some studies 40–50% reduced MTHFR activity *in vitro* and a biochemical profile similar to that seen among 677T homozygotes with increased homocysteine levels and decreased folate levels (van der Put *et al*., 1998 ; Weisberg *et al*.,1998).

However, results indicate that the MTHFR 1298A>C polymorphism does not contribute significantly to hyperhomocystinemia, neither by itself nor in combination with the 677C>T polymorphism (Friso *et al*.,2002; Zetterberg *et al* ., 2002), and the phenotypic effect of the polymorphism has also been questioned from a biochemical point of view (Yamada *et al*., 2001). It must, however, be borne in mind that the absence of a biochemical phenotype in vitro does not necessarily rule out the possible importance of the 1298A>C polymorphism in vivo, for instance during times of high folate requirement, such as pregnancy (Zetterberg *et al*., 2002).

1.2.14. Drug Used to Treatment of Polycystic Ovary Syndrome.

Fertility drugs are often used alone as initial treatment to induce ovulation. If they fail as sole therapy, they may be used with assisted reproductive procedures, such as invitro fertilization, to produce multiple eggs, a process called **superovulation**. According to the American Society for Reproductive Medicine, fertility drugs can be divided into three main categories:

- Medications for Ovarian Stimulation. Clomiphene (Clomid, Serophene); follicle stimulating hormone (FSH) [Follistim, Gonal-F]; human menopausal gonadotropin (hMG) [Humegon]; luteneizing hormone (LH).
- Medications for Oocyte Maturation. Human chorionic gonadotropin (hCG).
- Medications to Prevent Premature Ovulation. GnRh agonists (Lupron and Synarel); GnRH antagonists (Antagon, Cetrotide) (Boostanfar *et al*., 2001 ; Hughes *et al*., 2004).

1.2.14.1. Clomiphene.

Clomiphene citrate (Clomid, Serophene) is usually the first fertility drug of choice for women with infrequent periods and long menstrual cycles. Unlike more potent drugs used in super ovulation, clomiphene is gentler and works by blocking estrogen, which tricks the pituitary into producing follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This boosts follicle growth and the release of the egg. Clomiphene can be taken orally, is relatively inexpensive, and the risk for multiple births (about 5%, mostly twins) is lower than with other drugs (Buvat *et al*., 1987).

Women with the best chances for success with this drug are those who have the following conditions:

- Polycystic ovarian syndrome (PCOS)
- Ability to menstruate but irregular menstrual cycle

Women with poorer chances of success with this drug have the following conditions:

- ➢ Infertility but with normal ovulation
- ➢ Low estrogen levels
- ➢ Premature ovarian failure (early menopause)

One or two tablets are taken each day for 5 days, usually starting 2 - 5 days after the period starts. If successful, ovulation occurs about a week after the last pill has been taken (Suginami *et al*., 1993). If ovulation does not occur, then a higher dose may be given for the next cycle. If this regimen is not successful, treatment may be prolonged or additional drugs may be added. Doctors usually do not recommend more than 6 cycles. Clomiphene often reduces the amount and quality of cervical mucus and may cause thinning of the uterine lining. In such cases, other hormonal drugs may be given to restore thickness. Other side effects of clomiphene include ovarian cysts, hot flashes, nausea, headaches, weight gain, and fatigue. There is a 5% chance of having twins with this drug, and a slightly increased risk for miscarriage (Yen *et al*., 1973).

1.2.14.2. Gonadotropin.

If clomiphene does not work or is not an appropriate choice, gonadotropin drugs are a second option. Gonadotropins include several different types of drugs that contain either a combination of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), or only FSH. Whereas clomiphene works indirectly by stimulating the pituitary gland to secrete FSH, (which prompts follicle production), gonadotropin hormones directly stimulate the ovaries to produce multiple follicles. Gonadotropins are given by injection. Gonadotropins include:

- Human Menopausal Gonadotropins (hMG), also called menotropins
- Human Chorionic Gonadotropins (hCG)
- Follicle Stimulating Hormone (FSH)
- Gonadotropin-releasing hormone (GnRH) analogs, which include GnRH agonist and GnRH antagonists.

This action helps prevent the premature release of the eggs before they can be harvested for assisted reproductive technologies (Levene *et al*., 1992).

GnRH agonists and antagonists:

- GnRH agonists include leuprolide (Lupron), nafarelin (Synarel), and goserelin (Zoladex).
- GnRH antagonists include ganarelix (Antagon) and cetrorelix (Cetrotide). GnRH antagonists suppress FSH and LH more than GnRH agonists, and they may require fewer injections (Shushan *et al*., 1996 ; Mosgaard *et al*., 1997).

1.2.14.3. Insulin Sensitizers.

Insulin sensitizers are intended to help the body effectively process insulin again. These types of medications are approved for treatment of adult-onset diabetes, but have also been shown to successfully treat women with PCOS who have insulin resistance. As insulin levels normalize, the ovaries resume normal function and many PCOS symptoms diminish. Insulin sensitizers may help restore menstrual cycles and alleviate excessive hair growth, acne, and weight gain. Metformin (brand name Glucophage) is an insulin sensitizer that seems to alleviate PCOS symptoms for many women(Epstein and Randy Hutter , 2000) But, because this drug is not approved by the FDA for treatment of PCOS, getting health insurance coverage for insulin sensitizers for a woman who does not have diabetes is difficult, at best.

1.2.15. Geography of Polycystic Ovary Syndrome genetic markers.

The distribution of the allelic variants associated to PCOS is different among the populations worldwide, as observable by Human Genome Diversity Project (HGDP) selection browser (http://hgdp.uchicago.edu/cgi-bin/gbrowse/HGDP) (Coop *et al*., 2009 ; Pritchard *et al*., 2010), a web-based software, which calculates the geographic distributions of user-selected markers from Stanford

SNP genotyping data (Cann *et al*., 2002 ; Cavalli-Sforza , 2005). A different genetic pattern distribution of PCOS markers could be reflected in different phenotypic features of the disease, resulting from adaptive evolution (Welt *et al*., 2012 ; Louwers *et al*., 2013 ; Cui *et al*., 2013) or from genetic drift generated by a serial founder effect occurring during the ancient human migrations out of Africa (Ramachandran *et al*., 2005). Accordingly, the decay of expected heterozygosity as measure of genetic variation accompanies the increase of genetic and geographic distance from Africa (Cann *et al*., 2002 ; Cavalli-Sforza , 2005). But the overall constant prevalence of the disease remains unexplained. The determination of the genetic background and its relationship with the phenotype may be relevant to optimize the pharmacological treatment of the disease and protocols for assisted reproduction. To define the degree of similarity of the PCOS genotypes, we show a population genetics analysis by Bayesian clustering and an evaluation of pairwise genetic distance using SNPs data from different populations, available in online databases. The genotype-phenotype link and the correlation between genetic and geographic data are discussed from an evolutionary point of view as show in Figure (1-4): (Li *et al*., 2008).

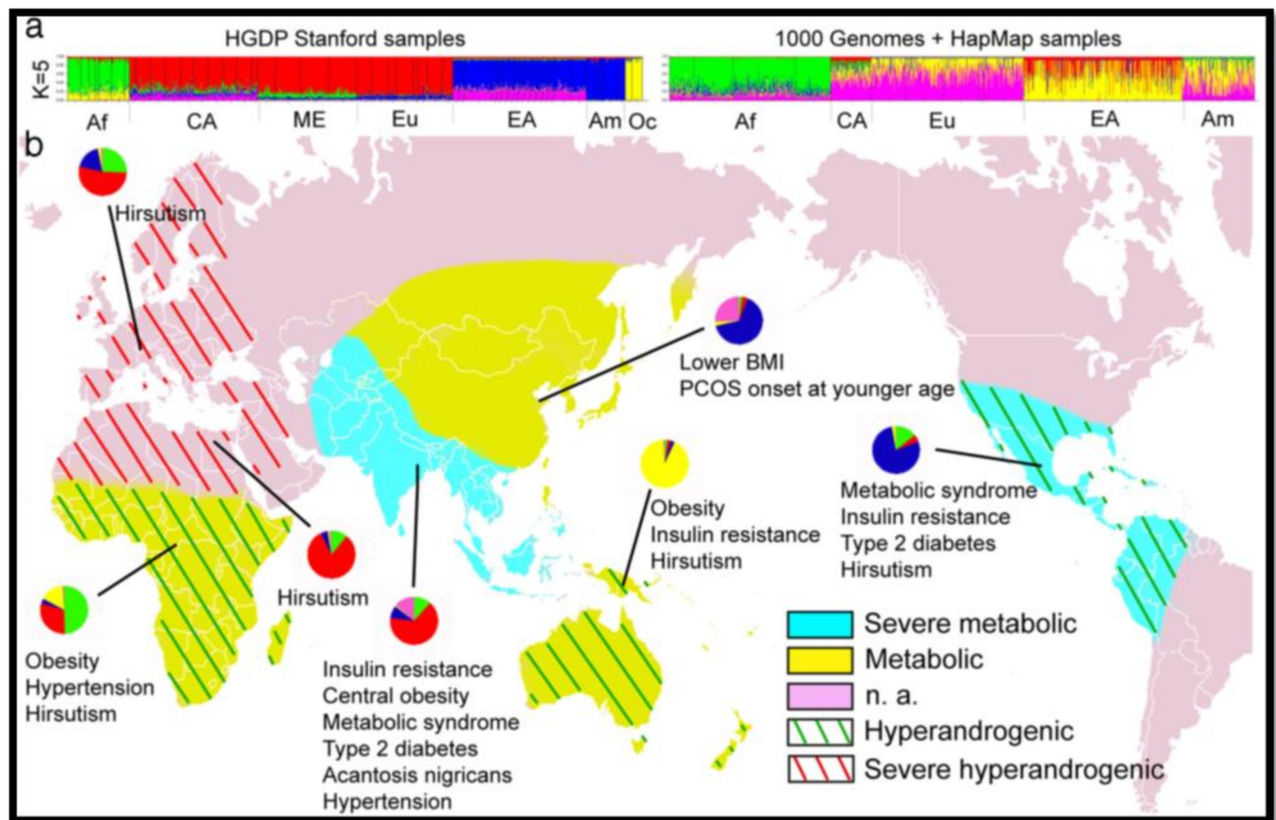


Figure (1-4): World distribution of the affinity to the genetic clusters and PCOS phenotypes prevalence. A, Bar plots of individual Q values calculated by the STRUCTURE software assign each individual to different subpopulations that matches the main world areas, with a certain degree of admixture. The analysis was performed differentially for the HGDP and the 1000 Genomes merged together with the Hap Map samples. Each color indicates the membership of individuals in a genetic cluster (K=5); Af , African ; CA , Central Asian; ME , Mediterranean/Middle Eastern ; Eu , European ; Am , American ; Oc , Oceanian. B, Pie chart of cluster affinity among continents, indicating the frequencies of the PCOS susceptibility markers. The charts were obtained as the means of the Q values by merging the HGDP, 1000 Genomes, and Hap Map populations for each genetic cluster (colors of the pie charts do not refer to panel A). The overall prevalence of a genetic cluster is different between the geographic area, suggesting a link with the corresponding PCOS phenotype and clinic features, which were obtained by a review of the literature; green, cluster 1; red, cluster 2; blue, cluster3; yellow, cluster 4; magenta, cluster 5; n.a., data not available or not assessed (Cann *et al*., 2002 ; Cavalli-Sforza , 2005).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Apparatus

Various apparatus were used in this study as listed in Table (2-1).

Table (2-1): Apparatus used in this study.

Apparatus	Company / country
Autoclave	HIRAYAMA/Japan
Bench centrifuge	Uni-Media / Korea
Conventional PCR	Techne / U.K.
Distillator	GFL / Germany
Electrophoresis equipment	JUNY1-JY200C/USA
Eppendorff-cooling centrifuge	Eppendorf / Germany
Gradient PCR thermal cycler	Techne / U.K.
Heater – magnetic stirrer	Stuart / England
Microcentrifuge	MIKRO120 – Hettich/Germany
Microtom	Lipshaw / MFG
Microwave	LG / Korea
Nanodrop spectrophotometer	Thermo / USA
Oven	Sanyo / Japan
pH meter	Martini / Japan
Sensitive balance	Mettler / Switzerland
U.V. Transilliuminator	Flowgen / U.K.
Vortex	Scientific industries / U.S.A.
Water bath	Memmert / Germany

2.1.2. Chemicals and biological materials

Various chemicals were used in the study as listed in Table (2-2).

Table (2-2): Chemicals and biological materials used in this study.

Chemicals	Company / country
Absolute ethanol	Phamacia / sweeden
Agarose	Biobasic / Canada
DNA ladder	Bioneer / Korea
Ethedium bromide	Promega /USA
Free nuclease D.W	Promega /USA
Green master mix	Promega /USA
Loading dye	Biobasic / Canada
Primers	Bioneer / Korea
Tris Borate EDTA (TBE)10X	Biobasic / Canada
xylene	Sigma/ USA

2.1.3. Kits

kits used in the study are listed in Table (2-3).

Table (2-3): The kits used in this study.

Kit	Company	Country
FavorPrep™ Tissue DNA Extraction Mini Kit	Favorgen	Taiwan
gSYNC™ DNA Extraction Kit	Geneaid	Taiwan
LH , FSH and Testosterone	Bio-merieux	France

2.1.4. Primers

The sequences of the primers used in Table (2-4).

Table (2-4):Sequences of The Primers Used.

No	Name	Oligonucleotides	Tm °C	GC%	Product size	Sequence (5'-3')	Ref.
1-	MTHFR-F (exon2)	Forward primer	59.5	65.0	198bp	AGGACGGTGCGGTGAGAGTG	Banu *et al*., 2010
	MTHFR-R	Reverse primer	63.7	56.5	-	TGAAGGAGAAGGTGTCTGCGGGA	
2-	PCO-F5 (exon5-6)	Forward primer	50.5	40.0	278bp	GGTTTTAATCCATGCCTGTT	Designed in this study*
	PCO-R5	Reverse primer	48.1	7202	-	AGTCTTGCTCAACAGGGTAG	
3-	PCO-F (exon7-8)	Forward primer	47.5	40.0	278bp	CATGTGTGTTTCCTTCTTTG	Designed in this study*
	PCO-R	Reverse primer	51.0	40.0	-	TTTCCTTCCTCAATCCAGAA	
4-	PCO-F (exon9-10)	Forward primer	36.0	50.0	284bp	CTCATCACAGATGTATTATA	Designed in this study*
	PCO-R	Reverse primer	73.9	45.0	-	GGCAGCAAGGTTAAAAATCG	

* Designing primer used in this study by Ass. Prof. Dr. Rebah Najah Jabbar/ Biotechnology research center at Al- Nahrain university

2.2. Study subjects.

This study included 50 blood samples of patients suffering from polycystic ovary syndrome and during the period from November 2015 to January 2016, from Kamal al-Samarrai Hospital/ Baghdad. Also 20

tissue samples of polycystic ovary syndrome patients were collected during period January 2016 till February 2016 from Madinat al-Amamin al-Kazimin al-Ttbbia Hospital/ Baghdad in which 20 samples were paraffin embedded tissues. 50 blood samples from healthy persons They were selected to be used as a control group. The main ages of patients were 20 to 50 and the same for the control (healthy) group under specialist of Gynecology (Appendix 1).

2.3. Collection of Samples.

2.3.1. Blood sample.

Five ml of blood was collected by vein puncture kept in EDTA tube and preserved at -20 °C until be used .

2.3.2. Paraffin embedded tissue (PE).

A total of twenty paraffinized polycystic ovary syndrome tissue samples with confirmed polycystic ovary syndrome was obtained from Madinat al-Amamin al-Kazimin al-Ttbbia Hospital. The main data and parameters included in this study were patient age, family history of polycystic ovary syndrome and pattern of the menstrual cycle.

2.4. Solutions and buffers.

2.4.1 Hormonal assay.(Appendix 2)

The hormones concentrations were measured with the VIDAS, (biomerieux, France).

2.4.1.1 FSH.

FSH levels were measured by the Bio – Merieux kit marcy I' Etoile – France, as instructed by the manufacturer.

2.4.1.2. LH.

LH levels were measured by the Bio – Merieux kit marcy I' Etoile – France , as instructed by the manufacturer.

2.4.1.3. Testosterone

Testosterone levels were measured by the Bio – Merieux kit marcy I' Etoile – France , as instructed by the manufacturer.

2.4.2 gSYNC™ DNA Extraction Kit.

The gSYNC™ DNA Extraction Kit from Geneaid (Taiwan) , ready to use that contained the following Component:

- GST Buffer
- GSB Buffer
- W1 Buffer
- Wash Buffer(100ml Ethanol was added before use)
- Elution Buffer (10 mM Tris-HCl, pH8.5 at 25ºC)
- Proteinase K
- GS Columns
- 2 ml Collection Tube

2.4.3 FavorPrep™ Tissue DNA Extraction Mini Kit.

The FavorPrep™ Tissue DNA Extraction Mini Kit from Favorgen (Taiwan) ready to use that contained the following Component:

- Proteinase K Powder
- FATG1Buffer
- FATG2 Buffer
- W1 Buffer (45 ml Ethanol was added before use)
- Wash Buffer (200 ml Ethanol was added before use)
- Elution Buffer
- FATG Columns
- 2 ml Collection tubes
- 1.5 ml Elution tubes
- Micropestles

2.5. Green Master Mix.

Go Taq® Green Master Mix is a premixed, ready to use solution containing bacterially derived Taq DNA polymerase, dNTPs, $Mgcl_2$ and reaction buffers at optimal concentration for effective amplification of DNA templates by PCR.

2.6. DNA ladder (100 bp)(Bioneer / Korea).

DNA ladder 100- 1500 bp containing 11 fragments was supplied in storage buffer 10 mM Tris-HCL pH (7.5), 0.1 mM EDTA.

2.7. Methods.

2.7.3. Sterilization methods.

- Autoclaving: Buffers and solutions were sterilized by pressure vessel (autoclave) at 121^0C and 15 bar for 15 minutes.
- Dry heat: A laboratory oven was used for glassware sterilization. Glassware was placed in the oven at 200^0C for 2 hours.

2.7.2. Specimen preparation.

- Preparation of blood sample:

  Blood sample was left for thawing then mixed thoroughly by a rotisserie shaker at room temperature.

- Preparation of PE tissues:

  1. Cut up to 25 mg paraffin-embedded tissue sample with microtome to a microcentrifuge tube (not provided).
  2. one ml of Xylene was added, mix well and incubated at room temperature for 30 min.
  3. Centrifuged at full speed (14,000) for 5 min. supernatant wasRemoved by pipetting.
  4. one ml of absolute Ethanol (96- 100 %) was added to the deparaffined tissue, mixed gently by vortexing.

5. Centrifuged at full speed (14,000) for 3 min. supernatant was Removed by pipetting.
6. Repeated step 4 and 5.
7. Incubated at 37 °C for 10 min to evaporate Ethanol residue completely.
8. Grinded the tissue sample by micropestle.

2.7.5. Extraction of DNA from samples.

2.7.5.1. Protocol for the extraction of DNA from blood samples. (Appendix 3)

The Extraction was briefly carried out as follow depending on manufacture company:

- The blood Sample was mixed thoroughly for at least 10 minutes in a rotisserie at room temperature.
- Aliquot of 20 µl of Proteinase K Solution was added into Microcentrifuge tube contain blood.
- Aliquot of 200 µl of blood was added to the Proteinase K Solution and incubate at 60°C for 5 minutes.
- Aliquot of 200 µl of GSB Buffer was added to the tube and mix by shaking vigorously by vortex. Incubate at 60°C for 5 minutes, inverting the tube every 2 minutes.
- Aliquot of 200 µl of absolute ethanol was added to the sample lysate and mix immediately by shaking vigorously for 10 seconds.
- Placed GD colum in a 2 ml collection tube.the mixture was transfer to the GD column and centrifuge for 1 minute.
- The collection tube containing the supernatant was discarded and replaced by a fresh collection tube.
- Aliquot of 400 µl of W1 Buffer was added to the GD column Centrifuged for 30 seconds.

- The collection tube containing the supernatant was discarded and replaced by a fresh collection tube.
- Aliquot of 600 µl of Wash Buffer was added to the GD Column.centrifuged for 30 seconds.
- The collection tube containing the supernatant was discarded and replaced by a fresh collection tube.
- Centrifuged again for 3minutes.
- The dried GD Column was transfer to a clean 1.5 ml microcentrifuge tube.Aliquot of 100 µl of pre-heated Elution Buffer was added to the column. Let stand for 3 minutes.Centrifuge for 30 seconds to elute the purified DNA.
- The column was discarded and the eluted was stored at -20^{0}C.

2.7.3.2. Protocol for the extraction of DNA from Paraffin-Embedded tissue. (Appendix 4)

- Aliquot of 200 µl FATG1 Buffer was added to a microcentrifuge tube contain tissue and mix well by Micropestle or pipette tip.
- Aliquot of 20 µl Proteinase K (10mg/ml) was added to the sample mixture. Mix thoroughly by vortexing.
- Incubated at 60 °C until the tissue is lysed completely (1h). Vrotex occasionally during incubation.Sample can be incubated overnight as well for complete lysis.
- Aliquot of 200 µl FATG2 Buffer was added to the sample mixture, mix thoroughly by pulse-vortexing and incubate at 70 °C for 10 min.
- Aliquot of 200 µl Ethanol (96-100%) was added to the sample mixture. Mix thoroughly by pulse-vortexing.
- Briefly spined the tube to remove drops from the inside of the lid.

- Placed a FATG Mini Column in a Collection Tube. Transfer the mixture (including any precipitate) carefully to the FATG Mini Column. Centrifuge for 1 min then place the FATG Mini Column to a new Collection Tube.
- Aliquot of 500 µl W1 Buffer was added to the FATG Mini Column. Centrifuged at full speed for 1 min then discard flow-through.
- Aliquot of 750 µl Wash Buffer was added to the FATG Mini Column. Centrifuged at full speed for 1 min then discard flow-through.
- Centrifuged at full speed for an additional 3 min to dry the column.
- Aliquot of 100 µl of preheated Elution Buffer or ddH2O (pH 7.5-9.0) was added to the membrane of the FATG Mini Column. Stand the FATG Mini Column for 3 min.
- Centrifuged at full speed for 2 min to elute DNA.
- Store total extracted DNAat 4°C.

2.7.5.3. Quantitation of DNA Concentration.

Ananodrop spectrophotometer (Thermo / USA) was used to estimate the concentration and purity of the extracted DNA (from blood ,tissue of patients and control) according to the following procedure:

Concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50µg/ml

2.7.5.3.1. Sample measurement.

1- Two µl of the sample was pipetted on to the measurement pedestal while the sample arm is opened.

2- The sampling arm was laid down and the solution was observed bridging the gap between both optical fibers.

3- The button (Measure) was clicked and the measurement result appeared on the screen within 3 sec.

4- When any sample gives purity less than 1.5 and /or concentration less than 10 ng/µl DNA extraction from the blood of that sample re-performed until the desired purity and concentration were obtained.

2.9.6. Amplification of MTHFR and PCO genes.

For PCR amplification of MTHFR and PCO genes the following PCR protocols was followed:

1. The Go Taq® Master (Promega / USA) Mix was thawed at room temperature. The master mix was mixed by over taxing then it was spined briefly in a microcentrifuge.

Component of Taq® Master mix are illustrated in Table (2-5) .

Table (2-5): Component of Taq® Master mix.

No.	Component	concentration
1-	Taq polymerase	2.5µl
2-	dNTP(dATP, dCTP, dGTP, dTTP)	250µM
3-	Tris – BASE (pH 9.0)	10 mM
4-	KCl	30mM
5-	$MgCl_2$	1.5mM

40The reaction mixture was prepared by adding the components listed in Table (2- 6).

Table (2-6): The Optimal Values of Various Steps in The Amplification.

Components	Volume	Concentration
Go Taq® Green Master Mix(2X)	12.5 µl	1x
Forward primer	0.2 µl	0.2 µM

Reverse primer	0.2 µl	0.2 µM
DNA template	3 µl	> 250 ng
Nuclease free water	9.1 µl	-
Final volume	25 µl	

3.The mixture was put in microcentrifuge and spinned for better mixing.

4.After mixing the master mix tubes were transferred to the thermocycler (Techne 32 thermal block / UK) which was previously programmed with the above protocol according to the MTHFR and PCO genes.

2.7.4.1. Optimal protocol of PCR amplification.

For PCR amplification of MTHFR and PCO genes annealing temperature in optimizing the following PCR protocols as followed :

PCR Amplification using R1 primer

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^{0}C for 5 minute
Denaturation	35cycle	94^{0}C for 30 second
Annealing		61C for 30 second
Extension		72^{0}C for 30 second
Final Extension	1 cycle	72^{0} C for 10 min.

PCR Amplification using R2 primer

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^{0}C for 5 minute
Denaturation	35cycle	94^{0}C for 30 second
Annealing		55^{0}C for 30 second

Extension		72^0C for 30 second
Final Extension	1 cycle	72^0 C for 10 min.

PCR Amplification using R3 primer

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 5 minute
Denaturation	35cycle	94^0C for 30 second
Annealing		53°C for 30 second
Extension		72^0C for 30 second
Final Extension	1 cycle	72^0 C for 10 min.

PCR Amplification using R4 primer

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 5 minute
Denaturation	35cycle	94^0C for 30 second
Annealing		52^0C for 30 second
Extension		72^0C for 30 second
Final Extension	1 cycle	72^0 C for 10 min.

2.8. Agarose Gel Electrophoresis (Sambrook and Russel, 2001).

1. Two percentage of agarose gel was prepared by adding: 100ml TBE 1X and 2 gm agarose in a glass bottle. A glass bottle was heated on hot plate with heater until the components were dissolved in microwave.
2. This solution was cooled to 60°C, 2 µl ethidium bromides was added from stock solution and mixed thoroughly.

3. The clean glass mold was set on a horizontal section of the bench. The comb was set in position 0.5-1.0 mm above the surface of the mold so that a complete well was formed when agarose was added.
4. The warm agarose – solution was poured into the mold.
5. After the gel was completely set (20-30 min. at room temperature), the comb was carefully removed and the gel mounted in the electrophoresis tank which contain previously small amounts of 1X TBE buffer.
6. A volume of 600ml of(1X TBE and D.W.)was added to cover the gel in depth about 1mm.
7. A volume of 10 µl of the sample of DNA (PCR product) was added slowly into the slots of the submerged gel using a micropipette.
8. A volume of 4 µl of DNA marker was set slowly into the slots of the submerged gel using a micropipette.
9. The gel was subjected to electrophoresis at 70 volts until the bromo phenol blue tracking dye, migrated at least two-thirds of the way down the gel.
10. The gel was examined by ultraviolet light using a UV transilluminator then photographed.

2.9. DNA sequencing.

The purified PCR products of the analyzed MTHFR and PCO genes regions and primers were sent to Macrogen company in Korea for DNA Sequencing. The obtained sequences of these samples were aligned using (Mega-6) software. Furthermore, the nucleotide sequences were compared to the information in gene bank of the National Center for Biotechnology Information (NCBI) web site databases using the BLAST search tool and examined for the presence of SNPs.

2.10. Statistical analysis.

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters . Least significant difference –LSD test (T-test) was used to significant compare between means in this study.

Chapter Three

Results

and

Discussion

3. Result and discussion.

3.1. The distribution of the studied groups.

In this study,fifty samples of blood have been collected from female patients who had Polycystic ovary syndrome which showed the difference in factors of the Patients age and hormones. Also fifty samples have been collected from healthy female which age was from (20-50) years. Chi-square test was used to significant compare between percentages in this study depending on SAS (SAS, 2012).

The 50 patients were divided into three groups according to the ages as shown in the Table (3-1).

Table (3-1): Level of LH, FSH and Testosterone in blood samples from Polycystic Ovary syndrome women at different ages.

Parameters	Age group (year)			LSD	
	20-30	31-40	41-50		Control
LH(IU/mI)	5.32±0.83	5.51 ± 0.79	7.48 ± 1.40	0.974	2.78±0.26
FSH(IU/mI)	4.97±0.64	3.13 ± 0.32	2.30 ± 0.46	0.627	5.41±0.37
Testosterone(ng/ml)	0.585±0.09	0.696 ±0.09	0.518 ± 0.08	0.825	0.205±0.04

Test of fertility hormones, (LH, FSH and Testosterone) were done for all patients groups to check the effect of PCOS on their level. It was shown from the Table (3-1) that the elevation in LH hormone was more in the age group (41-50) years old among the other two groups (20-30), (31-40),with a mean ±standard deviation of 7.48 ± 1.40 , but the elevation was less in the group (20-30) in which the mean was5.23 ± 0.83 . In contrast, for the hormone FSH it was noticed that this hormone decreased significantly in the age groups (41-50) as the difference of level of this hormone was more than the LSD value which

was 0.627.for the hormone Testosterone it was noticed that this hormone increased significantly in the age groups (31-40) as the difference of level of this hormone was more than the LSD value which was 0.825. The results agreed with (Yildiz *et al*., 2012) who reported that PCOS is a complex, multifactorial endocrine disorder affecting approximately 5% to 10% of all women of reproductive age. It is believed that the reason for this is due to genetic differences and geographic locations in addition to the environmental conditions and surrounded by physical and chemical effects. The etiology of PCOS is still unclear, but environmental and genetic factors may contribute to the pathogenesis of PCOS. It may be explained by the existence of a vicious perpetual circle of pathological effects, where androgen excess favoring visceral abdominal fat disposition facilitates an increased secretion of androgens by the ovaries and/or the adrenal glands (Escobar-Morreale and San Millan , 2007).

3.2. Effect of Polycystic Ovary Syndrome on Fertility Hormones.

3.2.1. Luteinizing Hormone (LH).

Table (3-2) showed the mean distribution of the glycoprotein hormone concentrations Luteinizing Hormone (LH) to the females patients when compared with the mean concentration of the normal controls, a highly significant difference ($P<0.01$). The LH hormone level increased in PCOS patients.

Table (3-2): Level of LH in blood samples of PCOS women and healthy controls

Group	No.	Mean ± SE (IU/ml)
Patients	50	5.99 ± 0.58
Control (Healthy)	50	2.78 ± 0.23
T-test	---	1.873 *
P-value	---	0.00248
* (P<0.01).		

From the same Table (3-1) it is found that the mean of Luteinizing Hormone (LH) hormone level in PCOS patients is 5.99 ± 0.58, while for control healthy is 2.78 ± 0.23. This higher difference is statically significant as the difference between the mean value of two groups (control and patients) is higher than the LSD (T-test) value which is 1.873**.The presence of hypothalamic dysfunction is suggested by the increase in LH pulse frequency that has been described in this and numerous previous studies (Arroyo *et al.,* 1997 ; Allahbadia and Merchant, 2011) listed additional probable causes of LH hypersecretion:

- Aromatization of Androgens to Estrogens, resulting in permanent estrogen overproduction, which favors LH hypersecretion.
- Direct leptin-induced GnRH modulation.
- An insulin-mediated increase in serum LH pulse amplitude.

3.2.2. Follicle Stimulating Hormone (FSH).

Table (3-3) showed the mean distribution of the glycoprotein hormone concentrations Follicle Stimulating Hormone (FSH) to the females patients when compared with the mean concentration of the normal controls, a highly significant difference ($P<0.05$).

Table (3- 3): Level of FSH in blood samples PCOS women and healthy controls

Group	No.	Mean ± SE (IU/ml)
Patients	50	4.44 ± 0.19
Control (Healthy)	50	5.42 ± 0.26
T-test	---	0.633 **
P-value	---	0.0419
** (P<0.05).		

The FSH hormone level decreased in PCOS patients. From the same table it is found that the mean of FSH hormone level in PCOS patients is 4.44 ± 0.19 , while for control healthy is 5.42 ± 0.26. This higher difference is statically significant as the difference between the mean value of two groups (control and

patients) is higher than the LSD (T-test) value which is 0.633. The current results agreed with (Nawras , 2010) who showed that no significant differences (p>0.05) in level of FSH. The reduction levels of FSH can be explained by the increase of the conversion of androstenedione in adipose tissue which additionally stimulates LH and inhibits FSH (Marx and Mehata , 2003).

On the other hand, the FSH/LH ratio was significantly high in PCOS patients 1.165± 0.08 rather than in the healthy control groups 0.693 ± 0.10 when the difference of mean value is higher than of LSD (T-test) which is0.352 *. In contrast the ratio was not significant between POCS age groups and healthy control. This is in agreement with (Mukherjee et al., 1996 ; Taylor *et al*., 1997 ; Nawras , 2010) who detect non significant FSH\LH ratio .

3.2.3. Testosterone hormone.

The same was also for the other fertility hormone (Testosterone), shown Table (3-4). In which it increased in PCOS patients rather than in the healthy control groups when the difference of mean value is higher than of LSD (T-test) which is 0.277 *.

Table (3-4): Level of Testosterone in blood samples PCOS women and healthy controls

Group	No.	Mean ± SE (ng/ml)
Patients	50	0.620 ± 0.04
Control (Healthy)	50	0.205 ± 0.03
T-test	---	0.277 *
P-value	---	0.0061
* (P<0.01).		

Androgen overproduction (testosterone) often results from overproduction of LH (luteinizing hormone) which is produced by the pituitary gland. Excess of LH will stimulate ovaries to produce more testosterone when insulin level in the blood is high (Yousouf *et al*., 2012).

3.3. Molecular Detection of Polycystic Ovary Syndrome by PCR technique.

In this study four primer sets were designed using the NCBI primer-Design online tool in order to amplify a specific region(exon 2) in the MTHFR and (exon 5,6,7,8,9,19) PCO genes by using a routine PCR technique.

3.3.1. Concentration and purity of DNA extracted from blood samples and Paraffin embedded tissues (PE).

Genomic DNA was extracted from all samples of patients using gSYNC™ DNA extraction kit for frozen blood and FavorPrep™ Tissue DNA Extraction Mini Kit, for Paraffin embedded tissues (PE) the concentration and purity of DNA using Nano drops as shown in the Table (3-5).

Table (3-5): Concentration and purity of DNA from patient samples

Type of patient sample	DNA conc.	DNA purity
PE tissue	50-100 ng/ µl	1.6
Frozen blood	50-120 ng/ µl	1.7-2.0

It is clear that this quantity and purity are suitable for amplification by PCR because higher amounts of DNA template increase the risk of generating nonspecific PCR products and lower amounts of product reduce the accuracy of the amplification. Also, DNA samples have been analyzed by gel electrophoresis in which the bands give an indicator that DNA pure and ready to be subjected to PCR amplification.

The genomic DNA, which extracted from blood and tissue of polycystic ovary syndrome women, showed good concentration, as shown in Figure (3-4).

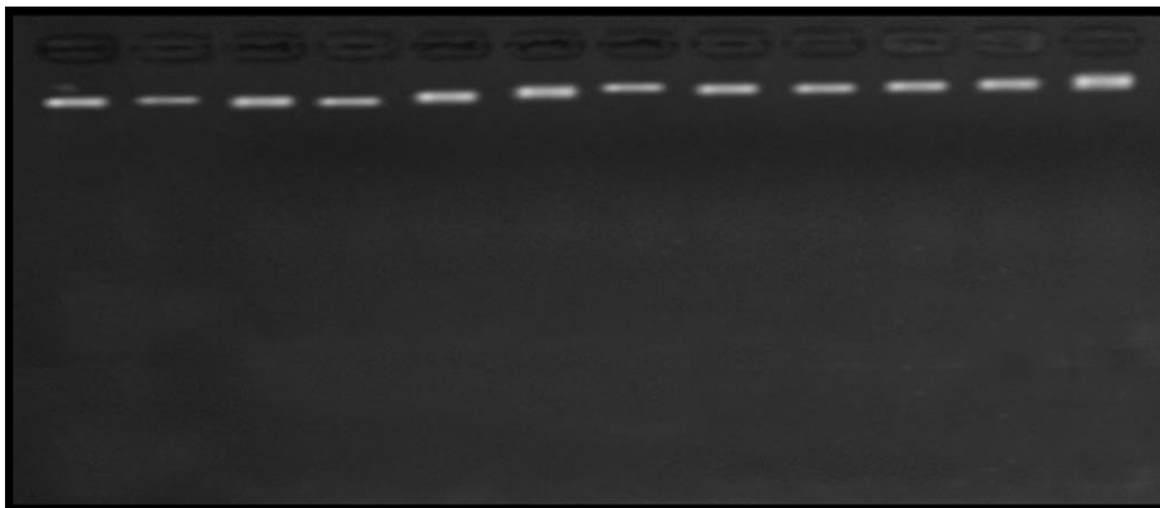


Figure (3-4): Chromosomal DNA Bands on agarose gel electrophoresis (0.8 % agarose gel, at 100volt, 60 minutes).Visualized under U.V light after staining with Ethidium Bromide.

3.3.2 MTHFR and PCO genes amplifications.

All samples (blood and Paraffin embedded tissues (PE) were subjected to molecular detection through PCR amplification of the MTHFR and PCO genes by using four specific primer. The first primer was used in this PCR technique (MTHFR), the amplifies in exon (2) specific for MTHFR gene design with product lengths(198bp) which is shown in figure (3-5).

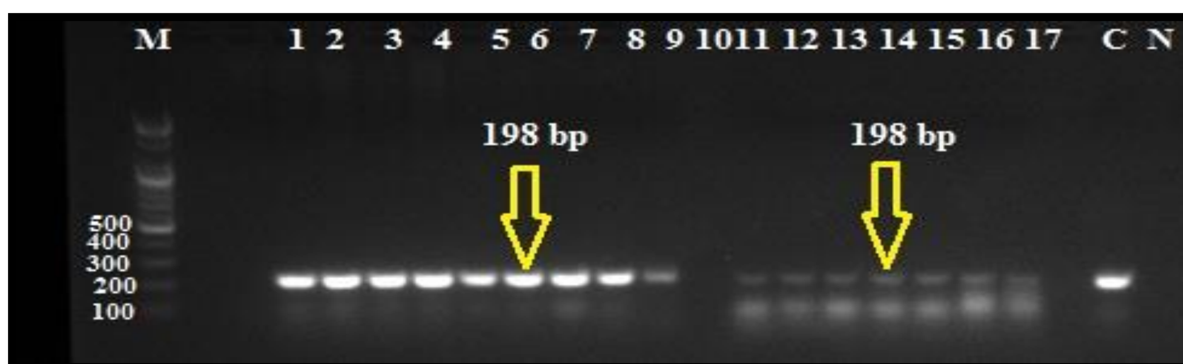


Figure (3-5): PCR product for MTHFR primer, agarose gel electrophoresis (2% agarose gel, 10 minutes at 100 voltage and then lowered to 70 Volts, 80 minutes). Visualized under U.V light after staining with ethidium bromide Lane M: 100bp marker, lane1-9: DNA isolated from blood samples of patients, lane 11-17 DNA isolated from PE tissue, lane C: from control (healthy) and lane N: from negative control.

Primer (MTHFR) used in this study were previously designed to amplify the MTHFR gene, show the blood samples give band in PCR product (198bp).and PE tissue samples give band in PCR product (198bp).

The second primer set used in this PCR technique (PCO) the amplifies in exon (5) and exon (6) of PCOS from NCBI with product size (278bp) which is shown in the figure (3-6).

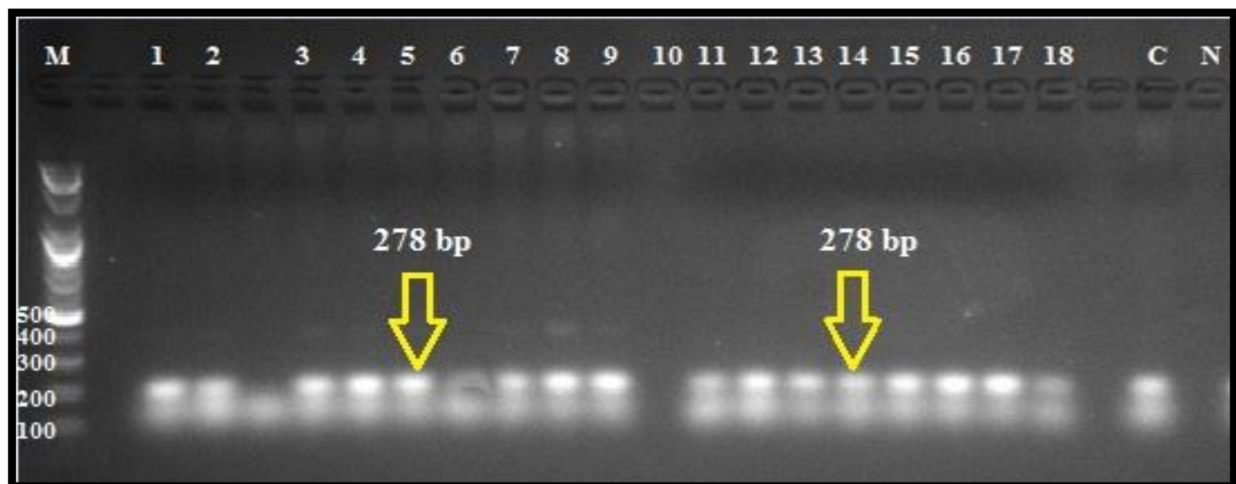


Figure (3-6): PCR product for PCO primer, agarose gel electrophoresis (2% agarose gel, 10 minutes at 100 voltage and then lowered to 70 Volts, 80 minutes). Visualized under U.V light after staining with ethidium bromide Lane M: 100bp marker, lane1-9: DNA isolated from blood samples of patients, lane 11-18 DNA isolated from PE tissue, lane C: from control (healthy) and lane N: from negative control.

Primer (PCO) used in this study were previously designed to amplify the PCOS, show the blood samples give band in PCR product (278bp).and PE tissue samples give band in PCR product (278bp).

The third primer set used in this PCR technique (PCO) the amplifies in exon (7) and exon (8) of PCOS from NCBI with product size (278bp) which is shown in the figure (3-7).

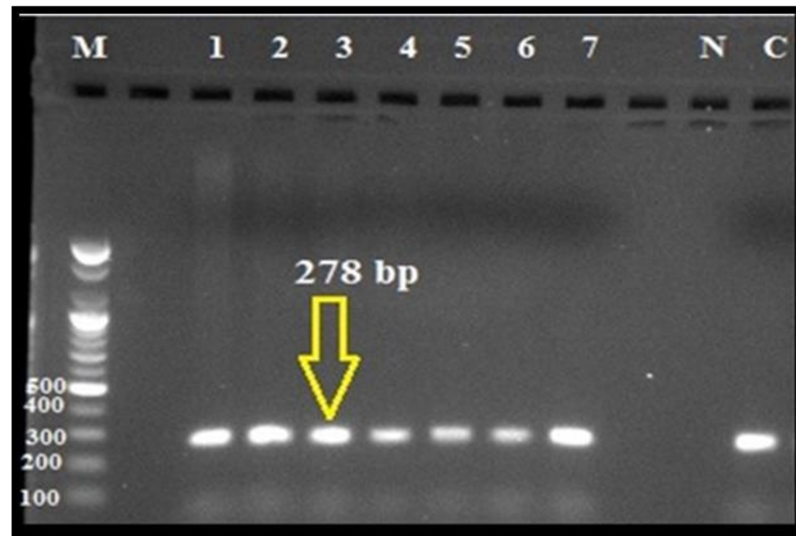


Figure (3-7): PCR product for PCO primer, agarose gel electrophoresis (2% agarose gel, 10 minutes at 100 voltage and then lowered to 70 Volts, 80 minutes). Visualized under U.V light after staining with ethidium bromide Lane M: 100bp marker, lane1-4: DNA isolated from blood sample of patients, lane 5-7 DNA isolated from PE tissue, lane N: from negative control and lane C: from control (healthy).

Primer (PCO) used in this study were previously designed to amplify the PCOS, show the blood samples give band in PCR product (278bp).and PE tissue samples give band in PCR product (278bp).

The fourth primer set used in this PCR technique (PCO) the amplifies in exon (9) and exon (10) of PCOS from NCBI with product size (284bp) which is shown in the figure (3-8).

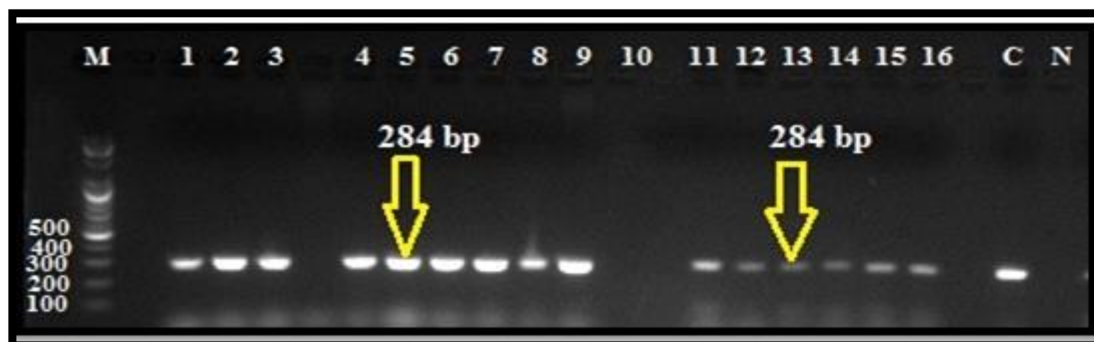


Figure (3-8): PCR product for PCO primer, agarose gel electrophoresis (2% agarose gel, 10 minutes at 100 voltage and then lowered to 70 Volts, 80 minutes). Visualized under U.V light after staining ethidium bromide Lane M: 100bp marker, lane1-9: DNA isolated from blood sample of patients, 1ane11-16: DNA isolated from PE tissue, lane C: from control (healthy) and lane N: from negative control.

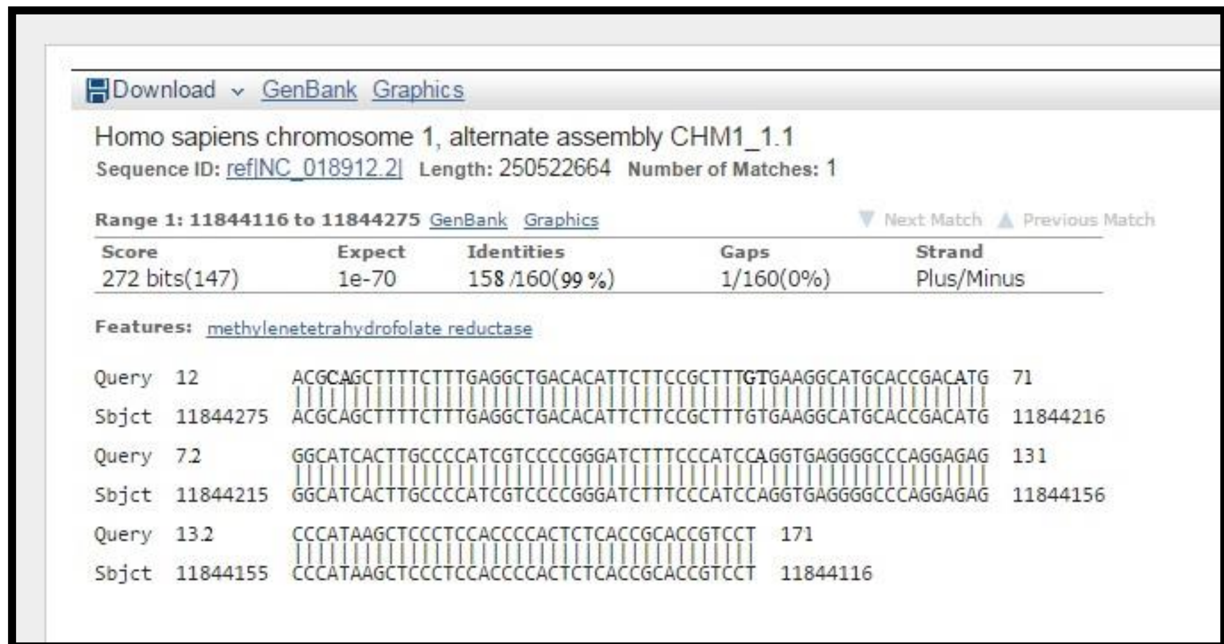
Primer (PCO) used in this study were previously designed to amplify the PCOS, show the blood samples give band in PCR product (٢84bp). and PE tissue samples give band in PCR product (284bp).

3.4. Detection of MTHFR Gene and PCO Gene Mutations by sequencing.

After amplification of genomic fragments corresponding to exon (2) of the MTHFR gene and exon(5,6,7,8,9,10) for PCO gene the PCR products were (198bp) for the MTHFR gene and (278, 278, 284bp) for the PCO as shown in the figures. By using the DNA of the above cases good quality products (pure and concentrate) were selected to be sequenced in order to evaluate if any genetic variation in the MTHFR gene and PCO were known as predictors of PCOS high risk.

3.4.1. Type of mutations.

The First DNA sequence of the MTHFR gene located in exon (2) was taken from blood samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (3-9A,B and C).



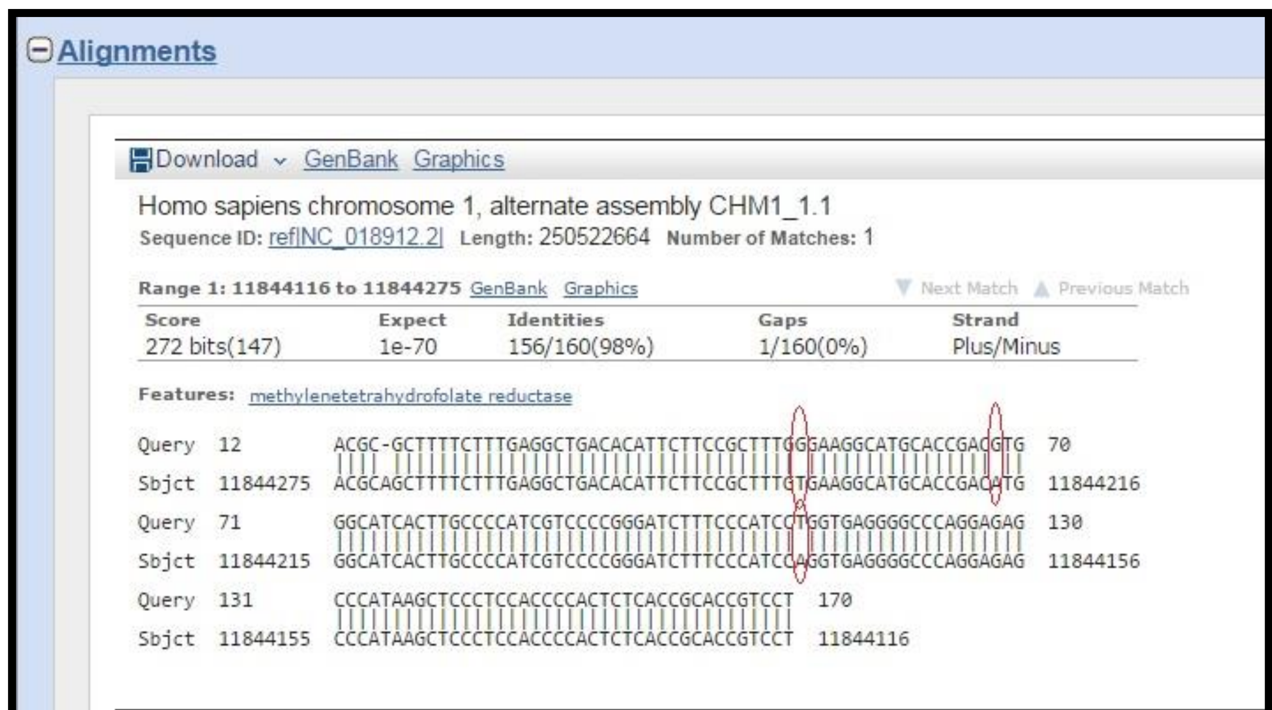


Figure (3-9A): The automated sequencing comparison of MTHFR gene for DNA isolated from blood and healthy samples, red circle indicate the position at which mutation occur.

Representation of the sample by query and the subject representation of database of the National Center Biotechnology Information.

The region of the gene of MTHFR, there are many SNPs, 3 SNPs in which are the first encode for a missense mutation (substitution) T/G in position 51 that convert a.a Cys to Pro, the other substitution A/G in position 68 that convert a.a His to Arg, also in position 111 substitution occur A/ T which convert a.a Pro to Ser.

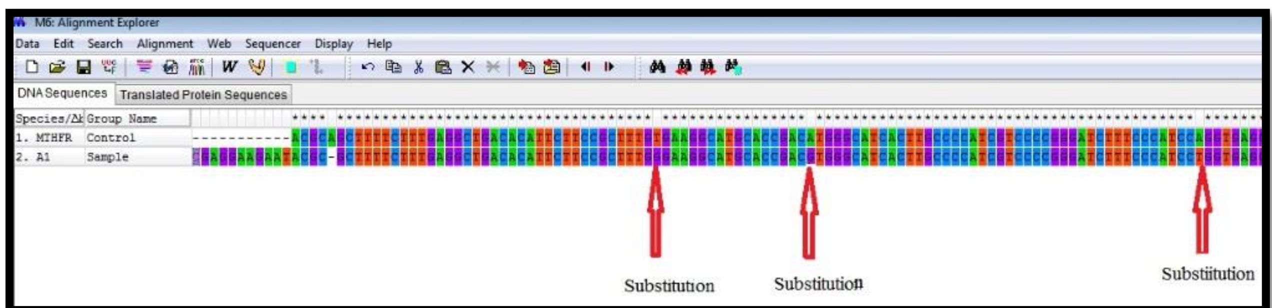
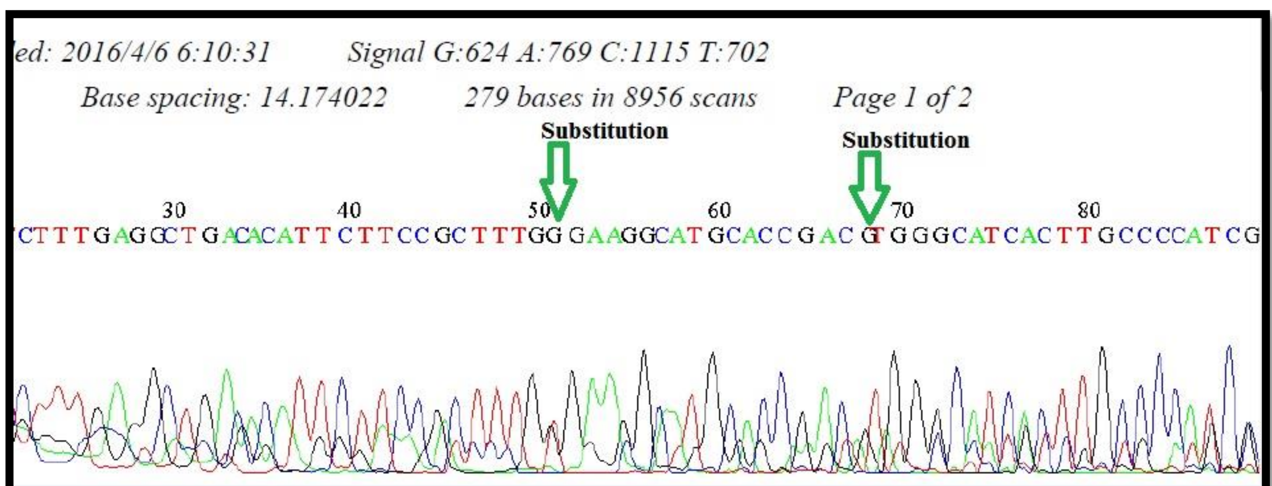
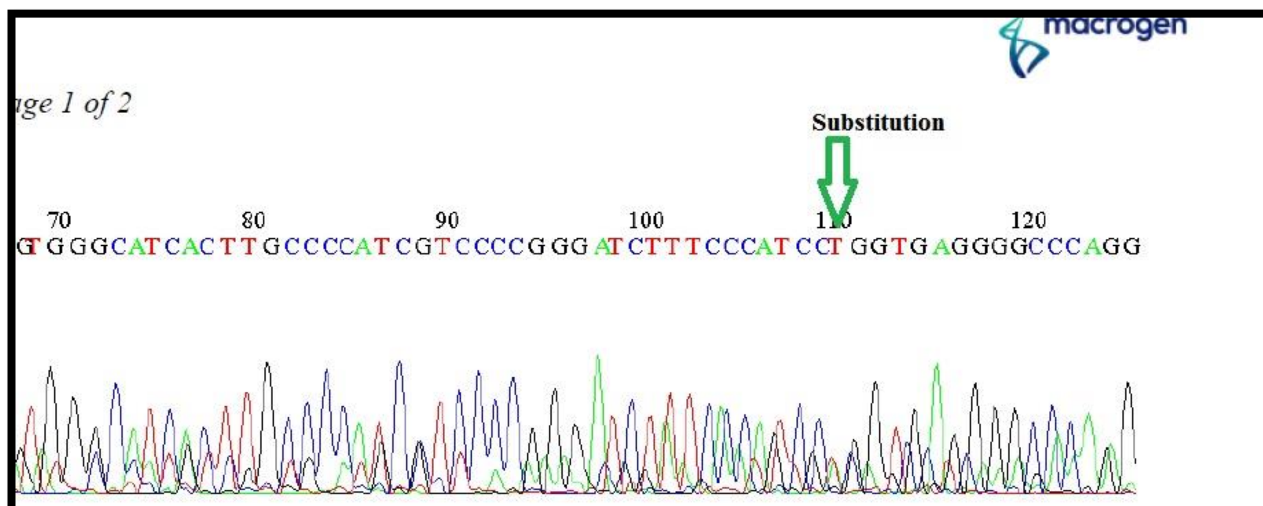


Figure (3-9B) :comparing between control (NCBI nucleotide blast) and Polycystic ovary syndrome patient in the MEGA 6 program(for blood).

The peaks that appear the mutations are clear in figure (3-9C).





CTTTGAGGCTGACACATTCTTCCGCTTTGGGAAGGCATGCACC

GACGTGGGCATCACTTGCCCATCG

GTGGGCATCACTTGCCCATCGTCCCCGGGATCTTTCCCATCCT

GGTGAGGGGCCCAGG

Figure (3-9C): A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution region. (Appendix 5)

The First DNA sequence of the MTHFR gene located in exon (2) was taken from tissue samples of patient and was compared using the NCBI nucleotide blast,as shown in the figure (3-10 a,b and c).

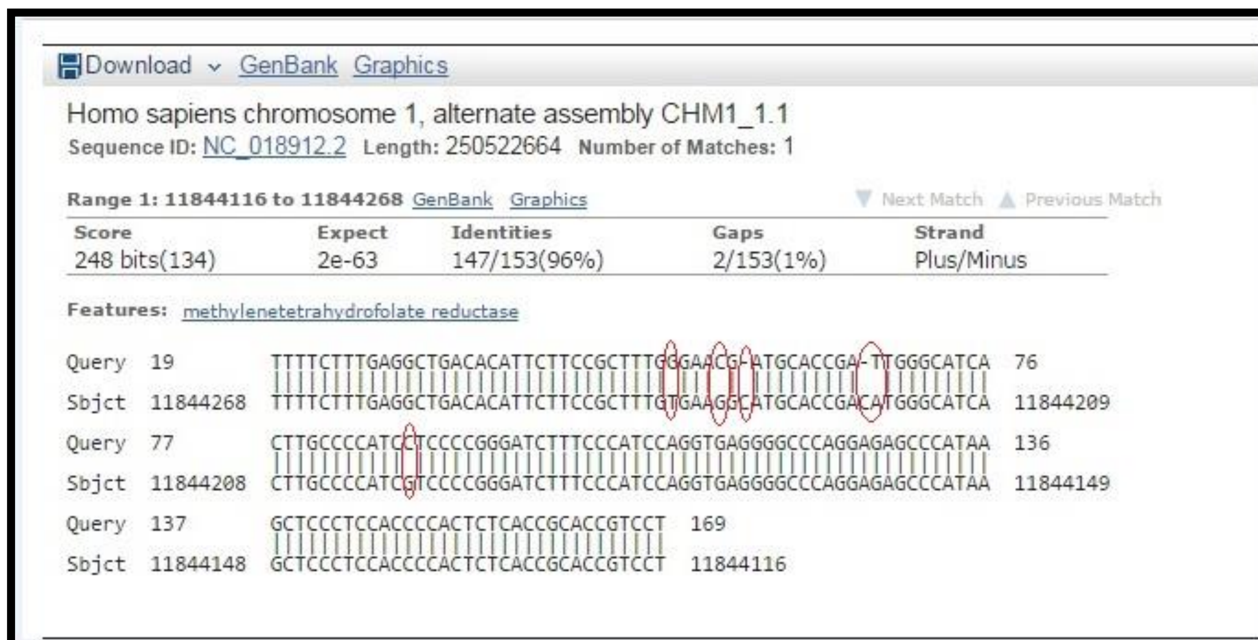


Figure (3-10a): The automated sequencing of MTHFR gene for DNA isolated from tissue.

There are many heterozygous SNPs in patient samples 6 SNPs in this region of the gene are: in position 51 T/ G which convert a.a Val to Ala, but in position 55 the G changed to C that convert a.a Arg to Thr. In position 56-57 C / - that convert a.a His to deletion. In position 65-66 C/- that converts a.a Thr to deletion, and the other common SNP is A/T in position 66 which converts a.a Thr to Thr. The last SNP was G/C in position 87 that convert a.a Arg to Pro.

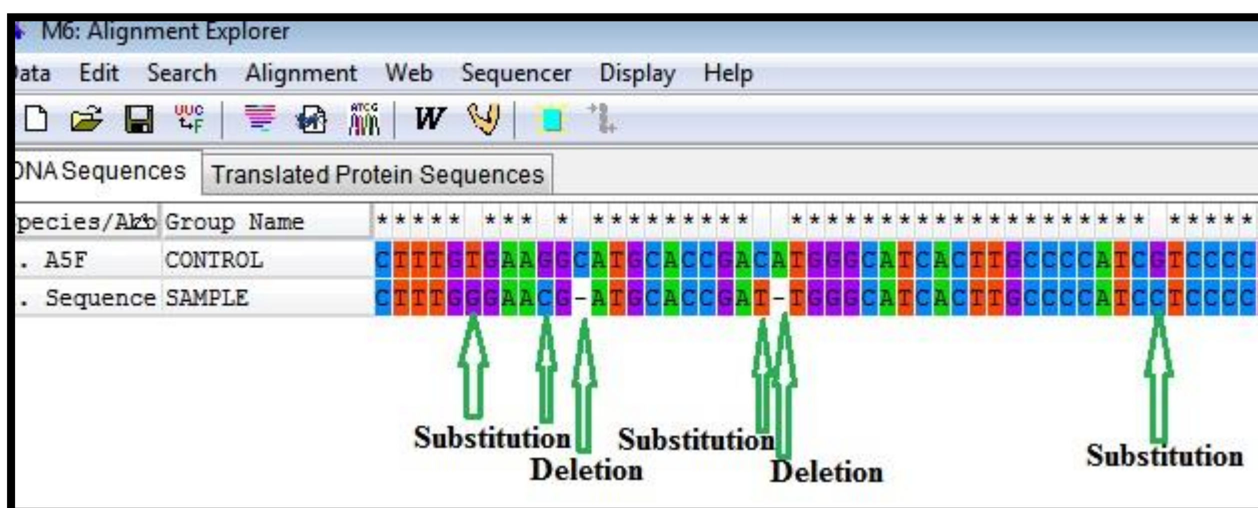
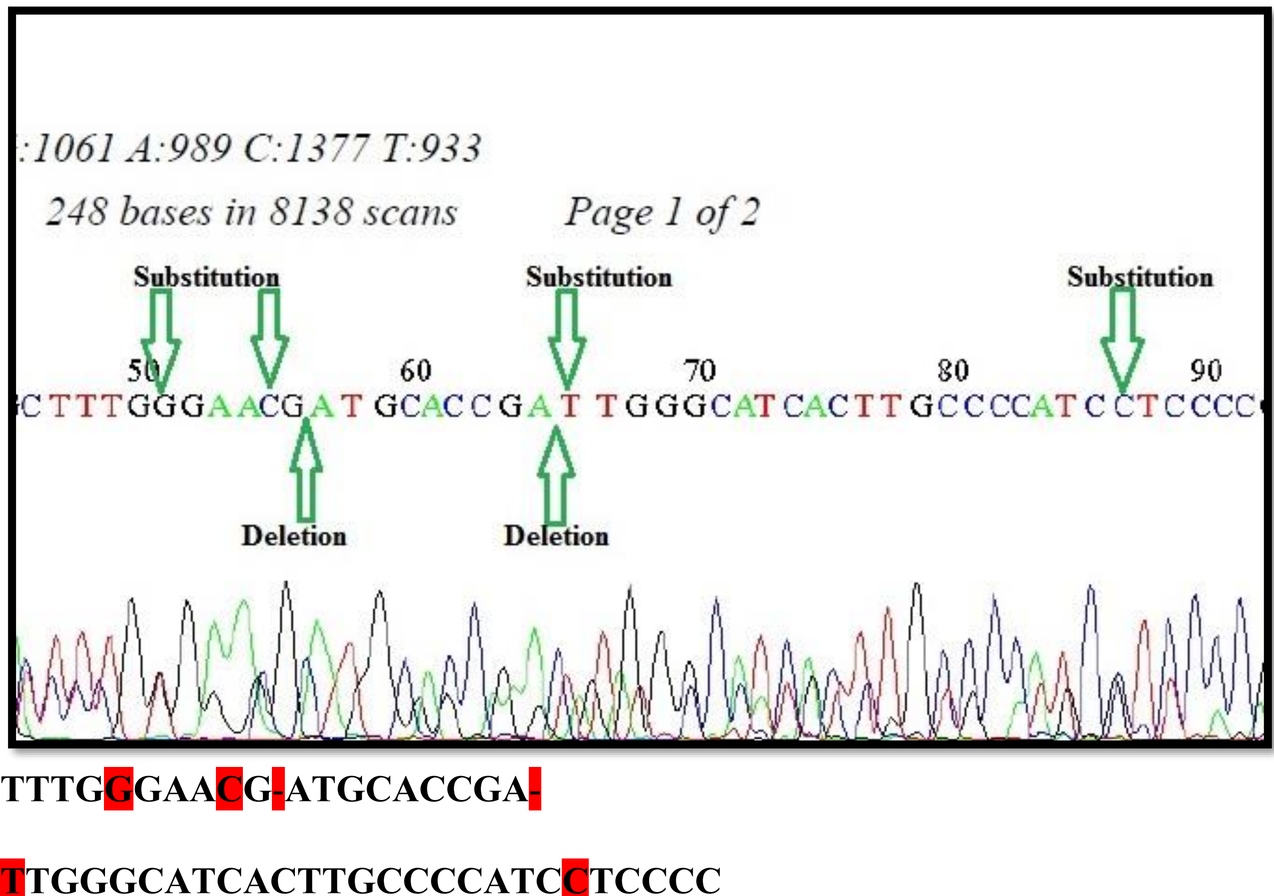


Figure (3-10b) :comparing between control (NCBI nucleotide blast) and Polycystic ovary syndrome patient in the MEGA 6 program(for tissue).



TTTGGGAACG-ATGCACCGA-

TTGGGCATCACTTGCCCATCCTCCCC

Figure (3-10c): A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution and deletion region. (Appendix 6)

The Second DNA sequence of PCO gene located in exon (5) and (6) was taken from blood samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (3-11 A, B and C).

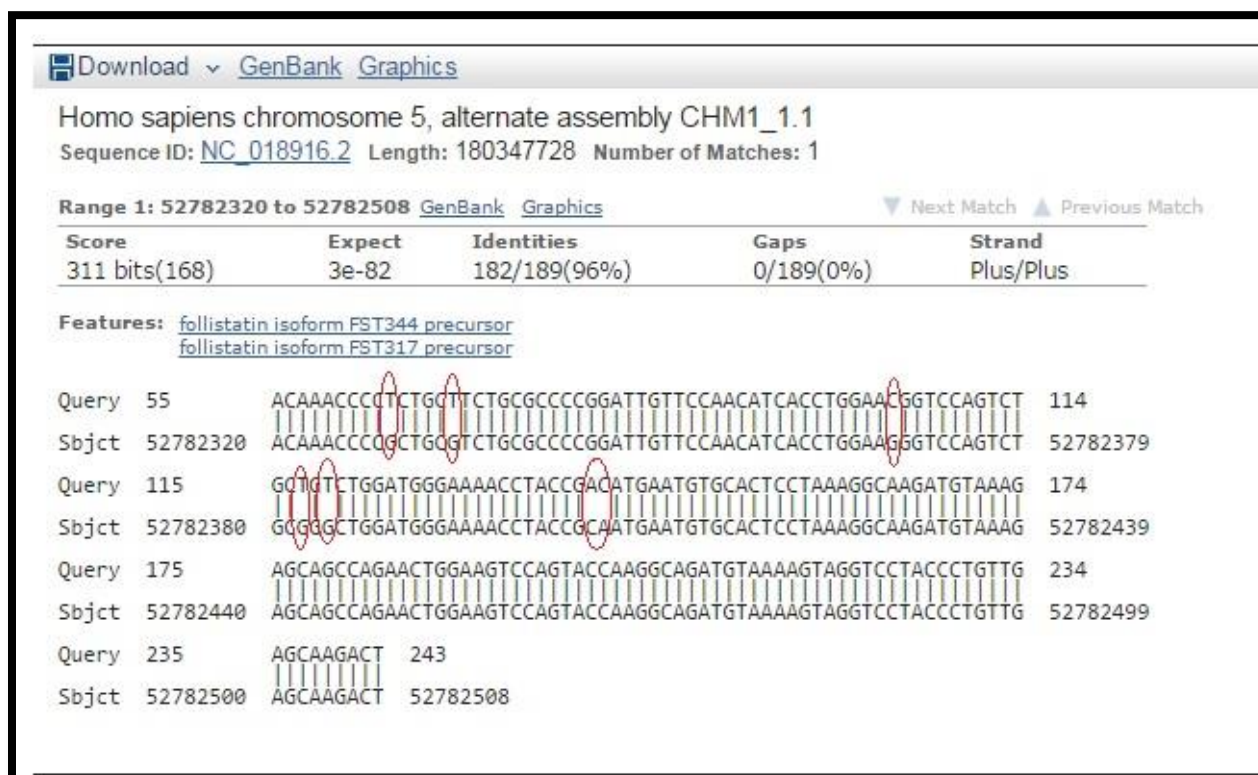


Figure (3-11A): The automated sequencing of PCO for DNA isolated from blood red circle indicate the position at which the mutation occur.

The 7 SNPs of PCOS patients are as follows: where one is substitution mutation in which G/T in position 64 that convert a.a Ala to Ser. In the same region of PCOS patients , there is a substitution mutation in position 69 G/T that convert Arg to Leu. Substitution mutation is in the position104 G/C that convert Gly toVal. Also Substitution in G/T in position 117 that convert a.a Ala to Asp.in position 119 there is substitutin G/T that convert a.a Gly to Val.another substitution mutation is in position 140 and 141CA/AC that convert a.a Gln to Thr.

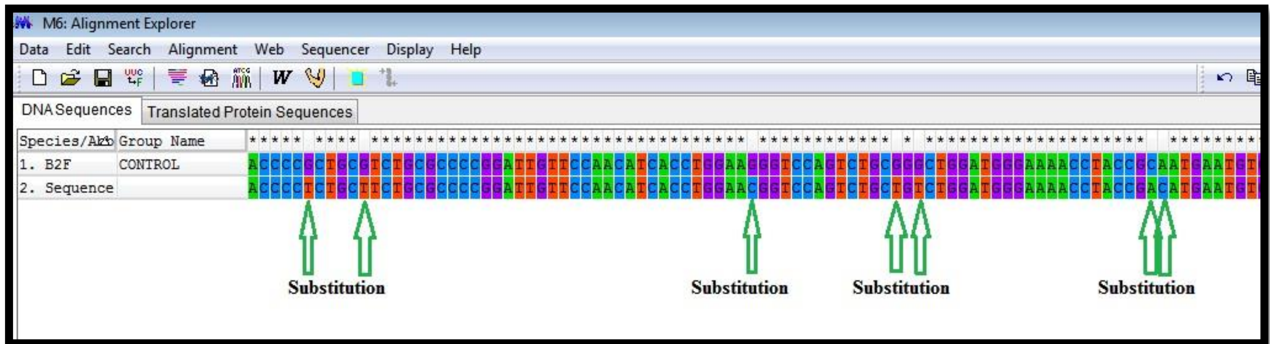
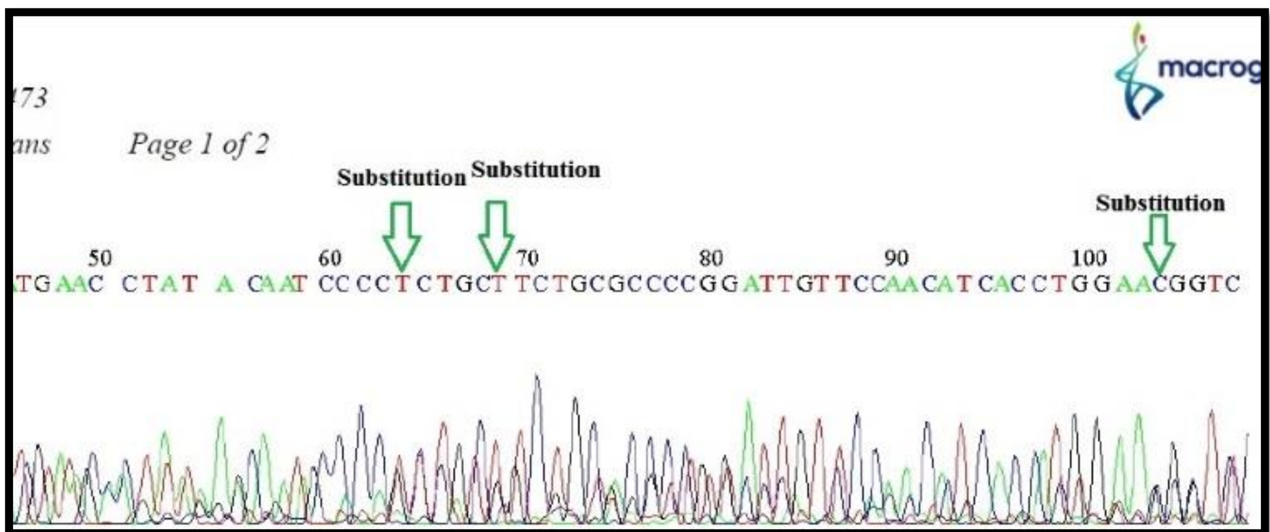
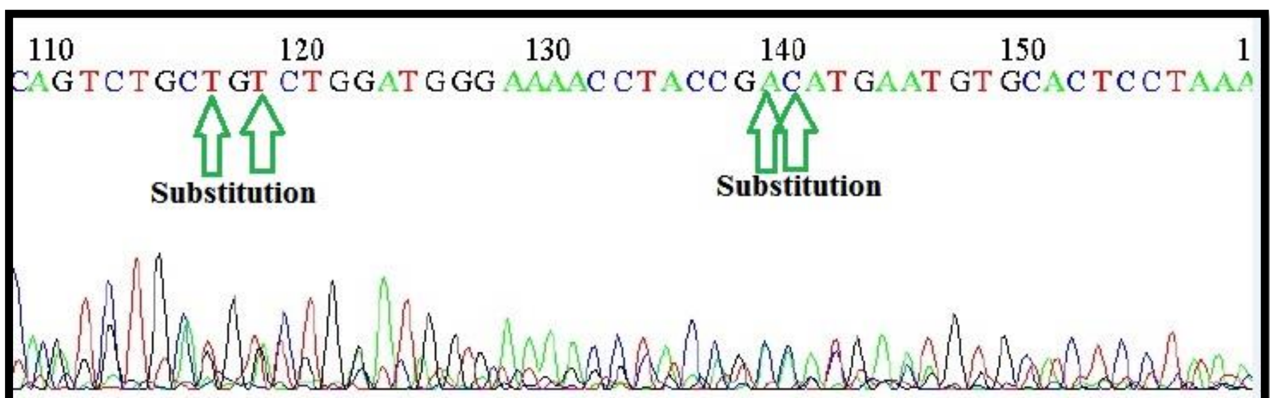


Figure (3-11B) :comparing between control (NCBI nucleotide blast) and Polycystic ovary syndrome patient in the MEGA 6 program(for blood).





TGAACCTATACAAACCCCTCTGCTTCTGCGCCCCGGATTGTTCCA ACATCACCTGGAACGGTC

CAGTCTGCTGTCTGGATGGGAAAACCTACCGACATGAATGTGCA CTCCTAAA

Figure (3-11C): A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution region. (Appendix 7)

The Second DNA sequence of PCO gene located in exon (5)and(6) was taken from tissue samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (3-12 a,b and c).

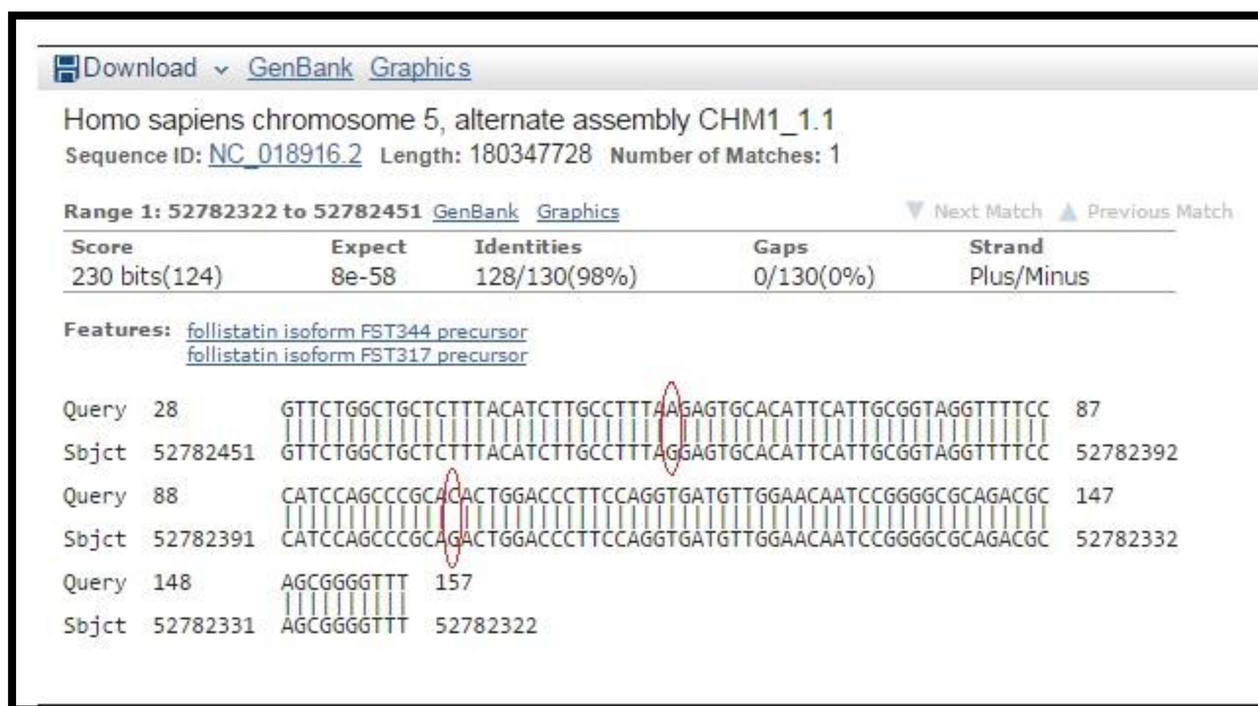


Figure (3-12a): The automated sequencing of PCO for DNA isolated from tissue.

The region of the gene of PCOS patients, there are two SNPs, in which are G/A, in position 58 that convert a.a Gly to Arg, the other G/C in position 101that convert a.a Arg to Thr.

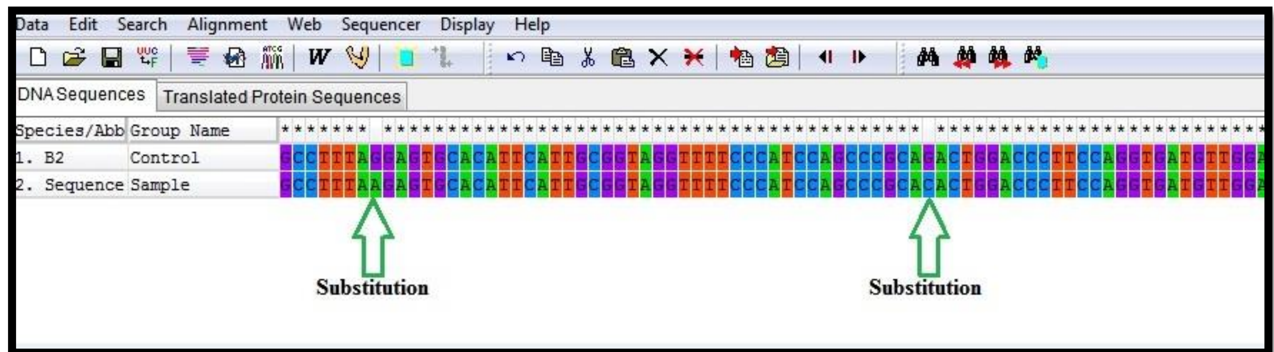
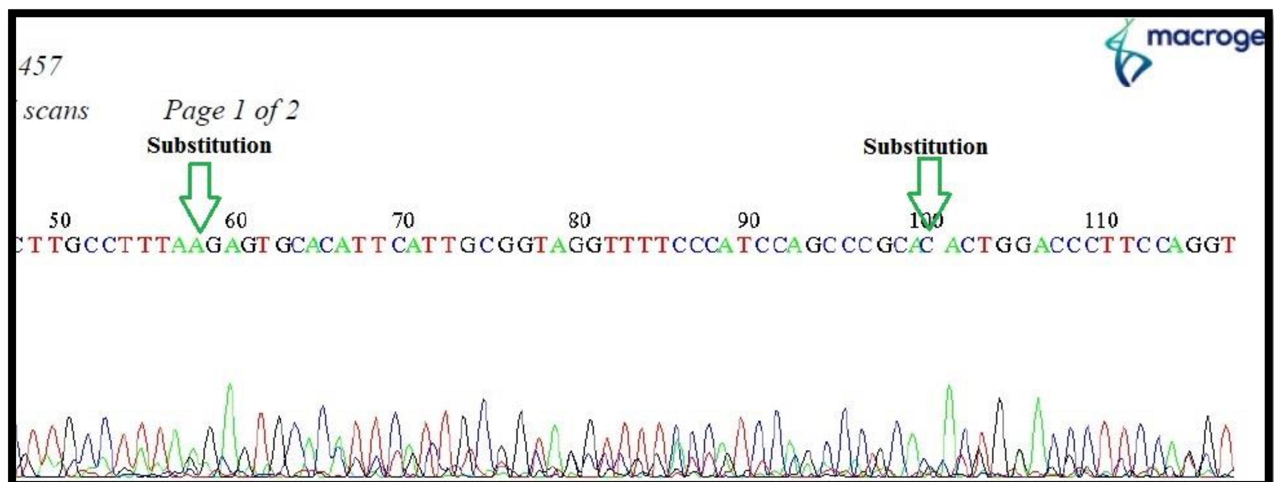


Figure (3-12b) :comparing between control (NCBI nucleotide blast) and Polycystic ovary syndrome patient in the MEGA 6 program(for tissue).



CTTGCCTTTAAGAGTGCACATTCATTGCGGGTAGGTTTT

CCCATCCAGCCCGCACACTGGACCCTTCCAGGT

Figure (3-12c): A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution region. (Appendix 8)

The third DNA sequence of PCO located in exon (7) and (8) was taken from blood samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (3-13A,B and C).

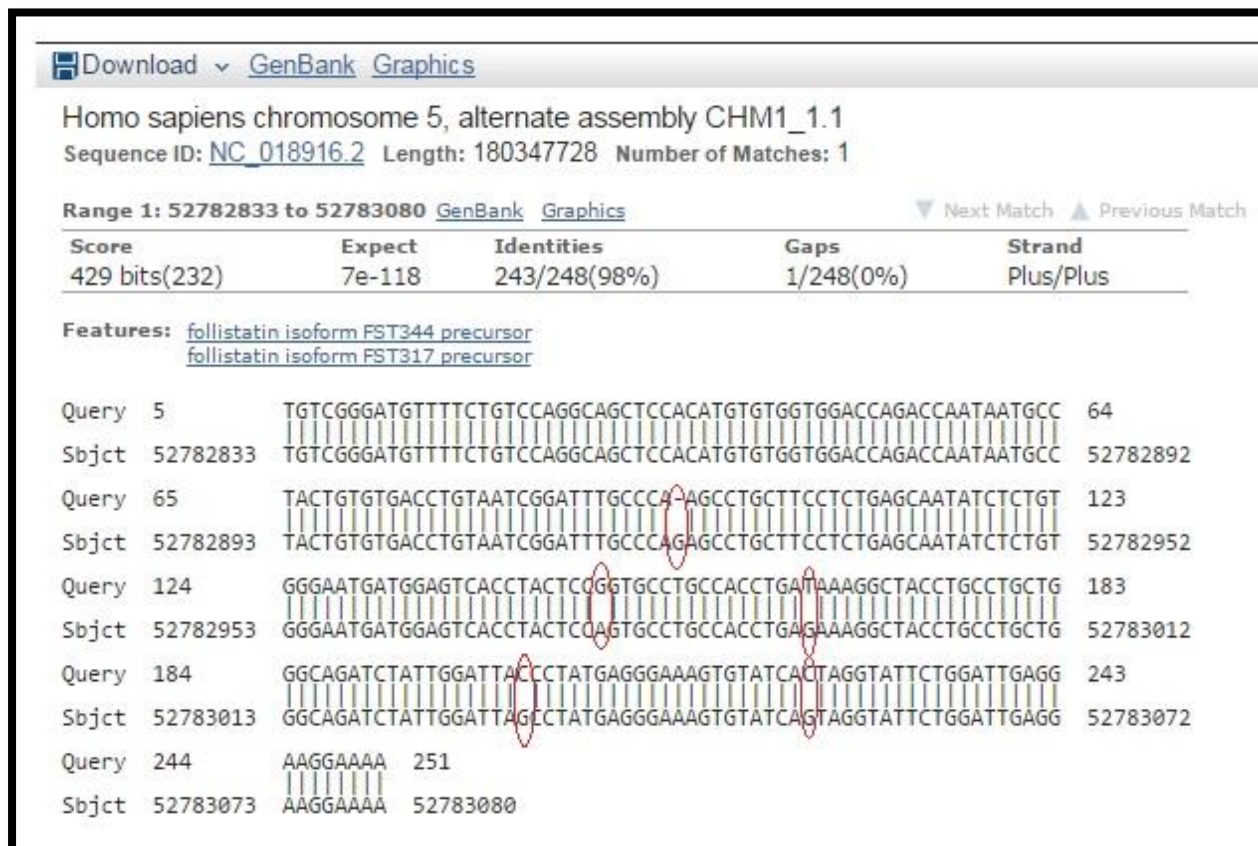


Figure (3-13A): The automated sequencing of PCO for DNA isolated from blood red circle indicate the position at which the mutation occur.

The SNPs of PCOS patients are as follows: in position 88-89 deletion occur AGA/A-A that convert a.a Arg to deletion, also in position 141 substitution mutation occur CAG/CGG that convert a.a Gln to Arg,in position 157 GAG/GAT substitution convert a.a Glu to Asp, the last two mutation AGC/ACC in position 195 that convert a.a Ser to Thr, in position 217substitution convert CAG/CAC a.a Gln to His.

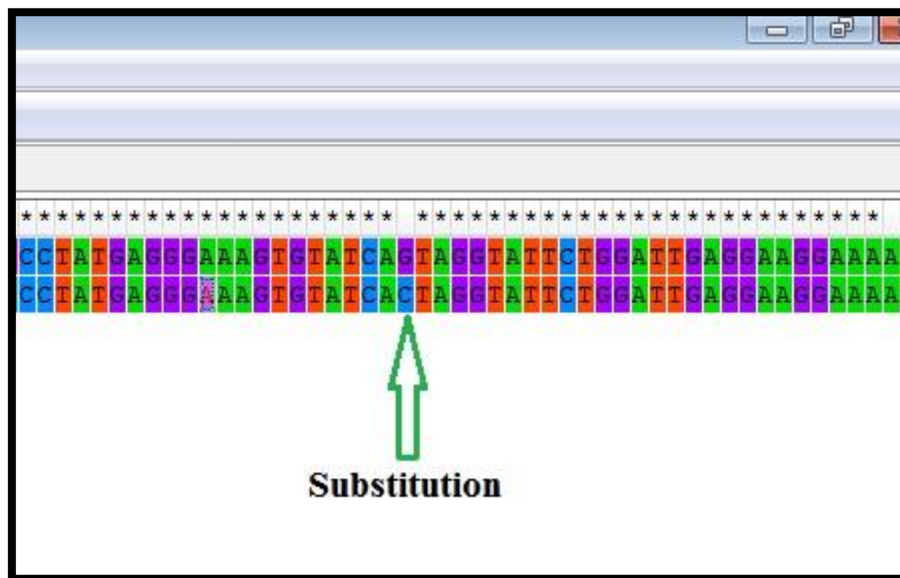
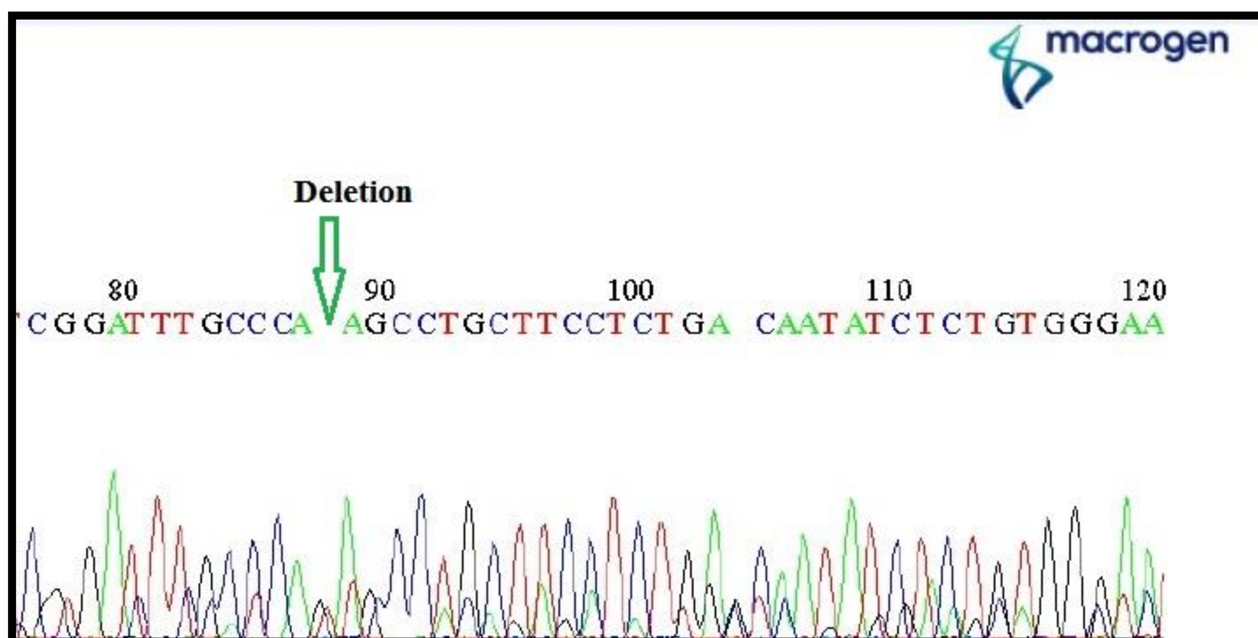
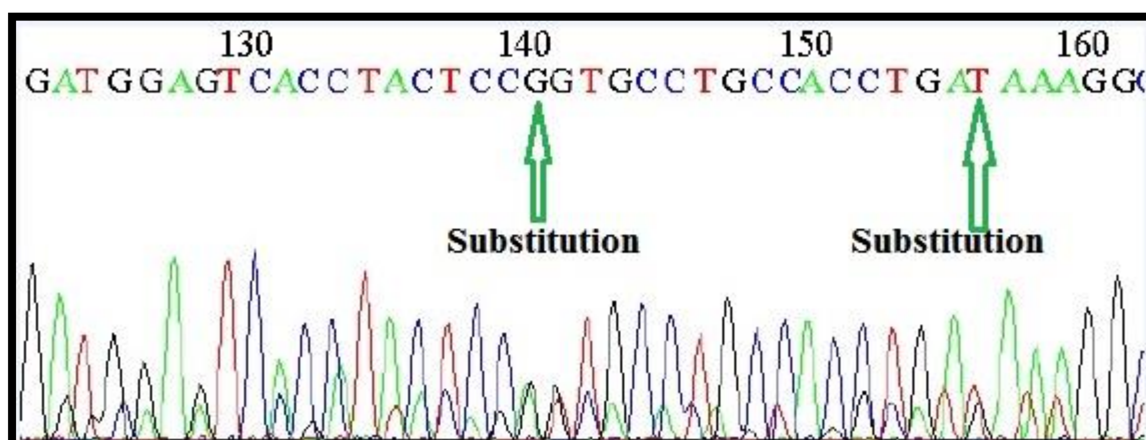
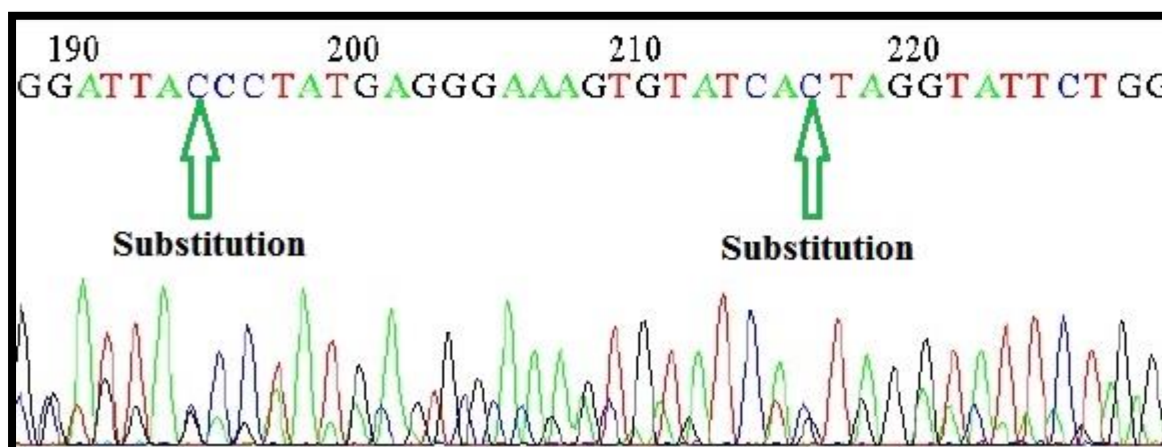


Figure (3-13B) :comparing between control (NCBI nucleotide blast) and Polycystic ovary syndrome patient in the MEGA 6 program(for blood).







CGGATTTGCCCA-

AGCCTGCTTCCTCTGACAATATCTCTGTGGGAA

GATGGAGTCACCTACTCCGGTGCCTGCCACCTGATAAAGG

GGATTACCCTATGAGGGAAAGTGTATCACTAGGTATTCTGG

Figure (3-13C): A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution and deletion region.

The third DNA sequence of PCO located in exon (7) and (8) was taken from tissue samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (3-14 a, b and c).

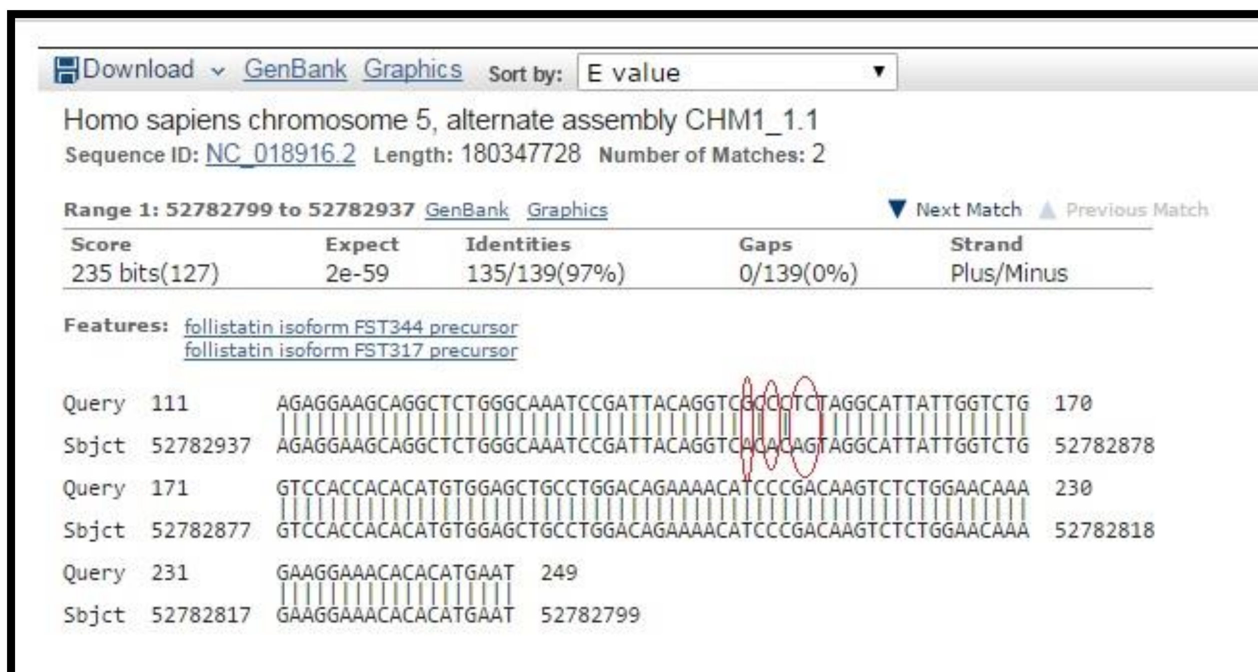


Figure (3-14a): The automated sequencing of PCO for DNA isolated from tissue.

More than one SNPs found in this automated sequencing, the first one is substitution in position 148 and 150 ACA/GCC that convert a.a Thr to Ala, in position 152 and 153 CAG/CTC that convert a.a Gln to Leu.

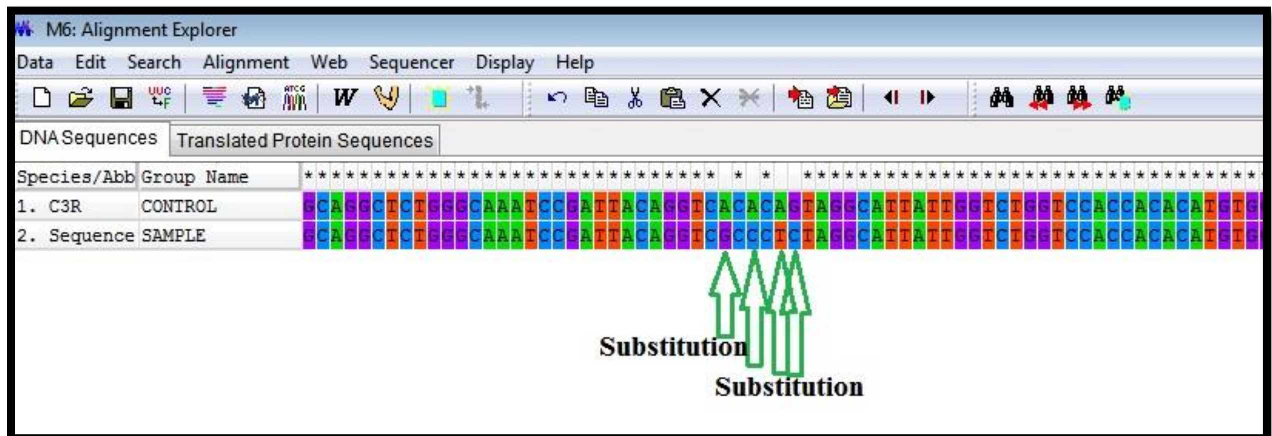
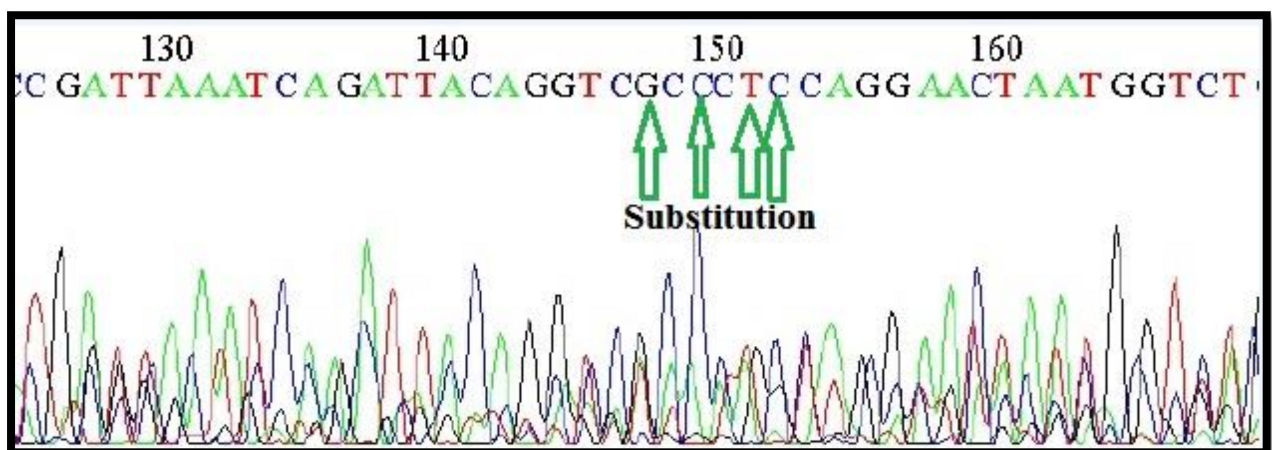


Figure (3-14b) :comparing between control (NCBI nucleotide blast) and Polycystic ovary syndrome patient in the MEGA 6 program(for tissue).



CGATTAAATCAGATTACAGGTCGCCCTCCAGGAACTAATGGTCT

Figure (3-14c): A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution region.

The fourth DNA sequence of PCO located in exon (9) and (10) was taken from blood samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (3-15 A,B and C).

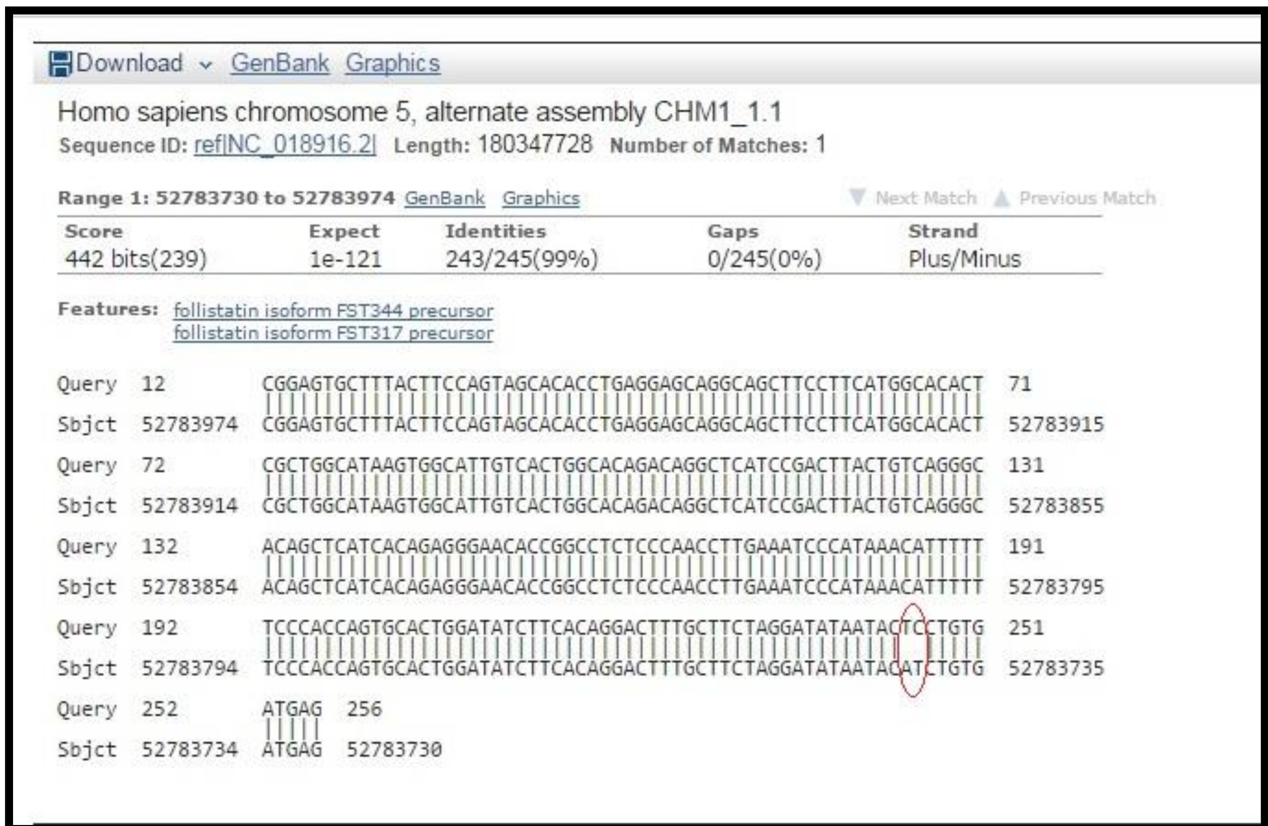


Figure (3-15A): The automated sequencing of PCO for DNA isolated from blood red circle indicate the position at which the mutation occure.

The SNPs in this region are substitution AT/TC in position 246 and 247 of PCOS patient which convert a.a His to Leu.

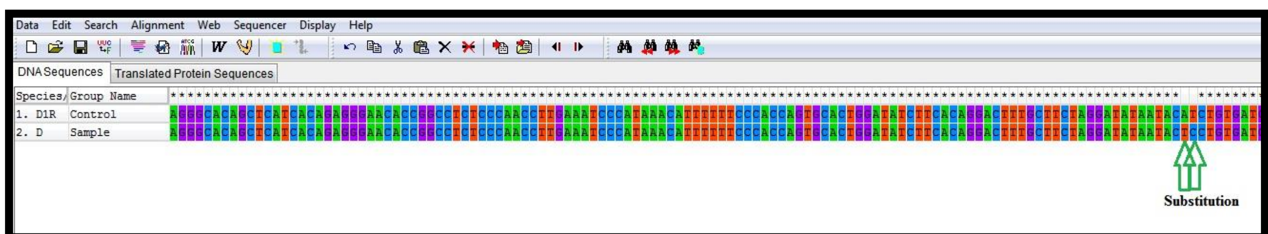
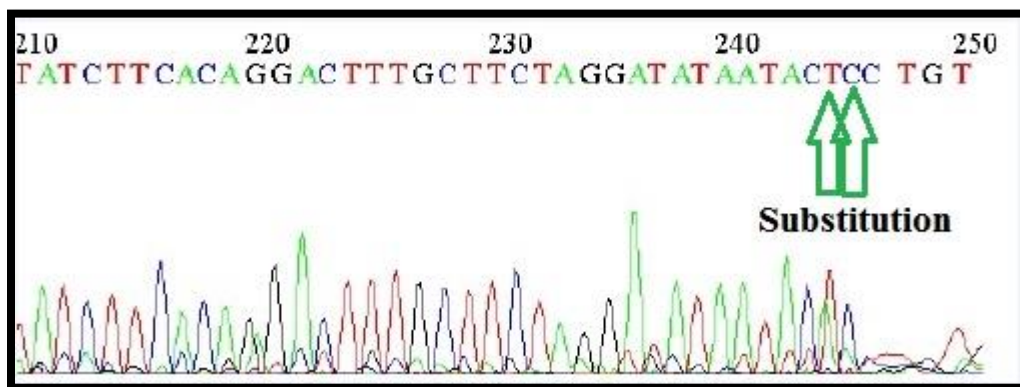


Figure (3-15B) :comparing between control (NCBI nucleotide blast) and Polycystic ovary syndrome patient in the MEGA 6 program(for blood).



TATCTTCACAGGACTTTGCTTCTAGGATATAATACTCCTGT

Figure (3-15C): A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution and region.

The fourth DNA sequence of PCO located in exon (9) and (10) was taken from tissue samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (3-16 a,b and c).

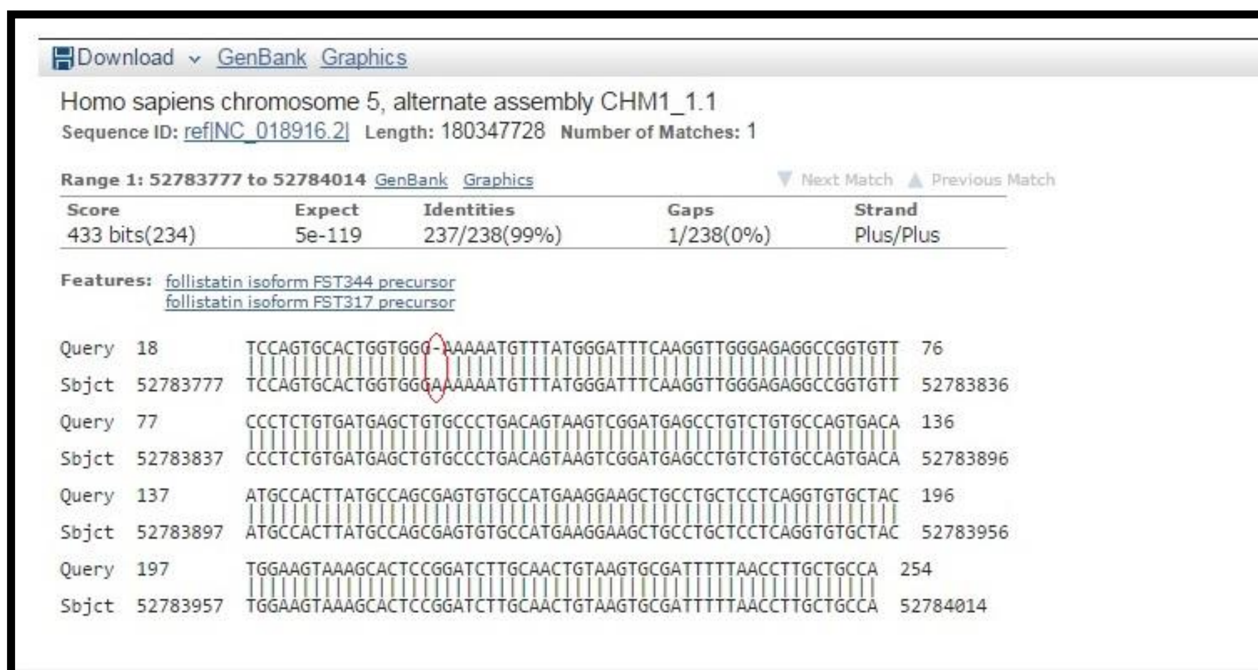


Figure (3-16a): The automated sequencing of PCO for DNA isolated from tissue.

The SNPs in this region are deletion A/- in position 34-35 of PCOS patient which convert a.a Met to deletion.

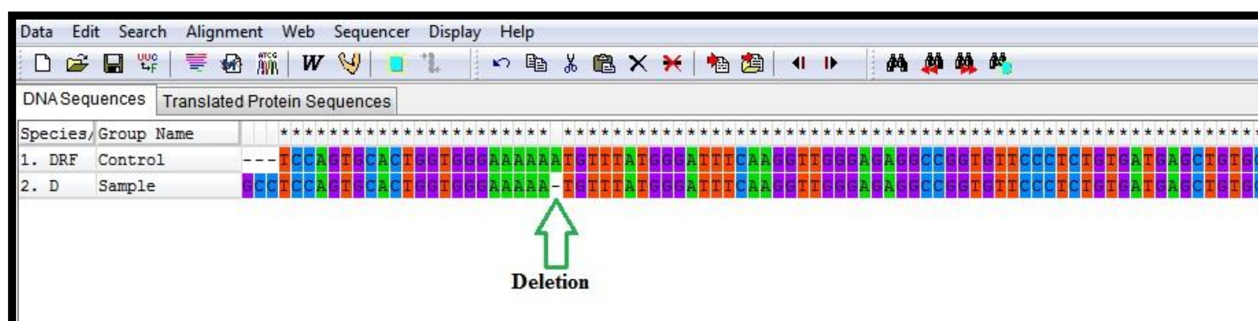
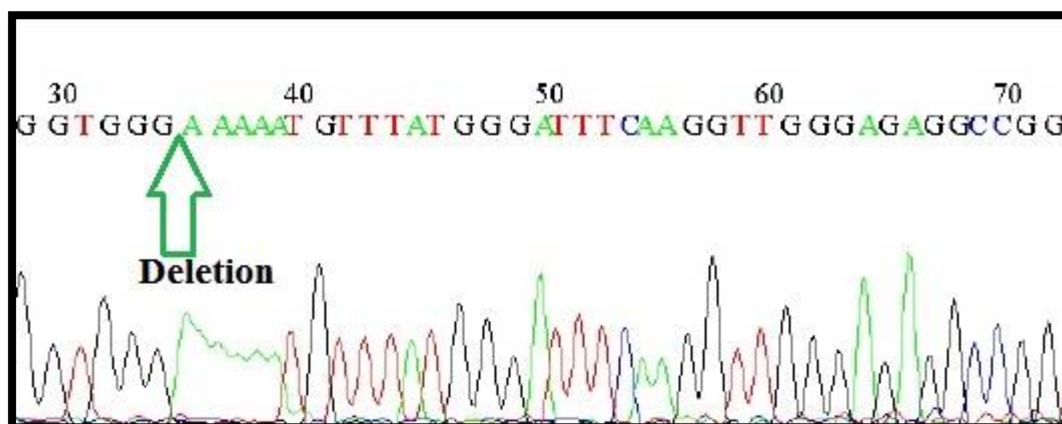


Figure (3-16b) :comparing between control (NCBI nucleotide blast) and Polycystic ovary syndrome patient in the MEGA 6 program(for tissue).



GGTGGG-

AAAAATGTTTATGGGATTTCAAGGTTGGGAGAGGCCGG

Figure (3-16c): A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the deletion region.

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy affecting women of childbearing age causing not only reproductive but also metabolic anomalies. PCOS women present with ovulatory dysfunction, abnormal hormones, hyperandrogenemia, obesity, and hyperinsulinemia (Franks *et al.*, 2006). It is a heterogeneous disorder which results from interaction of multiple genes along with environmental factors (Xita *et al.*, 2002). For a number of genes altered patterns of expression have been detected, suggesting that the genetic abnormality in PCOS affects signal transduction pathways controlling the expression of multiple genes rather than abnormal expression of a single gene (Jakubowski, 2005). Circulating Hcy

(Homocysteine) levels could be influenced by many determinants (Refsum *et ali.*,1998). Genetic factors play an important role in the metabolic pathway of Hcy synthesis. Enzymatic defects caused by genetic mutations induce a significant increase in Hcy concentrations (Jacobsen , 1998). In the general population, the most common cause of abnormal serum Hcy levels seems to be a reduced efficiency of methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in the folate-dependent remethylation of Hcy to methionine (Sills *et al*., 2001).The C677T mutation, *i.e*. alanine to valine substitution at nucleotide 677, in MTHFR gene causes an impairment of its enzymatic activity (Rozen , 1997 ; McQuillan *et al*., 1999).When C677T mutation is present in a homozygous state, this polymorphism results in a variant of MTHFR enzyme, referred to as the thermolabile MTHFR (Sills *et al*., 2001), and in elevated circulating total Hcy levels (Tawakol *et al*., 1997 ; Herrmann , 2001).

Different mutation of one or more than located gene region. However substitution and deletion affected the MTHFR and PCO genes in Iraqi patients as show in Table (3-6).

Table (3-6):point mutations detect in patient samples.

Name of Primers	Wild type	Mutant type	Change in amino acids	Site Of N.A.	Type of Mutation	Effect on translation
(R1 for blood)	TGT	CCG	Cys-Pro	51	Substitution	Missense
	CAT	CGT	His-Arg	68	Substitution	Missense
	CCA	TCA	Pro-Ser	111	Substitution	Missense
(R1 for tissue)	GTG	GGG	Val-Ala	51	Substitution	Missense
	AGG	ACG	Arg-Thr	55	Substitution	Missense
	CAT	-AT	His-Deletion	56-57	Deletion	Frame shift
	ACA	-TT	Thr-Deletion	65-66	Deletion	Frame shift
	ACA	ACT	Thr-Thr	66	Substitution	Missense
	CGT	CCT	Arg-Pro	87	Substitution	Missense

(R2 for blood)	GCT	TCT	Ala-Ser	64	Substitution	Missense
	CGT	CTT	Arg-Leu	69	Substitution	Missense
	GGG	CGG	Gly-Val	104	Substitution	Missense
	GCG	GCT	Ala-Asp	117	Substitution	Missense
	GGC	GTC	Gly-Val	119	Substitution	Missense
	CAA	ACA	Gln-Thr	140-141	Substitution	Missense
(R2 for tissue)	GGA	AGA	Gly-Arg	58	Substitution	Missense
	AGA	ACA	Arg-Thr	101	Substitution	Missense
(R3 for blood)	AGA	A-A	Arg-Deletion	88-89	deletion	Frame shift
	CAG	CGG	Gln-Arg	141	Substitutin	Missense
	GAG	GAT	Glu-Asp	157	Substitutin	Missense
	AGC	ACC	Ser-Thr	195	Substitutin	Missense
	CAG	CAC	Gln-His	217	Substitutin	Missense
(R3 for tissue)	ACA	GCC	Thr-Ala	148-150	Substitution	Missense
	CAG	CTC	Gln-Leu	152-153	Substitution	Missense
(R4 for blood)	CAT	CTC	His-Leu	246-247	Substitution	Missense
(R4 for tissue)	ATG	-AA	Met-Deletion	34-35	Deletion	Frame shift

Genetic factors are important for the disease in many samples of patients, the mutations detected in exons region of Poly cystic ovary syndrome (PCO) gene of Polycystic ovary syndrome (PCOS) patients give evidence that these mutation play a part in this syndrome.

Conclusions and Recommendatios

Conclusions and Recommendations

1. Conclusions

1. The statistical analysis of fertility hormone measurements showed that the level of the Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and Testosterone hormone were significantly difference in Polycystic ovary syndrome (PCOS) patients when compared to the control, when Polycystic ovary syndrome (PCOS) showed a high elevation Luteinizing Hormone (LH), Testosterone while Follicle Stimulating Hormone (FSH) decreased.

2. The Polycystic ovary syndrome (PCOS) patients were divided in to three age groups, the more decreased in Follicle Stimulating Hormone (FSH) and the greatest increased of Luteinizing Hormone (LH) were in the age group (41-50)years old, while increased in Testosterone hormone was in the age group (31-40) years old.

3. Mutation was detected in methylene tetrahydrofolate reductase (MTHFR) gene of Polycystic ovary syndrome (PCOS) patients.

4. For some patients there are more one mutation in more than one exon of Polycystic ovary syndrome (PCOS) gene detected, including deletion and substitution.

2. Recommendations

1. Determination of gene expression regarding MTHFR in patients with Polycystic ovary syndrome (PCOS).

2. Detection of other types of mutations by amplifying another regions of the Polycystic ovary (PCO) genes by using other primers.

References

-A-

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Appendices

Appendix-1-

Case profile:

Sample No.: *Date: / /*

- Name:

- Age:

- Smoking: Yes ☐ *NO* ☐

- Duration of marriage: ☐ *years* ☐ *months*

- Parity : Yes ☐ *NO* ☐

-Miscarriage : Yes *Numbers:* *NO* ☐

- PCOS Familial history: Yes ☐ *NO* ☐ *Who is my:*

- Type of infertility: Primary: *Secondary:*

- Height (m): *Weight(kg):*

- Past Surgical history: Yes ☐ *NO* ☐ *e:* *Date: / /20……*

- Menstrual history: *Ultrasound test: Yes PCOS* **case** ☐ *NO* ☐

_Symptoms:

-Hirsutism

- Acne

Address:

-Time of disease diagnosis:

_Medication:

Appendix-2-

Hormonal & Biochemical levels :

Tests			Normal values
LH	:	**IU/L**	**Females: follicular: 2 – 14** **mid-cycle: 4 – 70** **luteal: 0.5 – 20** **postmenopausal: 14 – 60** **Males : 2.0 – 12.0**
FSH	:	**IU/L**	**Females: follicular: 4 – 11** **mid-cycle: 4 – 21** **luteal: 1 – 11** **post menopausal: 36 – 170** **Males : 2 – 18**
Testosterone:		**ng/ml**	**Females: 0.1 – 0.6** **Males (adult): 2.0 – 9.0**

Appendix-3-

DNA Extraction Procedure from Blood.

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Blood Protocol Procedure

1. Sample Preparation

Transfer up to **200 µl of whole blood, serum, plasma, buffy coat or body fluids to a 1.5 ml microcentrifuge tube**. **Using PBS adjust the volume to 200 µl**. Add **20 µl of Proteinase K** then mix by pipetting. Incubate at 60°C for 5 minutes.

NOTE: Fresh blood is recommended. However, frozen or blood treated with anticoagulants (EDTA etc.) can also be used. Increased storage length decreases DNA yield. If using nucleated blood (e.g. bird or fish) use up to 10 µl then adjust volume to 200 µl with PBS.

2. Cell Lysis

Add **200 µl of GSB Buffer** then mix by shaking vigorously. Incubate at 60°C for 5 minutes, inverting the tube every 2 minutes.

NOTE: It is essential that the sample and GSB Buffer are mixed thoroughly to yield a homogenous solution. Inverting the sample occasionally during incubation will facilitate Proteinase K digestion and cell lysis. Using an auto shaking system is more convenient when incubating samples. During incubation, transfer the required volume of Elution Buffer (200 µl/sample) to a 1.5 ml microcentrifuge tube and heat to 60°C (for Step 5 DNA Elution).

Optional RNA Removal Step

For RNA-free gDNA, following GSB Buffer addition and 60°C incubation, add 5 µl of RNase A (50 mg/ml) and mix by shaking vigorously. Incubate at room temperature for 5 minutes to ensure efficient RNA degradation.

3. DNA Binding

Add **200 µl of absolute ethanol** to the sample lysate and mix **IMMEDIATELY** by shaking vigorously for 10 seconds. If precipitate appears, break it up as much as possible with a pipette. Place a **GD Column in a 2 ml Collection Tube**. Transfer all of the mixture (including any insoluble precipitate) to the GD Column. Centrifuge at 14-16,000 x g for 1 minute. Following centrifugation, if the mixture did not flow through the GD Column membrane, increase the centrifuge time until it passes completely. Discard the 2 ml Collection Tube containing the flow-through then transfer the GD Column to a new 2 ml Collection Tube.

NOTE: It is important that the lysate and ethanol are mixed thoroughly to yield a homogeneous solution.

4. Wash

Add **400 µl of W1 Buffer** to the GD Column. Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through. Place the GD Column back in the 2 ml Collection Tube. **Add 600 µl of Wash Buffer (make sure absolute ethanol was added)** to the GD Column. Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through. Place the GD Column back in the 2 ml Collection Tube. Centrifuge again for 3 minutes at 14-16,000 x g to dry the column matrix.

NOTE: Additional centrifugation at 14-16,000 x g for 5 minutes or incubation at 60°C for 5 minutes will completely dry the GD Column to avoid any residual ethanol carryover and ensure the most effective downstream applications.

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5. Elution

Standard elution volume is 100 µl. If less sample is to be used, reduce the elution volume (30-50 µl) to increase DNA concentration. If higher DNA yield is required, repeat the DNA Elution step to increase DNA recovery and the total elution volume to approximately 200 µl.

Transfer the dried GD Column to a clean 1.5 ml microcentrifuge tube. Add **100 µl of pre-heated Elution Buffer**[1], TE Buffer[2] or water[3] into the **CENTER** of the column matrix. Let stand for at least 3 minutes to allow Elution Buffer, TE Buffer or water to be completely absorbed. Centrifuge at 14-16,000 x g for 30 seconds to elute the purified DNA.

[1]Ensure that Elution Buffer (10 mM Tris-HCl, pH8.5 at 25°C) is added into the center of the GD Column matrix and is completely absorbed.

[2]Using TE (10 mM Tris-HCl, 1 mM EDTA, pH8.0) for elution is beneficial as EDTA preserves DNA for long term storage. However, EDTA will affect PCR and other sensitive downstream applications. Ensure that TE is added into the center of the GD Column matrix and is completely absorbed.

[3]If using water for elution, ensure the water pH is between 7.0 and 8.5. ddH_2O should be fresh as ambient CO_2 can quickly cause acidification. Ensure that water is added into the center of the GD Column matrix and is completely absorbed. DNA Eluted in water should be stored at -20°C to avoid degradation.

Cultured Cell Protocol Procedure

1. Sample Preparation

Trypsinze adherent cells prior to harvesting. Transfer **cells (up to 1 x 10^7)** to a 1.5 ml microcentrifuge tube then centrifuge for 5 minutes at 300 x g. Discard the supernatant then resuspend cells in **200 µl of PBS** by pipette. Add **20 µl of Proteinase K** then mix by pipetting. Incubate at 60°C for 5 minutes.

2. Cell Lysis

Add **200 µl of GSB Buffer** then mix by shaking vigorously. Incubate at 60°C for 5 minutes, inverting the tube every 2 minutes.

NOTE: It is essential that the sample and GSB Buffer are mixed thoroughly to yield a homogenous solution. Inverting the sample occasionally during incubation will facilitate Proteinase K digestion and cell lysis. Using an auto shaking system is more convenient when incubating samples. During incubation, transfer the required volume of Elution Buffer (200 µl/sample) to a 1.5 ml microcentrifuge tube and heat to 60°C (for Step 5 DNA Elution).

Optional RNA Removal Step

For RNA-free gDNA, following GSB Buffer addition and 60°C incubation, add 5 µl of RNase A (50 mg/ml) and mix by shaking vigorously. Incubate at room temperature for 5 minutes to ensure efficient RNA degradation.

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Appendix-4-

DNA Extraction Procedure from tissue.

FAVORGEN BIOTECH CORP.

FavorPrep™ Tissue Genomic DNA Extraction Mini Kit

Cat.: FATGK001, 50 Preps
FATGK001-1, 100 Preps
FATGK001-2, 300 Preps
(For Research Use Only) v.1211

Kit Contents:

	FATGK001 (50 preps)	FATGK001-1 (100 preps)	FATGK001-2 (300 preps)
FATG1 Buffer	15 ml	30 ml	70 ml
FATG2 Buffer	15 ml	30 ml	70 ml
W1 Buffer*	22 ml	44 ml	124 ml
Wash Buffer**	10 ml	20 ml	50 ml
Elution Buffer	15 ml	30 ml	90 ml
Proteinase K	11 mg	11 mg x 2	11 mg x 6
FATG Column	50 pcs	100 pcs	300 pcs
Elution Tube	50 pcs	100 pcs	300 pcs
Micropestle	50 pcs	100 pcs	300 pcs
Collection Tube	100 pcs	200 pcs	600 pcs

*Add 8 / 16 / 45 ml ethanol (96-100%) to W1 Buffer when first open.
**Add 40 / 80 / 200 ml ethanol (96-100%) to Wash Buffer when first open.

Sample → Grind the sample; Cell lysis (FATG1); Protein degradation (Proteinase K) → Cell lysis (FATG2) → Binding → centrifuge → Washing (W1 Buffer) (Wash Buffer) → centrifuge → Elution (Elution Buffer) → centrifuge → Pure genomic DNA

Important Notes:

1. Buffers provided in this system contain irritants. Wear gloves and lab coat when handling these buffers.
2. Add 1.1 ml sterile ddH_2O to Proteinase K tube to make a 10 mg/ml stock solution. Vortex and make sure that Proteinase K has been completely dissolved. Store the stock solution at 4 °C.
3. Add 8 / 16 / 45 ml ethanol (96- 100 %) to W1 Buffer when first open.
4. Add 40 / 80 / 200 ml ethanol (96- 100 %) to Wash Buffer when first open.
5. Prepare two dry baths or two water baths before the operation: one to 60 °C for step 4 and the other to 70 °C for step 7.
6. All centrifuge steps are done at full speed (14,000 rpm or 10,000 x g) in a microcentrifuge.

Genernal Protocol:

Please Read Important Notes Before Starting The Following steps.
For other special samples, please refer to Special Protocol and choose the appropriate one.

1. (For fresh sample) Cut up to 25 mg tissue sample to a microcentrifuge tube (not provided). Use provided Micropestle to grind the tissue sample a few times. Or you can grind the tissue sample in liquid nitrogen then transfer the powder to a new microcentrifuge tube. (not provided)

   (For frozen sample) Weigh up to 25 mg tissue sample and grind the tissue sample in liquid nitrogen then transfer the powder to a new microcentrifuge tube. (not provided)
   ---If DNA is prepared from spleen tissue, no more than 10 mg should be used.
2. Add 200 µl FATG1 Buffer and mix well by micropestle or pipette tip.
3. Add 20 µl Proteinase K (10mg/ml) to the sample mixture. Mix thoroughly by vortexing.
4. Incubate at 60 °C until the tissue is lysed completely. Vortex every 10~15 min during incubation to break up the tissue sample.
5. Briefly spin the tube to remove drops from the inside of the lid.
6. (Optional) If RNA-free genomic DNA is required, add 4 µl of 100 mg/ml RNase A and incubate for 2 min at room temperature.
7. Add 200 µl FATG2 Buffer to the sample mixture, mix thoroughly by pulse-vortexing and incubate at 70 °C for 10 min.
8. Briefly spin the tube to remove drops from the inside of the lid.
9. Add 200 µl ethanol (96-100%) to the sample. Mix thoroughly by pulse-vortexing.
10. Briefly spin the tube to remove drops from the inside of the lid.

1

11. Place a FATG Mini Column in a Collection Tube. Transfer the mixture (including any precipitate) carefully to FATG Mini Column. Centrifuge for 1 min **then place FATG Mini Column to a new Collection Tube.**
12. Wash FATG Mini Column with 500 µl W1 Buffer by centrifuge for 1 min then discard the flow-through.
13. Wash FATG Mini Column with 750 µl Wash Buffer by centrifuge for 1 min then discard the flow-through.
    ---Make sure that ethanol has been added into Wash Buffer when first open.
14. Centrifuge for an additional 3 min to dry the column.
    Important Step! The residual liquid can affect the quality of DNA and inhibit subsequent enzymatic reactions.
15. Place FATG Mini Column to Elution Tube.
16. Add 50~200 µl of Elution Buffer or ddH_2O (pH 7.5-9.0) to the membrane center of FATG Mini Column. Stand FATG Mini Column for 3 min.
    Important Step! For effective elution, make sure that the elution solution is dispensed onto the membrane center and is absorbed completely.
    ---Standard volume for elution is 200 µl. If less sample to be use, reduce the elution volume (50~150 µl) to increase DNA concentration.
17. Centrifuge for 2 min to elute total DNA.
18. Store total DNA at 4°C or -20°C.

Troubleshooting

Possible reasons	Solutions
Poor cell lysis because of insufficient incubation time	Repeat the extraction procedure with a new sample. Extend the incubation time and make sure that no residual particulates remain.
Ethanol is not added into the lysate before transferring into FATG Mini Column Set	Repeat the extraction procedure with a new sample.
Incorrect preparation of Wash Buffer	
Ethanol is not added into Wash Buffer when first open	Make sure that the correct volumes of ethanol (96- 100 %) is added into Wash Buffer when first open. Repeat the extraction procedure with a new sample.
The volume or the percentage of ethanol is not correct before adding into Wash Buffer	Make sure that the correct volumes of ethanol (96- 100 %) is added into Wash Buffer when first open. Repeat the extraction procedure with a new sample.
Genomic DNA is contaminated	Do not wet the rim of the column during sample and buffer loading.
A_{260}/A_{280} ratio of eluted DNA is high	
A lot of residual RNA in eluted DNA	Follow the General Protocol step 6 to remove RNA.
FATG2 Buffer added to the sample before adding RNase A	Make sure that RNase A has been added to the sample before adding FATG2 Buffer when using optional RNase A step.
A_{260}/A_{280} ratio of eluted DNA is low	
Poor cell lysis because of insufficient Proteinase K activity	Repeat the extraction procedure with a new sample. Use a fresh or well-stored Proteinase K stock solution.
	Do not add Proteinase K directly to FATG2 Buffer.
Poor cell lysis because of insufficient mixing with FATG2 Buffer	Mix the sample and FATG2 Buffer immediately and thoroughly by pulse-vortexing.

Possible reasons	Solutions
Column is clogged	
Lysate contains insoluble residues	Remove insoluble residues (e.g. filter paper, bone or hair) before centrifuging.
Sample is too viscous	Reduce the sample volume.
Insufficient activity of Proteinase K	Use a fresh or well-stored Proteinase K stock solution.
	Repeat the extraction procedure with a new sample. Do not add Proteinase K into FATG2 Buffer directly.
Elution of genomic DNA is not efficient	
pH of water (ddH_2O) for elution is acidic	Make sure the pH of ddH_2O is between 7.5- 9.0.
	Use Elution Buffer (provided) for elution.
Elution Buffer or ddH_2O is not completely absorbed by column membrane.	After Elution Buffer or ddH_2O is added, stand the GDE Mini Column for 5 min before centrifugation.
Ethanol is not added into the lysate before transferring into ALS GDE Mini Column Set	Repeat the extraction procedure with a new sample.
Degradation of eluted DNA	
Sample is old	Always use fresh or well-stored sample for genomic DNA extraction.
Buffer for gel electrophoresis contaminated with DNase	Use fresh running buffer for gel electrophoresis.
Paraffin-embedded tissue is used as sample	Genomic DNA extracted from paraffin-embedded tissue is usually degraded. It is still suitable for PCR reaction, but is not recommened for Southern blotting and restriction analysis.

2

Special Protocol (For FavorPrep™ Tissue Genomic DNA Extraction Mini Sample Kit)

For Bacterial

I. For bacterial cultures

1. Transfer 1 ml well-grown bacterial culture to a microcentrifuge tube (not provided).
2. Descend the bacterial cells by centrifuging at full speed for 2 min and discard the supernatant completely.
3. Follow the General Protocol starting from step 2.

II. For bacterial in biological fluids

1. Collect bacteria by centrifuging biological fluids at 7,500 rpm (5,000 x g) for 10 min.
2. Follow the General Protocol starting from step 2.

III. For bacteria from eye, nasal, pharyngeal, or other swabs

1. Soak the swabs in 2 ml PBS at room temperature for 2- 3 hr.
2. Collect bacteria by centrifuging at 7,500 rpm (5,000 x g) for 10 min.
3. Follow the General Protocol starting from step 2.

IV.For Gram-positive bacterial

HINT: Preheat two dry baths or two water baths before the operation: one to 60 °C and the other to 95 °C for step 7.

1. Transfer 1 ml well-grown bacterial culture to a microcentrifuge tube (provided by user).
2. Descend the bacterial cells by centrifuging at full speed for 2 min and discard the supernatant completely.
3. Resuspend the cell pellet in **200 µl lysozyme reaction solution** (20 mg/ml lysozyme; 20 mM Tris-HCl, pH 8.0; 2mM EDTA; 1.2 % Triton).
4. Incubate at 37 °C for 30 min.
5. (Optional): If RNA-free genomic DNA is required, add 4 µl of 100 mg/ml RNase A and incubate for 2 min at room temperature.
6. Add 20 µl Proteinase K to the sample, and then add 200 µl FATG2 Buffer to the sample. Mix thoroughly by pulse-vortexing.
7. Incubate at 60 °C for 30 min and then for a further 15 min at 95 °C.
8. Follow the General Protocol starting from step 8.

For Fixed Tissues

I. For paraffin-embedded tissues

1. Cut up to 25 mg paraffin-embedded tissue sample to a microcentrifuge tube (not provided).
2. Add 1 ml xylene, mix well and incubate at room temperature for 30 min.
3. Centrifuge at full speed for 5 min. Remove the supernatant by pipetting.
4. Add 1 ml ethanol (96- 100 %) to the deparaffined tissue, mix gently by vortexing.
5. Centrifuge at full speed for 5 min. Remove the supernatant by pipetting.
6. Incubate at 37 °C for 10 min to evaporate ethanol residue.
7. Grind the tissue sample by micropestle or liquid nitrogen and follow the General Protocol starting from step 2.

II. For formalin-fixed tissues

1. Wash 25 mg tissue sample twice with 1 ml PBS to remove formalin.
2. Grind the tissue sample by micropestle or liquid nitrogen and follow the General Protocol starting from step 2.

For Yeast

1. Transfer 3 ml log-phase (OD600 = 10) yeast culture to a microcentrifuge tube (not provided).
2. Descend the yeast cells by centrifuging at 7,500 rpm (5,000 x g) for 10 min and discard the supernatant completely.
3. Resuspend the cell pellet in 600 µl **sorbitol buffer** (1M sorbitol; 100 mM EDTA; 14 mM ß-mercaptoethanol). Add 200 U zymolase or lyticase at 30 °C for 30 min.
4. Centrifuge at 7,500 rpm (5,000 x g) for 5 min. Remove the supernatant by pipetting.
5. Follow the General Protocol starting from step 2.

For Dried Blood Spot

HINT: Preheat three dry baths or three water baths before the operation: one to 85 °C for step 2, another to 60 °C for step 5 and the other to 70 °C for General Protocol step 7.

1. Cut the filter paper (e.g. S&S903) with dried blood spot into a microcentrifuge tube.
2. Add 200 µl FATG1 Buffer and incubate at 85 °C for 10 min.
3. Briefly spin the tube to remove drops from the inside of the lid.
4. Add 20 µl Proteinase K to the sample mixture. Mix thoroughly by vortexing.
5. Incubate at 60 °C for 1 hr. Vortex every 10- 15 min during incubation.
6. Follow the General Protocol starting from step 5.

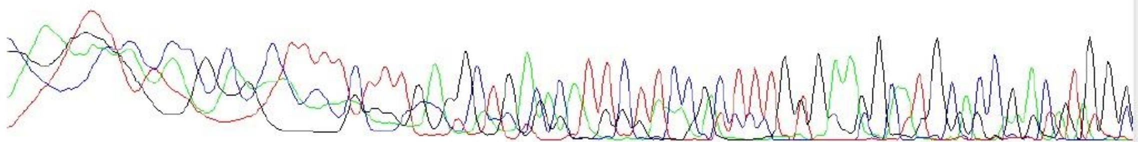
3 (Special Protocol)

Appendix-5-

The First DNA sequence of the MTHFR gene located in exon (2) was taken from blood samples of patient

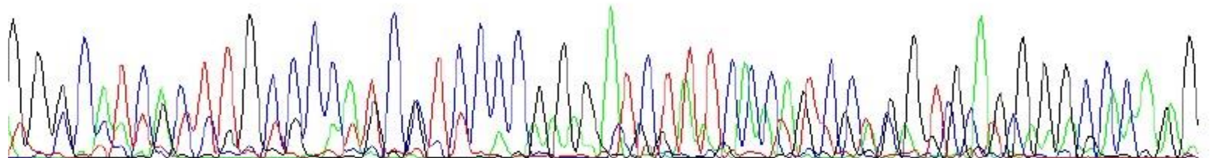
File: A1_AR.abl   Run Ended: 2016/4/6 6:10:31   Signal G:624 A:769 C:1115 T:702
Sample: A1_AR   Lane: 29   Base spacing: 14.174022   279 bases in 8956 scans   Page 1 of

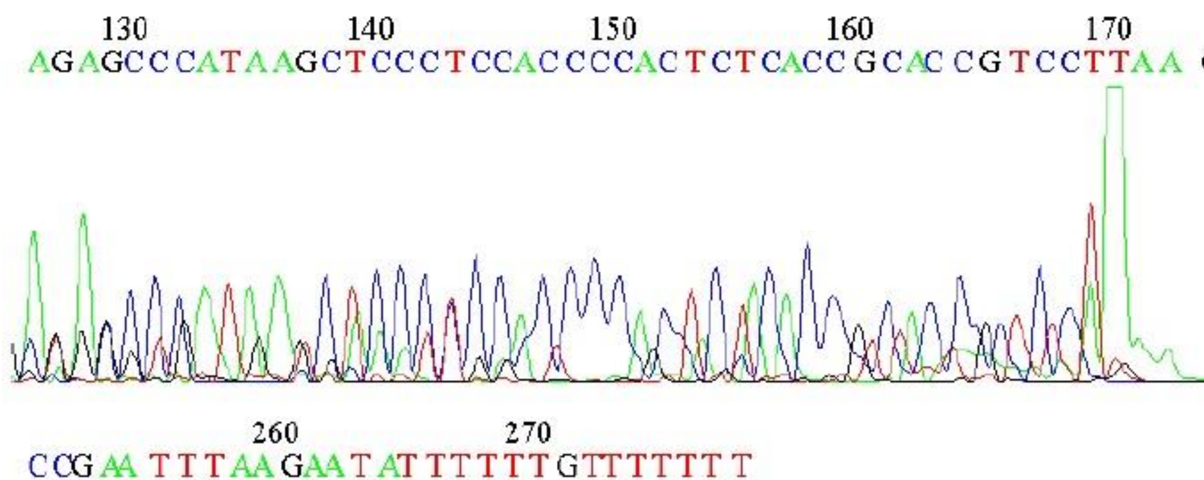
10 20 30 40 50 60 70
CGAGGAAGAA TACGCG CTTTTCTTTGAGGCTGACACATTCTTCCGCTTTGGGAAGGCATGCACCGACGTGGG



1 of 2

70 80 90 100 110 120
GGGCATCACTTGCCCCATCGTCCCCGGGATCTTTCCCATCCTGGTGAGGGGCCCAGG



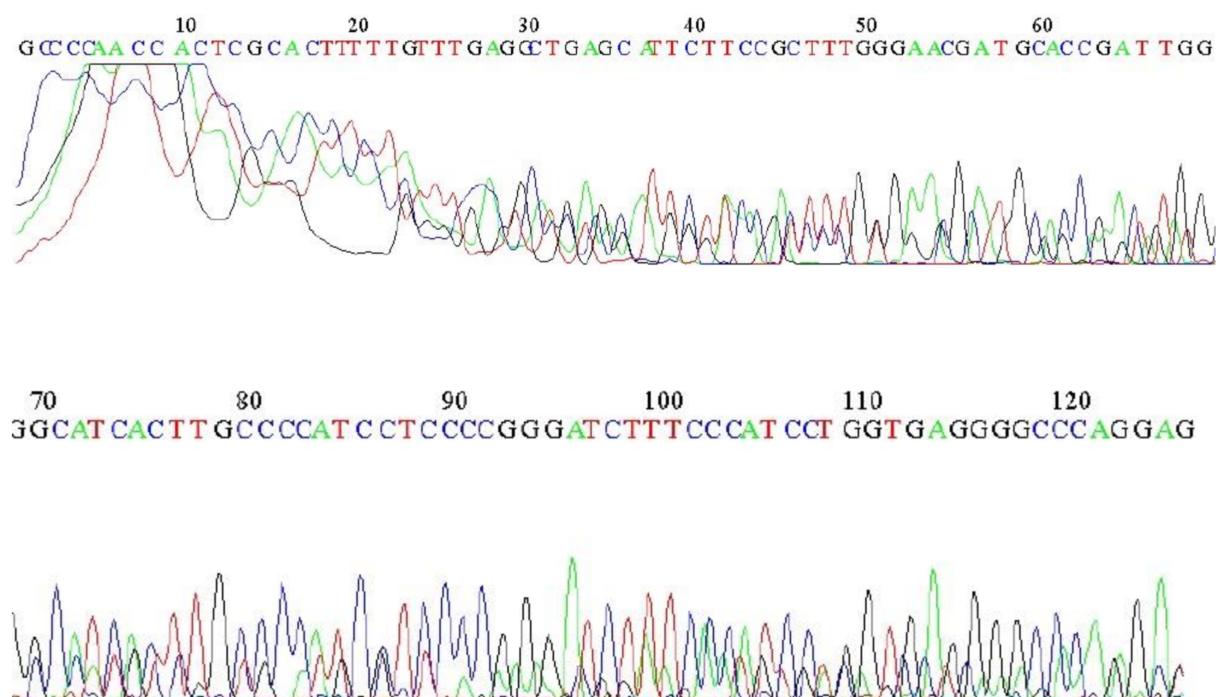


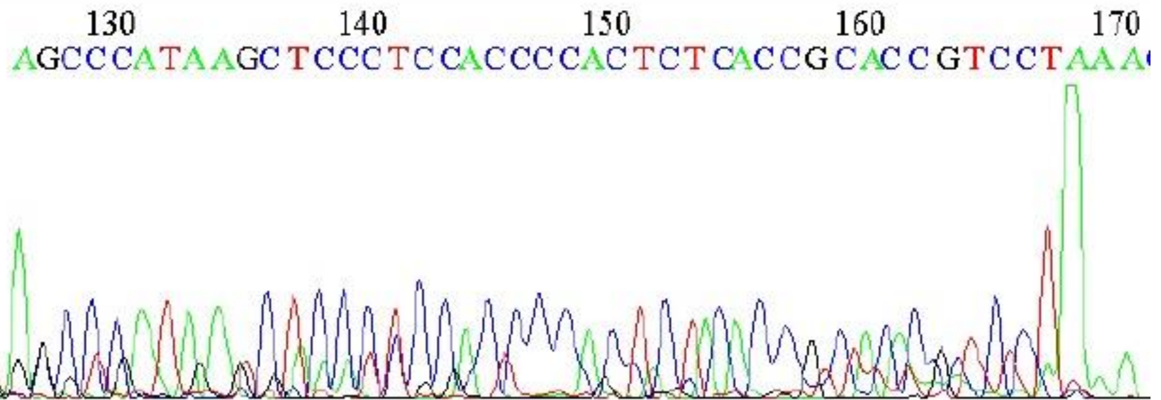
Appendix-6-

The First DNA sequence of the MTHFR gene located in exon (2) was taken from tissue samples of patient.

File: A5_AF.ab1 *Run Ended: 2016/4/6 3:21:34* *Signal G:1061 A:989 C:1377 T:933*

Sample: A5_AF *Lane: 30* *Base spacing: -15.016356* *248 bases in 8138 scans* *Page 1*

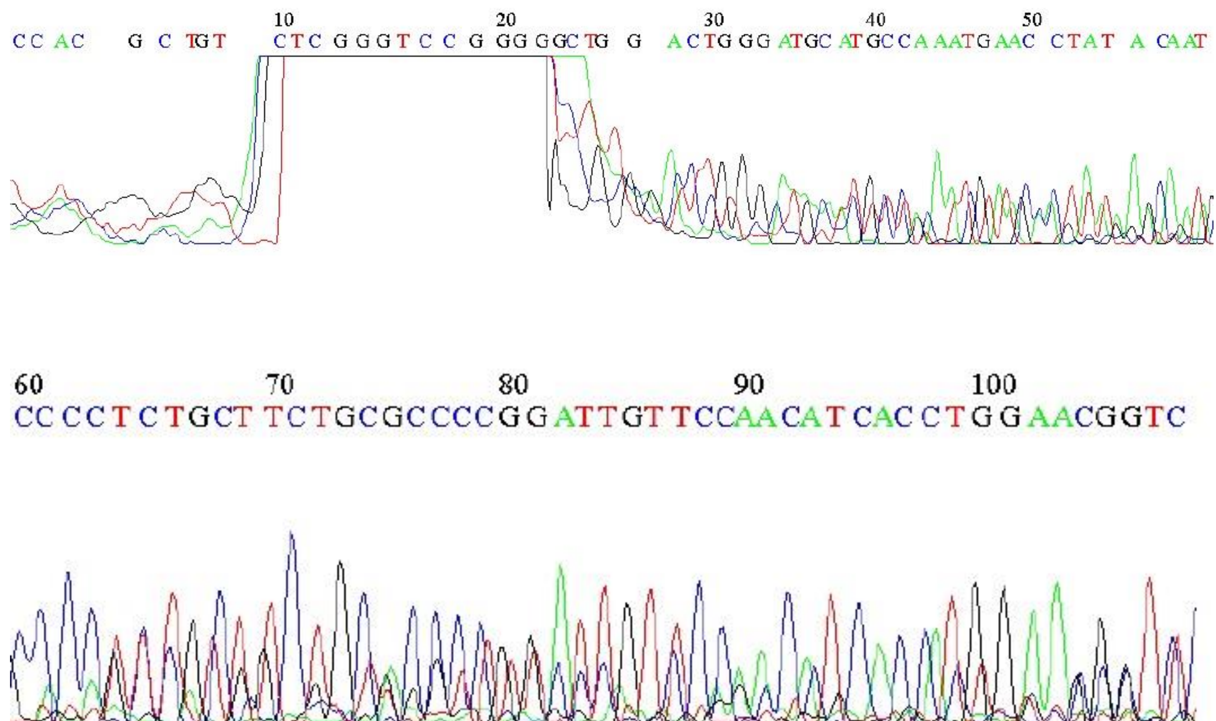


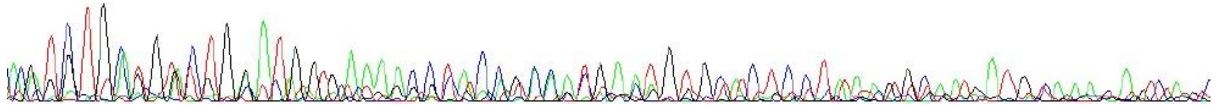


Appendix-7-

The Second DNA sequence of PCOS gene located in exon (5) and (6) was taken from blood samples of patient.

File: B2_BF.ab1 *Run Ended: 2016/4/6 3:21:34* *Signal G:420 A:860 C:770 T:473*
Sample: B2_BF *Lane: 26* *Base spacing: 14.248376* *250 bases in 8806 scans* *Page 1 of 2*



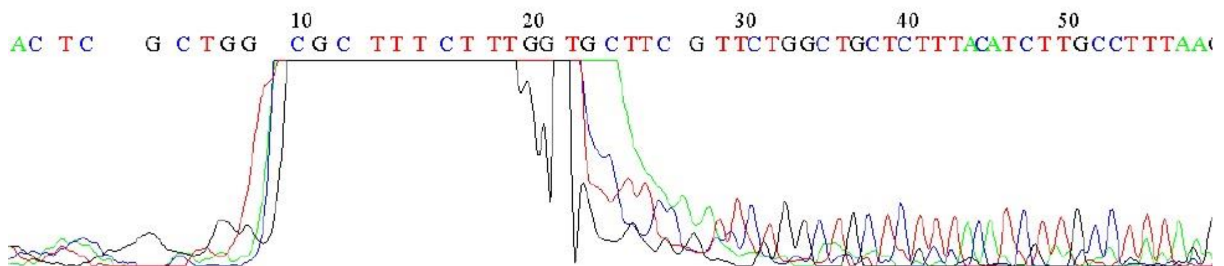


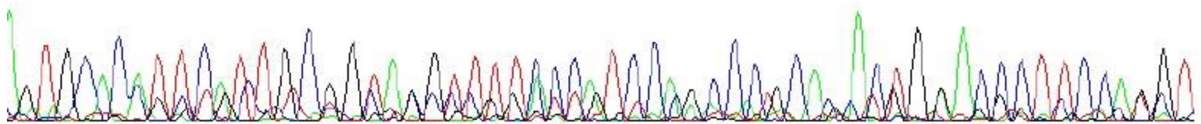
Appendix-8-

The Second DNA sequence of PCOS gene located in exon (5) and (6) was taken from tissue samples of patient.

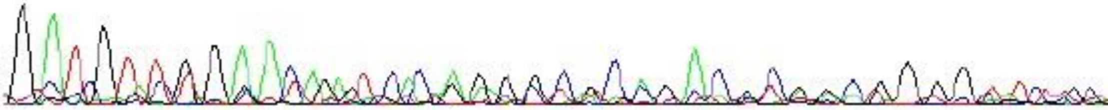
File: B2_BR.ab1 Run Ended: 2016/4/6 5:14:3 Signal G:417 A:829 C:830 T:457

Sample: B2_BR Lane: 26 Base spacing: -15.016356 255 bases in 8267 scans Pag





120 130 140 150
GATGTTGGAACAATCCAGGGCGCAGACGCGACGGGGTTTTC



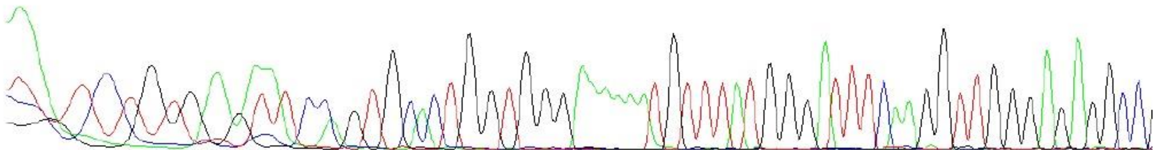
Appendix-9-

DNA sequance for DNA collected from healthy female.

File: D5_DF.ab1 Run Ended: 2016/4/6 3:21:34 Signal G:2297 A:1753 C:1972 T:1792

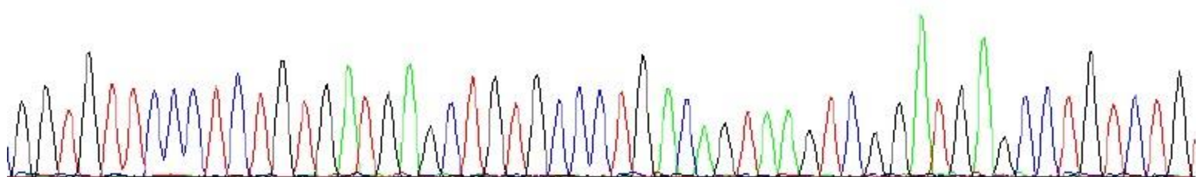
Sample: D5_DF Lane: 48 Base spacing: 14.237049 253 bases in 9387 scans Page 1

10 20 30 40 50 60
CC GG TCC T G TG A GAAT CCA GT G CACT G GT G GG AAAAAAT GT T TAT G GGATTT CAAGGTTGGGAGAGGCC

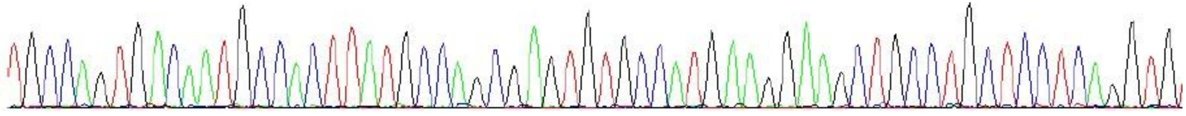


' of 2

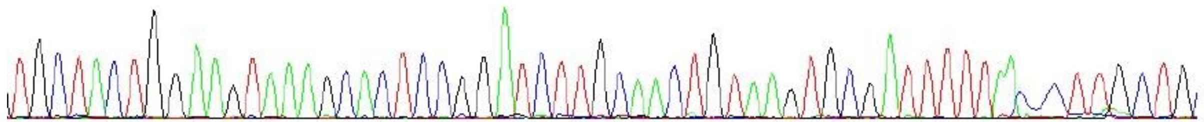
70 80 90 100 110 120
GGTGTTCCCTCTGTGATGAGCTGTGCCCTGACAGTAAGTCGGATGAGCCTGTCTG



130 140 150 160 170 180
TGCCAGTGACAATGCCACTTATGCCAGCGAGTGTGCCATGAAGGAAGCTGCCTGCTCCTCAGGTG



190 200 210 220 230 240
GTGCTACTGGAAGTAAAGCACTCCGGATCTTGCAACTGTAAGTGCGATTTTTAC CTTGCTG



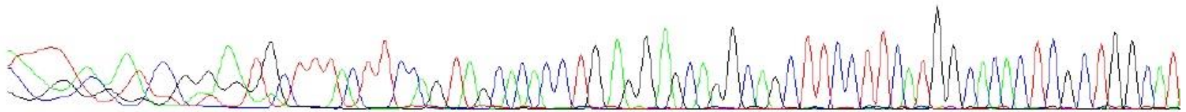
250
CCCA

Appendix-10-

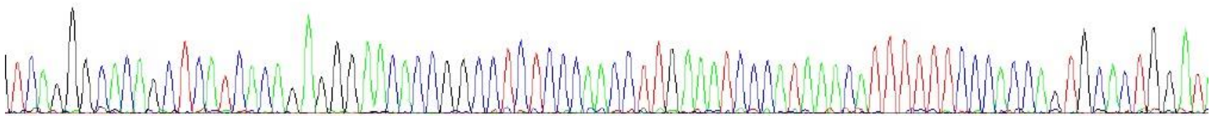
Another DNA sequance for DNA collected from healthy female.

File: D5_DR.ab1 *Run Ended: 2016/4/6 5:14:3* *Signal G:2135 A:2623 C:3764 T:2461*
Sample: D5_DR *Lane: 48* *Base spacing: 14.333696* *353 bases in 8699 scans* *Page 1 of 2*

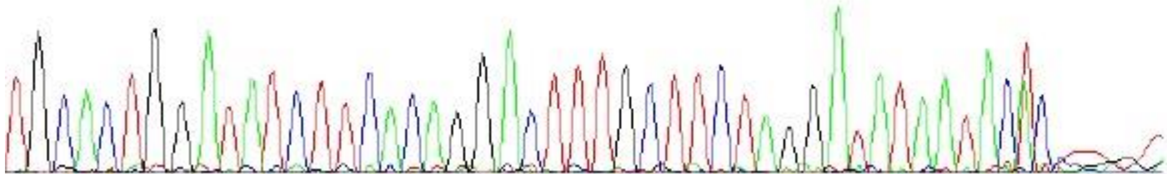
10 20 30 40 50 60 70
CAAAG A GAT C GAG AG TGCTTTACTT CCAG TAGCACACC TG AGG AGCAGGCAGCTTCCTTCATGGCACACTCGCTGGCAT



130 140 150 160 170 180 190 200
TCAGGGCACAGCTCATCACAGAGGGAACACCGGCCTCTCCCAACCTTGAAATCCCATAAACATTTTTTCCCACCAGTGCACTGGAT



200 210 220 230 240
TGCACTGGATATCTTCACAGGACTTTGCTTCTAGGATATAATACACCG GT



250 260 270
GATGAGAA T CGCCCTGAC GGTT ACT CGGAT

Appendix-11-

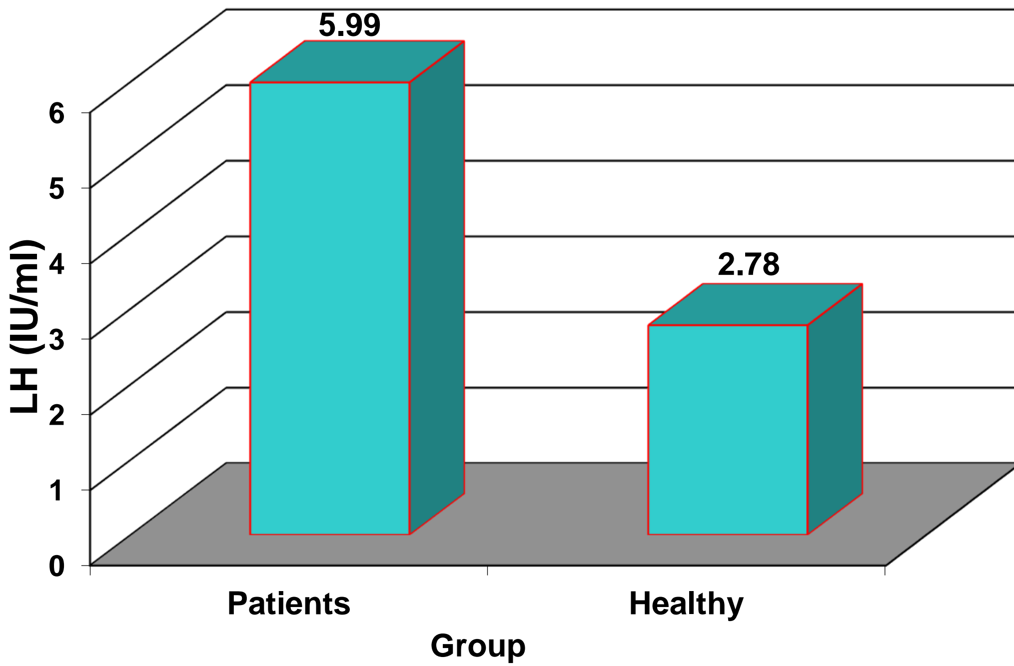


Figure 1. Compare between ptients and control in LH (IU/ml)

Appendix-12-

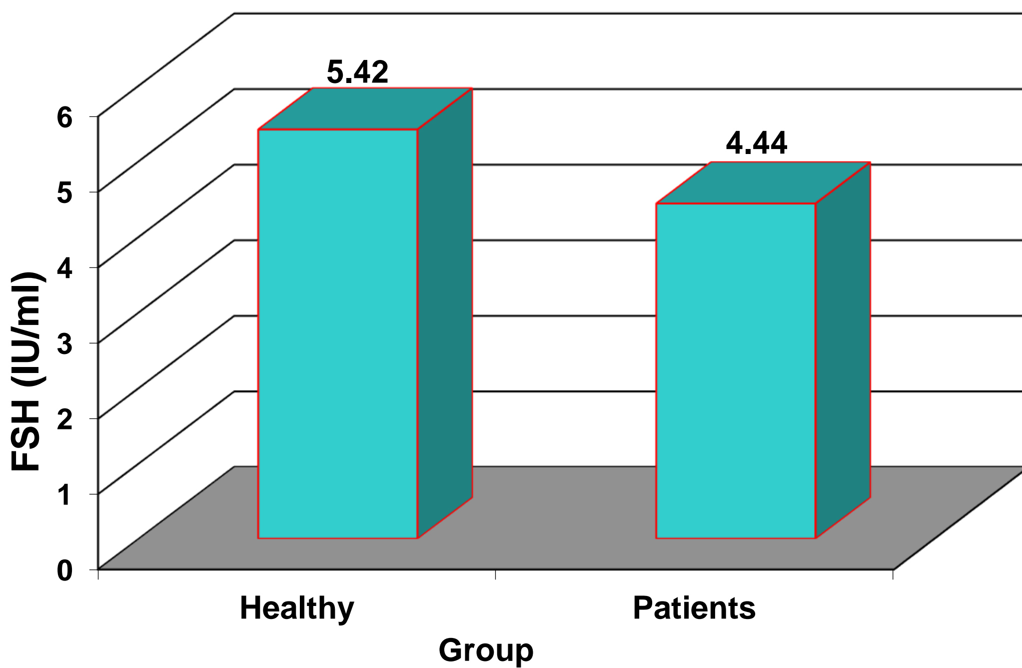


Figure 2. Compare between ptients and control in FSH (IU/ml)

Appendix-13-

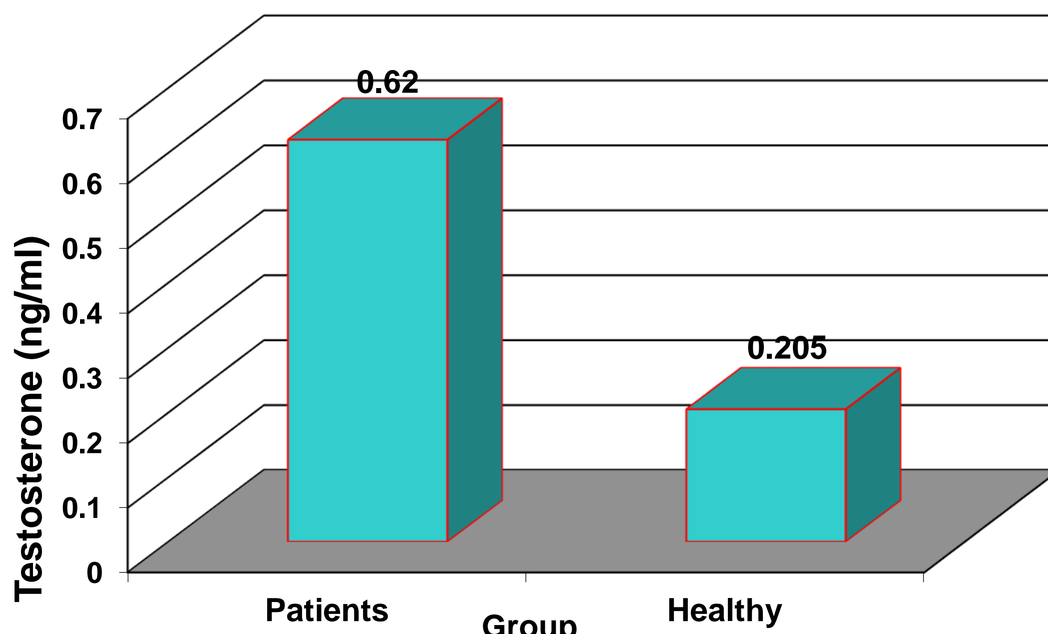


Figure 3. Compare between ptients and control in Testosterone (ng/ml)

اظهرت النتائج ظهور طفرات نقطية منذ حذف واستبدال وكانت نسبة طفرات الاستبدال ٨٨% بينما بلغت نسبة طفرات الحذف ١٢% ومن هذا نلاحظ ان حدوث هذه الطفرات في المناطق المشفرة للجين مسؤولة عن حدوث المرض.

الملخص

هدفت هذه الدراسة لدراسة موضلاء لاالات عدم الخصوبة الناتجة عن ملاثلالا تكيس المبايض وذلك بواسطة التحري عن الاساس الكيميائي الحيوي لهذا المرض فضلا عن ايجاد العلاقة بين الطفرات بجين (methylene tetrahydrofolate reductase (MTHFR وجين تكيس المبايض.

جمعت عينات دم من ٥٠ امراة مصابة بمرض متلازمة تكيس المبايض من شهر تشرين الثاني ٢٠١٥ الى كانون الثاني ٢٠١٦ من مستشفى كمال السامرائي وجمعلا ٢٠ عينة من انسجة المبايض من مستشفى مدينة الامامين الكاظمين الطبية وجمعت ٥٠ عينة دم من نساء صحيحات استخدمت كمجموعة سيطرة و كان معدل اعمار العينات المجموعة سواء للمرضى و الاصحاء تتراوح بين ٢٠-٥٠ ..ة.

لدراسة الكيمياء الحيوية لهذا المرض قسمت مجموعة المريضات (٥٠ مريضة)الى لالاث مجاميع عمرية و لاي ٢٠-٣٠ ،٣١-٤٠ ، ٤١-٥٠ سنة.

عينات المصل لمريضات متلازمة تكيس المبايض تم استخدامها لقياس هرمونات الخصوبة وهم:

(LH)الهرمون اللوتيني.

(FSH)الهرمون المحفز للجريبات.

(Testosterone)الهرمون الذكري.

ولقد وجد ان هناك اختلاف معنوي في تركيز الهرمون المحفز للجريبات لدى النساء المصابات بمتلازمة تكيس المبايض بالمقارنة مع الصحيحات حيث كان قليل عند المريضات بينما سجل كل من الهرمون اللوتيني والهرمون الذكري ارتفاع ملحوظ مما يدل على تاثير المرض وهذا التفسير كان ملحوظ بشكل كبير في الفئة العمرية (٤١-٥٠)بالنسبة للهرمون اللوتيني ، (٣١-٤٠) بالنسبة للهرمون الذكري.

لدراسة الجانب الجزيئي لمرض متلازمة تكيس المبايض ولدراسة التغييرات الحاصلة على الجين المسؤول تم استخلاص المادة الوراثية (الحامض النووي) الديوكسي رايبوزي من عينات المريضات وتم تضخيم جين MTHFR و PCOبجميع العينات بواسطة تفاعل سلسلة البلمرة على الحامض النووي المستخلص باستخدام مجموعة من البادئات المتخصصة.

زوج بادئ واحد لجين MTHFR و(٣) ازواج بادئات لجين تكيس المبايض وارسلت نواتج تفاعل سلسلة البلمرة الى شركة ماكروجين في كوريا من اجل اجراء فحص ودراسة تتابع تسلسل القواعد النتروجينية على قطع جين موضوع البحث.

وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

التباين الوراثي في جينات PCO و MTHFR المصاحبة للاعتلال لامتلازمة تكيس المبايض في عينة من النساء العراقيات

رسالة

مقدمة الى مجلس كلية العلوم/جامعة النهرين

كجزء من متطلبات نيل درجة الماجستير في كلية العلوم/التقانة الاحيائية

من قبل

ريم جعفر علي

بكالوريوس تقانة احيائية (٢٠١٤)

أشراف

د. رحاب صبحي رمضان

استاذ مساعد

ربيع الاول ١٤٣٨ كانون الاول ٢٠١٦