**Republic of Iraq**
**Ministry of Higher Education**
**and Scientific Research**
**University of Baghdad**
**Institute of Genetic Engineering**
**and Biotechnology for Postgraduate Studies**

# *Study of Vitamin D Receptor Gene Polymorphisms and Cytogenetic Changes in a sample of Iraqi infertile women with Polycystic Ovary Syndrome*

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By

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بسم الله الرحمن الرحيـــــــــم

﴿ إِنَّ فِي خَلْقِ السَّماواتِ وَالْأَرْضِ وَاخْتِلافِ اللَّيْلِ وَالنَّهارِ لَآياتٍ لِأُولِي الألباب (190) الَّذِينَ يَذْكُرُونَ اللَّهَ قِيامًا وَقُعُودًا وَعَلى جُنُوبِهِمْ وَيَتَفَكَّرُونَ فِي خَلْقِ السَّماواتِ وَالْأَرْضِ رَبَّنا ما خَلَقْتَ هذا باطِلًا سُبْحانَكَ فَقِنا عَذابَ النَّارِ (191) ﴾

صدق الله العلي العظيم

سورة آل عمران: الآيات 190- 191

# *Certification*

I certify that thesis entitled " ***Study of vitamin D receptor gene polymorphisms and cytogenetic changes in a sample of Iraqi infertile women with polycystic ovary syndrome"*** has been conducted under my supervision as partial fulfillment of the requirements for the degree of Doctor of philosophy in Genetic Engineering and Biotechnology for Postgraduate studies , University of Baghdad .

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# EXAMINING COMMETTE CERTIFICATION

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# Dedication

To.....

Greater ..... Allah

My Homeland..... Iraq

My father.....with my whole respect

The pulsated heart ....My mother

My life partner.....My Husband

To supporter.....My brother and sisters

My Lovely children .....Assal & Abo-AL-Fahdel

Sanaa

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*Sanaa*

Polycystic ovary syndrome (PCOS) is the most frequent endocrinological disorder , occurring in young women of reproductive age . Single nucleotide polymorphisms (SNPs) in many studied genes have been related to metabolic co morbidities in general population. This study was carried out to investigate whether the polymorphisms of two exonic and one intronic SNPs of the *VDR* gene polymorphisms are associated with susceptibility to PCOS, investigating the effect of these polymorphisms on the levels of Luteinizing hormone (LH), Follicle stimulating hormone (FSH), Thyroid stimulating hormone (TSH) and Prolactin hormones and the increased of micronucleus frequencies as well as the enhancement of sister chromatid exchanges .

This study was carried out in the Laboratories of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies - University of Baghdad through the period from 1 November 2016 until the end of August 2017, The PCOS patients were taken from the Kamal Al-Samarrai infertility treatment Hospital in Baghdad.

Women with PCOS (n=50) and control group (n=50), were enrolled . Genotyping of *VDR gene* SNP exon 2 (T to C; rs2228570) ; intron 8 (C to A; rs 7975232), and exon 9 (T to C; rs731236) were determined using Taqman genotyping assay by RT-PCR.The results showed that the distribution of genotypes and alleles frequencies at rs2228570T>C SNP of *VDR* gene indicated no significant differences in frequency percentage which were noted between control subjects and patients with polycystic ovary syndrome. Whereas, the frequency of mutant CC genotype was significantly ($p<0.05$) lower in patients with polycystic ovary syndrome than in control subjects . Also in the distribution of genotypes and alleles frequencies at rs7975232 C>A polymorphism ,the frequency of wild CC

genotype was significantly ($p<0.05$) lower in PCOS patients than in apparently healthy subjects .In contrast, the frequency of heterozygous mutant CA genotype was significantly ($p<0.05$) higher in PCOS patients when compared with control subjects. The frequency of wild TT genotype at rs731236 T>C polymorphism was significantly ($p<0.05$) lower in PCOS patients than in control subjects . While as related with TC, CC and combined TC+CC genotypes, no significant differences in frequency percentage were observed between apparently healthy subjects and PCOS patients. Further the three *VDR* SNPs, were presented eight possible haplotypes defined by rs731236 , rs2228570 and rs7975232 with TTA and TTC being the most common in both groups (patients and controls). In particular, the TCC haplotype showed statistically significant ($p<0.05$) distribution between PCOS patients and controls. And the results showed that the frequency of TTC / CCA haplotype combination was significantly ($p<0.05$) higher in PCOS patients than in control subjects (8% *versus* 16% , respectively). This study found that no association between both heterozygous and homozygous mutants at rs2228570 and rs 731236 of *VDR* gene with the incidence of PCOS , while heterozygous at rs 7975232 of *VDR* gene showed a risk for susceptibility of the development of PCOS .

Hormonal analysis for LH, FSH, TSH and Prolactin was performed by using Automated Immune Assay (AIA).The results indicate that serum LH , FSH and TSH concentrations were unaffected by the studied SNPs of *VDR* gene, within carriers of genotypes of rs2228570 , rs7975232 and rs731236 SNPs of *VDR* gene. At the same time serum prolactin levels were significantly ($p<0.05$) higher in PCOS patients *versus* controls.

The genomic instability was evaluated by measuring the frequency of micronucleus and sister chromatid exchange in 66 PCOS patients (the

number of patients =50 but sometimes one patient have homozygous mutant in one SNP and heterozygous mutant in other SNP) and 14 as control women. PCOS patients were classified depending on the results of the genotyping assay. The results showed significant differences in the level of micronuclei and sister chromatid exchange in patients with PCOS for each of *VDR* gene polymorphisms compared with controls.

This study found that no association between both heterozygous and homozygous mutants at rs2228570 and rs 731236 of *VDR* gene with the incidence of PCOS , while heterozygous at rs 7975232 of *VDR* gene showed a risk for susceptibility of the development of PCOS .Also it indicates that serum LH , FSH and TSH concentrations were unaffected by the studied SNPs of *VDR* gene within carriers of genotypes of rs2228570 , rs7975232 and rs731236 SNPs in *VDR* gene, while serum prolactin levels were significantly ($p<0.05$) higher in PCOS patients *versus* controls. In addition results showed significant differences in the level of micronuclei and sister chromatid exchange in patients with PCOS for each of *VDR* gene polymorphisms compared with normal women.

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| | |
|---|---|
| **AE-PCOS** | Androgen Excess and pcos society |
| **AHI** | Apnea-Hypopnea Index |
| **AIA** | Automated Immune Assay |
| **BMI** | Body Mass Index |
| **BrdU** | Bromodeoxyyridine |
| **Ca-P** | Calcium –Phosphate |
| **CVD** | Cardiovascular disease |
| **CML** | Chronic Myelogenous Leukemia |
| **CBMN** | Cytokinesis-block micronuclei |
| **DHEAS** | Dehydroepiandrosterone |
| **DMSO** | Dimethyl Sulfoxide |
| **ELFA** | Enzyme Linked Fluorescent |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **FISH** | Fluorescent insitu hyberdization |
| **FSH** | Follicle Stimulating hormone |
| **FMR1** | Fragile X mental retardation 1 |
| **GnRH** | Gonadotrophin releasing hormone |
| **HDL** | High density lipoprotein |
| **25-(OH)D** | 25-hydroxy vitamin D |
| **IGF-1** | Insulin – like growth factor-1 |
| **IGFBP-1** | Insulin – like growth factor binding protein-1 |
| **IR** | Insulin resistance |
| **LDL** | Low density lipoprotein |
| **LH** | Luteinizing hormone |
| **MN** | Micronucleus |
| **MGB** | Minor groove binder |

| | |
|---|---|
| **MI** | Mitotic Index |
| **Mfg** | Modified Ferriman – Gallwey |
| **NCBI** | National center for biotechnology information |
| **NIH** | National Institutes of Health |
| **NCEP** | National Cholesterol Education Program |
| **NR1I1** | Nuclear receptor subfamily 1,group I , member 1 |
| **OSA** | Obstructive Sleep Apnea |
| **OD** | Optical Density |
| **OV** | Ovarian Volume |
| **PTH** | Parathyrod hormone |
| **P.B.S.** | Phosphate Buffer Saline |
| **PHA** | Phytoheamagglutinin |
| **PCOm** | Polycystic Ovarian morphology |
| **PCOS** | Polycystic Ovary Syndrome |
| **PRI** | Proliferation Rate Index |
| **qRT-PCR** | quantitative real-time PCR |
| **RXR** | Retinoid X receptor |
| **SHBG** | Sex hormone – binding globulin |
| **SNPs** | Single nucleotide polymorphisms |
| **SCE** | Sister chromatid exchange |
| **SRC** | Steroid receptor activator |
| **TSH** | Thyroid stimulating hormone |
| **T2DM** | Type 2 Diabetes Mellitus |
| **UV** | Ultraviolet |
| **VDR** | Vitamin D receptor |
| **WHO** | World Health Organization |

# *Chapter One*

## *Introduction*

# 1. Introduction:

Polycystic ovary syndrome (PCOS) is the most common endocrinological disorder that affects women of reproductive age and is characterized by anovulation, polycystic ovaries, hyperandrogenism and other symptoms such as hirsutism , acne alopecia and menstrual irregularity.(Azziz *et al*.,2009;Walters *et al*., 2012) .

Etiological studies have shown that several complicating factors play critical roles in the pathogenesis of PCOS (Farmakiotis *et al*., 2007). In addition , insulin resistance (IR) is common in PCOS women ( Wehr *et al*., 2009) . who are at an increased risk of type 2 diabetes mellitus , dyslipidaemia, and cardiovascular diseases (Ethrmann , 2005) .

Another common feature of PCOS is the higher obesity prevalence, including a higher waist-hip ratio (visceral obesity), which exacerbates the magnitude of irregular menstrual cycles and other metabolic alterations, including a high frequency of type 2 diabetes mellitus (Norman *et al*., 2007) . The change in lifestyle focusing on weight loss can reduce these abnormalities and improve fertility, inducing ovulation . In many countries, it represents the leading cause of female infertility (Spritzer , 2015).

Various susceptibility genes of PCOS interact with each other and with the environmental factors and influence of the development and manifestation of the syndrome , It is not possible to point out a single gene responsible for the development of the PCOS, as several studies suggest multigenic factors that could contribute to this syndrome , Candidate genes include those involved in insulin pathway, in steroidogenesis, and genes associated with chronic inflammation ( Diamanti –Kandarakis *et al*., 2004) .

Vitamin D levels play a critical role in metabolic modulations including calcium-phosphate (Ca-P) homeostasis, specifically in the regulation of insulin secretion by the β-cells , Abnormalities in calcium balance may also be responsible, in part, for the arrested follicular development in women with PCOS and may even contribute to the pathogenesis of the PCOS syndrome (Pittas *et al* ., 2007)

Some studies suggest that vitamin D deficiency may be a causal factor in the pathogenesis of Insulin resistance ( IR) and the metabolic syndrome in PCOS. However it is not established clearly, vitamin D is also related to endocrine parameters and fertility in PCOS (Wehr *et al*., 2009; Yildizhan , 2009 ). Low serum 25-hydroxy vitamin D [25(OH) D] status is correlated with PCOS features such as testosterone and dehydroepiandrosterone (DHEAS) levels, luteinizing hormone/follicle stimulating hormone (LH/FSH) ratio, free androgen index, sex hormone-binding globulin (SHBG) and hirsutism score (Yildizhan , 2009). Hence, genes involved in insulin signaling pathway and vitamin D metabolism have been suggested as candidates for PCOS.

Vitamin D receptor (*VDR*) gene is considered to be an important candidate gene for PCOS(Morteza *et al* ., 2013). It is a ligand-activated transcription factor that mediates the genomic actions of vitamin D regulating several endocrine functions and cell functions including bone metabolism and calcium-phosphate homeostasis( Serge *et al* .,1998 ; Fariba *et al* ., 2010). It is expressed in various tissues including skeletal, parathyroid as well as reproductive, and modulates the expression of several target genes to produce a variety of biological effects. The *VDR* gene is mapped to chromosomal locus 12q12.14.

Studies on VDR polymorphisms among PCOS women are sparse. Several of them show the polymorphisms of the *VDR* gene might be associated with PCOS and biochemical markers in a relation to PCOS (Mahmoudi , 2009 ; Ranjzad *et al*. 2011, ).

The identification of predisposing factor to PCOS and its complications can provide ways to good understanding the PCOS pathogenesis and result in better prevention , diagnosis and treatment in future .

Several studies report that PCOS is an oligogenic disorder, and more studies are necessary to define its genetic basis (Escobar *et al*. 2005). Different combinations of multiple gene polymorphisms and environmental factors explain the heterogeneity of PCOS (Escobar *et al*. 2005). The cytogenetics provide several biomarkers for chromosomal instability assessment , one of which is the sister chromatid exchange (SCE) frequency in cells. SCE is a natural process that implicates the exchange of homologous genetic segments as a mode of repair mechanism ( Wilson *et al*., 2007). The other biomarker of chromosome damages , such as micronucleus (MN) frequencies in cultured peripheral blood lymphocytes have been used for many years . MN are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind at the anaphase stage of cell division. Their presence in cells reflects structural and/or numerical chromosomal aberrations arising in mitosis (Fenech and Morley 1985 ; Kirsch *et al* ., 2003).

## Aims of the study :

1- Evaluate three SNPs polymorphisms frequency in *VDR* gene of Iraqi women with PCOS in comparison with controls.

2- Study the association between the polymorphisms of these SNPs and the incidence of PCOS in Iraqi women .
3- Study the association of these SNPs as a hapoltype with the incidence of PCOS in Iraqi women .
4- Study the effect of *VDR* gene polymorphisms on the level of LH , FSH , TSH and prolactin hormone.
5- Study some of the cytogenetic parameters such as micronucleus and sister chromatid exchanges in Iraqi women with PCOS.

# Chapter Two

## Literature Review

# 2 Literature review

## 2.1. Polycystic ovary syndrome (PCOS)

The polycystic ovary syndrome is a disorder that characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphologic features. As defined by the diagnostic criteria of the National Institutes of Health (i.e., hyperandrogenism plus ovulatory dysfunction), "classic" polycystic ovary syndrome affects 6 to 10% of women of reproductive age, but the prevalence may be twice as high under the broader Rotterdam criteria Manifestations of androgen excess (e.g., hirsutism) may cause substantial distress in patients, and the polycystic ovary syndrome is the most common cause of anovulatory infertility ( Dumesic *et al*.,2015) .

### 2.1. 1. Definition of PCOS

Since its first description in 1935 by Stein and Leventhal (Stein and Leventhal,1935) a variety of histologic, biochemical, sonographic and clinical characteristics has been associated with polycystic ovarian syndrome.

There is no general agreement,however, about its definition. A 1990 consensus conference on the diagnostic criteria for polycystic ovarian syndrome, convened by the National Institute of Child Health and Development, revealed that there was no consensus; rather, there was a wide variety of views among the assembled experts regarding the clinical and endocrinologic features that characterize polycystic ovarian syndrome. Which report that, a practical and useful clinical definition of polycystic ovarian syndrome has emerged in the United States. Women are defined to have polycystic ovarian syndrome if they have chronic anovulation and

evidence of androgen excess for which there is no other cause (Zawadski and Dunaif ,1992).

Polycystic ovary syndrome is a condition with a range of reproductive and metabolic features that affects 4–18% of reproductive-age women, depending on the diagnostic criteria used ( Moran *et al* .,2011 ; Sirmans and Pate, 2014). PCOS typically involves hormonal imbalances, insulin resistance, and metabolic abnormalities, which significantly increase the risk of infertility, type 2 diabetes, and cardiovascular disease (CVD) and affect quality of life (Cinar *et al*. 2011). Women with PCOS suffer from greater body dissatisfaction and are also at increased risk of mood, generalized anxiety, and eating disorders (Himelein and Thatcher,2006 ; Dokras , 2012). Despite its prevalence and implications for reproductive, metabolic, and psychological health, PCOS is underdiagnosed, in part because of the diversity of phenotypes manifested by this condition (Sirmans and Pate, 2014 ).

However, during the 2003 Rotterdam Consensus workshop, the syndrome was widely accepted as the commonest cause of anovulatory infertility with clinical and/or biochemical signs of excess androgen secretion, associated with hyperinsulinemia and high prevalence of significant metabolic abnormalities, with long-term sequelae which may affect the women's long-term health, therefor PCOS was defined as a multi-system network of abnormalities that included obesity, insulin resistance, hyperandrogenism, elevated LH concentrations, increased risk of type 2 diabetes mellitus, cardiovascular events and menstrual irregularities (Rotterdam ESHRE/ASRM,2004) .

### 2.1. 2. Prevalence of PCOS

PCOS being considered the most common endocrine disorder in women of reproductive age ( Kauffman *et al*., 2008).

Prevalence estimates are highly variable, ranging from 2.2% to as high as 26% (Knochenhauer *et al*., 1998; Diamanti-Kandarakis *et al*., 1999; Michelmore *et al*., 1999). This variability is due to several factors. Firstly, diagnosing the disorder is logistically difficult, with the necessity to carry out blood or ultrasound tests. This has resulted in prevalence studies being based on convenience samples and generally not exceeding 400 participants. For example, participants in commonly cited prevalence studies have been University employees (Knochenhauer *et al*.,1998) , or blood donors but there has been no indication of the representativeness of these subgroups in the studies. Secondly, considerable heterogeneity in the presentation of symptoms has contributed to a lack of agreement over the diagnostic criteria used to define the condition.

Estimations of the prevalence of PCOS depend on the population being assessed, as there are ethnic differences in the clinical and biochemical features of PCOS (Fauzia *et al*, 2007). The reported prevalence of PCOS ranges between 2.2% to 26% in various countries, depending on the recruitment method, the study population, the criteria used for its definition and the method used to define each criterion. The prevalence of PCOS can be as high as 30% in women with secondary amenorrhea, 40% in women with infertility, 75% in women with oligomenorrhea and 90% in women with hirsutism (Joyce , 2007).

### 2.1.3. Signs and symptoms

In addition to the three features used to diagnose PCOS (absence of ovulation, high levels of androgens, and ovarian cysts), PCOS has many signs and symptoms, some of which may not seem to be related:(Teede *et al* ., 2011) .

- Menstrual irregularities:
  - No menstrual periods—called amenorrhea .
  - Frequently missed periods—called oligomenorrhea .
  - Very heavy periods .
  - Bleeding but no ovulation—called anovulatory periods
- Infertility
- Excess hair growth on the face, chest, belly, or upper thighs—a condition called hirsutism .
- Severe, late-onset, or persistent acne that does not respond well to usual treatments
- Obesity, weight gain, or trouble losing weight, especially around the waist
- Pelvic pain
- Oily skin
- Patches of thickened, dark, velvety skin—a condition called acanthosis nigerians.

Because many women do not consider problems such as oily skin, extra hair growth, or acne to be symptoms of a serious health condition, they may not mention these things to their health care providers. As a result, many women are not diagnosed with PCOS until they have trouble getting pregnant or if they have abnormal periods or missed periods.

Although PCOS is a leading cause of infertility, many women with PCOS can and do get pregnant. Pregnant women who have PCOS, however, are at higher risk for certain problems, such as miscarriage.

## 2.2. PCOS and Reproductive Health

PCOS is the most common cause of an ovulatory infertility; ~ 90–95% of an ovulatory women seeking treatment for infertility have PCOS (Teede *et al.* , 2010). Women may learn they have PCOS only after seeking infertility treatment. Most women with PCOS have elevated levels of luteinizing hormone and reduced levels of FSH , coupled with elevated levels of androgens and insulin (Haqq *et al.*,2014). These imbalances can manifest as oligo menorrhea or amenorrhea (infrequent or lack of menstruation). Underproduction of estrogen and overproduction of androgens (testosterone, dehydroepiandrosterone, and androstenedione) by the ovaries can result in a number of additional clinical features, including tiny cysts on the surface of the ovaries (polycysts) and hair and skin symptoms ( Salley *et al.*, 2007). Women with PCOS who become pregnant are at higher risk than those without PCOS of developing gestational diabetes mellitus or suffering a first-trimester spontaneous abortion (Salley *et al.*, 2007 ;
Sirmans and Pate, 2014 ) .

## 2.3. Diagnosing PCOS

Diagnosing and treating PCOS is important to preserve or restore fertility, reduce symptoms, and prevent complications that can develop in women with PCOS from adolescence to the postmenopausal period. Obstacles to timely diagnosis include the presence of multiple PCOS phenotypes and significant individual variation in clinical features, as well as competing diagnostic criteria from the National Institutes of

Health (NIH), the European Society for Human Reproduction and Embryology and American Society for Reproductive Medicine (ESHRE/ASRM Rotterdam), and the Androgen Excess and PCOS Society (AE-PCOS) (Badawy and Elnashar, 2011). To be diagnosed with PCOS under the ESHRE/ASRM Rotterdam criteria, which are considered to be a compromise between those of NIH and AE-PCOS, a woman must have at least two of three criteria after other related health conditions are ruled out: oligo-ovulation and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries visible by ultrasound (Rotterdam,2003 ; Broekmans and Fauser,2006). Although approximately three out of four women with PCOS have polycysts on their ovaries, this clinical feature is no longer deemed necessary or sufficient for diagnosis. One recommendation from the NIH Evidence-Based Methodology Workshop on (PCOS) held in December 2012 ( in U.S. Department of Health and Human Service) was that PCOS be renamed to more accurately reflect the complex nature of this syndrome and its implications for women's reproductive and metabolic health (Sirmans and Pate, 2014).

**Table 2- 1.** Diagnostic criteria for PCOS (NIH final report,2012).

| | |
|---|---|
| **NIH 1990** | **1- Chronic anovulation**<br>**2- Clinical and/or biochemical signs of hyperandrogenism (with exclusion of other etiologies, e.g., congenital adrenal hyperplasia)** ***(Both criteria needed)*** |
| **Rotterdam 2003** | **1- Oligo- and/or anovulation**<br>**2- Clinical and/or biochemical signs of hyperandrogenism**<br>**3- Polycystic ovaries** ***(Two of three criteria needed)*** |
| **AE-PCOS Society 2006** | **1- Oligo-anovulation and/or polycystic ovarian morphology(Ovarian dysfunction)**<br>**2- Clinical and/or biochemical signs of hyperandrogenism** ***(Both criteria needed)*** |

**Abbreviations:** NIH, National Institutes of Health; AE-PCOS, Androgen Excess and PCOS

## 2.4. Pathogenesis of PCOS

Polycystic ovaries develop when the ovaries are stimulated to produce excessive amounts of male hormones (androgens), particularly testosterone, by either the release of excessive luteinizing hormone by the anterior pituitary gland, high levels of insulin in the blood (hyperinsulinaemia) in women whose ovaries are sensitive to this stimulus or reduced levels of sex-hormone binding globulin (SHBG) resulting in increased free androgens (Strauss,2003). The syndrome has acquired its name due to the common sign on ultrasound examination of multiple ovarian cysts which represent immature follicles. The follicles have developed from primordial follicles but the development has stopped at an early antral stage due to the disturbed ovarian function. The follicles may be oriented along the ovarian periphery appearing as a ‘string of pearls’ on ultrasound examination (Lee and Rausch, 2012). Patients with PCOS have higher gonadotrophin releasing hormone

(GnRH), which in turn results in an increase in LH/FSH ratio in females with PCOS. The majority of patients with PCOS have insulin resistance and/or obesity. Their elevated insulin levels contribute to or cause the abnormalities seen in the hypothalamic-pituitary-ovarian axis that lead to PCOS. Hyperinsulinemia increases GnRH pulse frequency, LH over FSH dominance, increased ovarian androgen production, decreased follicular maturation and decreased SHBG binding. All these factors contribute to the development of PCOS (Sharquie *et al*., 2007; Nafiye *et al*.,2010). PCOS is characterized by a complex positive feedback of insulin resistance and hyperandrogenism. In most cases, it cannot be determined which of those two should be regarded to be the causative agent. Experimental treatment with either anti-androgens or insulin sensitizing agents improves both hyper-androgenism and insulin resistance (Pasquali & Gambineri,2007). Adipose tissue possesses aromatase, an enzyme that converts androstenedione to estrone and testosterone to estradiol. The excess of adipose tissue in obese patients causes them to have both excess androgens (which are responsible for hirsutism and virilization) and estrogens (which inhibit FSH via negative feedback) (Sathyapalan and Atkin,2010). PCOS may be associated with chronic inflammation of the ovary which may induce conformational, endocrinal and metabolic changes which may predispose to PCOS. Several studies correlate the inflammatory mediators and oxidative stress with anovulation and other PCOS symptoms (González,2006). It has been previously suggested that the excessive androgen production in PCOS could be caused by a decreased serum level of insulin-like growth factor binding protein-1 (IGFBP-1), in turn increasing the level of free IGF-1 which stimulates ovarian androgen production, but recent data concludes this mechanism to be unlikely. PCOS has also been associated with a specific fragile X

mental retardation 1 (FMR1) sub-genotype. Many studies suggested that women who have heterozygous-normal/low FMR1 have polycystic-like symptoms of excessive follicle-activity and hyperactive ovarian function (Gleicher *et al*., 2010; Kelly *et al*., 2011).

## 2.5. Etiology of PCOS

Although the exact cause of PCOS is unknown, it is understood to be a multifactorial condition with a genetic component. Approximately 20–40% of first-degree female relatives of women with PCOS go on to develop PCOS themselves, compared to an estimated 4–6% prevalence in the general population (Goodarzi, 2011). Many women with PCOS have female relatives with PCOS, even if it has been never diagnosed (Sirmans and Pate, 2014). As with type 2 diabetes, it is likely that numerous genes each make a small contribution to the etiology of PCOS; and genome-wide association studies have identified candidate genes (Goodarzi, 2011 ; Sirmans and Pate, 2014). Any underlying genetic predisposition is likely complicated by epigenetic and environmental factors such as an unhealthy diet and lack of physical activity. (Goodarzi, 2011) .

## 2.6. Reproductive consequences of PCOS

### 2.6.1. Infertility in PCOS

PCOS is characterized by anovulation due to a developmental defect of follicles beyond 10 mm in size. The clinical manifestations, including infertility, are related to the hypersecretion of LH (70%) present in women with hyperandrogenism anovulatory women, (the ratio of LH/FSH ratio and high increase in ovarian androgen production). Most of the cycles are anovulatory, making it essential to induce ovulation, Infertility has been considered by the World Health Organization (WHO)

as a public health problem . The agency defines infertility as the absence of pregnancy after two years of regular intercourse, without using any contraception method. However, there is a consensus that, after one year, a process of assessment of possible factors involved should begin (Izzo,2013). The Rotterdam ESHRE/ASRM (2004a) recommends that, before startig any intervention, acounseling before conception should emphasize the importance of lifestyle changes, especially weight loss and regular exercises in overweight patients, smoking cessation, and reducing alcohol consumption. Regarding ovulation inducing drugs, all are associated with the increase in multiple pregnancies, obstetric and neonatal risks (ACOG, 2009 ). Among the most commonly found female's causes of infertility, it is possible to observe structural changes, ovulatory changes, immune disorders and endometriosis. Infertility patterns may be influenced by many factors, such as woman's age, frequency in sexual activity, woman's weight and smoking, among others. This way, different techniques should be used to reach an accurate diagnosis (Martins *et al.* , 2009). About 50% of infertile women have also obesity. There is a clear association between obesity and menstrual irregularities, since the adipose tissue is the largest peripheral area for the aromatization of androgens to estrogens, contributing to estrogen production (Izzo,2013).

The progesterone dosage may be useful as an additional screening test (Xin *et al*., 2014). It is also recommended to exclude other infertility causes besides anovulation, in couples in which a woman has PCOS. The diagnosis of PCOS is very important, because it identifies the metabolic risks, the potential cardiovascular risk and mainly because such a diagnosis interferes directly with the fertility status of these patients (Legro *et al.* , 2013).

### 2.6.1.1 Ovarian dysfunction

With the advent of trans-vaginal ultrasonography, assessment of ovarian follicle number has become the main item of polycystic ovarian morphology (PCOm). An increase in ovarian volume (OV), as well as an increased ovarian area, are also considered as accurate markers of PCOm, provided the measurements are carried out on median sections of the ovaries. There is an almost universal consensus on the choice of follicular excess and ovarian enlargement as the main criteria to define PCOm by ultrasound ( Dewailly *et al*., 2014)**.**

### 2.6.1.2. Menstrual irregularity

Menstrual regulation may be indicated in those women who do not wish to conceive. Many patients suffer occasional distressing episodes of prolonged and/or heavy vaginal bleeding. Adolescents frequently exhibit physiological menstrual irregularities such as oligomenorrhea (Powers *et al*., 2015), usually during the first 2 years after menarche, due to lack of maturation of hypothalamic-hypopituitary-ovarian axis (Tfayli and Arslanian, 2008). As such, menstrual irregularity can be sometimes an unreliable criterion for diagnosis of PCOS in adolescents (Kamangar *et al*., 2015). Through close observation of the menstrual cycle patterns, clinicians have to differentiate physiological anovulation associated with puberty from pathological anovulation as a dysfunction identified in PCOS (Franks, 2002; Wiksten-Almstromer *et al*., 2008). It has been suggested to postpone diagnosis at least 2 years after menarche to establish a persistent menstrual irregularity (Hardy and Norman, 2013). However, this may delay the initiation of appropriate treatment (Powers *et al*., 2015). Even in patients without menstrual symptoms, treatment may be advisable because of the long-term risk of unopposed oestrogen (endometrial hyperplasia and cancer). (Legro *et al*., 2013) **.**

### 2.6.1.3. Miscarriage

Miscarriage rates are believed to be higher in PCOS women than in normal women, although it is discussed whether it is the PCOS presence or the associated overweight/obesity that is the actual cause. A meta-analysis verified the results of several other studies and showed an increased prevalence in PCOS women of gestational diabetes, gestational hypertension, preeclampsia and premature births. In addition, the infants of PCOS women were more often admitted to a neonatal intensive care unit and the perinatal mortality was higher, independently of multiple pregnancies/deliveries (Boomsma *et al*.,2006) .

## 2.7. Long-term consequences of PCOS

### 2.7.1. Type 2 Diabetes Mellitus (T2DM)

Most women with PCOS, particularly those presenting with overweight or obesity (Gambineri *et al*.,2002), do in fact have insulin resistance and compensatory hyperinsulinemia (Dunaif,1997; Poretsky *et al*.,1999), partly attributed to intrinsic insulin resistance mechanisms (Ciaraldi *et al*.,1997 ; Corbould *et al*.,2005 ; Bremer and Miller ,2008). Insulin resistance is a known key factor in the development of type 2 diabetes (Puavilai *et al*.,1999). Several studies have demonstrated that type 2 diabetes occurs with increased frequency in women with PCOS (Moran *et al*.,2010 Tomlinson *et al*.,2010), so that PCOS recently has been identified as a significant nonmodifiable risk factor associated with type 2 diabetes by the International Diabetes Federation and by the American Diabetes Association (American Diabetes Association,2004 ; Alberti *et al*.,2007).

### 2.7.2. Obesity and PCOS

Obesity is considered one of the most important features of PCOS. Its prevalence in diseased women varies between 61 and 76% (Glueck *et al*., 2005). The prevalence of obesity reaches 80% in the United States (Ehrmann *et al*., 1999) and 50% outside (Balen *et al*., 1995) which indicates that figure 2-1 depends on local environmental factors, ethnic backgrounds, and lifestyle, and not on the mere presence of PCOS itself. Childhood obesity is a well-documented risk factor for PCOS. Obese girls are at a higher risk of developing insulin resistance, metabolic syndrome, and PCOS later on in life (Pasquali *et al*., 2011). On the other hand, women with PCOS are at a higher risk of developing obesity (Randeva *et al*., 2012). Kirschner *et al*. (1990) explain that females with PCOS have increased visceral and subcutaneous body fat distribution due to increased androgen production rates. This central obesity follows a masculinized body fat distribution (Borruel *et al*., 2013) where the amount of visceral fat correlates with the degree of insulin resistance (Karabulut *et al*., 2012). Moreover, obesity plays a significant role in expressing the metabolic features of PCOS. Women with PCOS have an atherogenic lipid profile, associated with elevated levels of low-density lipoprotein, triglycerides and cholesterol, along with decreased levels of high-density lipoprotein. They are also at a higher risk of developing atherosclerosis, arterial stiffness, and altered vascular endothelium (Hart and Norman, 2006). In addition, women with PCOS show a worsened cardiovascular profile and associated complications (Randeva *et al*., 2012). However, obesity by itself is not the main reason behind these features. This is evident in lean women with PCOS who demonstrate the same metabolic features as those who are obese (Balen *et al*., 1995) .

Whether obesity leads to PCOS or PCOS leads to obesity, it is still debatable (Kamangar *et al*., 2015).

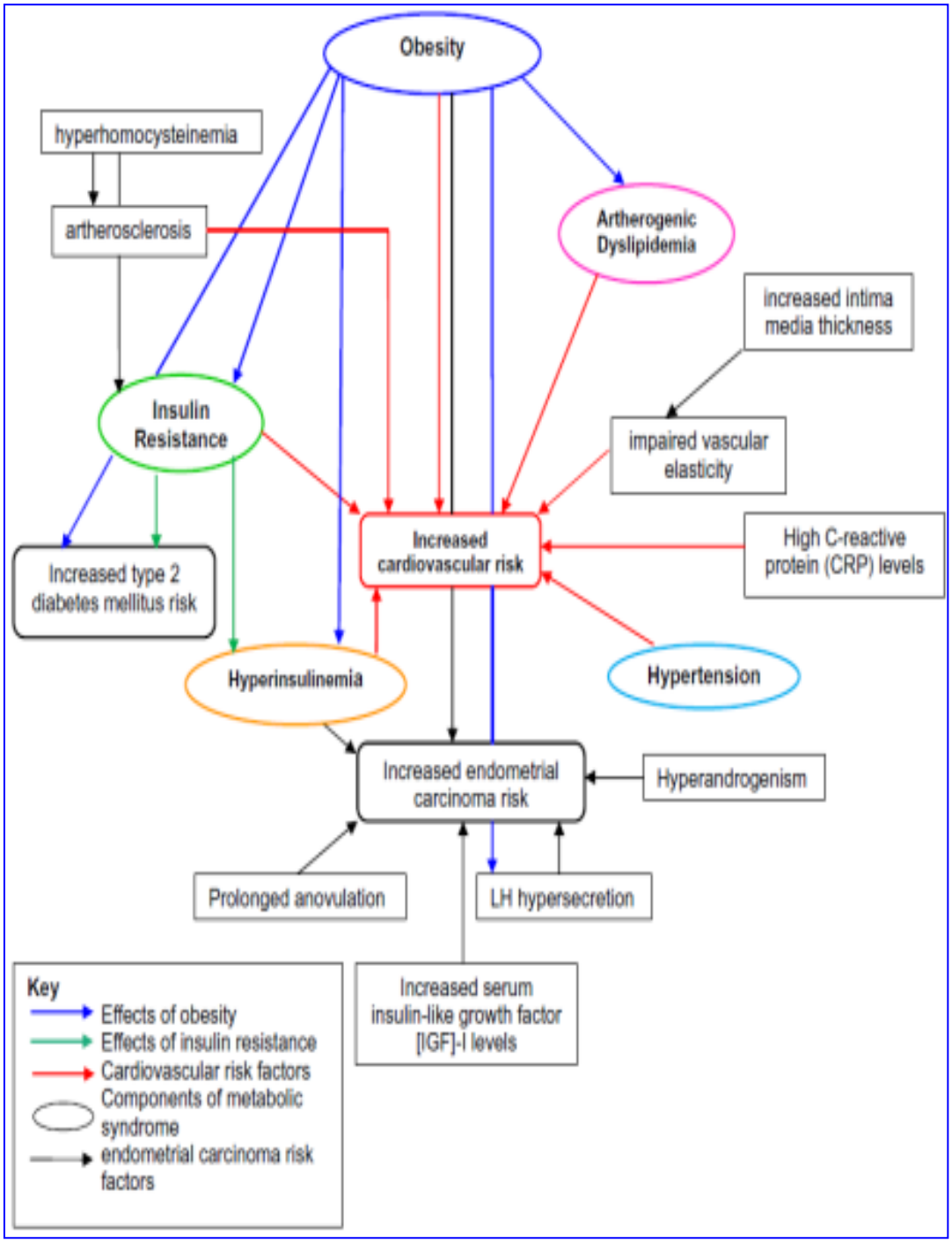


**Figure 2-1. Metabolic dysfunction in PCOS (Allahbadia and Merchant, 2011).**

### 2.7.3. Hyperlipidemia and PCOS

Elevated serum lipids are well known risk factors for CVD, while high density Lipo protein (HDL) may have a protective effect (Mineo *et al*.,2006). According to the National Cholesterol Education Program (NCEP), ~70% of PCOS women have abnormal serum lipid levels (NCEP,2002), and dyslipidemia might be the most common metabolic abnormality in PCOS (Diamanti-Kandarakis *et al*.,2007). The dyslipidemia in PCOS includes elevated levels of LDL and triglycerides and decreased levels of HDL (Talbott *et al*.,1998 ; Diamanti-Kandarakis *et al*.,2007). Some studies have shown low Apo A1, high ApoB and a high Apo B/Apo A1 ratio to be superior predictors of acute MI (Mass Index) in both sexes and at all ages (Yusuf *et al*.,2004; McQueen *et al*.,2008). Apo A1 is the major protein component of HDL and promotes cholesterol efflux from the liver. Apo B, a lipoprotein of LDL, is a ligand for LDL receptors and facilitates the LDL cholesterol distribution to tissues. In PCOS, studies on Apo levels have included only young women and the results indicate lower Apo A1 and similar Apo B as in non-PCOS women (Diamanti-Kandarakis *et al*.,2007). As PCOS women tend to have increased abdominal fat, they are more predisposed to dyslipidemia, as the centrally located adipocytes seem to exert an adverse effect on blood lipids (Yildirim *et al*.,2003). Within the adipocytes in PCOS, insulin resistance and hyperandrogenemia, result in increased catecholamine-induced lipolysis and the release of free fatty acids into the circulation. Elevated free fatty acids flux to the liver and stimulate the assembly and secretion of very low-density lipoprotein producing hypertriglyceridemia (Diamanti-Kandarakis *et al*.,2007). Centrally located fat is more insulin-resistant and recycles fatty acids more rapidly through lipolysis compared with peripheral fat (Bjorntorp,1992; Diamanti-Kandarakis *et al*.,2007) .

### 2.7.4. Cardiovascular disease

CVD is estimated to be the major cause of death worldwide (Murray *et al*.,1996). Several risk factors for CVD/MI exist and they can be grouped into non-modifiable and modifiable factors. The major non-modifiable CVD risk factors in women are age and family history of premature CVD. Modifiable risk factors for CVD/MI are high cholesterol, diabetes, hypertension, abdominal obesity, smoking, alcohol, physical inactivity and psychosocial stress, and these explain more than 90% of the risk of acute MI (Yusuf *et al*.,2004). Similar odds ratios were found regarding all the risk factors in both sexes, except that the increased risk associated with hypertension and diabetes, and the protective effect of exercise, seemed to be greater in women than in men (Yusuf *et al*.,2004). Multiple studies showed an increased frequency of CVD risk factors in PCOS women of fertile/premenopausal ages. It has also been shown that mothers of women with PCOS had a higher risk of CVD events than controls (Cheang *et al*.,2008) and, in addition, that PCOS women have an increased prevalence of the metabolic syndrome (Glueck *et al*.,2003). The metabolic syndrome is strongly associated to diabetes, CVD (Isomaa *et al*.,2001) and all-cause mortality (Trevisan *et al*.,1998) in the general female population, although it has been stressed that it is not more powerful in predicting CVD events than any other single risk factor (Nilsson *et al*.,2007). Hence, women with PCOS would be expected to have a greater risk of fatal and non-fatal CVD events during the postmenopausal period. However, due to the lack of prospective follow-up studies in postmenopausal PCOS women and the fact that the few existing studies show deficiencies, such as small sample sizes, wide age ranges, studies of women of young ages, cross-sectional designs, variations in PCOS criteria and different definitions and choices of

outcome measures, evidence is still lacking (Wild *et al*., 2010; Schmidt *et al*., 2011; Sathyapalan and Atkin, 2012). Several biomarkers of CVD risk are increased in women with PCOS. Morphological markers has been found in PCOS women; for example, increased arterial calcification in the carotid artery (increased intima media thickness) (Carmina *et al*.,2006 ), in coronary arteries (Talbott *et al*.,2004) and in the aorta (Talbott *et al*.,2004), and reduced vascular compliance (in the brachial artery, measured as flow-mediated dilation (Kelly *et al*.,2002) or increased arterial stiffness (Carmina *et al*.,2006 ; Orio *et al*.,2004), in the internal carotid artery (Lakhani *et al*.,2000).

### 2.7.5. Prevalence of obstructive sleep apnea(OSA) in PCOS

The most recent estimate of the prevalence of OSA in the general adult population is approximately 17%. In overweight individuals (body mass index [BMI] R25 kg/m2 ), the proportion of mild to moderate OSA is 41% to 58% (Young *et al*.,2005). The prevalence of OSA is particularly low in premenopausal women and increases after menopause (Young *et al*.,2003). Among women who have PCOS, however, OSA is considerably more common than expected (Fogel *et al*.,2001; Vgontzas *et al*.,2001; Gopal *et al*.,2002). Vgontzas *et al*.,2001 assessed the prevalence of OSA in 53 premenopausal women who had PCOS compared with 452 control women , they found that PCOS women were 30 times more likely to have OSA than control subjects and that the difference between the two groups remained significant even after controlling for BMI (Vgontzas *et al*.,2001) . An independent study published the same year, comparing 18 overweight women who had PCOS with 18 age- and weightmatched control subjects, showed that PCOS women were significantly more likely to suffer from symptomatic OSA (based on an apnea-hypopnea index (AHI) >5 and the presence of

excessive daytime sleepiness) than control women (44.4% *versus* 5.5%) (Fogel *et al*.,2001) . In another cohort of 23 obese PCOS women, the prevalence of OSA was found to be 70%, and there was no association between obesity (as assessed by BMI) and the severity of OSA (Gopal *et al*.,2002) . Survey assessments of the prevalence of sleep apnea risk (using the Berlin questionnaire) in a cohort of 40 women who had PCOS, revealed that three of four women were at high risk for sleep apnea ( Tasali *et al*.,2006). About two thirds of these PCOS women had poor sleep quality as assessed by the Pittsburgh Sleep Quality questionnaire and 45% had chronic daytime sleepiness as defined by the Epworth Sleepiness Scale. Less than 8% of this cohort of 40 women who had PCOS were free of sleep complaints ( Tasali *et al*.,2006). Sleep disturbances thus appear to be an important feature of PCOS. Given the high prevalence of OSA among women who have PCOS, it may be warranted to systematically evaluate them for sleep disorders. Findings from a recent survey, however, suggest that more than 90% of physicians who manage PCOS patients rarely (Vgontzas *et al*.,2001) .

## 2.8. Cancer and PCOS

Endometrial, ovarian and breast cancer are mainly discussed as having a possible association with PCOS. Risk factors for endometrial cancer are excessive weight, hyperinsulinemia, nulliparity, and a longer time of exposure of estrogens (Amant *et al*.,2005), and these factors are also associated with PCOS. In addition, increasing age and a sedentary lifestyle add to the already mentioned risk factors (Amant *et al*.,2005). The mechanism behind endometrial cancer in PCOS 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). However, A meta-analysis has showed an almost three times higher risk of developing endometrial cancer for PCOS women (OR 2.70;95% CI 1.00-7.29) compared to women without PCOS (Chittenden *et al*.,2009). Regarding breast cancer, the cause of an association with PCOS, is that of obesity, hyperandrogenism, the longer time-periods of unopposed estrogen and of infertility . 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*. (Chittenden *et al*.,2009). The risk of ovarian cancer seems to be increased in women with multiple ovulations i.e., nulliparity, late menopause and early menarche (Balen,2001). 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. Most studies seem to show no association between ovarian cancer and PCOS (Pierpoint *et al*.,1998 ; Balen,2001), but one small population-based case control study reported a ~2-fold increased risk (Schildkraut *et*

*al*.,1996). The studies of cancer and a possible association with PCOS have been performed on women below the age of 50 years. The risk of the cancer types mentioned above increases with age, as most of the cases of endometrial cancer are diagnosed after the menopause, with the highest incidence around the age of 70 years (Amant *et al*.,2005).

## 2.9. Vitamin D & Vitamin D deficiency

Vitamin D (calciferol) comprises a group of fat soluble seco-sterols is found naturally only in a few foods, such as fish-liver oils, fatty fish, mushrooms, egg yolks, and liver. The two major physiologically relevant forms of vitamin D are D2 (ergocalciferol) and D3 (cholecalciferol). Vitamin D3 is photosynthesized in the skin of vertebrates by the action of solar ultraviolet (UV) B radiation on 7-dehydrocholesterol (Fieser 1959). Vitamin D2 is produced by UV irradiation of ergosterol, which occurs in molds, yeast, and higher-order plants. Under conditions of regular sun exposure, dietary vitamin D intake is of minor importance. However, latitude, season, aging, sunscreen use, and skin pigmentation influence the production of vitamin D3 by the skin. Vitamin D without a subscript represents either D2 or D3 or both and is biologically inert. Vitamin D from the skin or diet is only short-lived in circulation (with a half-life of 1–2 days), as it is either stored in fat cells or metabolized in the liver (Mawer , 1972). In circulation, vitamin D is bound to vitamin D-binding protein and transported to the liver, where it is converted to 25-hydroxyvitamin D (DeLuca , 1984). This major circulating form of vitamin D is a good reflection of cumulative effects of exposure to sunlight and dietary intake of vitamin D (Haddad , 1973; Holick , 1995) and is, therefore, used by clinicians to determine vitamin D status. To be biologically activated at physiologic concentrations, 25(OH)D must be

converted in the kidneys to 1,25-dihydroxyvitamin D , which is thought to be responsible for most, if not all, of the biologic functions of vitamin D (DeLuca 1988; Reichel 1989). The production of 25(OH)D in the liver and of 1,25(OH)2 D in the kidney is tightly regulated. In the liver, vitamin D-25-hydroxylase is down-regulated by vitamin D and its metabolites, thereby limiting any increase in the circulating concentration of 25(OH)D following intakes or following production of vitamin D after exposure to sunlight. In the kidney, in response to serum calcium and phosphorus concentrations, the production of 1,25(OH)2 D is regulated through the action of parathyroid hormone (PTH) (DeLuca , 1988; Reichel , 1989). Active vitamin D functions as a hormone, and its main biologic function in people is to maintain serum calcium and phosphorus concentrations within the normal range by enhancing the efficiency of the small intestine to absorb these minerals from the diet (DeLuca , 1988; Reichel , 1989). When dietary calcium intake is inadequate to satisfy the body's calcium requirement, 1,25(OH)2 D, along with Parathyroid hormone (PTH) , mobilizes calcium stores from the bone. In the kidney, 1,25(OH)2 D increases calcium reabsorption by the distal renal tubules. Apart from these traditional calcium-related actions, 1,25(OH)2 D and its synthetic analogs are increasingly recognized for their potent antiproliferative, prodifferentiative, and immunomodulatory activities (Nagpal , 2005).

In adults, vitamin D deficiency is defined as a serum 25-hydroxyvitamin D level of less than 20 ng per mL (50 nmol per L), and insufficiency is defined as a serum 25-hydroxyvitamin D level of 20 to 30 ng per mL (50 to 75 nmol per L) ( Holick, 2007).

Vitamin D deficiency in adults was previously thought to be limited to older persons living in institutions . A group of international experts concluded that approximately 50 percent of persons , 65 years and older in North America and 66 percent of persons internationally (all ages) failed to maintain healthy bone density and tooth attachment because of inadequate vitamin D levels . ( Norman *et al*. 2007**).**

A common cause of deficiency is medication use, such as anticonvulsants or glucocorticoids, which can increase catabolism and actively destroy vitamin D ( Holick , 2007)**.**

One study has found that 25-hydroxyvitamin D levels were inversely related to the following cardiovascular risk factors: blood pressure greater than 140/90 mm Hg, blood glucose level above 125 mg per dL (6.95 mmol per L), and body mass index of 30 kg per m2 or greater.( Martins *et al.* , 2007).

**2.10. Vitamin D receptor**

The calcitriol receptor, also known as the vitamin D receptor (VDR) and also known as NR1I1 (nuclear receptor subfamily 1, group I, member 1), is a member of the nuclear receptor family of transcription factors. ( Bollag , 2007 ).

Vitamin D ( $1,25(OH)_2$ D ) is the main ligand for the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcriptional regulators (DeLuca,1984). The 25(OH)D precursor is widely used in assessment of vitamin D repletion and has a slower rate of clearance from the circulation than $1,25(OH)_2$D. Because the VDR is expressed in a large number of tissues, it is not surprising that ligand-activated VDR modulates the expression of many genes (MacDonald ,1999).

## 2.11. Vitamin D and vitamin D receptor relationship with PCOS

Women with PCOS may also be at elevated risk of vitamin D deficiency (VDD). In contrast to a prevalence of 20%–48% among the general adult population (Forrest *et al.* , 2011; Hovsepian *et al.* , 2011), a relative higher prevalence of VDD is observed among women with PCOS (approximately 67%–85% women with PCOS have VDD) (Thomson *et al.* , 2012) .

Vitamin D receptors are expressed in 2776 genomic positions and modulate the expression of 229 genes in more than 30 different tissues, such as skeleton, brain, breast, pancreas, parathyroid glands, immune cells, cardiomyocytes, and ovaries (Holick, 2007 ; Ramagopalan *et al.* , 2010). Thus, deficiency in this vitamin, in addition to its well-described role in calcium homeostasis and bone metabolism, may cause a wide range of extra-skeletal effects with impact on glucose homeostasis, cardiovascular disease, cancer, autoimmune diseases and psychological disorders (Freedman *et al.* , 2007 ; Holick,2007; Dobnig *et al.* , 2008). Vitamin D may play a role in glucose metabolism by enhancing insulin synthesis and release, and increasing insulin receptor expression or suppression of proinflammatory cytokines that possibly contribute to the development of insulin resistance (Teegarden and Donkin, 2009). The effect of vitamin D on metabolic and reproductive dysfunctions in PCOS may be mediated by insulin resistance. Reproductively, insulin resistance increases hyperandrogenism through insulin increasing ovarian androgen production, and reducing sex hormone-binding globulin (SHBG) production (Plymate *et al.* , 1988). Metabolically, insulin resistance is associated with an increased risk for impaired glucose tolerance, type 2 diabetes mellitus and cardiovascular disease (; Lillioja *et al.* , 1993 ;

Ruige *et al*. , 1998; Haffner *et al.* , 1999; Rutter *et al*. , 2005) . Therefore, vitamin D may play a key role in the development of PCOS.

## 2.12. Genetic effects

There is evidence of a genetic component based on the existence of familial clustering (Legro *et al*.,1998; Diamanti-Kandarakis *et al*.,2006) and twin studies have displayed a two fold increased concordance of PCOS in genetically identical twins compared with non-identical twins (Vink *et al*.,2006). In spite of numerous association studies (mainly focusing on genes associated with the synthesis and metabolism of androgens and insulin), the way in which PCOS is inherited remains unclear (Balen ,2010). An efforts, using modern mapping techniques, have made some progress to identify promising candidate genes. Two promising candidate genes have so far emerged. The first, a locus on chromosome 19p13.2, is associated with high susceptibility to PCOS (Urbanek *et al*.,2005) and the second is the fat-mass and obesity associated gene, whose polymorphism has been found to be associated with PCOS (Barber *et al*.,2008). However, the studies implicating these two locus, needs to be confirmed in larger studies and in other populations.

### 2.12.1. Genetic etiology of PCOS

PCOS is a complex oligogenic disorder in which, a small number of key genes interact with environmental factors (notably obesity), the balance of which , determine the typically heterogeneous , clinical and biochemical phenotype (Franks *et al*., 2000).

Multiple biochemical pathways have been implicated in the pathogenesis of PCOS and several genes from these pathways have been tested, including genes according to Jakubowski , (2005) :

1- Steroid hormone biosynthesis and metabolism (*StAR , CYP11, CYP17, CYP19, HSD17B1-3 and HSD3B1-2*) .
2- Gonadotropin and gonadal hormones action (*ACTR1, ACTR2A-B, FS, INHA, INHBA-B, INHC, SHBG, LHCGR, FSHR, MADH4* and *AR*).
3- Obesity and energy regulation (*MC4R, OB, OBR, POMC and UCP2-3*).
4- Insulin secretion and action (*IGF-1, IGF-1R ,IGFBPI1-3, INS VNTR, IR, INSL, IRS1–2 and PPARG*) , and many others.

Several lines of evidence suggest that there is an underlying genetic cause for PCOS (Strauss , 2003) . Collectively, these findings are consistent with the concept that a gene or several genes are linked to PCOS susceptibility. A strong genetic basis for PCOS also comes from the fact that the syndrome clusters in families (Jakubowski , 2005) . However, many candidate genes have been evaluated for a causal role in PCOS during the past decade, because of genetic and phenotypic heterogeneity and underpowered studies, the results of many of these studies remain inconclusive (Urbanek , 2007). Most of these genes represent only minor modifying loci; the evidence supporting linkage is not overwhelming and needs to be buttressed in larger studies. Because the mutations/genotypes associated with PCOS are rare, and their full impact on the phenotype incompletely understood, routine screening of women with PCOS or stigmata of PCOS for these genetic variants is not indicated at that time (Legro and Strauss , 2002).

### 2.12.2. Mode of inheritance for PCOS

The mode of inheritance of the disorder is still uncertain, although the majority of studies are consistent with an autosomal dominant pattern (Legro and Strauss, 2002) with incomplete penetrance, modified perhaps by environmental factors. Studies of first-degree relatives of women diagnosed with PCOS reveal familial clustering of the disease, particularly hyperandrogenemia, with 46% of ascertainable sisters of PCOS women are reported as hyperandrogenemic, suggesting a dominantly inherited trait controlling androgen levels ( Strauss, 2003) .

The probability of finding a metabolic disorder in the families of PCOS patients has been reported to be 2.7-fold higher than in families of the control group and metabolic disorders were more frequent in parents and grandparents of PCOS patients than in those of normal women (Arora and Allahbadia , 2006). The increase of total, low-density lipoprotein and triglyceride levels and an increased prevalence of Metabolic Syndrome (MBS) has been observed in affected sisters of women with PCOS compared with unaffected sisters, consistent with a heritable trait (Arora and Allahbadia , 2006). Insulin resistance has also been demonstrated in brothers of women with PCO, comparable to that associated with a family history of type2 DM and associated with elevations of blood pressure, abnormalities in serum lipid concentrations and impaired endothelial cell function (Kaushal *et al*., 2004). Significant phenotypic and genetic heterogeneity have been observed both within and between families. However, the difficulties in classifying female family members as clearly affected or unaffected and lack of a definitive male phenotype complicate the use of linkage analysis to identify the PCOS genes. Each family is best considered on an individual basis to identify genetic markers that segregate with the clinical features of androgen excess (Sanders *et al*.,

2002). Therefore, PCOS is caused by a combination of genetic susceptibility and environmental exposures.

Although it is known that multiple genes are involved in PCOS onset and the development, the pathophysiological basis of PCOS remains unclear despite its growing global importance. To date, a number of genes have been reported to be associated with PCOS. Most of the genes are investigated because they are presumed to be relevant to the pathogenesis of PCOS based on their functions.

### 2.12.3. Vitamin D receptor gene

This gene encodes the nuclear hormone receptor for vitamin $D_3$. This receptor also functions as a receptor for the secondary bile acid lithocholic acid. The receptor belongs to the family of trans-acting transcriptional regulatory factors and shows similarity of sequence to the steroid and thyroid hormone receptors. (Germain *et al*.,2006).

Downstream targets of this nuclear hormone receptor are involved principally in mineral metabolism though the receptor regulates a variety of other metabolic pathways, such as those involved in the immune response and cancer. (Adorini *et al*. , 2006).

Mutations in this gene are associated with type II vitamin D-resistant rickets. A single nucleotide polymorphism in the initiation codon results in an alternate translation start site three codons downstream. Alternative splicing results in multiple transcript variants encoding the same protein.The vitamin D receptor plays an important role in regulating the hair cycle. Loss of VDR is associated with hair loss in experimental animals, The vitamin D receptor (VDR) is a ligand-dependent transcriptional factor that binds to vitamin D-responsive elements as a

heterodimer with retinoid X receptor (RXR) to regulate target gene transcription. The steroid receptor coactivator (SRC) proteins are coactivators that interact with the AF-2 domain of VDR to augment 1,25-dihydroxyvitamin D3-dependent transcription (figure 2.2) (Zhang et al , 2001).

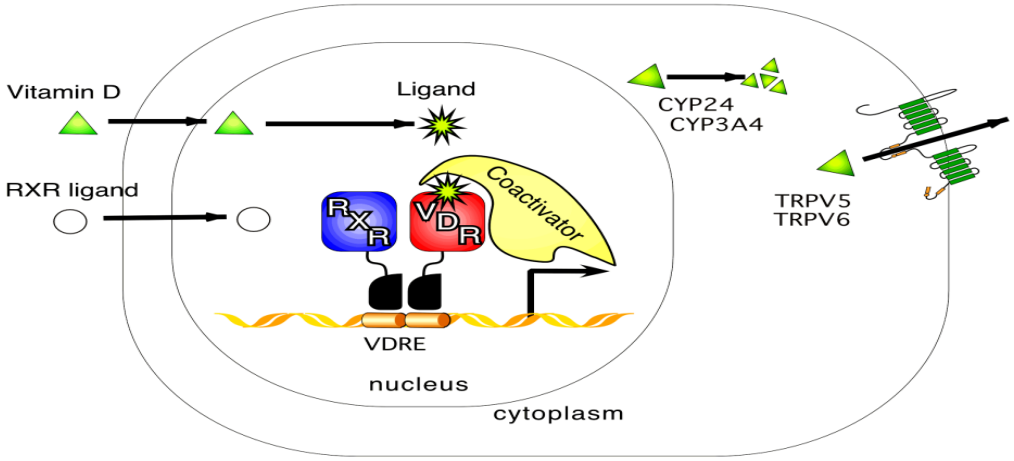


**Figure 2.2. Primary action of VDR is transcriptional regulation (Zhang *et al* , 2001)**

### 2.12.3.1. *VDR* gene chromosomal location

Cytogenetic Location: 12q13.11, which is the long (q) arm of chromosome 12 at position 13.11 (Figure 2.3) .

Molecular Location: base pairs 47,841,537 to 47,905,031 on chromosome 12 (Homo sapiens Annotation Release 108, GRCh38.p7) (Feldman and Malloy , 2014).

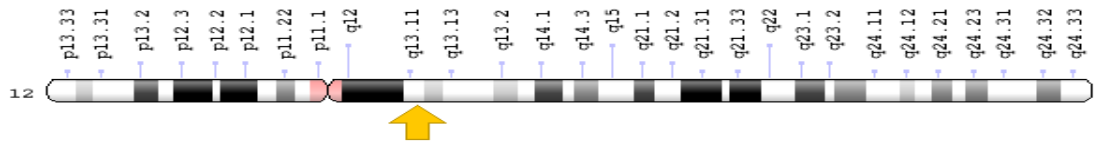


**Figure 2.3. *VDR* gene chromosomal location (Feldman and Malloy , 2014).**

### 2.12.3.2. *VDR* gene polymorphisms

*VDR* gene contains 14 exons and is mapped on chromosome 12q12.14. Several allelic variations have been reported in the *VDR* gene such as the following restriction fragment length polymorphisms: *FokI* in exon 2 (C/T) (rs10735810), *BsmI* in intron 8 (G/A) (rs1544410), *ApaI* in intron 8 (C/A) (rs7975232), *Tru9I* in intron 8 (G/A) (rs757343) and *TaqI* in exon 9 (T/C) (rs731236). *TaqI* based restriction fragment length polymorphism is located at the 3' end of the *VDR* gene. The function of the *TaqI*-specific hypervariable polymorphism is unclear (Uitterlinden , 2004). *VDR* gene variants have been associated to breast cancer risk (Guy, 2004), prostate cancer progression (Xu , 2003), colorectal cancer (Yaylim-Eraltan , 2007), diabetes (Motohashi , 2003), primary hyperparathyroidism (Carling *et al*.,1995), coronary artery disease (Van Schooten *et al* .,1998) and PCOS (Grulet *et al*.,1993 ; Mahmoudi , 2009 ; Ranjzad *et al*.,2011 ; Lerchbaum and Obermayer-Pietsch,2012).

The results of some studies imply that VDR genetic variants may impact PCOS and IR in women with PCOS (Urbanek, 2007 ). Vitamin D and calcium repletion predict reproductive success following fertilization (Brannon and Picciano, 2011 ; Grundmann and Von Versen-Hoynck, 2011). Regulation of the egg activation, oocyte maturation, follicular development and mammalian embryo development is $Ca^{2+}$ dependent (Liang *et al*., 2011). The VDR is defined as the nuclear steroid hormone

receptor resulting in gene expression regulation through binding to specific response elements within the promoter of some genes (Jones *et al*., 1998). Cellular ligandactivated transcription factors are encoded by the VDR gene (MacDonald *et al*., 1995). These transcription factors have different functions including calcium homeostasis. The mechanism of gene expression regulation by VDR is not well characterized. It has been demonstrated that VDR produces a specific protein which interacts with the basal transcription factor TFIIB (MacDonald *et al*., 1995). Also, VDR regulates gene transcription with other different mechanisms including interaction with co-activator or co-repressor molecules. VDR may influence the acetylation of histones as well as chromatin remodeling (Jenster *et al*., 1997 ; Chen *et al*., 1997 ; Collingwood *et al*., 1999 ; Lin and White , 2004) .

Grulet *et al*. (1993) have indicated that there is an association between insulin resistance and hyperandrogenism as well as luteinizing hormone (LH) and insulin sensitivity in PCOS (Ranjzad *et al*., 2011 ; Lerchbaum and Obermayer- Pietsch, 2012).

The findings of Ranjzad *et al*. (2011) have demonstrated that there is a significant association between VDR TaqI CC genotype and serum concentrations of LH in women with PCOS. Whereas there is a association between VDR TaqI CC genotype with serum level of LH (Ranjzad *et al*., 2011) and LH with insulin sensitivity in PCOS (Lerchbaum and Obermayer- Pietsch, 2012) .

## 2.13. Cytogenetics

Cytogenetics is the branch of genetics that studies the structure of DNA within the cell nucleus. This DNA is condensed during cell division and form chromosomes. The cytogenetic studies the number and morphology of chromosomes. Using chromosome banding techniques (classical cytogenetics) or hybridization fluorescently labeled probes (molecular cytogenetics). The number and morphology of chromosomes in a cell of a particular species are always constant, in most cells of the body (with the exception of reproductive cells and others such as the liver). This is a characteristic of each specie, in humans such as the number of chromosomes is 46.(NHGRI,2018).

### 2.13.1. Human abnormalities and medical applications

A chromosome abnormality, disorder, anomaly, aberration, or mutation is a missing, extra, or irregular portion of chromosomal DNA (NHGRI,2018) , It can be from an atypical number of chromosomes or a structural abnormality in one or more chromosomes. Chromosome mutation was formerly used in a strict sense to mean a change in a chromosomal segment, involving more than one gene (Rieger, *et al*., 1968) The term "karyotype" refers to the full set of chromosomes from an individual; this can be compared to a "normal" karyotype for the species via genetic testing. A chromosome anomaly may be detected or confirmed in this manner. Chromosome anomalies usually occur when there is an error in cell division following meiosis or mitosis. There are many types of chromosome anomalies. They can be organized into two basic groups, numerical and structural anomalies. Numerical anomalies is called aneuploidy (an abnormal number of chromosomes), and occurs when an individual either is missing a chromosome from a pair (monosomy) or has more than two chromosomes of a pair (trisomy, tetrasomy, etc.) (Santaguida and Amon , 2015).

When the chromosome's structure is altered, this can take several forms (Governini *et al* ., 2014) :

**Deletions**: A portion of the chromosome is missing or deleted. Known disorders in humans include Wolf-Hirschhorn syndrome, which is caused by partial deletion of the short arm of chromosome 4; and Jacobsen syndrome, also called the terminal 11q deletion disorder.

**Duplications**: A portion of the chromosome is duplicated, resulting in extra genetic material. Known human disorders include Charcot-Marie-Tooth disease type 1A, which may be caused by duplication of the gene encoding peripheral myelin protein 22 (PMP22) on chromosome 17.

**Translocations**: A portion of one chromosome is transferred to another chromosome. There are two main types of translocations:

**Reciprocal translocation**: Segments from two different chromosomes have been exchanged.

**Robertsonian translocation**: An entire chromosome has attached to another at the centromere - in humans these only occur with chromosomes 13, 14, 15, 21, and 22.

**Inversions**: A portion of the chromosome has broken off, turned upside down, and reattached, therefore the genetic material is inverted.

**Insertions**: A portion of one chromosome has been deleted from its normal place and inserted into another chromosome.

**Rings**: A portion of a chromosome has broken off and formed a circle or ring. This can happen with or without loss of genetic material.

**Isochromosome**: Formed by the mirror image copy of a chromosome segment including the centromere.

### 2.13.2. Fragile X syndrome and PCOS

publications reported associations between number of triple CGG nucleotide repeats on the fragile X mental retardation 1 (FMR1) gene and risk towards premature ovarian senescence (Hundscheid *et al* ., 2001).

studies were reported evidence that different FMR1 genotypes vary in rate of follicle recruitment and, therefore, at least partially, affect functional ovarian reserve, as assessed by anti-Müllerian hormone (Gleicher *et al*., 2010).

A normal triple nucleotide (CGG) count range of 26 to 34 repeats (median 30), in respect to ovarian function, allows definition of distinct FMR1 genotypes, depending on whether both (normal), only one (heterozygous) or neither (homozygous) allele is in normal range(Gleicher *et al*., 2010) . In a small pilot study a heterozygous-normal/low sub-genotype appeared associated with a lean polycystic ovary (PCO)-like phenotype with rapidly depleting ovarian reserve (Barad *et al* ., 2009).

### 2.13.3. Cytogenetic biomarkers

#### 2.13.3.1. Sister chromatid exchanges assay (SCEs)

The methodology of SCEs has been proved to be a very useful tool with predictive value, for detecting harmful effects on DNA, caused by various physical and chemical factors. Increased frequency of this index reflects the existence of genotoxicity in cells and the subsequent failure of repair mechanisms to recover the damaged site. Several studies have reported that SCE analysis is a very sensitive method, able to detect mutagens and/or carcinogens (Wilson and Thompson, 2007) and may be more sensitive than other cytogenetic endpoints, such as micronuclei and chromosome aberrations(Lasne *et al*., 1984). Additionally, Proliferation Rate Index (PRI) and Mitotic Index (MI) are sensitive indices of cellular toxicity by antimutagenic and chemotherapeutic agents (Lialiaris *et al*., 1988 ; Maskaleris *et al*., 1998 ) .

Sister chromatid exchanges frequencies in peripheral blood lymphocytes provide an additional diagnostic tool for identifying accumulated chromosomal damage occurring during the lifespan of the

cells. Evagelia *et al*. (2015) suggest that there is chromosomal instability in lymphocytes of PCOS patients, which is associated with phenotypic characteristics .

### 2.13.3.2. Micronucleus assay (MN)

The quantification of MN assay for the evaluation of chromosome damage offers several advantages: the method is simple and fast, and it dose not require the presence of metaphasic cells. Another advantage of the MN assay is the relative ease of scoring and the statistical power obtained from scoring larger numbers of cells than are typically used for metaphase analysis (Fenech *et al*., 1999). Fenech and Morley (1985) have presented a Cytokinesis – block micronuclei (CBMN) assay that resolved earlier problems experienced with differences in cell growth by monitoring cell division with the aid of cytochalasin B. This agent allows division of the cell nucleus (karyokinesis), but prevents cell division (cytogenesis), thus resulting in binucleated cells (Fenech and Morley, 1985 ; Fenech, 1993 ; Fenech *et al*., 1999 ).

Carmina (2009) reported that women with PCOS had statistical increase in genomic instability which demonstrated by a significant elevated number of binucleated lymphocytes containing micronuclei, micronuclei total number, elevated proportion of aneuploidy X chromosome signals (2:1 X and 3:1 X) and a lower proportion of normal X chromosome segregation signals (2:2 X) in binucleated lymphocytes than women without PCOS. Association between increased micronucleus frequency and PCOS , that there is genetic instability in peripheral blood lymphocytes in women with PCOS. Elif *et al*., (2006) suggest that hyperandrogenism, hyperinsulinemia and perhaps oxidative stress are factors contributing to increased MN frequency and chromosomal damage.

# Chapter Three

## Materials & Methods

# 3.Subjects , Maerials and Methods

## 3.1. Subjects

This study was carried out in the laboratories of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies - University of Baghdad through the period from 1 November 2016 until the end of August 2017.Two study groups were investigated :

### 3.1.1. Patients groups ( PCOS group )

This study has one patients group including : 50 patients with PCOS Iraqi women . Patients groups were selected from Kamal Al-Samarrai infertility treatment and *in vitro* fertilization hospital , there are excluded samples were taken from women with PCOS because they take medicines or in inappropriate times (rather than 2-3 days) , The questionnaire form has been filled for each patient as shown in appendix (1).

### 3.1.2. Apparently Healthy control group ( fertile )

Apparently healthy control group consists of 50 healthy fertile women , subjects aged 16-45 years, and have been chosen depending on the following criteria (Macklon and Fauser, 2000):

1. Regular menstrual cycle (26 to 30 days)
2. Age 16 to 45 years
3. No history of endocrine disease
4. No use of medication or oral contraceptives.

### 3.1.3. Criteria used for the diagnosis PCOS subjects

To confirm the subjects with PCOS should include at least two of the following three features according to the Rotterdam 2003 criteria (Rotterdam ESHRE/ASRM Consensus, 2004b) :

1. Presence of clinical and/or biochemical signs of hyperandrogenism.
2. Infrequent periods with intermenestrual interval of more than 35 days.
3. Polycystic ovaries , (an ovary with ultrasound appearance of more than 10 subcapsular follicles (< 10 mm in diameter)

## 3.2. Materials :

### 3.2.1. Instruments and Equipments

The general instruments and equipments used in this study are listed in table (3-1).

**Table 3 -1 : Instruments and equipment used in the study**

| Equipment | Company | Country |
|---|---|---|
| **Autoclave** | **Bypa** | **Spain** |
| **Blue tips (50-1000 µL)** | **Human** | **Germany** |
| **Centrifuge tubes (Sterile)- 1.5ml** | **Bio basic** | **Canada Inc** |
| **Digital camera** | **Sony** | **Japan** |
| **Distllater** | **GIF** | **Germany** |
| **EDTA test tubes (5ml)** | **AFCO** | **Jordon** |
| **Electrone Balance** | **Sartorius** | **Germany** |
| **Eppendorf Centrifuge** | **Hattich** | **Germany** |
| **Gel electrophoresis apparatus and power supply** | **Wealtec Europe** | **England** |
| **Heparin test tube** | **AFCO** | **Jordon** |
| **Incubator** | **Taisite** | **Korea** |
| **Laminar air flow** | **Bioair** | **Thailand** |

| | | |
|---|---|---|
| **Light Microscope** | **Humoscope** | **Germany** |
| **LH , FSH , TSH and Prolactin analyzer** | **VIDAS(bioMérieux)** | **France** |
| **Microwave** | **Shownic** | **China** |
| **Micropipette 1.0-10*u*l** | **NEXTY-10** | **Japan** |
| **Micropipette 10 – 100ul** | **Slamed** | **Japan** |
| **Micropipette 100-1000ul** | **Slamed** | **Japan** |
| **Micropipette 50 – 200ul** | **Slamed** | **Japan** |
| **Nanodrope** | **Quawell** | **Hongkong** |
| **PCR Tubes flat cup (Sterile) -0.2ml** | **Bio basic** | **Canada Inc** |
| **PH meter** | **HANNA** | **USA** |
| **Powder Free Violet Blue Gloves** | **Broche** | **Malaysia** |
| **Real Time PCR** | **QIAGENE** | **Armenia** |
| **Spin mix Hot plate with Magrnetic stirrer** | **LABINCO** | **Netherland** |
| **Syringe 5 and 10 mL** | **Homecare** | **China** |
| **Vaccum Sterile Glasses Gel & Clot Activator 6ml tube** | **Afco-Dispose** | **Jordon** |
| **Vaccum Sterile Glasses 10ml tube** | **AFCO** | **Jordon** |
| **Vortex** | **Bionex** | **Korea** |
| **UV. Transiluminator** | **Elettrofor** | **Italy** |
| **Yellow tips (2-200µL)** | **Human** | **Germany** |

### 3.2.2. Reagents and Chemicals

General reagents and chemicals used throughout the study are listed in table (3-2).

**Table 3-2: The reagents and chemicals used in the study.**

| Chemicals | Sources | Country |
|---|---|---|
| **Absolute ethanol** | **Sigma** | **USA** |
| **Agarose** | **Dsbio** | **Korea** |
| **5-bromodeoxy-uridine (Brdu)** | **Sigma** | **USA** |
| **Colcemide Solution** | **PAA** | **Austria** |
| **Cytochalasin – B** | **Santacruz Biotecnology** | **Canada** |
| **Ethedium Bromide** | **Bioneer** | **South korea** |
| **Geimsa stain** | **PAA** | **Austria** |
| **Glacial acetic acid** | **Fluka** | **Germany** |
| **Hoechst stain** | **Sigma** | **USA** |
| **Loading Dye** | **Promega** | **USA** |
| **Potassium chloride** | **BDH** | **UK** |
| **Phospphate buffer saline (PBS)** | **PAA** | **Austria** |
| **RPMI-1640- Medium with PHA** | **Genomics** | **Germany** |
| **Sodium chloride** | **BDH** | **UK** |
| **TBE buffer (10x)** | **Dsbio** | **Korea** |
| **UltraPure DNase/RNase-Free Distilled Water** | **Dsbio** | **Korea** |

### 3.2.3. Kits used in the study

Kits used in the study included:

**3.2.3.1. The WizPrep™ DNA Extraction Kit components are presented in Table (3-3) .**

**Table 3-3 : components of DNA Extraction Kit (WizPrep™) .**

| Components | Quantity |
|---|---|
| **Binding Buffer (BBA)** | **1 x 27.5ml** |
| **Cell Lysis Buffer (CLD)** | **1 x 22ml** |
| **Collection Tubes** | **10 40/pk** |
| **Column Wash Solution (CWD)** | **1 x 165ml** |
| **Nuclease-Free Water** | **1 x 25ml** |
| **Proteinase K (PK) Solution** | **2 x 1.1ml** |
| **ReliaPrep™ Binding Columns** | **2 x 50/pk** |

### 3.2.3.2. qPCRMaster (PROBE)

The components of WizPure qPCRMaster (PROBE) include :

One eppendorf tube (1 ml) 2X qPCRMaster (PROBE) contains:-

- HS- Taq DNA Polymerase .
- PCR buffer buffer , $MgCl_2$ and dNTPs , except DNA template and primers
- ROX™ Dye .

### 3.2.3.3. Hormonal assay kits :

Hormonal analysis kits are presented in table (3-4).

**Table 3-4 : Hormonal assay Kits .**

| Kit | Sources | Country |
|---|---|---|
| **LH** | **VIDAS** | **France** |
| **FSH** | **VIDAS** | **France** |
| **TSH** | **VIDAS** | **France** |
| **Prolactin** | **VIDAS** | **France** |

## 3.3. Primers and Probes:

Primers and Probes used in the study with their sequences are shown in table (3-5):

**Table (3-5): Primers and Probes used in the study**

| Primer/probe | Sequence (5′ →3′ direction) |
|---|---|
| ***VDR* gene exon 9 (rs731236)** | |
| **Forword** | TTCTTCTCTATCCCCGTGCC |
| **Reverse** | GTCGGCTAGCTTCTGGATCA |
| **FAM- probe** | TGATTGAGGCCATCCAG |
| **VIC-probe** | ATCGAGGCCATCCAGG |
| ***VDR* gene exon 2(rs2228570)** | |
| **Forword** | GGCCTGCTTGCTGTTCTTAC |
| **Reverse** | TGCTTCTTCTCCCTCCCTTT |
| **FAM- probe** | ATGGAGGCAATGGCG |
| **VIC-probe** | GGACGGAGGCAATGG |
| ***VDR* gene intron 8(rs7975232)** | |
| **Forword** | GGGATAGAGAAGAAGGCACAG |
| **Reverse** | GGATCCTAAATGCACGGAGA |
| **FAM- probe** | GCCCCTCACTGCTCAATC |
| **VIC-probe** | GGGCACCTCACTGGCT |

## 3.4. Methods:

### 3.4.1. Clinical data:

Every participant woman has been interviewed and asked to answer information including sociodemographic data, menstrual history, gynecological surgery , obstetric, PCOS family histories. They have been also subjected to medical checkup for signs of hyperandrogenism and polycystic ovary .

### 3.4.2. Menstrual cycle:

Menstrual irregularity mean long cycle of >35 days. Anovulation has been considered as a history of fewer than eight menstrual cycles per year. Anovulation severity has been identified according to these three anovulation sub groups:

1- Mild ; menstrual cycle length between 40-60 days ( 6-9 times\year) ,
2- Moderate ; menstrual cycle length between 61-180 days (2-6 times\year),
3- Severe ; amenorrhea or menstrual cycle length >180days (2-less times\year),(Usadi *et al*., 2012) .

### 3.4.3. Hirsutism

The hirsutism has been considered as the indicator for hyperandrogenism . Modified Ferriman-Gallwey (mFG) score test taken as significant in locations (upper lip , face , jaw and neck , upper back, lower back , upper arm , thigh , chest , upper abdomen , lower abdomen and perineum) when the hair distribution score ≥8 of 36 (Tehrani *et al*., 2013) .

### 3.4.4. Body Mass Index (BMI)

Body mass index (BMI) has been estimated by measuring an individual's weight and height to lean body mass. The BMI is thus an index of weight adjusted for stature. Body mass index has been figured by dividing weight in kilograms by height in meters squared.

$BMI = Mass\ (Kg) / \ height\ (m)^2$

The BMI is a diagnostic tool for both obesity and protein-energy malnutrition ( Maskarinec, 2000 ) .

### 3.4.5. Ultrasound examination

In $3^{rd}$ or $4^{th}$ day of the menstrual cycle, ultrasonic evaluation has been performed with transvaginal ultrasound to check the morphological appearance of ovaries. Test has been done by ultrasound specialist physician . Ovarian volume and total number of ovarian follicles have been performed for both patients and control groups (Usadi *et al*., 2012) .

### 3.4.6. Blood Samples collection

The blood samples have been collected during the follicular phase (day 2, 3) of the menstrual cycle from each women of both studied groups (patients and healthy control) . five ml of venous blood samples have collected and divided into three portions :

A. First portion: 2ml EDTA containing tubes for DNA isolation (Molecular genetic studies) .

B. Second portion: 3 ml The serum obtained by putting the blood samples in a vacuum sterile glasses gel and clot activator 6ml tube and allowed to clot at 37 ºC for 30 minutes before centrifugation. The tubes centrifuged at 6000

rpm for 5 minutes, serum has been collected and kept in freezer until used for hormonal assays.

C. Third portion : Heparin containing tubes for cytogenetic studies , this portion has been collected depending on the results of genotyping assay and the blood samples has been collected immediately before the beginning of the cytogenetic analysis .

### 3.4.7. Genomic DNA isolation

Total genomic DNA isolated from the whole fresh blood have been collected in EDTA containing tubes for molecular studies, was applied using genomic DNA purification kits (The WizPrep™ DNA Extraction Kit.).

#### 3.4.7.1. Genomic DNA Isolation protocol

The protocol supplied by Wizbiosolutions . Company has been used for DNA isolation as follow:

1. A volume 300 µl of peripheral whole blood has been transferred to a 1.5 ml sterile microcentrifuge tube .
2. A volume of 20µl of Proteinase K (PK) has been dispensed into a 1.5ml microcentrifuge tube.
3. Then 900 µl RBC lysis buffer has been added (3X from the sample volume) and mixed by inversion (Do not vortex).
4. The tube (Blood and RBC lysis buffer) has been incubated for 10 minutes at room temperature.
5. The incubated tube has been centrifuged for 5 minutes at 3,000xg then completely removed the supernatant.

6. A volume 100 µl of RBC lysis buffer was added to resuspend the leukocyte pellet.
7. A volume 200 µl of GB buffer has been added to the 1.5 ml microcentrifuge tube then shaked vigorously.
8. The sample lysate has been incubated at 60ºC for 10 minutes to ensure the lysate was clear.
9. During incubation, invert the tube every 3 minutes,( At this time, preheat)
10. The required elution buffer (200 µl per sample) to 60ºC (for Step of DNA Elution).
11. Absolute ethanol ( 200) µl has been added to the lysate then immediately mixed by shaking vigorously for 10 seconds.
12. The GD column has been placed in a 2 ml collection tube.
13. The mixture (including any precipitate) then transferred to the GD column.
14. The mixture then has been centrifuged at 14,000 x g for 5 minutes.
15. The 2 ml collection tube then discarded and the GD column place in a new 2 ml collection tube.
16. A volume 400 µl of W1 buffer has been added to the GD column then centrifuged at 14,000 xg for 30 seconds.
17. The flow has been discarded then the GD column placed back in the 2 ml collection tube.
18. A volume 600 µl of wash buffer has been added to the GD column (make sure ethanol was added).

19. The GD column has been centrifuged at 14,000 x g for 30 seconds then the flow discarded.
20. The GD column has been placed back in the 2 ml collection tube.
21. To dry the column matrix the GD column has been centrifuged again for 3 minutes at 14,000 xg.
22. The dried GD column has been transferred to a clean 1.5 ml eppendorf tube .
23. A volume 100 µl of pre-heated elution buffer or TE has been added to the center of the column matrix.
24. The elution buffer or TE has been let for at least 3 minutes to ensure completely absorbed.
25. Then centrifuged at 14,000 x g for 30 seconds to elute the purified DNA.
26. Then genomic DNA has been stored in freezer at -20℃.

### 3.4.8. Estimation of DNA Concentration

Nanodrop (2000C apparatus , Thermo Scientific,USA) has been used to estimate the purity and the concentration for DNA samples in this study by putting 1µl of the extracted DNA in the Nanodrop apparatus to detect concentration in ng/µl and the purity was detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The purity of DNA has been ranged 1.7 – 1.9 and that accepted 260/280 ratio with previous literatures (Sambrook *et al*., 1989) .

### 3.4.9. Agarose Gel Electrophoresis

After genomic DNA extraction, agarose gel electrophoresis has been adopted to confirm the presence and integrity of the extracted DNA (Sambrook *et al*., 1989) .

#### 3.4.9.1. Reagents of Gel Electrophoresis

- Agarose .
- 1 X TBE Buffer.
- Bromophenol Blue in 1 % glycerol(loading buffer).
- Ethidium Bromide.

#### 3.4.9.2. Gel Electrophoresis protocol as described by Sambrook *et al*. (1989)

#### 3.4.9.2.1. Agarose gel preparation

Agarose gel (1%) was prepared by :

1- Agarose powder (0.5 gm) has been taken in a cleaned dry beaker.

2- The amount of 1X TBE (50 ml) has been added to the agarose powder.

3- The solution has been heated to boiling using the microwaves oven until all gel particles has been dissolved completely without any bubbles.

4- The solution was left to cool down at 50 – 60 $^{o}$C.

5- Ethidium bromide (1µl) of (10mg/ml) has been added to the cooled agarose solution to enable the visualization .

### 3.4.9.2.2. Casting of the horizontal agarose gel

The agarose solution has been poured into a pre cleaned dry gel tray. The agarose has been allowed to solidify at room temperature for 30 minutes (depending on room temperature) . The comb has been positioned for about 1 cm from the top edge of the tray. After polymerization the fixed comb has been carefully removed and the gel tray has been placed in the gel tank. The tank has been filled with 1 X TBE buffer until it reached 1-2 mm over the surface of the gel (Sambrook *et al*., 1989).

### 3.4.9.2.3. DNA Loading and Electrophoresis

DNA (7µl) has been mixed with 3µl of bromophenol blue dye (loading buffer ). Samples were loaded carefully into the individual wells of the gel , Then electrical power has been turned on at 50 volt for 40 minutes or 100 volt for 25 minutes (5 volt/$cm^2$ ), afterwards the DNA moved from cathode (-) to anode (+) poles. The ethidium bromide stained bands in the gel has been visualized using UV transiluminator at wave-length 350 nm and photographed (Sambrook *et al*., 1989).(Appendix 2).

### 3.4.10 . Genotyping \allelic discrimination principle:

The TaqMan probe contains a reporter dye (FAM and VIC) at the 5´ end of the probe and a quencher dye (MGB) at the 3´ end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, if

the target of interest is present, the probe specifically anneals to the target. The 5´ to 3´ nucleolytic activity of the AmpliTaq Gold, polymerase enzyme cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target.

The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3´ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle, and it does not interfere with the exponential accumulation of product.

The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and if it is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

### 3.4.10.1. Primer and Probe sequence matching:

TaqMan fluorescent oligonucleotide probes and primers sequences were prepared according to William *et al*., (2004) , synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at (-23°C). The sequences of each of the probes and primers used in the allelic discrimination experiments are shown in table (3-4) they included *VDR gene* SNP intron 8 (C to A; rs 7975232), exon 9 (T to C; rs731236) and exon 2 (T to C; rs2228570).

### 3.4.10.2. Probe Optimization Reaction

Probes were optimized as follow :

1. Master stock of probe at 100 μM was prepared,the stock was made into aliquots and stored at –23°C.
2. The probe was diluted to 5 μM working solution and stored at –23°C.
3. A concentration of 1 μM probe stock was used to set up the probe matrix.
4.

### 3.4.10.3. Real-time PCR run

DNA samples from PCOS patients (n=50) and apparently healthy subjects (n=50) were genotyped for the *VDR* gene SNPs (rs7975232, rs 731236 and rs 2228570) with a Taqman SNP genotyping assay using real time thermocycler according to the protocol recommended by the manufacturer , the kit content as show in table (3-6):

Table (3-6): components of Real-time PCR/ allelic discrimination reaction

| No. | Components | Volume | Final conc. | Volume (µl) | |
|---|---|---|---|---|---|
| 1 | qPCR Master (PROBE) | 10 µl | 1X | 10 | |
| 2 | Forward primer | 0.2-2.0 µl | 0.1-1.0 µM | 10 µl | 0.5 |
| 3 | Reverse primer | 0.2-2.0 µl | 0.1-1.0 µM | 10 µl | |
| 4 | Fluorescence Probe | Variable | ≤500ng/reaction | 20 µl | |
| 5 | Template DNA | Variable | - | 4 | |
| 6 | Water , RNase free | Up0 to 20 | - | 5.5 | |
| | Final Volume | | | 20 | |

The thermal profile of allelic discrimination Real-time PCR program is shown in table (3-7) (Appendix 3).

**Table (3-7) Real Time PCR program for *VDR* gene SNPs (rs7975232, rs 731236 and rs 2228570).**

| Cycle step | | Temp. (°C) | Time | Cycle |
|---|---|---|---|---|
| **Hold1** | | 50 | 15 min. | 1 |
| **Hold2** | | 95 | 15min. | |
| **Cycling 1** | **Denaturation** | 95 | 5 sec. | 5 |
| | **Annealing** | 60 | 20 sec. | |
| | **Extension** | 72 | 15 sec. | |
| **Cycling 2** | **Denaturation** | 95 | 5 sec. | 40 |
| | ***Annealing** | 60 | 20 sec. | |
| | **Extension** | 72 | 15 sec. | |

*** In this step the acquiring Green and Yellow (FAM and VIC) were added.**

### 3.4.11. Hormonal assays

Hormonal analysis for LH, FSH, TSH and Prolactin was performed by using Automated Immune Assay **(AIA)** by the VIDAS auto analyzer, (bioMérieux Company ) France.

#### 3.4.11.1. Principle

VIDAS hormonal assay is an automated quantitative test for use on the VIDAS instrument for the quantitative measurement of human serum using the ELFA (enzyme linked fluorescent assay) technique.

The solid phase receptacle (SPR) serves as the solid phase as well as the pipetting device for the assay. Reagents for the assay are ready-to-use and predisposed in the sealed reagents strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times.

The principle of FSH and LH estimation combine a one step enzyme immunoassay sandwich method with a final fluorescent detection by ELFA .

**The competition method:** a sample is taken and transferred into the well containing the hormonal antigen labeled with alkaline phosphatase (conjugate). Competition occurs between the antigen present in the sample and the labeled antigen for the specific anti-hormonal antibodies coated on the interior of the SPR.

**The sandwich method:** a sample is transferred into the well containing the anti hormonal antibody labeled with alkaline phosphatase (conjugate). The sample/ conjugate mixture are cycled in and out of the SPR. The antigen binds to antibodies coated on the SPR and to the conjugate forming a “sandwich”. Unbound components are eliminated during washing steps.

During the **final step:** the substrate (4-Methyl-umbelliferly phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methylumbelliferone) the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is inversely proportional to the concentration of antigen present in the sample in the competition method. In the sandwich method, the intensity of the fluorescence is proportional to the concentration of antigen present in the sample.

Results are automatically calculated by the instrument in relation to the calibration curve stored in memory, and then printed out.

**3.4.11.2. Procedure**

**1-** The required reagents were removed from the refrigerator and were allowed to come to room temperature for 30 minutes.

**2-** One FSH strip and one FSH-SPR were used from the kit for each sample, (control or calibrator) to be tested. The storage pouch was resealed after the required SPRs removed.

**3-** FSH on the instrument test code was selected. The calibrator must be identified by (S1), and tested. If the control needed to be tested, it should be identified by C1.

**4-** The samples, the calibrator and /or the control were mixed, using a vortex –type mixer.

**5-** Pipetting 200 µl of sample, calibrator or control into the sample well.

**6-** The VIDAS SPRs and strips were inserted into the positions indicated on the screen. The color labels with the three letters assay code on the SPRs and the reagent strip should be matched.

**7-** The assay processing was initiated as directed in the Operator's Manual.

**8-** After the assay was completed, the SPRs and strips were removed from the instrument. All the assay steps were performed automatically by the

instrument. The assay was completed within approximately 60 minutes .The same procedure was carried out on the LH, Prolactin and TSH .

The values of hormonal assays for the subjects were compared with the next normal range: FSH = 3.9-12 μIU/ml, LH =1.5- 8 μ IU/ml, TSH = 0.5-5 μIU/ml and Prolactin 4-25 μg/l .

## 3.4.12. Cytogenetic analysis

### 3.4.12.1. Blood sampling

Two milliliters(2ml) of blood were collected by venipuncture from (12 , 10 and 10 ) PCOS patients who have homozygous mutant result in genotyping assay for *VDR* gene SNP (rs 7975232, rs 731236 and rs 2228570) respectively , and from (10 ,10 and 14) PCOS patients who have heterozygouse result assay for *VDR* gene SNP (rs 7975232, rs 731235 and rs 228570) respectively. as well as two milliliters of blood were collected from (14) apparently healthy women for control . Each collected blood sample was dispensed into heparinzed tubes for cytogenetic studies

## 3.4.12.2. Preparation of stock solutions and media

### 3.4.12.2.1. Quantum PBL medium

Modified RPMI 1640, Phytohaemagglutinin (PHA) high quality, pretested FBS, buffered with $HCO_3$, with antibiotics penicillin and streptomycin and L-glutamine (Fenech, 2000) were purchased and kept in a deep freeze at -20 ºC until use.

### 3.4.12.2.2. Colcemid solution

The colcemid solution was supplied at a concentration of 10 μg/ml. It was used at a concentration of 1 μg/mL as recommended by the supplier to bind the

tubulin protein and obstruct the spindle fiber formation. This solution was kept at -20 ºC until use .

### 3.4.12.2.3. Hypotonic solution (0.075 and 0.087 M KCl )

The hypotonic solutions 0.075 M KCl and 0.087 M KCl were prepared by dissolving 2.8 gm and 3.25 gm of KCl , respectively, for chromosomal analysis and micronucleus , respectively in 500 ml distilled water (Wojcik *et al.*, 2000).

### 3.4.12.2.4. Fixative solution

Three volumes of methanol were mixed with one volume of glacial acetic acid; the solution was prepared instantly before use. (Wojcik *et al.*, 2000).

### 3.4.12.2.5. Phosphate buffer saline solution (P.B.S)

It consisted of the following components : NaCl , 8 gm ; KCL , 0.2 gm ; Na2HPO4 , 1.15gm and KH2PO4 , 0.2gm and weights diluted in 1 liter distilled water. The pH of the buffer was adjusted to 7.2 then stored at $4^0$C.

### 3.4.12.2.6. Giemsa stain

This solution was prepared by dissolving two grams of Giemsa powder with 100 ml of absolute methanol, stirred for two hours at 60°C on a hot plate, then mixed well and filtered using Watman No.1 filter paper. The stock solution was kept in a dark bottle at room temperature till use. Final concentration was (2%) (Wojcik *et al.*, 2000).

### 3.4.12.2.7. Cytochalasin B solution

It was prepped by dissolving 5mg of cytochalasin B in 1ml dimethyl sulfoxide (DMSO) and divided to aliquots of 0.1ml and placed in eppendorff tubes, then stored at -20°C. The volume was completed to 1ml by adding PBS before use and added to the medium to give a final stock concentration of 500µg /ml, then stored at -20°C (Wojcik *et al.,* 2000).

### 3.4.12.2.8. Bromodeoxyuridine (BrdU):

5-bromo- 2-deoxyuridin powder 50 mg was dissolved in 37.3 ml of D.W. The solution was sterilized by filtration through 0.22 µm sterile filter, distributed in sterile tubes with final concentration 1.33 mg/ml and stored at -20ºC until use (Schvartzman and Gutierrez, 1980).

### 3.4.12.3. Blood culture

### 3.4.12.3.1. Micronucleus assay

- **Principle (Fenech, 2000)**

Cytokinesis block micronucleus assay (CBMA) in peripheral blood lymphocytes was used as a useful technique to evaluate cytogenetic damage. The *in vitro* micronucleus assay is a mutagenic test system for the detection of chemicals that induce the formation of small membrane bound DNA fragments.

The purpose of the micronucleus assay is to detect those agents that modify chromosome structure and segregation in such a way leading to the formation of micronuclei in cells at interphase. The principle of the test method is by adding cytochalasin B to the cell cultures. This blocks cytokinesis in cultured cells,

which in turn allows cells to grow for a reasonable period of time. Consequently, this will allow for chromosomal damage leading to the formation of micronuclei in bi- or multinucleated cells at interphase phase. Harvested and stained these cells are then analyzed microscopically for the presence of micronuclei.

- **Protocol**

Experimental design of culture technique was implemented according to Fenech, (2000) as follows:

1. The culture tubes were prepared by placing 5ml of Quantum PBL medium in each tube.
2. Aliquot of 0.6ml of blood sample was added to each culture tube.
3. The contents of each culture tube were mixed gently by inverting for a few minutes, the culture tubes were then incubated for 44 hrs at $37^0$C in a slant position (this position creates more surface area between the liquid and gaseous phases and allow cells to settle over a larger area of the culture tubes which provide optimal culture conditions for cell growth and proliferation).
4. Aliquot of 500 µl of Cytochalasin B was then added to each culture at a concentration of 5µg/ml to block cells cytokinesis and cultures re-incubated at $37^0$C for 28 hrs before cells harvest.

Note: All these steps were done under aseptic conditions.

- **Cell harvest**

1. After the end of incubation period, culture tubes were centrifuged at 10000 *rpm* for 10 min.
2. The supernatant was discarded by pipetting the medium, leaving a little medium as possible over the cell pellet.
3. Cell pellets were re-suspended in 10 ml of hypotonic warm solution of (0.075M and 0.087M) KCl for chromosomal analysis and micronucleus respectively and incubated for 30 min. in a water bath at 37 $^{0}$C.
4. Tubes were centrifuged at 10000 rpm for 10 min.
5. The supernatant was discarded and the pellets were disturbed thoroughly by tapping at the bottom of the tubes, and the pellet was re-suspended in 5ml of fixative solution. Tubes were kept in refrigerator for about 24hrs.
6. Again, the tubes were centrifuged, the supernatant was discarded. This step was repeated for three more times.
7. After a final centrifugation, the supernatant was aspirated and 1ml of the precipitate was shaken very gently then the samples were dropped on clean slides from 30-80cm. The slides were left until drying then labeled (Micale, 2010).
8. 

- **Slides staining**

1. Slides were stained in freshly giemsa staining solution .
2. The slides were allowed to dry and then become ready for microscopic examination .

- **Slide examination**

Slides were prepared in order to examine the micronuclei formation by light microscope.

- **Assessment of micronucleus frequency**

At least 1000 cells were scored to assess the frequency of MNi, the cells were classified as mononucleate, binucleates, trinucleates or tetranucleates. The increase in the cell number containing micronuclei is classified as a positive result. The positive result from the *in vitro* micronucleus test indicates chromosome damage or damage in the cell division apparatus. While negative results have to be confirmed (Kirsch-Volders, 1997).

### 3.4.12.3.2. Sister chromatid exchange

Sister chromatid exchange can be found in any cell that has replicated twice in the presence of 5-bromodeoxy- uridine (BrdU).( Schvartzman and Gutierrez,1980) It is known that SCE represents the interchange of DNA replication products, which maintain their polarity, (Tice *et al.,* 1975; Sasaki, 1977**)** at homologous loci. The precise molecular mechanism is not known.

About 10 µg per ml concentrations of each sample 24 h after initiation of cultures for two consecutive cell cycles were put in a slide. Slides were stained with Hoechst stain (33258) for the analysis of cell cycle progression and sister chromatid exchange. according to the method of Freshney, (2000)**.**

**1-** The slides were immersed in Hoechst 33258 at a concentration of (20 µg/ml) for 10min in a coplin jar.

**2-** The slides were transferred to a slide rack, and on them (500µl) of 2 x SSC were dropped.

**3-** The slides were covered with a 22-mm x 50-mm cover slips, and the edges were sealed with a temporary seal, such as cow gum, to prevent evaporation.

**4-** The covered slides in the slide rack (cover slip facing downwards) were placed on a short-wave UV box. A distance of approximately 4cm between the slides were maintained and the UV source. The pale chromatid will become, when the slides were exposed for about 24-60min.

**5-** The cover slips were removed from the slides, and the slides were washed three times in water for 5 min wash. The slide holder was covered with aluminum foil.

**6-** The slides were air dried in the dark.

**7-** The slides were stained in a coplin jar containing 3.5% Giemsa solution in PBS buffer for 3-5min (PH, 6.8).

**8-** The slides were carefully rinsed in tap water, and drained using a paper tissue.

**9-** The slides were air dried on the bench for 1hour and dipped into xylene. Four drops of DPX mountant were dropped onto the slide and a 22-mm x 50-mm cover slip was lowered, expressing any air bubbles with tissue.

**10-** The slides were air dried in a fume hood overnight.

### 3.4.13. Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to affect different factors in study parameters . Chi-square test was used to significant comparison between percentage and Least significant difference –LSD test was used to significant comparison between means in this study. (SAS,2012).

# Chapter Four

## Results & Discussion

# 4 . Results and Discussion

## 4.1 Subjects

A total of 100 blood samples were collected from two groups of Iraqi women include patients ( 50 PCOS) and (50) apparently healthy control

### 4.1.1 Age distribution

The age of all patients group was ranging from (17-40) years. Table 4-1 revealed that 64.00% of patients age was less than 25 and 36.00% of the PCOS patients aged higher than 35 years. While 68.00% of control age was less than 25 and 32.00% of the control aged higher than 35 years.

**Table 4-1. Age distribution of PCOS**

| Variable | No. | | Percentage (%) | |
|---|---|---|---|---|
| **Age (year)** | | | | |
| | **control** | **PCOS** | **control** | **PCOS** |
| (17-25) | 34 | 32 | 68.00 | 64.00 |
| (26-40) | 16 | 18 | 32.00 | 36.00 |
| Total | 50 | 50 | 100% | 100% |

This is in consistence with results of current study, that 64.00% of the PCOS patients in this study were of age < 35 years; while the results agreed with Nabag *et al.*,( 2014) who reported that 73% of PCOS were Young( 20-29 years) , because, in Iraq, diagnosis of PCOS often associated with infertility and mostly start in the first year after the marriage; therefore, the results revealed the high percentage in age <35 years.

### 4.1.2.Questionnaires Distribution

Data listed in table 4-2 revealed the percentage of the PCOS women with irregular menstrual cycle , type of infertility and miscarriage

The percentage of menstruation irregularity, in PCOS women is significantly higher than the percentage of regular menstrual (62.00% *versus* 38.00%, respectively, $\chi^2$= 9.273, *P*-value = 0.0016).

The primary infertility percentage was significantly higher than the percentage of the secondary infertility (76.00% *versus* 24.00% , respectively, $\chi 2$= 12.092, *P*-value = 0.0001).

The percentage of positive miscarriage was significantly ($p< 0.05$) higher than the negative in PCOS women ( 80.00% *versus* 20.00% , respectively, $\chi^2$= 13.250, *P*-value = 0.0001).

These results were agreed with Haleem *et al*., (2014) on the infertile women in Baghdad found that from 110 married infertile women, 85.4% belong to age less than 35 years " 33.33 % with PCOS; 41.3 % had hormonal problems ; 86.8 % had problems in ovulation function; 11.8 % had Dysfunction of fallopian tube " . Regarding the period of marriage 9.1 % of 110 women one year married period, 33.6% of women from Baghdad , 39.1% from Northern and 27.3% of women from Southern governorates .

The results of the current study were agreed with other reports (Haleem *et al*.,2014 and ). Scientists considered PCOS syndrome as one of the main causes of the infertility, and have many dimensions that in addition to genetic causes include environmental, social and economical causes.

The Iraqi environment suffered from pollution that might causes women infertile, and increase the infertility in females, because the hormonal system in females is more sensitive to environmental variations (Mahmoud *et al*.,2014) . Also, the Iraqi society suffered from many psychological difficulties that affect

women's behavior which leads to disturb the female hormonal system that might be lead to PCOS ( Haleem *et al*.,2014 ) .

**Table 4-2. Clinical features of women with PCOS .**

| Variable | No. | Percentage (%) |
|---|---|---|
| **Menstrual state** | | |
| Irregular –I | 31 | 62.00 |
| Regular –R | 19 | 38.00 |
| Chi-Square- $\chi^2$<br>P-value | --- | 9.273 **<br>0.0016 |
| **Type of infertility** | | |
| Primary | 38 | 76.00 |
| Secondary | 12 | 24.00 |
| Total | 50 | 100% |
| Chi-Square- $\chi^2$<br>P-value | --- | 12.092 **<br>0.0001 |
| **Miscarriage** | | |
| Positive | 40 | 80.00 |
| Negative | 10 | 20.00 |
| Total | 50 | 100% |
| Chi-Square- $\chi^2$<br>P-value | --- | 13.250 **<br>0.0001 |

## 4.2. Primer and Probe sequence matching:

Primer and probe sequence were matched by National Center for Biotechnology Information (NCBI) as shown in Figure (4-1) , The wild type

detecting probe was labeled with FAM in the 5′ end and Minor groove binder probe (MGB) in the 3′ end. While the probe prepared for the mutant allele (SNP)was labeled with VIC in the 5′ end and MGB in the 3′ end.(Figure 4-2) .

*exon 2 (T to C; VDR gene , rs 2228570)*

**Rs2228570 , Band size : 221 bp , g.30920T>C**

**Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), RefSeqGene on chromosome 12**

NCBI Reference Sequence: NG_008731.1

GenBank Graphics

>NG_008731.1:30895-31115 Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), RefSeqGene on chromosome 12

**Probe 1 FAM**

**GGCCTGCTTGCTGTTCTTACAGGGATGGAGGCAATGGCGGCCAGCACTTCCCT GCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGGATCTGTGGGGTGTGTG GAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAAGGCTGCAAA GGCTTCTTCAGGTGAGCCCTCCTCCCAGGCTCTCCCCAGTGGAAAGGGAGGGA GAAGAAGCA**

**Probe 2 VIC**

**GGCCTGCTTGCTGTTCTTACAGGGACGGAGGCAATGGCGGCCAGCACTTCCCT GCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGGATCTGTGGGGTGTGTG GAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAAGGCTGCAAA GGCTTCTTCAGGTGAGCCCTCCTCCCAGGCTCTCCCCAGTGGAAAGGGAGGGA GAAGAAGCA**

*Intron 8 (C to A; VDR gene , rs 7975232)*

**Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), RefSeqGene on chromosome 12**

NCBI Reference Sequence: NG_008731.1

GenBank Graphics

>NG_008731.1:64834-65015 Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), RefSeqGene on chromosome 12

**Probe 1 FAM**

GGGATAGAGAAGAAGGCACAGGAGCTCTCAGCTGGGCCCTCACTGCTCA
ATCCCACCACCCCCCACCCACACAGACACTCAACGGCAGCACCCCCTAGG
CCACCCCTCTATGACTGCTGACCGGTGATACCACTGCCTGGCCCCAAAGC
CCTCCAGTGACTTCTCCGTGCATTTAGGATCC
<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<

**Probe 2 VIC**

GGGATAGAGAAGAAGGCACAGGAGCTCTCAGCTGGGCACCTCACTGCTCA
ATCCCACCACCCCCCACCCACACAGACACTCAACGGCAGCACCCCCTAGG
CCACCCCTCTATGACTGCTGACCGGTGATACCACTGCCTGGCCCCAAAGC
CCTCCAGTGACTTCTCCGTGCATTTAGGATCC
<<<<<<<<<<<<<<<<<<<<<<<<<<<<<<

*exon 9(T to C; VDR gene , rs 731236)*

**Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), RefSeqGene on chromosome 12**

NCBI Reference Sequence: NG_008731.1

GenBank Graphics

>gi|209447082:64765-65509 Homo sapiens vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR), RefSeqGene on chromosome 12

**Probe 1 FAM**

TTCTTCTCTATCCCCGTGCCCACAGATCGTCCTGGGGTGCAGGACGCCGC
GCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACACTGCAGACGTACA
TCCGCTGCGCCACCCGCCCCCGGGCAGCCACCTGCTCTATGCCAAGATGA
TCCAGAAGCTAGCCGACC

**Probe 2 VIC**

TTCTTCTCTATCCCCGTGCCCACAGATCGTCCTGGGGTGCAGGACGCCGC
GCTGATCGAGGCCATCCAGGACCGCCTGTCCAACACACTGCAGACGTACAT
CCGCTGCGCCACCCGCCCCCGGGCAGCCACCTGCTCTATGCCAAGATGAT
CCAGAAGCTAGCCGACC

**Figure (4-1):** Matching of the primers and probes sequences on the bioinformatic programs/ NCBI

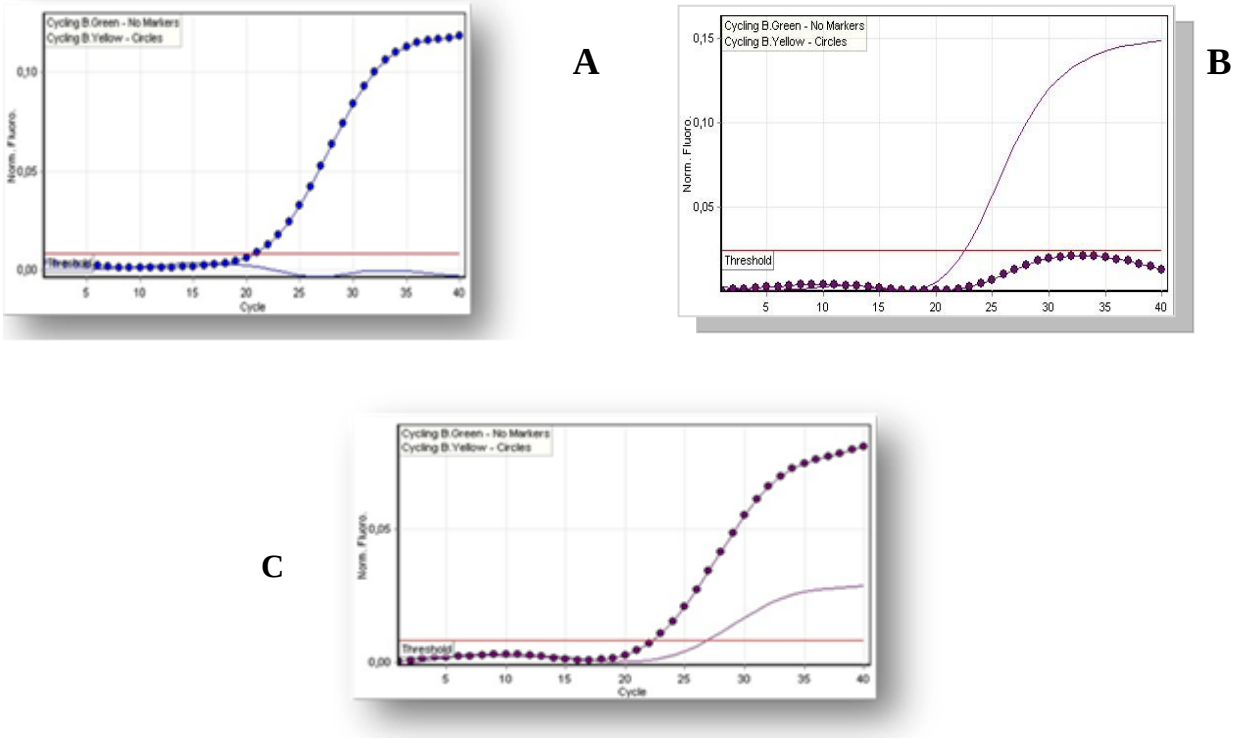


**Figure (4-2) . Showed the(A= Homozygous mutant genotype , B= Homozygous Wild genotype and C= heterozygous genotype )The photograph was taken directly from Agilent qPCR machine .**

## 4.3. Polymorphisms of vitamin D receptor (*VDR*) gene

### 4.3.1. Genotype and allele frequency distributions

This study was examined three VDR polymorphism (rs2228570 T>C in exon 2, rs7975232 C>A in intron 8 and rs731236 T>C in exon 9) among Iraqi women with PCOS and in apparently healthy controls and tested for their association with the PCOS phenotype. The genotype and allele frequency distributions for each of the three *VDR* polymorphisms are presented in Tables 4-3, 4-4 and 4-5, respectively.

### 4.3.1.1. Rs2228570 T>C polymorphism

This variant is commonly known as *FokI* a restriction enzyme (endonuclease) and is located in exon 2. The distribution of genotypes alleles frequency at rs2228570 SNP of *VDR* gene presented in table 4-3.(Appendix 4)

As related with TT, TC and combined TC+CC genotypes, no significant differences in frequency percentage were noted between apparently healthy subjects and patients with polycystic ovary syndrome. Whereas, the frequency of CC genotype was significantly ($p<0.05$) lower in patients with polycystic ovary syndrome than in apparently healthy subjects (20% *versus* 28%, respectively, $\chi^2= 4.027$, *OR* = 0.615, $p<0.05$). Generally, the genotypes of *VDR* gene at rs2228570 SNP have no role in the incidence of PCOS in Iraqi women. The frequencies of T and C alleles were 0.36 and 0.64 in apparently healthy subjects and 0.42 and 0.58 in patients with PCOS, respectively.

Vitamin D receptor polymorphism rs2228570 T > C, defines the presence of T to C transition (ATG (methionine) to ACG (threonine)) in exon2 of *VDR* gene that result in eliminates the translation start site in exon 2, and the encoded protein is shortened by three amino acids (Saijo *et al*., 1991). The Smaller protein exhibits greater transcription activity because of its greater binding efficiency to transcription factor IIB (Jurutka *et al.*, 2000).

The results of the present study are in disagreement with other recent study in India (Dipanshu and Chakravorty, 2015) who has found that the polymorphism of rs2228570 SNP of vitamin D receptor gene is associated with PCOS and seems to modulate ovarian steroid secretion. Also, Sudhesna *et al.* (2013) has found that VDR polymorphism in exon 2 (rs2228570) might be a risk factor for the development of ovarian cancer in Indian population. Fei-fei *et al.* (2017) have found an association between insulin resistance related diseases

and VDR (rs2228570) variant and was more obvious in dark-pigmented Caucasians and Asians but not in Caucasian with white skin.

**Table 4-3. The frequency of genotypes and alleles at rs2228570 SNP in exon 2 of *VDR* gene in Iraqi women with PCOS and controls.**

| Genotypes | Frequency, n(%) | | $\chi^2$ | *OR*[3] |
|---|---|---|---|---|
| | Control[1] | PCOS[2] | | |
| TT | 0 (0%) | 2 (4%) | 0.731 NS | 0.047 |
| TC | 36 (72%) | 38 (76%) | 0.731 NS | 0.047 |
| CC | 14 (28%) | 10 (20%) | 4.027 * | 0.615 |
| TC+CC | 50 (100%) | 48 (96%) | 0.306 NS | 0.022 |
| **Alleles frequencies** | | | | |
| T | 0.36 | 0.42 | - | - |
| C | 0.64 | 0.58 | - | - |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome. [3] Odd ratios.
NS: No significant. *: Significant at 0.05 level.

An earlier studies related to these results , Ames *et al.* (1999) have observed that children with wild TT genotype (rs2228570) of *VDR* gene were found to have lower calcium absorption. In addition, Abrams *et al.* (2005) have found that individuals with wild TT genotype (rs2228570) of *VDR* gene had less total calcium absorption and less calcium accretion to the skeleton in early pubertal adolescents. In a study on 36 white postmenopausal women with osteoporosis or osteopenia who have been treated with calcium and vitamin D supplements for three months, the heterozygous TC genotype (rs2228570) of *VDR* gene was more common in responders compared with nonresponders (Elnenaei *et al.*, 2011). Roth *et al.* (2004) have examined the VDR genetic effect on treatment response of antimycobacterial chemotherapy against tuberculosis in Peruvian

patients, carriers of the mutant CC genotype (rs2228570) of *VDR* gene had faster conversion of sputum mycobacterial culture from positive to negative compared with non-CC genotype carriers.

In this study, T allele frequency was within 36-42% in the studied sample of Iraqi population and this range is close to the frequency of Asian (40%) and more than that of sub-Saharan Africans (20%) (International HapMap Consortium, 2003).

#### 4.3.1.2. Rs7975232 C>A polymorphism

This variant is commonly known as *ApaI* of Vitamin D receptor restriction fragment and is located in intron 8. At present, no known functional consequence of this variant has been described. The distribution of genotypes alleles frequency at rs7975232 SNP of *VDR* gene presented in table 4-4.(Appendix 5).

The frequency of wild CC genotype was significantly ($p<0.05$) lower in PCOS patients than in apparently healthy subjects (2% *versus* 12%, respectively, $\chi^2= 4.825$, *OR*= 0.692, $p<0.05$). In contrast, the frequency of heterozygous CA genotype was significantly ($p<0.05$) higher in PCOS patients when in comparison with apparently healthy subjects (56% *versus* 46%, respectively, $\chi^2=4.825$, *OR*=0.692, $p<0.05$). We believe that the effect of heterozygous CA genotype of *VDR* gene SNP (rs7975232) in intron 8 on the susceptibility of the development of PCOS in Iraqi women could be better clarified with a large sample.

As shown in table 4-4, no significant differences in frequency percentage of AA and combined CA+AA genotypes between apparently healthy subjects and PCOS patients. The frequencies of C and A alleles were 0.35 and 0.65 in apparently healthy subjects and 0.30 and 0.70 in PCOS patients, respectively.

**Table 4-4. The frequency of genotypes and alleles at rs7975232 SNP in intron 8 of *VDR* gene in Iraqi women with PCOS and controls.**

| Genotypes | Frequency, n(%) | | $\chi^2$ | *OR*[3] |
|---|---|---|---|---|
| | Control[1] | PCOS[2] | | |
| CC | 6 (12%) | 1 (2%) | 4.825 * | 0.692 |
| CA | 23 (46%) | 28 (56%) | 4.825 * | 0.692 |
| AA | 21 (42%) | 21 (42%) | 0.000 NS | 0.000 |
| CA+AA | 44 (88%) | 49 (98%) | 1.735 NS | 0.198 |
| **Alleles** | | | | |
| C | 0.35 | 0.30 | - | - |
| A | 0.65 | 0.70 | - | - |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome. [3] Odd ratios.
NS: No significant. *: Significant at 0.05 level.

Also, the results of the present study were disagreed with the results of Dasgupta *et al.* (2015) who indicated that the CC genotype of rs7975232 of *VDR* gene have showed a risk for infertility while variant genotype AA was associated with testosterone levels which were in contrast to a study on Austrian women with PCOS as they have demonstrated AA genotype to be associated with low testosterone levels (Wehr *et al.*, 2011). Chunming *et al.*, (2016) reported that VDR polymorphism in rs7975232 was associated with increased risk of renal cell carcinoma in Chinese population.

In a study that has investigated the effect of dairy intake and colorectal cancer recurrence, Hubner *et al.* (2008) have found an interaction between rs7975232 genotypes and dairy product intake. In particular, individuals with at least one copy of allele A and who consumed a large amount of dairy products

had the lowest risk of colorectral cancer recurrence compared with CC genotype carriers who consumed lower amounts of dairy products.

### 4.3.1.3. Rs731236 T>C polymorphism

This variant is commonly known as TaqI and is located in exon 9. The distribution of genotypes alleles frequency at rs731236 SNP of *VDR* gene presented in table 4-5.(Appendix 6).

The frequency of TT genotype was significantly ($p<0.05$) lower in PCOS patients than in apparently healthy subjects (2% *versus* 12%, respectively, $\chi^2= 4.825$, *OR* = 0.692, $p<0.05$). As related with TC, CC and combined TC+CC genotypes, no significant differences in frequency percentage were observed between apparently healthy subjects and PCOS patients. Generally, both heterozygous and homozygous mutants at rs731236 of *VDR* gene have no association with the incidence of PCOS in Iraqi patients. The frequencies of T and C alleles were 0.49 and 0.51 in apparently healthy subjects and 0.41 and 0.59 in PCOS patients, respectively.

As related with rs731236 SNP of *VDR* gene, the results of the present study are in agreement with Jedrzejuk *et al.* (2015) who have found that classic PCOS phenotype was not associated with *VDR* gene polymorphism at rs731236 SNP. Also, agree with Mahmoudi *et al.* (2015) who have found no significant differences for VDR gene in exon 9 (rs731236 T>C) polymorphism between the women with PCOS and controls. Other studies were in contrast with the results of the present study. In Iranian Azeri patients, Bagheri *et al.* (2013) have observed that the CC genotype of VDR in exon 9 (rs731236 T>C) is associated with the incidence of PCOS. Also, in Egyptian women, El-Shal *et al*. (2013) have found that CC genotype and C allele were associated with increased risk of PCOS.

**Table 4-5. The frequency of genotypes and alleles at rs731236 SNP in exon 9 of *VDR* gene in Iraqi women with PCOS and controls.**

| Genotypes | Frequency, n(%) | | $\chi^2$ | *OR*[3] |
|---|---|---|---|---|
| | Control[1] | PCOS[2] | | |
| TT | 6 (12%) | 1 (2%) | 4.825 * | 0.692 |
| TC | 37 (74%) | 39 (78%) | 0.731 NS | 0.047 |
| CC | 7 (14%) | 10 (20%) | 1.921 NS | 0.217 |
| TC+CC | 44 (88%) | 49 (98%) | 1.735 NS | 0.198 |
| **Alleles** | | | | |
| T | 0.49 | 0.41 | - | - |
| C | 0.51 | 0.59 | - | - |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome. [3] Odd ratios.
NS: No significant. *: Significant at 0.05 level.

The VDR polymorphism in rs731236 associated with the rate of gene expression, is a T to C substitution (ATT (isoleucine)to ATC (isoleucine)) leading to a synonymous change (silent) at codon 352 (Köstner *et al.*, 2009). The rs731236 and rs7975232 polymorphism of *VDR* gene are known to be linked with poly (A) microsatellite repeat in the gene and can influence gene activity (McCullough *et al*., 2007).

### 4.3.2. Haplotypes

The results of haplotype frequency defined by rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls are shown in table 4-6.

Considering the three VDR SNPs, this study have observed eight possible haplotypes, with TTA and TTC being the most common in both groups

(patients and controls). In particular, the TCC haplotype showed statistically significant ($p<0.05$) distribution between PCOS patients and apparently healthy controls (8% *versus* 16%, respectively).

### 4.3.3. Haplotypes combination

The results of haplotype combination frequently defined by rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls are shown in table 4-7.

The frequency of TTC / CCA haplotype combination was significantly ($p<0.05$) higher in PCOS patients than in apparently healthy subjects (28% *versus* 18%, respectively, $\chi^2=4.825$, *OR*= 0.692, $p<0.05$). This haplotype combination means that all studied SNPs were absent. Also, as shown in the table 4-5, 15 of 16 possible haplotype combinations showed no statistically significant differences between PCOS patients and control groups. Both TTA / CCA and TTC / CCA haplotype combinations being the most common.

Polycystic ovary syndrome is among the most common disorders in women of reproductive age and have a strong genetic component (Rotterdam ESHRE/ASRM, 2004). Vitamin D regulates about 3% of the human genomic, including genes that are crucial for glucose and lipid metabolism, via its nucleoprotein receptor that binds to vitamin D response elements found in the promoter region of responsive genes (Darwish and DeLuca, 1993).

Also, vitamin D is the key regulating hormone in calcium homeostasis. It has been shown that calcium plays a role in oocyte activation and maturation resulting in the progression of follicular development (DeFelici *et al.,* 1991). Vitamin D and calcium repletion might lead to normalization of menstrual cycles and restoration of ovulation in PCOS women (Thys-Jacobs *et al.,* 1999).

**Table 4-6. The frequency of haplotypes defined by rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls.**

| Haplotypes | Frequency, n(%) | | $\chi^2$ | *OR*[3] |
|---|---|---|---|---|
| | Control[1] | PCOS[2] | | |
| TTA | 16 (32%) | 15 (30%) | 0.368 NS | 0.026 |
| TCA | 4 (8%) | 4 (8%) | 0.000 NS | 0.000 |
| TCC | 8 (16%) | 4 (8%) | 4.027 * | 0.615 |
| TTC | 15 (30%) | 17 (34%) | 0.731 NS | 0.047 |
| CTC | 6 (12%) | 7 (14%) | 0.368 NS | 0.026 |
| CCA | 1 (2%) | 0 (0 %) | 0.368 NS | 0.026 |
| CCC | 0 (0%) | 1 (2%) | 0.368 NS | 0.026 |
| CTA | 0 (0%) | 2 (4%) | 0.731 NS | 0.047 |
| Total | 50 (100%) | 50 (100%) | | |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome. [3] Odd ratios.

NS: No significant. *: Significant at 0.05 level.

Most of biological actions of vitamin D are considered to be exerted through the nuclear vitamin D receptor (VDR)-mediated control of target genes. VDR belongs to the nuclear hormone receptor superfamily and acts as a ligand-inducible transcription factor. VDR mediates most effects of vitamin D on gene expression via formation of a heterodimer with the retinoid X receptor that binds to promoter regions of many target genes (Pike and Meyer, 2010).

VDR polymorphisms have been associated with vitamin D levels, glucose metabolism, insulin secretion and peripheral action in different populations (McGrath *et al.*, 2010).

**Table 4-7. The frequency of haplotype combinations defined by rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls.**

| Haplotype combinations | Frequency, n(%) | | $\chi^2$ | *OR*[3] |
|---|---|---|---|---|
| | Control[1] | PCOS[2] | | |
| TTA / CCA | 14 (28%) | 14 (28%) | 0.000 NS | 0.000 |
| TCA / CCA | 3 (6%) | 4 (8%) | 0.368 NS | 0.026 |
| TCC / CCA | 6 (12%) | 5 (10%) | 0.368 NS | 0.026 |
| TTC / CCA | 9 (18%) | 14 (28%) | 4.825 * | 0.692 |
| TTC / CCC | 3 (6%) | 0 (0%) | 1.921 NS | 0.217 |
| CTC / CCA | 5 (10%) | 6 (12%) | 0.368 NS | 0.026 |
| TCC / CCC | 2 (4%) | 0 (0%) | 0.731 NS | 0.047 |
| TTC / TCA | 2 (4%) | 0 (0%) | 0.731 NS | 0.047 |
| CCA / CCA | 1 (2%) | 0 (0%) | 0.368 NS | 0.026 |
| TCC / TCA | 1 (2%) | 0 (0%) | 0.368 NS | 0.026 |
| CTC / CCC | 1 (2%) | 1 (2%) | 0.000 NS | 0.000 |
| TTA / TCA | 2 (4%) | 1 (2%) | 0.368 NS | 0.026 |
| TCA / TCA | 1 (2%) | 0 (0%) | 0.368 NS | 0.026 |
| CCC / CCA | 0 (0%) | 1 (2%) | 0.368 NS | 0.026 |
| CTA / CCA | 0 (0%) | 2 (4%) | 0.731 NS | 0.047 |
| TTC / CTA | 0 (0%) | 2 (4%) | 0.731 NS | 0.047 |
| Total | 50 (100%) | 50 (100%) | - | - |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome. [3] Odd ratios.

NS: No significant. *: Significant at 0.05 level.

In addition, *VDR* gene polymorphism are associated with vitamin D deficiency in PCOS and its metabolic and endocrine disturbances (Mahmoudi, 2009; Wehr *et al*., 2011). Although they are limited by modest sample sizes, several studies have suggested associations between VDR polymorphisms and the development of PCOS as well as insulin resistance (Mahmoudi, 2009; Ranjzad *et al.*, 2010; Ranjzad *et al.*, 2012).

## 4.4. Hormonal assays

In general , the data of body mass index (BMI) and hormonal traits for control and PCOS groups in this study were presented in table 4-8.

Follicle stimulating hormone (FSH) values were significantly ($p$<0.05) higher in control subjects compared with PCOS patients (6.89±0.70 *versus* 5.27±0.25 mIU/ ml).The BMI values were significantly ($p$<0.05) higher in PCOS patients compared with control subjects 29.17±0.71 *versus* 26.97±0.44 kg / $m^2$, respectively). LH, prolactin and TSH values were unaffected in both PCOS and diabetic PCOS patients.

Recently, Nabag *et al*., (2014) have found that Sudanese women with PCOS were obese with BMI>30 Kg\$m^2$. Mulhim *et al*.,(2014) have found that Saudi women with PCOS had higher testosterone but lower FSH and significantly higher LH and prolactin hormones levels.

Study of Schmidt *et al*.,(2011) on reproductive hormone levels and anthropometry in postmenopausal women with polycystic ovary syndrome (PCOS), have found high levels in testosterone and lower FSH than apparently healthy controls**.**

Previously, Akbarzadeh *et al*., (2012) have found a positive relationship between plasma testosterone and insulin levels with incidence of PCOS in women with normal BMI. Results of TSH hormones in current study agreed with Al-Deresawi (2012) who have found no significant differences of TSH PCOS Iraqi patients women with PCOS.

Result of this study found that Iraqi women with PCOS had lower FSH levels than apparently healthy controls . These results disagree to those reported by others studied by Rotterdam ESHRE/ASRM,(2004 b) and Schmidt *et al*. (2011).

LH and prolactin hormones, were significantly higher in patient groups (Rotterdam ESHRE/ASRM, 2004). Women with PCOS are generally overweight or obese (Al-Mulhim *et al*., 2014)

**Table 4-8. Hormonal traits and Body mass index in study groups (Mean ± SE) .**

| Hormones | Control[1] | PCOS[2] | P-value |
|---|---|---|---|
| **LH (mIU/ ml )** | 5.36 ± 0.40 | 4.96 ± 0.54 | 0.556 NS |
| **FSH (mIU/ ml )** | 6.89 ± 0.70 | 5.27 ± 0.25 | 0.0336 * |
| **TSH (mIU/ ml )** | 1.66 ± 0.10 | 2.16 ± 0.38 | 0.218 NS |
| **Prolactin (ng/ml )** | 19.97 ± 7.74 | 25.38 ± 4.89 | 0.556 NS |
| **BMI ($kg/m^2$)** | 26.97 ± 0.44 | 29.17 ± 0.71 | 0.0097 ** |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

NS: No significant. *: Significant at 0.05 level.

### 4.4.1. Serum luteinizing hormone (LH).

Serum LH concentrations as affected by the genotypes of rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls are presented in table 4-9.

Generally, the results of the present study indicate that serum LH concentrations were unaffected by the studied SNPs of *VDR* gene. Although, serum LH concentrations were in PCOS patients (with wild TT genotype of rs731236 SNP in *VDR* gene) significantly ($p<0.05$) lower than those of apparently healthy subjects (2.10±0.00 *versus* 5.07±0.93(mIU/ ml), respectively). High levels of LH not only has an effect on oocyte maturity and human reproduction but also on lower fertility and higher miscarriage prevalence (Balen *et al.*, 1993).

Previous studies have found that PCOS patients show higher levels of LH than constant as compared with controls (Franks, 2002; Ranjzad *et al.*, 2011). High levels of LH leads to an elevated of LH: FSH ratio in PCOS patients (Franks, 2002). Ranjzad *et al.* (2011) have demonstrated that there is a significant association between VDR genotype CC of rs731236 and elevated concentrations of serum LH in PCOS women.

**Table 4-9. Serum LH concentrations as affected by the genotypes of rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls .**

| VDR SNPs | Genotypes | LH(mIU/ ml) | | *p*- value |
|---|---|---|---|---|
| | | Control[1] | Patients[2] | |
| rs2228570 T > C | TT | - | 6.00 ± 4.10 | - |
| | TC | 5.59 ± 0.48 | 4.58 ± 0.55 | 0.739 NS |
| | CC | 4.76 ± 0.75 | 6.22 ± 1.56 | 0.405 NS |
| | *p*- value | 0.353 NS | 0.458 NS | - |
| *rs7975232 C>A* | CC | 6.11 ± 1.23 | 10.10 ± 0.00 | 0.335 NS |
| | CA | 5.26 ± 0.63 | 4.47 ± 0.47 | 0.684 NS |
| | AA | 5.26 ± 0.60 | 5.37 ± 1.09 | 0.696 NS |
| | *p*- value | 0.785 NS | 0.301 NS | - |
| rs731236 T>C | TT | 5.07 ± 0.93 | 2.10 ± 0.00 | 0.047 * |
| | TC | 5.02 ± 0.43 | 4.86 ± 0.63 | 0.812 NS |
| | CC | 7.76 ± 1.48 | 5.65 ± 1.03 | 0.362 NS |
| | *p*- value | 0.096 NS | 0.641 NS | - |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

NS: No significant. *: Significant at 0.05 level.

In addition, Bagheri *et al*. (2013) have found that LH concentrations were significantly higher in PCOS patients with mutant CC genotype of *VDR* gene at rs731236 *versus* controls.

Some limitations to be considered for the present study include low sample size and lack of data regarding vitamin D status in contributors. Therefore, studies in large numbers of PCOS are necessary to validate our results.

### 4.4.2. Serum follicle stimulating hormone (FSH)

Serum FSH concentrations as affected by the genotypes of rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls are presented in table 4-10.

Generally, the results of the present study indicate that serum FSH concentrations were unaffected by the studied SNPs of *VDR* gene. Although, serum FSH concentrations were in PCOS patients (with mutant CC genotype of rs2228570 SNP in *VDR* gene) significantly ($p<0.05$) lower than those of apparently healthy subjects (5.72±0.73 *versus* 9.04±2.32 (mIU/ ml), respectively). Overall, the present results of serum FSH concentrations disagree with previous studies whose patients with PCOS show lower level of FSH as compared with controls (Franks, 2002; Ranjzad *et al*., 2011). Schmidt *et al*. (2011) have found lower FSH concentrations in postmenopausal women with PCOS than apparently healthy controls.

Mulhim *et al*. (2014) have found that Saudi women with PCOS had lower FSH concentrations versus controls. Recently, Mohammed (2015) have found that Iraqi women with PCOS and PCOS plus T2DM had lower FSH levels than apparently healthy subjects.

**Table 4-10. Serum FSH concentrations as affected by the genotypes of rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls.**

| VDR SNPs | Genotypes | FSH (mIU/ ml) | | *p*- value |
|---|---|---|---|---|
| | | Control[1] | Patients[2] | |
| **rs2228570 T > C** | **TT** | - | 5.30 ± 1.10 | - |
| | **TC** | 6.05 ± 0.35 | 5.15 ± 0.26 | 0.548 NS |
| | **CC** | 9.04 ± 2.32 | 5.72 ± 0.73 | 0.047 * |
| | ***p*- value** | 0.066 NS | 0.692 NS | - |
| ***rs7975232 C>A*** | **CC** | 6.13 ± 0.94 | 5.40 ± 0.00 | 0.552 NS |
| | **CA** | 6.32 ± 0.51 | 5.21 ± 0.28 | 0.781 NS |
| | **AA** | 7.73 ± 1.58 | 5.34 ± 0.47 | 0.712 NS |
| | ***p*- value** | 0.605 NS | 0.970 NS | - |
| **rs731236 T>C** | **TT** | 6.26 ± 0.86 | 6.60 ± 0.00 | 0.894 NS |
| | **TC** | 7.03 ± 0.91 | 5.28 ± 0.29 | 0.306 NS |
| | **CC** | 6.63 ± 0.96 | 5.06 ± 0.51 | 0.721 NS |
| | ***p*- value** | 0.932 NS | 0.728 NS | - |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

NS: No significant. *: Significant at 0.05 level.

### 4.4.3. Serum thyroid stimulating hormone (TSH)

Serum TSH concentrations as affected by the genotypes of rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls are presented in table 4-11.

The results of the present study indicate that serum TSH concentrations were unaffected by the studied SNPs of *VDR* gene. These results are in agreement with Mohammed (2015) and Al-Deresawi (2012) who have found no significant differences in TSH levels between Iraqi patients with PCOS and apparently healthy controls.

**Table 4-11. Serum TSH concentrations as affected by the genotypes of rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls.**

| VDR SNPs | Genotypes | TSH (mIU/ ml) | | *p*- value |
|---|---|---|---|---|
| | | Control[1] | Patients[2] | |
| **rs2228570 T > C** | **TT** | - | 1.980 ± 0.60 | - |
| | **TC** | 1.734 ± 0.12 | 2.268 ± 0.49 | 0.367 NS |
| | **CC** | 1.485 ± 0.24 | 1.760 ± 0.27 | 0.695 NS |
| | ***p*- value** | 0.312 NS | 0.873 NS | - |
| ***rs7975232 C>A*** | **CC** | 1.733 ± 0.11 | 1.270 ± 0.00 | 0.672 NS |
| | **CA** | 1.590 ± .18 | 1.738 ± .14 | 0.706 NS |
| | **AA** | 1.725 ± 0.17 | 2.75 ± 0.88 | 0.351 NS |
| | ***p*- value** | 0.826 NS | 0.436 NS | - |
| **rs731236 T>C** | **TT** | 1.316 ± 0.18 | 1.400 ± 0.00 | 0.283 NS |
| | **TC** | 1.781 ± 0.13 | 2.311 ± 0.48 | 0.359 NS |
| | **CC** | 1.273 ± 0.22 | 1.620 ± 0.12 | 0.872 NS |
| | ***p*- value** | 0.177 NS | 0.755 NS | - |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

NS: No significant. *: Significant at 0.05 level.

The results of this study are disagree with Dahiya *et al.* (2012) and GulabKanwar *et al*. (2015) who have found increased levels of TSH in PCOS women compared with controls.

Mueller *et al*. (2009) reported that serum TSH levels ≥ 2 m IU / L showed better sensitivity and specificity for the identification of women with PCOS.

### 4.4.4. Serum prolactin

Serum prolactin concentrations as affected by the genotypes of rs731236, rs2228570 and rs7975232 SNPs of VDR gene in Iraqi women with PCOS and controls are presented in table 4-12.

Within carriers of mutant CC genotype of rs2228570 SNP in *VDR* gene, serum prolactin levels were significantly ($p<0.05$) higher in PCOS patients *versus* controls (40.19±23.40 *versus* 13.14±1.11ng/ml , respectively).

Also, as related with rs7975232 SNP of *VDR* gene, serum prolactin levels of CC and CA genotypes carriers were significantly ($p<0.05$) higher in PCOS patients *versus* controls (44.80±0.00 *versus* 13.0±2.01 and 32.53±8.39 *versus* 12.39±0.96 ng/ml , respectively). Whereas, serum prolactin levels in mutant AA genotype were significantly ($p<0.05$) lower in PCOS patients versus controls (14.93±1.83 *versus* 30.26±18.4ng/ml , respectively). Moreover, AA genotype of rs7975232 led to significantly ($p<0.05$) increase in serum prolactin levels of apparently healthy controls, while, in PCOS patients led to significantly ($p<0.05$) decrease in serum prolactin levels .

In carriers of mutant CC genotype of rs731236 SNP in *VDR* gene, serum prolactin levels were significantly ($p<0.05$) higher in PCOS patients *versus* controls (22.34±4.07 *versus* 12.21±0.60 ng/ml , respectively). Also, heterozygous TC genotype of rs731236 led to significantly ($p<0.05$) increase in serum prolactin levels in both PCOS patients and apparently healthy subjects when compared with wild TT and mutant CC genotypes (22.72 10.17±*versus* 10.29±1.38 and 12.21±0.60 ng/ml , respectively, in apparently healthy subjects and 26.61±6.19 *versus* 8.30± 0.00and 22.34±4.07 ng/ml, respectively in PCOS patients).

Generally, the results of serum prolactin in the present study are in agreement with Mulhim *et al.* (2014) who have found that Saudi women with

PCOS had higher prolactin levels in comparison with apparently healthy controls.

**Table 4-12. Serum prolactin concentrations as affected by the genotypes of rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and controls.**

| VDR SNPs | Genotypes | Prolactin(ng/ml) | | *p*- value |
|---|---|---|---|---|
| | | Control[1] | Patients[2] | |
| **rs2228570 T > C** | **TT** | - | 33.40 ± 0.60 | - |
| | **TC** | 22.62 ± 10.79 | 21.07 ± 2.13 | 0.663 NS |
| | **CC** | 13.14 ± 1.11 | 40.19 ± 23.4 | 0.025 * |
| | ***p*- value** | 0.597 NS | 0.290 NS | - |
| ***rs7975232 C>A*** | **CC** | 13.00 ± 2.01 | 44.80 ± 0.00 | 0.257 * |
| | **CA** | 12.39 ± 0.96 | 32.53 ± 8.39 | 0.033 * |
| | **AA** | 30.26 ± 18.4 | 14.93 ± 1.83 | 0.038 * |
| | ***p*- value** | 0.051 * | 0.049 * | - |
| **rs731236 T>C** | **TT** | 10.29 ± 1.38 | 8.30 ± 0.00 | 0.315 NS |
| | **TC** | 22.72 ± 10.17 | 26.61 ± 6.19 | 0.553 NS |
| | **CC** | 12.21 ± 0.60 | 22.34 ± 4.07 | 0.041 * |
| | ***p*- value** | 0.038 * | 0.039 * | - |

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

NS: No significant. *: Significant at 0.05 level.

These results were agreed with Mohammed (2015) have observed that prolactin levels were significantly higher in PCOS and diabetic PCOS patients compared with control subjects.

## 4.5. Cytogenetic study

### 4.5.1. Micronucleus (MN).

The results of (MN) as affected by PCOS and the genotypes of *VDR* gene (rs7975232C>A) are shown in table (4-13) figure (4-3).

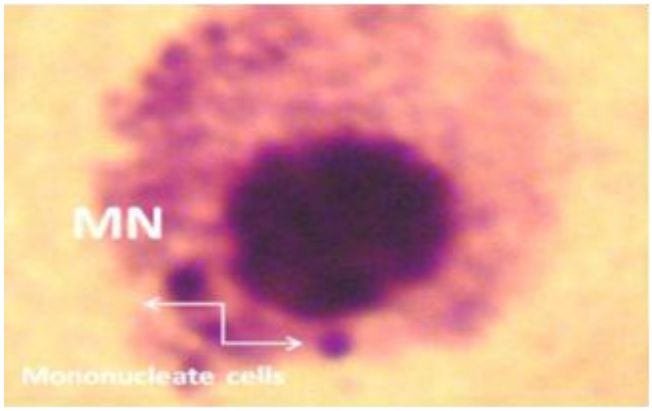


**Figure 4.3:-Mononuclear with Micronucleus in PCOS patients (X 100).**

The MN frequency was significantly ($p$<0.01) increased in PCOS patients compared with apparently healthy subjects (3.50±0.14 *versus* 1.07±0.10 MN/1000, respectively).

**Table (4-13). Effect of PCOS and VDR polymorphism (rs7975232 C>A) on the frequency of micronucleus (MN/1000). (Mean ± SE).**

| Group | | MN frequency(n/1000) |
|---|---|---|
| **Control**[1] | | 1.07± 0.10 b |
| **PCOS**[2] | | 3.50 ± 0.14 a |
| ***p*-value** | | 0.0001 ** |
| **Control** | | 1.07 ± 0.10 c |
| **PCOS** | **Heterozygous(CA)** | 2.95 ± 0.16 b |
| | **Homozygous (AA)** | 4.05 ± 0.20 a |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means. [1] apparently healthy subjects.

[2] Patients with polycystic ovary syndrome.

In PCOS patients, the MN frequency was significantly ($p$<0.01) higher in both heterozygous and homozygous mutants (rs7975232 of *VDR* gene) compared with apparently healthy subjects (2.95±0.16 and 4.05± 0.20 compared with 1.07±0.10 MN /1000, respectively). In addition, MN frequency was significantly ($p$<0.01) lower in heterozygous mutant (rs7975232 of *VDR* gene) than in homozygous mutant (2.95±0.16 *versus* 4.05±0.20 MN/1000, respectively).

The results of micronucleus (MN) as affected by PCOS and the genotypes of *VDR* gene (rs731236T>C) are shown in table (4-14).

**Table (4-14). Effect of PCOS and VDR polymorphism (rs731236 T>C) on the frequency of micronucleus (MN/1000). (Mean ± SE).**

| Group | | MN frequency(n/1000) |
|---|---|---|
| **Control[1]** | | 1.07 ± 0.10 b |
| **PCOS[2]** | | 3.26± 0.17 a |
| ***p*-value** | | 0.0001 ** |
| **Control** | | 1.07 ± 0.10 c |
| **PCOS** | **Heterozygous (TC)** | 3.66 ± 0.23 a |
| | **Homozygous (CC)** | 2.87 ± 0.24 b |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means. [1] apparently healthy subjects.

[2] Patients with polycystic ovary syndrome.

The MN frequency was significantly ($p$<0.01) increased in PCOS patients compared with apparently healthy subjects (3.26±0.17 *versus* 1.07±0.10 MN/1000, respectively). In PCOS patients, the MN frequency was significantly ($p$<0.01) higher in both heterozygous and homozygous mutants (rs731236 of

*VDR* gene) compared with apparently healthy subjects (3.66±0.23and 2.87±0.24 compared with 1.07±0.10 MN /1000, respectively).

In addition, MN frequency was significantly ($p$<0.01) higher in heterozygous mutant (rs731236 of *VDR* gene) than in homozygous mutant (3.66±0.23 *versus* 2.87±0.24 MN/1000, respectively).

The results of micronucleus (MN) as affected by PCOS and the genotypes of *VDR* gene (rs2228570T>C) are shown in table (4-15).

**Table (4-15). Effect of PCOS and VDR polymorphism (rs2228570 T>C) on the number of micronucleus (MN/1000). (Mean ± SE).**

| Group | | MN frequency(n/1000) |
|---|---|---|
| **Control[1]** | | 1.07 ± 0.10 b |
| **PCOS[2]** | | 3.28 ± 0.15 a |
| ***p*-value** | | 0.0001 ** |
| **Control** | | 1.07 ± 0.10 b |
| **PCOS** | **Heterozygous (TC)** | 3.07 ± 0.22 a |
| | **Homozygous (CC)** | 3.57 ± 0.21 a |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means. [1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

The MN frequency was significantly ($p$<0.01) increased in PCOS patients compared with apparently healthy subjects (3.28± 0.15*versus* 1.07±0.10 MN/1000, respectively).

In PCOS patients, the MN frequency was significantly ($p$<0.01) higher in both heterozygous and homozygous mutants (rs2228570 of *VDR* gene) compared with apparently healthy subjects (3.07±0.22 and 3.57±0.21 compared with 1.07±0.10 MN /1000, respectively).

In addition, no significant differences are shown in MN frequency between heterozygous and homozygous mutants (rs2228570 of *VDR* gene), (3.07± versus 3.57± MN/1000, respectively).

### 4.5.2. Binucleated cells with micronucleus(BNMN).

The results of (BNMN) as affected by PCOS and the genotypes of *VDR* gene (rs7975232C>A) are shown in table (4-16), figure (4-4).

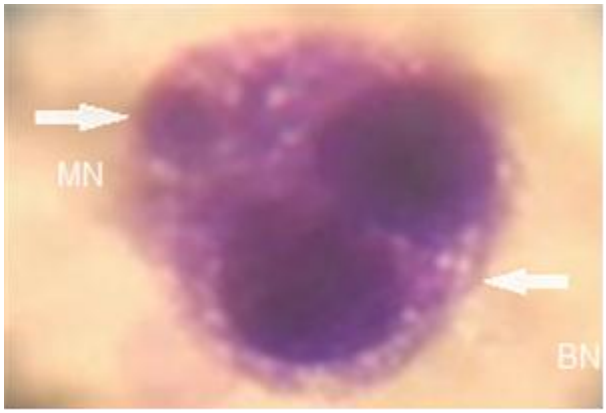


**Figure 4.4:- Binucleate (BN) cells with Micronucleus (MN) in PCOS patients (X 100).**

The BNMN frequency was significantly ($p$<0.01) increased in PCOS patients compared with apparently healthy subjects (4.24±0.14 *versus* 1.24±0.08 BNMN/1000, respectively). In PCOS patients, the BNMN frequency was significantly ($p$<0.01) higher in both heterozygous and homozygous mutant (rs7975232 of *VDR* gene) compared with apparently healthy subjects (3.78± and 4.70±0.17 compared with 1.24±0.08 BNMN /1000, respectively).

In addition, BNMN frequency was significantly ($p$<0.01) lower in heterozygous mutant (rs7975232 of *VDR* gene) than in homozygous mutant (3.78 ±0.20 *versus* 4.70±0.17 BNMN/1000, respectively).

**Table (4-16). Effect of PCOS and VDR polymorphism (rs7975232 C>A) on the frequency of binucleated cells with micronucleus (BNMN /1000). (Mean ± SE).**

| Group | | BNMN frequency(n/1000) |
|---|---|---|
| **Control[1]** | | 1.24 ± 0.08 b |
| **PCOS[2]** | | 4.24 ± 0.14 a |
| ***p*-value** | | 0.0001 ** |
| **Control** | | 1.24 ± 0.08 c |
| **PCOS** | **Heterozygous (CA)** | 3.78 ± 0.20 b |
| | **Homozygous (AA)** | 4.70 ± 0.17 a |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means. [1] apparently healthy subjects.

[2] Patients with polycystic ovary syndrome.

The results of binucleated cells with micronucleus (BNMN) as affected by PCOS and the genotypes of *VDR* gene (rs731236 T>C) are shown in table (4-17).

The BNMN frequency was significantly (P<0.01) increased in PCOS patients compared with apparently healthy subjects (4.02±0.16 *versus* 1.24±0.08 BNMN/1000, respectively). In PCOS patients, the BNMN frequency was significantly (*p*<0.01) higher in both heterozygous and homozygous mutants (rs731236 of *VDR* gene) compared with apparently healthy subjects (4.45±0.25and 3.60±0.18 compared with 1.24±0.08 BNMN /1000, respectively).

In addition, BNMN frequency was significantly (P<0.01) higher in heterozygous mutant (rs731236 of *VDR* gene) than in homozygous mutant (4.45 ±0.25 *versus* 3.60±0.18 BNMN/1000, respectively).

**Table (4-17). Effect of PCOS and VDR polymorphism (rs731236 T>C) on The number of binucleated cells with micronucleus (BNMN /1000). (Mean ± SE).**

| Group | | BNMN frequency(n/1000) |
|---|---|---|
| **Control[1]** | | 1.24 ± 0.08 b |
| **PCOS[2]** | | 4.02 ± 0.16 a |
| **P-value** | | 0.0001 ** |
| **Control** | | 1.24 ± 0.08 c |
| **PCOS** | **Heterozygous (TC)** | 4.45± 0.25 a |
| | **Homozygous (CC)** | 3.60 ± 0.18 b |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means. [1] apparently healthy subjects.

[2] Patients with polycystic ovary syndrome.

The results of binucleated cells with micronucleus (BNMN) as affected by PCOS and the genotypes of *VDR* gene (rs2228570 T>C) are shown in table (4-18).

The BNMN frequency was significantly ($p$<0.01) increased in PCOS patients compared with apparently healthy subjects (4.33±0.13 *versus* 1.24±0.08 BNMN/1000, respectively). In PCOS patients, the BNMN frequency was significantly ($p$<0.01) higher in both heterozygous and homozygous mutant (rs2228570 of *VDR* gene) compared with apparently healthy subjects

(4.19±0.16 and 4.53±0.22 compared with 1.24±0.08 BNMN /1000, respectively).

**Table (4-18). Effect of PCOS and VDR polymorphism (rs2228570 T>C) on the number of binucleated cells with micronucleus (BNMN /1000). (Mean ± SE).**

| Group | | BNMN frequency(n/1000) |
|---|---|---|
| **Control[1]** | | 1.24 ± 0.08 b |
| **PCOS[2]** | | 4.33 ± 0.13 a |
| **P-value** | | 0.0001 ** |
| **Control** | | 1.24 ± 0.08 b |
| **PCOS** | **Heterozygous (TC)** | 4.19 ± 0.16 a |
| | **Homozygous (CC)** | 4.53 ± 0.22 a |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means. [1] apparently healthy subjects.

[2] Patients with polycystic ovary syndrome.

In addition, no significant differences between BNMN frequency in heterozygous and homozygous mutant (rs2228570 of *VDR* gene), (4.19±0.16 *versus* 4.53±0.22 BNMN/1000, respectively).

### 4.5.3. Sister chromatid exchange (SCE)

The results of (SCE) as affected by PCOS and the genotypes of *VDR* gene (rs7975232C>A) are shown in table (4-19) figure (4-5).

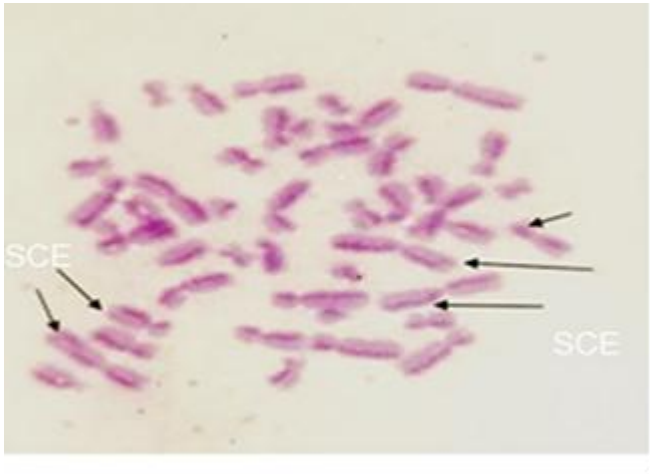


**Figure 4.5 Sister chromatid change (SCE) in PCOS patients (X 100).**

The SCE frequency was significantly ($p$<0.01) increased in PCOS patients compared with apparently healthy subjects (3.42 ±0.12*versus* 1.79±0.12 SCE/1000, respectively).

In PCOS patients, the SCE frequency was significantly ($p$<0.01) higher in both heterozygous and homozygous mutants (rs7975232 of *VDR* gene) compared with apparently healthy subjects (3.05±0.15and 3.78±0.18 compared with 1.79 ±0.12 SCE /1000, respectively).

In addition, SCE numbers were significantly ($p$<0.01) lower in heterozygous mutant (rs7975232 of *VDR* gene) than in homozygous mutant (3.05±0.15 *versus* 3.78±0.18 SCE/1000, respectively).

**Table (4-19). Effect of PCOS and VDR polymorphism (rs7975232 C>A) on the number of Sister chromatid exchange (SCE/1000). (Mean ± SE).**

| Group | | SCE frequency(n/1000) |
|---|---|---|
| **Control[1]** | | 1.79 ± 0.12 b |
| **PCOS[2]** | | 3.42 ± 0.12 a |
| ***p*-value** | | 0.0001 ** |
| **Control** | | 1.79 ± 0.12 c |
| **PCOS** | **Heterozygous(CA)** | 3.05 ± 0.15 b |
| | **Homozygous (AA)** | 3.78 ± 0.18 a |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means.

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

The results of sister chromatid exchange (SCE) as affected by PCOS and the genotypes of *VDR* gene (rs731236 T>C) are shown in table (4-20).

The SCE frequency was significantly ($p$<0.01) increased in PCOS patients compared with apparently healthy subjects (3.85±0.15 *versus* 1.79±0.12 SCE/1000, respectively). In PCOS patients, the SCE frequency was significantly ($p$<0.01) higher in both heterozygous and homozygous mutants (rs731236 of *VDR* gene) compared with apparently healthy subjects (3.76±0.22 and 3.93±0.19 compared with 1.79±0.12 SCE /1000, respectively).

In addition, no significant differences are shown SCE frequency between heterozygous and homozygous mutant (rs731236 of *VDR* gene), (3.76 ±0.22 *versus* 3.93 ±0.19 SCE/1000, respectively).

**Table (4-20). Effect of PCOS and VDR polymorphism (rs731236 T>C) on the number of Sister chromatid exchange (SCE/1000). (Mean ± SE).**

| Group | | SCE frequency(n/1000) |
|---|---|---|
| **Control[1]** | | 1.79 ± 0.12 b |
| **PCOS[2]** | | 3.85 ± 0.15 a |
| ***p*-value** | | 0.0001 ** |
| **Control** | | 1.79 ± 0.12 b |
| **PCOS** | **Heterozygous (TC)** | 3.76 ± 0.22 a |
| | **Homozygous (CC)** | 3.93 ± 0.19 a |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means.

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

The results of sister chromatid exchange (SCE) as affected by PCOS and the genotypes of *VDR* gene (rs2228570 T>C) are shown in table (4-21).

The SCE frequency was significantly ($p$<0.01) increased in PCOS patients compared with apparently healthy subjects (4.11±0.13 *versus* 1.79±0.12 SCE/1000, respectively). In PCOS patients, the SCE frequency was significantly ($p$<0.01) higher in both heterozygous and homozygous mutants (rs2228570 of *VDR* gene) compared with apparently healthy subjects (3.98±0.18 and 4.30±0.20 compared with 1.79±0.12 SCE /1000, respectively).

In addition, no significant differences are shown SCE frequency between heterozygous and homozygous mutant (rs2228570 of *VDR* gene), (3.98±0.18 *versus* 4.30 ±0.20 SCE/1000, respectively).

**Table (4-21). Effect of PCOS and VDR polymorphism (rs2228570T>C) on the number of Sister chromatid exchange (SCE/1000). (Mean ± SE).**

| Group | | SCE frequency(n/1000) |
|---|---|---|
| **Control[1]** | | 1.79 ± 0.12 b |
| **PCOS[2]** | | 4.11 ± 0.13 a |
| ***p*-value** | | 0.0001 ** |
| **Control** | | 1.79 ± 0.12 b |
| **PCOS** | **Heterozygous(TC)** | 3.98 ± 0.18 a |
| | **Homozygous (CC)** | 4.30 ± 0.20 a |
| ***p*-value** | | 0.0001 ** |

** means a significant difference at 0.01 level. different letters refer to a significant difference between means.

[1] apparently healthy subjects. [2] Patients with polycystic ovary syndrome.

In general, the results of this study predicted that the frequencies of BNMN, MN and SCE were significantly higher in PCOS patients than in controls regardless SNP type. Whereas, contradictory results were found in BNMN, MN and SCE frequencies comparison between heterozygous and homozygous genotypes within each SNP. The results of the present study are in agreement with several previous studies for MN (Hamuruca *et al*., 2010; Deepika, *et al.*, 2014; Al-Ahmed *et al.*, 2016) and for SCE (Nersesyan *et al*., 2010; Evagelia *et al*., 2015). Nersesyan *et al.* (2006) have studied 25 females with PCOS and found that the level of cells with MN was 1.54-fold higher in patients. Also, Elif *et al.* (2006) have reported about 3-fold increase in MN level in lymphocyte of women with PCOS. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind at the anaphase stage of cell division. Their presence in cells reflects chromosomal aberrations arising during mitosis

(Fenech *et al.,* 1999; Kirsch-Volders *et al.,* 2003). The quantification of MN for the evaluation of chromosome damage offers several advantages: the method is simple and fast, and it does not require the presence of metaphasic cells. Another advantage of the MN assay is the relative ease of scoring and the statistical power obtained from scoring larger numbers of cells than are typically used for metaphase analysis (Fenech *et al.,* 1999). Elif *et al.* (2006) have indicated that the hyperandrogenism and hyperinsulinemia in PCOS patients might play a central role in increased MN frequencies in peripheral blood lymphocytes. Trkova *et al.* (2000) have showed that MN frequencies are increased in couples with infertility or with two or more spontaneous abortions. Increased oxidative stress and decreased antioxidant capacity were reported in women with PCOS (Fenkci *et al.,* 2003). It was documented that oxidative stress induces chromosomal breakage and formation of bone-marrow MN (Simic, 1994). Pansarasa *et al.* (2002) have indicated that the main reason of oxidative stress for excess of testosterone in blood of females with PCOS because this hormone has such a property. Furthermore, some authors have shown a positive correlation between the extent of lipid peroxidation and genotoxicity reflected by increased MN formation (Chandra and Nagini, 2003; Subapriya *et al.,* 2004). Al-Ahmed *et al.* (2016) have reported that PCOS patients have genetic instability with increase in MN formation and they have attributed this increase to exposure to oxidative stress with PCOS which lead to increase DNA damage. Orio *et al.* (2004) have shown the detrimental effect of PCOS on the cardiovascular system. Botto *et al.* (2001) have demonstrated that the frequency of MN in peripheral blood is increased in patients with coronary artery disease. Women with PCOS are also thought to be at increased risk of cancer (Gadducci *et al.,* 2005). Some investigators have shown that in lymphocytes the MN levels are twofold higher in cancer patients than in control

individuals (Jagetia *et al.*, 2001). Hamurcu *et al.* (2010) have found that MN frequencies obtained from lymphocytes of the women with PCOS were significantly higher than those of controls (4.1 *versus* 2.1, respectively).

Cytogenetics provides several biomarkers for chromosomal instability assessment, one of which is the sister chromatid exchange (SCE) frequency in cells. SCE is a natural process that implicates the exchange of homologous genetic segments as a mode of repair mechanism. The methodology of SCEs has been proved to be a very useful tool with predictive value, for detecting harmful effects on DNA, caused by various physical and chemical factors. Increased frequency of this index reflects the existence of genotoxicity in cells and the subsequent failure of repair mechanisms to recover the damaged site. Several studies have reported that SCE analysis is a very sensitive method, able to detect mutagens and/or carcinogens (Wilson and Thompson, 2007) and may be more sensitive than other cytogenetic endpoints, such as micronuclei and chromosome aberrations (Lasne *et al.*, 1984). Evagelia *et al.* (2015) have used the frequency of sister chromatid exchange as an index of cytogenetic damage and they have found a significant increase in SCE levels in women with PCOS compared with controls. Also, Evagelia *et al.* (2015) have reported a positive correlation between DNA damage and PCOS phenotypes.

To the best of our knowledge this is the first study to investigate a possible association between chromosomal instability and clinical phenotypes of PCOS in Iraq. The results in this study revealed that the DNA of PCOS patients showed significant damage, as shown by the increased SCE frequency in lymphocytes, confirming some reports that deal with this issue (Moran *et al.*, 2008; Nersesyan and Chobanyan, 2010). The high rate of DNA damage in Iraqi PCOS patients suggests that the repair mechanisms are insufficient to genetic changes.

# *Conclusions & Recommendations*

## Conclusions :

According to the findings , this study concluded :

**1-** Both rs2228570 and rs731236 SNPs of vitamin D receptor gene have no role in the incidence of PCOS in Iraqi women .

**2-** As related with rs7975232 SNPs of *VDR* gene , Iraqi women with CA genotype were more susceptible to PCOS , but no risk in this study .

**3-** Considering the rs731236 , rs2228570 and rs7975232 SNPs of *VDR* gene,both TTA and TTC haplotypes were the most common in both study groups .

**4-** The increase of the TTC/CCA haplotype combination frequency in PCOS patients means that all studied SNPs have no relationship with the incidence of PCOS in Iraqi women .

**5-** Serum concentrations of LH , FSH and TSH hormones were unaffected by all studied SNPs of *VDR* gene, while , serum prolactin levels were affected by rs7975232 and rs731236 SNPs of *VDR* gene .

**6-** Increase the frequency of micronuclei and sister chromatid exchange in Iraqi women with PCOS .

**7-** The results of the present study indicate that Iraqi patients with PCOS were more affected by genetic instability than by the hot spot of *VDR* gene polymorphism studied.

## Recommendations:

In the light of the present results, we recommended to study :

**1-** Other molecular techniques such as qPCR may be used for evaluation of *VDR* gene expression in the case of PCOS in Iraqi patients then correlated with other parameters.

**2-** Screening for other SNPs of *VDR* gene that correlate with PCOS incidence.

**3-** Study of the *VDR* gene polymorphism on other diseases in Iraq.

**4-** Focusing on the cytogenetic studies in other genetic diseases in Iraq.

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# *Appendixes*

## Appendix I

### *Questionnaire form*

**1-Demographic characteristics & Patient's personal information :-**

***Sample No.:*** ***Hospital registration No.:*** ***Date: / / 2016***

***- Name:*** ***husband name &phone number:***

***- 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):*** ***Body mass index(BMI):*** ***Kg/(m)$^2$=***

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

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

**2- Hormonal & Biochemical levels :**

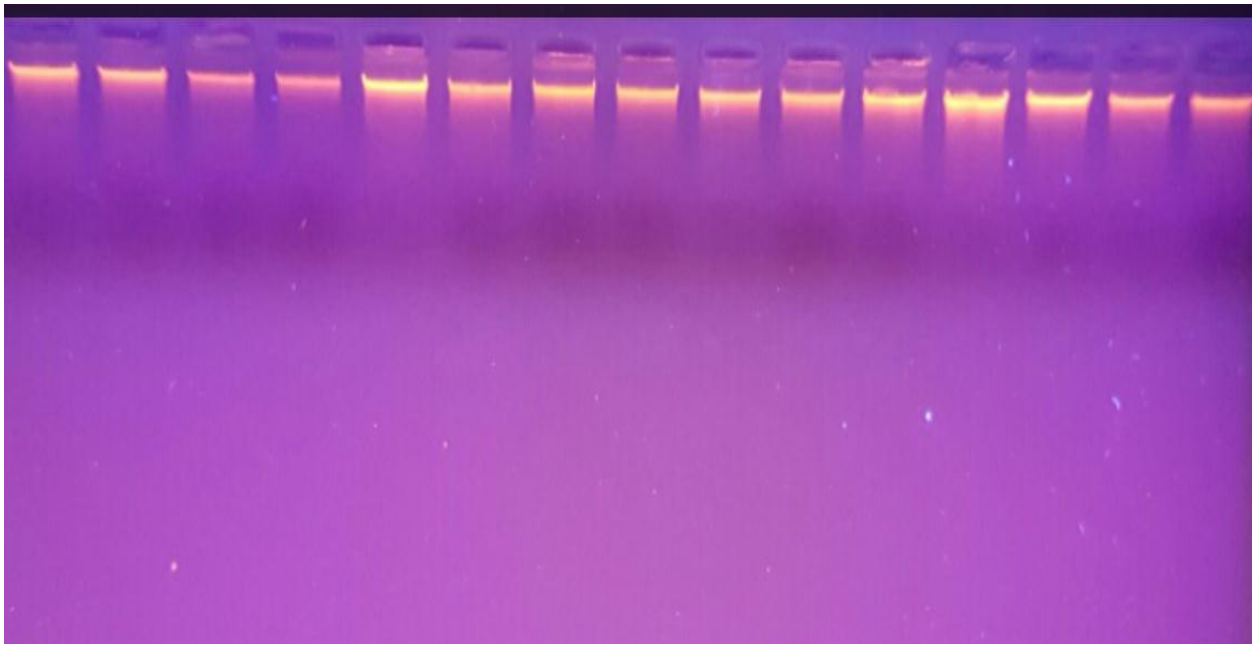
**LH:** **TSH:** **PCOS FBS:**

**FSH:** **T3 :** **Prolactin:**

**Testosterone:** **T4:** **Insulin:**

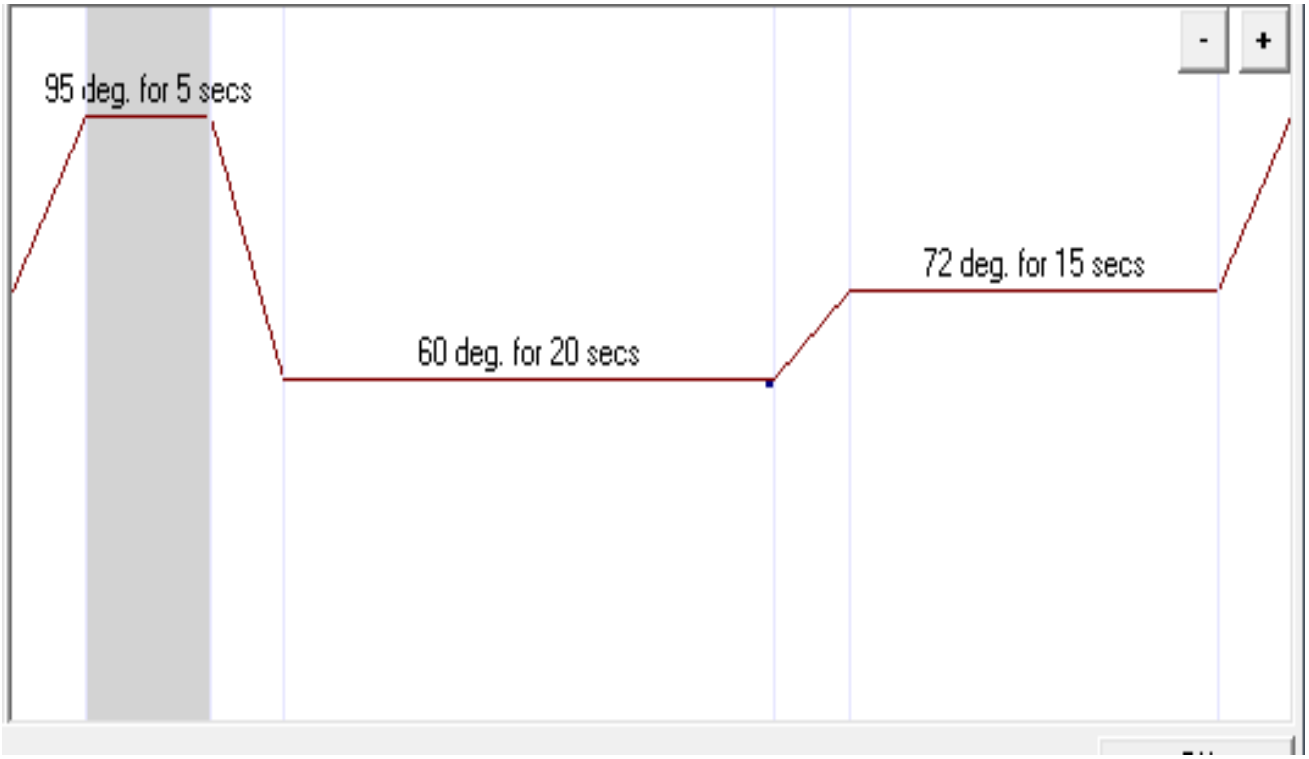
***Name of specialist physician: Dr. ……..***

**Appendix 2.** DNA Loading and Electrophoresis.



**Genomic DNA in Gel electrophoresis**

**Appendix 3.** The thermal profile of allelic discrimination Real-time PCR program



**Appendix 4.** The distribution of genotypes alleles frequency at rs2228570 SNP of *VDR* gene .

# Allelic Discrimination Report

### Experiment Information

| | |
|---|---|
| Run Name | sanaa 2017-03-28 (1) |
| Run Start | 2017-03-28 5:58:26 PD |
| Run Finish | 2017-03-28 7:44:17 PD |
| Operator | sanaa |
| Notes | |
| Run On Software Version | Rotor-Gene 2.1.0.9 |
| Run Signature | The Run Signature is valid. |
| Gain Green | 9,33 |
| Gain Yellow | 8, |

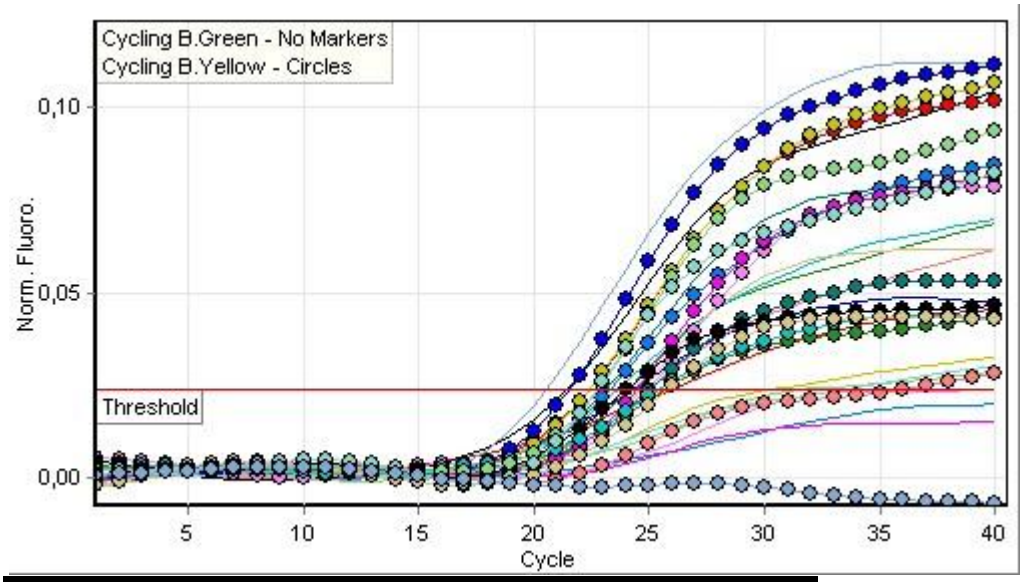
### Allelic Discrimination Information

| | |
|---|---|
| Digital Filter | Light |
| Imported Analysis Settings | |
| Left Threshold | 1 |
| No Template Control Threshold | % 0 |
| Noise Slope Correction | Yes |
| Normalisation Method | Dynamic Tube Normalisation |
| Reaction Efficiency Threshold | Disabled |
| Start normalising from cycle | 1 |
| Threshold | .02397 |

### Profile

| Cycle | Cycle Point |
|---|---|
| min secs Hold@50°,150 | |
| min secs Hold 2@95°,150 | |
| ( repeats) Cycling 5 | Step 1@95°, hold5secs |
| | Step 2@60°, hold20secs, acquiring toCycling A([Green][1][1],[Yellow][2][2]) |
| | Step 3@72°, hold15secs |
| ( repeats) Cycling 240 | Step 1@95°, hold5secs |
| | Step 2@60°, hold20secs, acquiring toCycling B([Green][1][1],[Yellow][2][2]) |
| | Step 3@72°, hold15secs |

## Allelic data for Cycling B.Green,Cycling B.Yellow




| No. | Colour | Name | Genotype | Cycling B.Green | Cycling B.Yellow |
|---|---|---|---|---|---|
| 1 | | | Heterozygous | Reaction | Reaction |
| 2 | | | Heterozygous | Reaction | Reaction |
| 3 | | | Heterozygous | Reaction | Reaction |
| 5 | | | Heterozygous | Reaction | Reaction |
| 6 | | | Mutant | No Reaction | Reaction |
| 7 | | | Heterozygous | Reaction | Reaction |
| 8 | | | Heterozygous | Reaction | Reaction |
| 9 | | | Heterozygous | Reaction | Reaction |
| 10 | | | Mutant | No Reaction | Reaction |
| 11 | | | Heterozygous | Reaction | Reaction |
| 12 | | | Heterozygous | Reaction | Reaction |
| 13 | | | Heterozygous | Reaction | Reaction |
| 14 | | | Heterozygous | Reaction | Reaction |
| 15 | | | Heterozygous | Reaction | Reaction |
| 16 | | | Wild Type | Reaction | No Reaction |

**Appendix 5.** The distribution of genotypes alleles frequency at rs7975232 SNP of *VDR* gene .

# Allelic Discrimination Report

### Experiment Information

| | |
|---|---|
| Run Name | sanaa 2017-03-23 (1) |
| Run Start | 2017-03-23 4:59:13 PD |
| Run Finish | 2017-03-23 6:44:49 PD |
| Operator | sanaa |
| Notes | |
| Run On Software Version | Rotor-Gene 2.1.0.9 |
| Run Signature | The Run Signature is valid. |
| Gain Green | 8, |
| Gain Yellow | 9,33 |

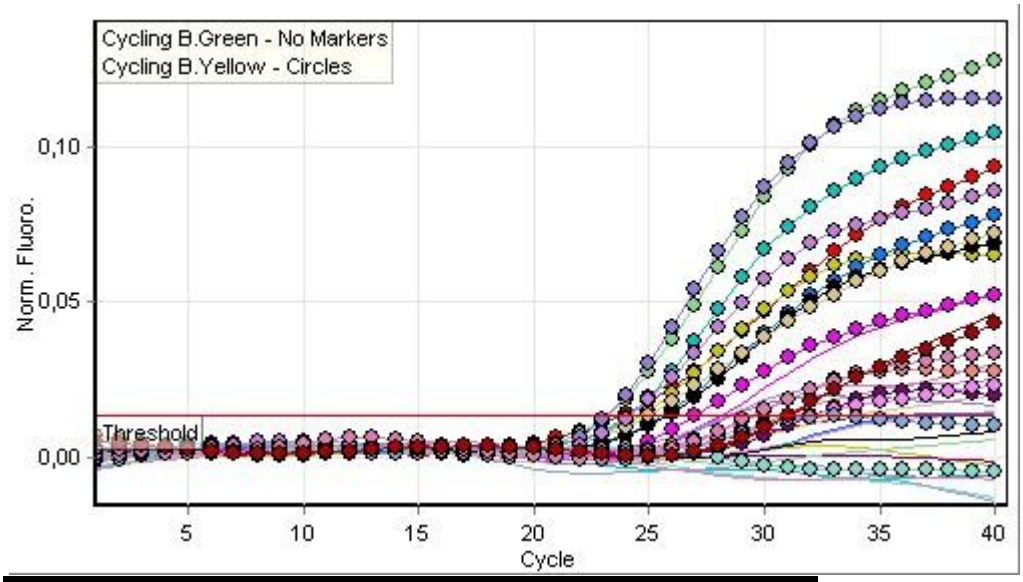
### Allelic Discrimination Information

| | |
|---|---|
| Digital Filter | Light |
| Imported Analysis Settings | |
| Left Threshold | 1 |
| No Template Control Threshold | % 0 |
| Noise Slope Correction | Yes |
| Normalisation Method | Dynamic Tube Normalisation |
| Reaction Efficiency Threshold | Disabled |
| Start normalising from cycle | 1 |
| Threshold | .01341 |

### Profile

| Cycle | Cycle Point |
|---|---|
| min secs Hold@50°,150 | |
| min secs Hold 2@95°,150 | |
| ( repeats) Cycling 5 | Step 1@95°, hold5secs |
| | Step 2@60°, hold20secs, acquiring toCycling A([Green][1][1],[Yellow][2][2]) |
| | Step 3@72°, hold15secs |
| ( repeats) Cycling  240 | Step 1@95°, hold5secs |
| | Step 2@60°, hold20secs, acquiring toCycling B([Green][1][1],[Yellow][2][2]) |
| | Step 3@72°, hold15secs |

## Allelic data for Cycling B.Green,Cycling B.Yellow




| No. | Colour | Name | Genotype | Cycling B.Green | Cycling B.Yellow |
|---|---|---|---|---|---|
| 1 | | | Heterozygous | Reaction | Reaction |
| 2 | | | Mutant | No Reaction | Reaction |
| 4 | | | Mutant | No Reaction | Reaction |
| 5 | | | Heterozygous | Reaction | Reaction |
| 6 | | | Heterozygous | Reaction | Reaction |
| 8 | | | Heterozygous | Reaction | Reaction |
| 10 | | | Heterozygous | Reaction | Reaction |
| 11 | | | Mutant | No Reaction | Reaction |
| 12 | | | Mutant | No Reaction | Reaction |
| 13 | | | Heterozygous | Reaction | Reaction |
| 14 | | | Mutant | No Reaction | Reaction |
| 15 | | | | No Reaction | No Reaction |
| 16 | | | | No Reaction | No Reaction |
| 17 | | | Heterozygous | Reaction | Reaction |
| 18 | | | Heterozygous | Reaction | Reaction |
| 19 | | | Mutant | No Reaction | Reaction |
| 20 | | | Heterozygous | Reaction | Reaction |

**Appendix 6.** The distribution of genotypes alleles frequency at rs731236 SNP of *VDR* gene **.**

# Allelic Discrimination Report

### Experiment Information

| | |
|---|---|
| Run Name | sanaa 2017-04-11 (snd run) |
| Run Start | 2017-04-11 8:51:36 PD |
| Run Finish | 2017-04-11 10:38:44 PD |
| Operator | sana |
| Notes | |
| Run On Software Version | Rotor-Gene 2.1.0.9 |
| Run Signature | The Run Signature is valid. |
| Gain Green | 6,67 |
| Gain Yellow | 8, |

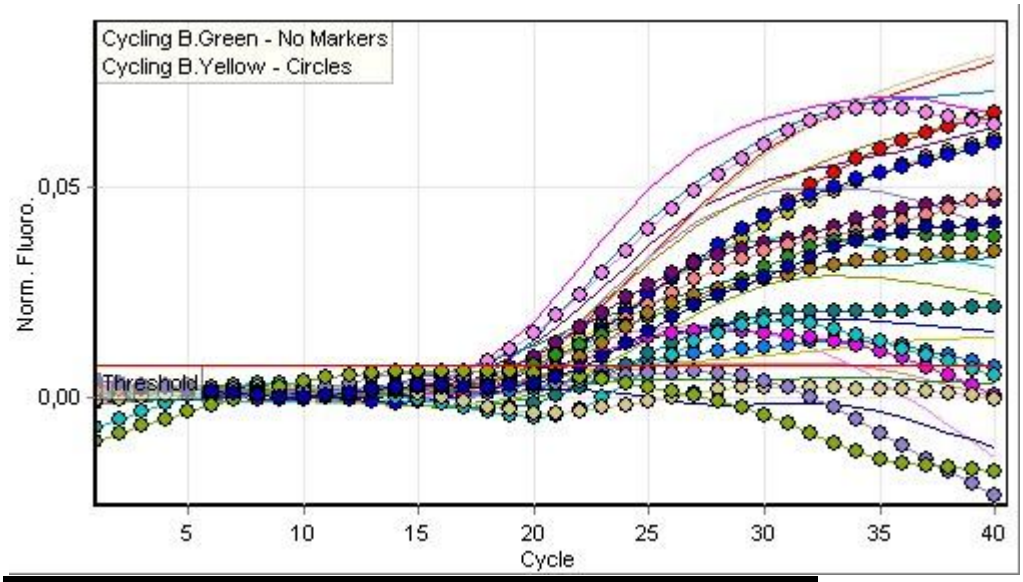
### Allelic Discrimination Information

| | |
|---|---|
| Digital Filter | Light |
| Imported Analysis Settings | |
| Left Threshold | 1 |
| No Template Control Threshold | % 0 |
| Noise Slope Correction | Yes |
| Normalisation Method | Dynamic Tube Normalisation |
| Reaction Efficiency Threshold | Disabled |
| Start normalising from cycle | 1 |
| Threshold | .00748 |

### Profile

| Cycle | Cycle Point |
|---|---|
| min secs Hold@50°,150 | |
| min secs Hold 2@95°,150 | |
| ( repeats) Cycling 5 | Step 1@95°, hold5secs |
| | Step 2@60°, hold20secs, acquiring toCycling A([Green][1][1],[Yellow][2][2]) |
| | Step 3@72°, hold15secs |
| ( repeats) Cycling  240 | Step 1@95°, hold5secs |
| | Step 2@60°, hold20secs, acquiring toCycling B([Green][1][1],[Yellow][2][2]) |
| | Step 3@72°, hold15secs |

## Allelic data for Cycling B.Green,Cycling B.Yellow




| No. | Colour | Name | Genotype | Cycling B.Green | Cycling B.Yellow |
|---|---|---|---|---|---|
| 1 | | | Heterozygous | Reaction | Reaction |
| 2 | | | Heterozygous | Reaction | Reaction |
| 3 | | | Heterozygous | Reaction | Reaction |
| 4 | | | Heterozygous | Reaction | Reaction |
| 5 | | | Heterozygous | Reaction | Reaction |
| 6 | | | Heterozygous | Reaction | Reaction |
| 7 | | | Heterozygous | Reaction | Reaction |
| 8 | | | Heterozygous | Reaction | Reaction |
| 9 | | | Mutant | No Reaction | Reaction |
| 10 | | | Heterozygous | Reaction | Reaction |
| 12 | | | Heterozygous | Reaction | Reaction |
| 13 | | | Wild Type | Reaction | No Reaction |
| 17 | | | Wild Type | Reaction | No Reaction |
| 21 | | | Heterozygous | Reaction | Reaction |
| 22 | | | Wild Type | Reaction | No Reaction |
| 25 | | | Mutant | No Reaction | Reaction |

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بغداد
معهد الهندسة الوراثية والتقنيات الاحيائية
للدراسات العليا

# دراسة بعض الاشكال الوراثية لجين مستقبل فيتأمين D والتغيرات الخلوية على النساء العراقيات العقيمات المصابات بتعدد الاكياس المبيضية

اطروحة مقدمة
الى مجلس معهد الهندسة الوراثية والتقنيات الاحيائية للدراسات العليا بجامعة بغداد
كجزء من متطلبات نيل درجة دكتوراه فلسفة في
الهندسة الوراثية والتقنيات الاحيائية

من قبل
**سناء جاسم كاظم البيضاني**
( ماجستير هندسة وراثية وتقنيات احيائية)
بإشراف

الاستاذ الدكتور
اسماعيل عبد الرضا عبد الحسن

جمادى الآخرة / 1439 هـ
شباط / 2018 م

# الخلاصة

متلازمة تعدد الاكياس المبيضية (PCOS) هي من بين الاضطرابات الصمية الأكثر تكراراً ، التي تحدث للنساء في عمر الإنجاب. درست العديد من الاشكال الوراثية ذات النيوكليوتيدة الواحدة (SNPs) في العديد من الجينات فيما يتعلق بعلاقتها بهذا الاضطراب ، أجريت هذه الدراسة للبحث في ما إذا كانت هناك علاقة بين الطرز في الانترون والاكسون(SNPs) لجين مستقبل فيتأمين D والتعرض لمتلازمة تعدد الأكياس المبيضية ودراسة تأثير هذه الطرز في مستويات هرمونات LH ,TSH,FSH والبرولاكتين وفي زيادة تكرار النويات الصغيرة وتعزيز استبدال الكروماتيدات الشقيقة .

أجريت هذه الدراسة في مختبرات معهد الهندسة الوراثية والتقانات الاحيائية للدراسات العليا – جامعة بغداد خلال الفترة من 1 تشرين الثاني 2016 حتى نهاية شهر أب 2017، تم الحصول على عينات مرضى متلازمة تعدد الاكياس المبيضية من مستشفى كمال السامرائي لعلاج العقم في بغداد .

شملت الدراسة 50 أمرأة مريضة بمتلازمة تعددالاكياس المبيضية و 50 من النساء الاصحاء ظاهرياً كمجموعة سيطرة ، تم تحديد طرز الطفرات rs2228570 و rs7975232 و rs731236 في جين مستقبل فيتأمين D باستخدام اختبار التنميط Taqman وبأستخدام RT–PCR.

أظهرت النتائج أن توزيع تكرار الطرز والاليلات للطفرة rs2228570 في جين مستقبل فيتامين D لم يظهر اختلافات معنوية بين مجموعة السيطرة ومجموعة مرضى تعدد الاكياس المبيضية. في حين، ان تكرار التركيب الوراثي CC كان أقل معنوياً ($p < 0.05$) في المرضى مقارنة بالسيطرة .

بالنسبة لتوزيع تكرارات اليلات الطفرة rs7975232 ، فأن تكرار التركيب الوراثيCC كان اقل معنوياً ($p < 0.05$) في المرضى مقارنة بمجموعة السيطرة .وعلى العكس فأن تكرار التركيب الوراثي المتغاير CA كان اعلى معنوياً ( $p < 0.05$) لدى المرضى مقارنة بالنساء الاصحاء . تكرار التركيب الوراثي TT للطفرة rs731236 كان أقل معنوياً ($p < 0.05$) لدى المرضى مقارنة بالسيطرة. في حين فيما يتعلق بالتراكيب الوراثية TC، CC و CC + TC ، فليس هناك فروق معنوية بين مجموعتي المرضى والاصحاء . ايضاً الطفرات الثلاثة لجين مستقبل فيتامين D المدروسة اظهرت 8 توليفات فردانية اكثرها شيوعاً TTA و TTC في مجموعتي الدراسة . التوليفة الفردانية TCC اظهرت فارق معنوي بين المرضى والسيطرة . وأظهرت نتائج تكرار التوليفة المركبة TTC / CCA بأن تكرارها اعلى معنويا ( $p < 0.05$) أعلى في المرضى مما في مجموعة السيطرة (8% مقارنة بـ 16% ، على التوالي). لم تجد هذه الدراسة علاقة بين الطفرات المتغايرة والمتماثلة عند rs 731236 , rs2228570 لجين مستقبل فيتامين D مع حدوث متلازمة تعددالاكياس المبيضية ، في حين ان الطفرة rs 7975232 لجين مستقبل فيتامين D تشكل خطرا تطور متلازمة تعدد الاكياس المبيضية.

تحليل هرمونات TSH, FSH, LH والبرولاكتين انجز باستعمال الاختبار المناعي AIA. تشير النتائج إلى أن تراكيز مصل الدم من هرمونات LH, FSH, TSH لم تتأثر بالطفرات المدروسة في جين مستقبل فيتأمين D. وفي نفس الوقت فأن مستويات البرولاكتين كانت اعلى معنوياً ($p < 0.05$) في المرضى مقارنة بالسيطرة .

تم قياس اضطراب الجينوم من خلال تكرار النويات الصغيرة وكذلك تعزيز استبدال الكروماتيدات الشقيقة في 66 مريضة و 14 من النساء الصحيحات ظاهرياً . وتم تصنيف المريضات بتعدد الاكياس

المبيضية اعتماداً على نتائج الطرز الوراثية ، اظهرت النتائج فروق معنوية في مستوى النويات الصغيرة وكذلك تعزيز استبدال الكروماتيدات الشقيقة في المرضى لكل طراز في جين مستقبل فيتأمين D مقارنة بالسيطرة .