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ASSOCIATION OF VITAMIN D RECEPTOR GENE POLYMORPHISM AT THREE SNPS AND THEIR HAPLOTYPES WITH POLYCYSTIC OVARY SYNDROME RISK IN IRAQI WOMEN

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ABSTRACT

Polycystic ovary syndrome (PCOS) is the most endocrinological disorder,in young reproductive age women , Vitamin D receptor (*VDR*) gene variants have been associated with metabolic co-morbidities in population. This study was carried out to examine whether the polymorphisms of *VDR* gene are correlated with the risk of PCOS. Polycystic ovary syndrome women (n=50) and apparently healthy control subject (n=50), were enrolled genotyping of *VDR* gene SNPs (rs2228570, rs7975232 and rs731236) were determined by using Taqman genotyping assay. The results showed that the distribution of genotypes and alleles frequencies at rs2228570 SNP of *VDR* gene, as related with TT, TC and combined TC+CC genotypes, no significant differences in frequency percentage were noted between PCOS patients and apparently healthy subjects with polycystic ovary syndrome. Whereas, the frequency of CC genotype was significantly ($p<0.05$) lower in PCOS patients compared with apparently healthy group. The genotypes and alleles frequencies distribution at rs7975232 C>A polymorphism, the frequency of wild CC genotype was significantly ($p<0.05$) lower in patients with PCOS than in apparently healthy subjects. In contrast, the frequency of heterozygous CA genotype was significant ($p<0.05$) higher in patients with PCOS compared with apparently healthy subjects. The frequency of TT genotype at rs731236 T>C polymorphism was significant ($p<0.05$) lower in patients with PCOS than in apparently healthy subjects. While as related with TC, CC and combined TC+CC genotypes, no significant differences in frequency percentage were detected between PCOS patients and apparently healthy subjects. Further, the three *VDR* SNPs presented eight possible haplotypes, with TTA and TTC being the most common in both groups (patients and controls). In particular, the TCC haplotype showed significant ($p<0.05$) distribution in PCOS patients compared with apparently healthy control and the frequency of TTC / CCA haplotype combination was significantly ($p<0.05$) higher in patients with PCOS than in apparently healthy subjects. This study found that no association between both heterozygous and homozygous mutants at rs2228570 and rs731236 of *VDR* gene with the incidence of PCOS, while heterozygosity at rs7975232 of *VDR* gene showed a risk for PCOS development susceptibility.

Keyword: poly cystic ovary syndrome, VDR gene polymorphisms.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common disorder that assumes women of reproductive age and is characterized by polycystic ovaries, anovulation, hyperandrogenism and its symptoms such as acne, hirsutism, menstrual irregularity and alopecia (Azziz *et al.*, 2009, Walters *et al.*, 2012). Studies have found that various factors play important roles in PCOS pathogenicity (Farmakiotis *et al.*, 2007). Another feature of PCOS is the obesity prevalence, including a visceral obesity, which intensifies the extent of menstrual cycles irregularity and other metabolic alterations, such as a high recurrence of diabetes mellitus type 2 (Norman *et al.*, 2007). The change in lifestyle concentrate on losing of the weight, improve fertility, especially ovulation. In many countries, it represents the leading cause of female infertility (Spritzer, *et al.*, 2015). There are Various PCOS genes interact with each other and with the factors of the environment causing the development of the syndrome. It is not conceivable to point out a single gene causing the PCOS development, as several researches found multigenic factors that could play a part in this syndrome. Possible genes include those implicated in the pathway of insulin, in steroidogenesis, and genes correlated with chronic inflammation (Xita *et al.*, 2003). The levels of vitamin D play a role in metabolic changes including calcium-phosphate homeostasis, specifically in insulin secretion regulation by the β-lymphocytes (Pittas *et al.*, 2007).

The vitamin D receptor (*VDR*) gene is considered to be an important candidate gene for PCOS (Morteza *et al.*, 2013). VDR, a nuclear protein, is a DNA binding transcription factor forms a heterodimer with a retinoid X receptor (RXR) and it is distributed throughout the tissues. When vitamin D binds to this complex, it strengthens the interaction between VDR and RXR, causing the transcription of several genes that regulated by vitamin D (Abdul Sahib, *et al.*, 2017; Haussler *et al.*, 2011). The chromosomal locus of *VDR* gene is 12q12-14. The

identification of exact genes which predisposing to PCOS and its complications can provide ways for good understanding of the PCOS pathogenesis leading to better prevention, diagnosis and treatment in future.

This study aimed to estimate the genotypes and alleles frequency in three SNPs of *VDR* gene and their haplotypes and study their association with the risk of PCOS in Iraqi women.

MATERIALS AND METHODS

Whole Blood samples were collected from PCOS patients (n=50) and apparently healthy subjects as control (n=50). Apparently healthy control group consists of fertile women at different ages. All of them were chosen depending on the menstrual cycle regularity (26-30 days), age (15-45) years) with no use of medication or oral contraceptives and no history of endocrine disease (Macklon and Fauser, 2000). The PCOS patients were chosen from Kamal Al-Samarraee hospital for infertility treatment in Baghdad. To confirm the patients with PCOS, they should have at least two of the following three features, according to Rotterdam 2003 criteria (Rotterdam ESHRE/ASRM Consensus, 2004): 1. The presence of polycystic ovary. 2. Clinical or biochemical features of hyperandrogenism. 3. Oligo or anovulation. Every participant woman was interviewed and asked to answer information including sociodemographic data, menstrual history, gynecological surgery, obstetric, PCOS family histories. They were also subjected to medical checkup for signs of hyperandrogenism and polycystic ovary**.** The blood samples were collected during the phase of follicular (day 3 or 4) of the menstrual cycle from each woman of both patients and healthy control. About three ml of venous blood samples were collected in tubes contain EDTA for DNA extraction.

Total genomic DNA was extracted from the whole fresh blood using genomic DNA extraction kit (WizPrep™ DNA Extraction Kit), Nanodrop (2000 C apparatus, Thermo Scientific, USA) was used to estimate the purity and the concentration for DNA samples**.** The purity of DNA should be between 1.7-1.9 (Sambrook *et al*., 1989).

Genotyping analyses were performed using Real Time PCR (The software program was used to analyze the genotypes comes from Rotor gene Q. QIAGEN Company\Armenia), TaqMan fluorescent oligonucleotide probes and primers (Alpha DNA Ltd., Canada) were prepared according to William *et al*.(2004), and stored lyophilized at -20°C. The sequence of each probe and primer used in the allelic discrimination experiments are shown in table 1. They include *VDR* gene SNP (rs731236) in exon 9 (T to C), SNP (rs 2228570) in exon 2 (T to C) and SNP (rs7975232) in intron 8 (C to A).

Primer and probe sequence were matched by the bioinformatic programs (NCBI). The probe prepared for the wild type was labeled with FAM at the 5′ end and MGB at the 3′ end. While the mutant allele detecting probe (SNP) was labeled with VIC at the 5′ end and MGB at the 3′ end, the normal wild type, mutant genotype and the heterozygous genotype are shown in figure.

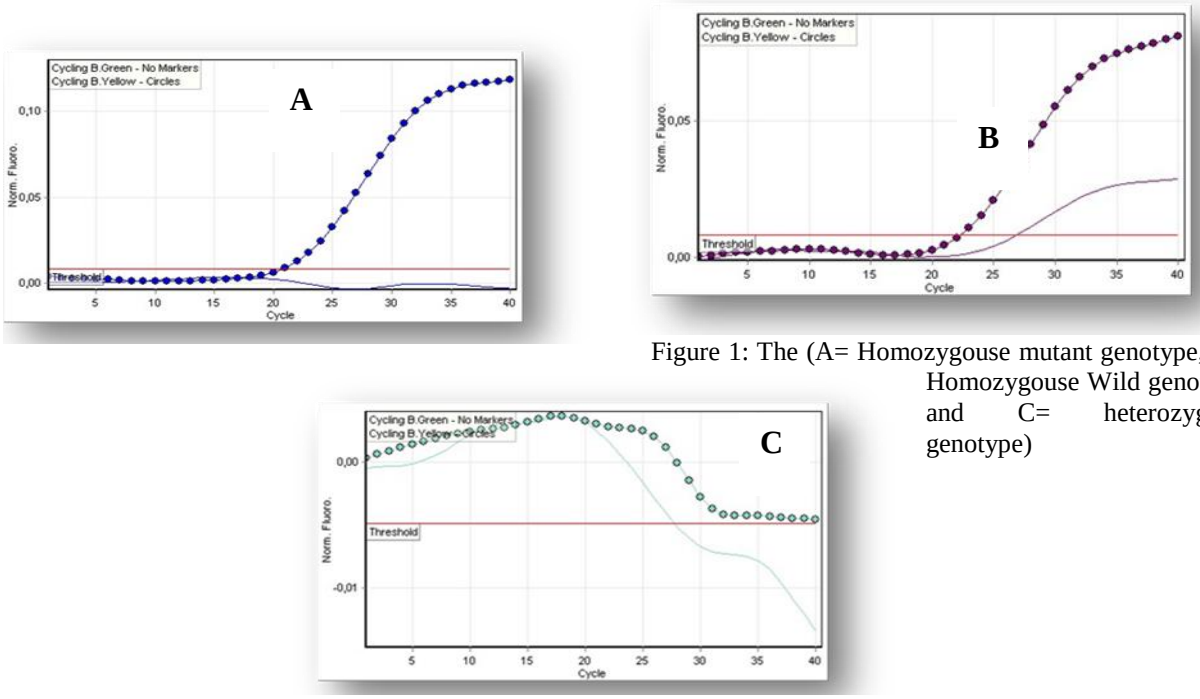

Figure 1: The (A= Homozygouse mutant genotype, B= Homozygouse Wild genotype and C= heterozygous genotype)

The System of Statistical Analysis - SAS (2012) program was used to evaluate the difference factors in parameters in this study. The test of Chi-square and Odd ratio were used for significant difference between percentage and least significant difference (LSD) test was used for significance comparison between means.

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

Table 1: Primers and Probes used in the stud

RESULTS AND DISCUSSION

This study examined three polymorphisms of VDR gene (rs 2228570 T>C in exon 2, rs 7975232 C>A in intron 8 and rs731236 T>C in exon 9) among Iraqi women suffered from PCOS and apparently healthy as control and tested their association with the phenotype of PCOS. The distributions of genotype and the frequency of allele were tested for each of the three *VDR* gene polymorphisms as shown in Tables 2, 3 and 4, respectively.

Rs2228570 T>C polymorphism: The variant is known as FokI in exon 2. The distribution of genotypes alleles frequency at rs2228570 SNP of *VDR* gene presented in **table 2**. As related with TT, TC and combined TC+CC genotypes, no significant differences in frequency percentage were noted between PCOS patients and apparently healthy subjects 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 T and C allele frequencies were 0.36 and 0.64 in apparently healthy subjects and 0.42 and 0.58 in patients with PCOS respectively. *VDR* polymorphism rs2228570 T > C, defines T transition to C (methionine) to (threonine) in *VDR* gene (exon 2) of that result in revoke the site of the translation start in exon2, and cropping the encoded protein by three amino acids (Saijo *et al*., 1991). Smaller protein displays greater transcription activity because of its high efficiency of binding to transcription factor IIB (Jurutka *et al.*, 2000).

The results of this study are in disagreement with other recent study in India (Dipanshu and Chakravorty, 2015) who found that the polymerphism of rs2228570 SNP of vitamin D receptor gene is correlated with PCOS and change ovarian steroid secretion.

Also, Sudhensa *et al.,* (2013) found that *VDR* polymorphism in exon 2 (rs2228570) might be a risk factor for the ovarian cancer development in Indian population. Fei-fei *et al.,* (2017) found a correlation between insulin resistance related diseases and *VDR* variant (rs2228570) and was conspicuous in Asians and Caucasians with dark-pigmented but not in Caucasian with white skin.

Genotypes	Frequency, n(%)		χ^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				
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 levels.

Ames *et al.*, (1999) observed that children with TT genotype (rs2228570) of *VDR* gene were fawned to have lower detected calcium. In addition, Abrams *et al.,* (2005) found that individuals with TT genotype (rs2228570) of *VDR* gene had less total detected calcium and less calcium increment to the skeleton in early pubertal adolescents. In a study on 36 white women in postmenopausal with osteopenia

or osteoporosis who were treated with vitamin D supplements and calcium for three months, the TC genotype (rs2228570) of *VDR* gene was more popular in responders compared with non-responders (Elnenaei *et al.*, 2011). Roth *et al.* (2004) tested the genetic effect of the VDR on anti-mycobacterial chemotherapy that has treatment effect against tuberculosis in Peruvian patients, patients with CC genotype (rs2228570) of *VDR* gene had faster transformation of sputum Rs 797- mycobacteria culture from positive to negative compared with non-CC genotype.

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%) (Belmont *et.al*, 2003)

5232 C>A polymorphism: It is variant known as ApaI in intron 8. Up to now, no important function of this variant has been noted. The distribution of genotypes alleles frequency at rs7975232 SNP of *VDR* gene are presented in table 3.

The frequency of wild genotype (CC) was significantly ($p<0.05$) lower in patients with PCOS than in apparently healthy subjects (2% and 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 patients with PCOS when compared with apparently healthy subjects (56% and 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 size.

Table 3: The frequency of genotypes and alleles at rs7975232 SNP in intron 8 of *VDR* gene in Iraqi women with PCOS and control.

Genotypes	Frequency, n(%)		χ^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.

As shown in table 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. 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 manifest the infertility risk while genotype AA variant was correlated with the levels of testosterone which were in difference with a study on PCOS Austrian women where they evidenced the association of AA genotype with the low levels of testosterone (Wehr *et al*., 2011). Chunming *et al*., (2016) reported that *VDR* polymorphism in rs7975232 was correlated with risk of renal cell carcinoma in Chinese population.

In a study that examined the dairy intake effect and the recurrence of colorectal cancer, Hubner *et al.* (2008) found a correlation between rs7975232 genotypes and the intake of dairy product. Particularly, persons with one copy of allele A (at least) and who consumed dairy products in a large amount, had the lowest colorectal cancer recurrence risk compared with carriers of CC genotype who consumed dairy products in lower amounts.

Rs731236 T>C polymorphism: This variant is called TaqI in exon 9. The distribution of genotypes alleles frequency at rs731236 SNP of *VDR* gene presented in table 4. The frequency of TT genotype was significantly ($p<0.05$) lower in patients with PCOS than in apparently healthy subjects (2% and 12%, respectively, $\chi^2= 4.825$, *OR* = 0.692, $p<0.05$). While as related with TC, CC and combined TC+CC genotypes, no significant differences in frequency percentage were found between apparently healthy subjects and PCOS patients. In general, both heterozygous and homozygous mutants at rs731236 of *VDR* gene have no association with the incidence of PCOS in Iraqi patients. The T and C alleles frequencies 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 this study are in coincidence with Jedrzejuk *et al.*, (2015) who found that classic PCOS phenotype is not associated with *VDR* gene

polymorphism at rs731236 SNP. Also, agree with Mahmoudi *et al.* (2015) who found no significant differences in *VDR* gene in exon 9 (rs731236 T>C) polymorphism between the PCOS women and apparently healthy subjects. Other studies were in contrast with the results of the present study. In Iranian Azeri patients, Bagheri *et al.*, (2013) observed that the genotype CC of *VDR* (rs731236 T>C) in exon 9 is correlated with PCOS incidence.

Table 4: The frequency of genotypes and alleles at rs731236 SNP in exon 9 of *VDR* gene in Iraqi women with PCOS and control

Genotypes	Frequency, n(%)		χ^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.

Also, in Egyptian women, El-Shal *et al*. (2013) found that genotype CC and C allele caused the increase of PCOS risk. The VDR polymorphism in rs731236 is correlated with the gene expression rate, T to C substitution (ATT (isoleucine) to ATC (isoleucine)) leading to a synonymous change at codon 352 (silent) (Köstner *et al*., 2009). The rs7-31236 and rs7975232 polymorphism of *VDR* gene are associated with poly (A) microsatellite repeat in the gene and has an effect on the activity of the gene (McCullough *et al*., 2007).

Haplotypes: The results of haplotype frequency defined by rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi PCOS women and apparently healthy subjects are shown in table 5. Considering the three *VDR* SNPs, we observed eight possible haplotypes, with TTA and TTC being the most common in both groups (patients and control). In particular, the TCC haplotype showed statistically significant ($p<0.05$) distribution between PCOS patients and apparently healthy control (28% and 18%, respectively).

Haplotypes combination: The results of haplotype combination frequency defined by rs731236, rs2-228570 and rs7975232 SNPs of VDR gene in Iraqi PCOS women and apparently healthy subjects are shown in table 6. The frequency of TTC/CCA haplotype combination was significantly ($p<0.05$) higher in patients with PCOS than in apparently healthy subjects (28% and 18%, respectively, $\chi^2=4.825$, *OR*= 0.692, $p<0.05$). This haplotype combination means that all studied SNPs were absent. In addition, as shown in the table 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 were the most common.

PCOS is most common in women of reproductive age and has strong genetic bases (Rotterdam ESHRE/ASRM, 2004). Vitamin D control about 3% of the human genomic, such as genes that are important for the metabolism of glucose and lipid, by its nucleoprotein receptor that binds to the elements of vitamin D response locate in the promoter region of the genes (Darwish and DeLuca, 1993). In addition, vitamin D is the regulating hormone in homeostasis of calcium. The calcium plays an important role in activation and maturation of oocyte that result from the follicular development progression (DeFelici *et al.,* 1991). Gratification of Vitamin D and calcium might affect the normalization of menstrual cycles and restoration of ovulation in women with PCOS (Thys-Jacob *et al.*, 1999). Most of vitamin D biological actions are exerted through the nuclear (VDR)-mediated control of target genes. VDR is a nuclear hormone receptor superfamily and play as a ligand-inducible transcription factor. VDR mediates most effects of vitamin D on gene expression by a heterodimer formation with the retinoid X receptor, that binds to regions called (promoter regions) of many genes (Pike and Meyer, 2010). The polymorphisms of VDR have been correlated with the levels of vitamin D insulin secretion glucose metabolism and peripheral action in various populations (Ortlepp *et al.,* 2003; McGrath *et al.,* 2010). In addition, the polymorphism of VDR gene is correlated with deficiency of vitamin D in PCOS and its endocrine and metabolic changes (Mahmoudi, 2009). Although they are restricted by small sample size, several researchers have found correlations between the

polymorphisms of VDR and PCOS development and insulin resistance (Chiu *et al.*, 2001; Mahmoudi 2009; Ranjzad *et al.*, 2010; Ranjzad *et al.*, 2012).

Table 5: The frequency of haplotypes defined by rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and control.

Haplotypes	Frequency, n(%)		χ^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 levels.

Table 6: The frequency of haplotypes combinations defined by rs731236, rs2228570 and rs7975232 SNPs of *VDR* gene in Iraqi women with PCOS and control.

Haplotype combinations	Frequency, n(%)		χ^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 levels.

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