DETECTIN OF VASPIN RS2236242 AND CHEMERIN RS17173608 GENE POLYMORPHSIMS IN A SAMPLE OF OBESE IRAQI POLYCYCTIC OVARY SYNDROME WOMEN

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ABSTRACT

This study was performed to detect assess Vaspin rs2236242 and Chemerin rs17173608 gene polymorphisms with its association with susceptibility of obese PCOS in Iraqi women .Blood samples from 100 women with obese PCOS and 75 healthy control women matched in age and BMI were collected from AL-Yarmouk Teaching hospital during February2018-July 2018 were enrolled in case control study. Tetra amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) was performed to detect the adipokine SNPs in patients and healthy women under study. Results demonstrated a significant variations in either genotype distribution and alleles frequencies between the obese PCOS and healthy women regarding vaspin rs2236242 polymorphism. The T allele increased the PCOS risk in comparison with the A allele which was the protective against PCOS, as well clear positive correlation between rs17173608 SNP of chemerin and susceptibility of PCOS was identified also, and The T allele had related with elevated PCOS risk as in comparison to the G allele. Data from this work determined for the first time a significant association between vaspin and chemerin SNPs and susceptibility PCOS risk in Iraqi obese women especially T allele .To confirm our findings new studies with large sample size and different ethnicities must be required.

Keywords: genetic study, adipokines , obesity ,insulin resistance , PCR
***Part of Ph.D Dissertation of 1st author**

التحري عن تعدد الأشكال الوراثية لجين الفاسبين والكميرين في عينة من النساء العراقيات البدينات المصابات بمتلازمة تعدد الأكياس المبيضية

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المستخلص

تهدف الدراسة الحالية إلى التحري عن وجود تعدد الأشكال الوراثية لجينات كل من الفاسبين والكميرين وعلاقته بامراضية تكيس المبايض في النساء العراقيات البدينات. تم جمع عينات دم من 100 أمراه بدينة مصابة بمرض تكيس المبايض و75 امرأة بدينة سليمة متصاحبة في العمر ومؤشر كتلة الجسم من مستشفى اليرموك التعليمي للفترة من شهر شباط 2018 إلى تموز 2018. صمم نظام تكاثر الطفرات الحرارية التضخمية مع تفاعل البلمرة المتسلسل للكشف عن وجود ظاهرة تعدد الأشكال الوراثية في الحر كيات الخلوية الدهنية قيد الدراسة الحالية. أظهرت النتائج وجود فروق معنوية ذات دلالة إحصائية في توزيع النمط الجيني وتردد الاليلات للفاسبين بين عينات مرضى تكيس المبايض مقارنة بمجموعة السيطرة. حيث وجد بان الاليل ت يزيد من خطر متلازمة تكيس المبايض على عكس الاليل أ الذي يعتبر وقائي ضد المرض. كذلك تم التعرف على وجود علاقة ايجابية بين تعدد الأشكال الوراثية للكميرين وخطر الإصابة بالمرض حيث يزيد وجود الاليل ت من المرض على عكس الاليل أ الذي يعتبر عامل وقائي ضد المرض. وفي الختام تشير نتائجنا لأول مرة بوجود ارتباط كبير بين كل من الاليل ت لجيني الفاسبين والكميرين ومتلازمة تكيس المبايض في عينة من النساء العراقيات وهنالك حاجة إلى مزيد من الدراسات مع حجم عينات اكبر واخذ التنوع العرقي لغرض تأكيد النتائج التي توصلنا إليها.

كلمات مفتاحيه: دراسة وراثية، الحر كيات الدهنية، السمنة، مقاومة الأنسولين, تفاعل البلمره المتسلسل
***البحث جزء من أطروحة دكتوراه للباحث الأول**

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INTRODUCTION

Polycystic ovary syndrome is a complicated condition characterized by excess androgen secretion, abnormal menstrual pattern, and/or small cysts on one or both ovaries (14,21,23).It affects according to the literature review 5-10% of females in age reproduction(7).PCOS can be considered as an oligogenic fault in which the interaction of many genetic and environmental factors determine the heterogeneous, clinical, and biochemical phenotypes (7,14,23).It have defects in both hepatic insulin action, as well pancreatic β-cell dysfunction and external, which reflects primarily skeletal muscle(7,23).The analysis of PCOS is very hard and unclear yet, and many genetic, environmental and lifestyle factors greatly contribute to the prediction of the PCOS (27). However, inherited factors play detracting role in the prognosis of PCOS. Currently, many studies have identified a positive association between adipcytokine and hazard of PCOS (21). Chemerin is a lately identified as adipokine that improved adiposity development and metabolic action as well as immunity activity (6,25,29).Chemerin, is a new adipokine identified as a chemo attractant protein by (22,27). Chemerin is also established as tazarotene-induced gene 2 (4,8).Chemerin may be one of the better essential relationship between obesity and insulin resistance(IR),and thus a good objective clinical marker for MS which is present in obese polycystic ovary (11),and there are many viarents of chemerin gene as seen in Fig.1, (22). **Vaspin** is also known as a serpin peptidase inhibitor and considered one of the serpin family (15,16). Vaspin was located on 14q32.13 and identified as a novel adipokine with insulating effects (4,24) and there are many variants of vaspin gene as observed in figure 2 below(4).There are many adiopkines like ghrelin which had important role of in promoting growth hormone sec retion and physiological functions in different organisms such as human, fish, mouse, rat and avian species were increased dramatically (5,12).In Current research many plants like back wheat used to reduce may metabolic disorders such as lipid and insulin resistance through reducing oxidative stress in PCOS patients(20). Till now in the literature ,there are rare studies regarding chemerin in addition vaspin gene SNP in PCOS and referred that chemerin and vaspin SNPs have been associated with visceral obesity (11) and T2DM/obesity risk (25). Thus, the goal of this work was performed to investigate the presence of a possible association between adipokine SNPs and risk to PCOS in a sample of Iraqi obese women compared with healthy Non obese women

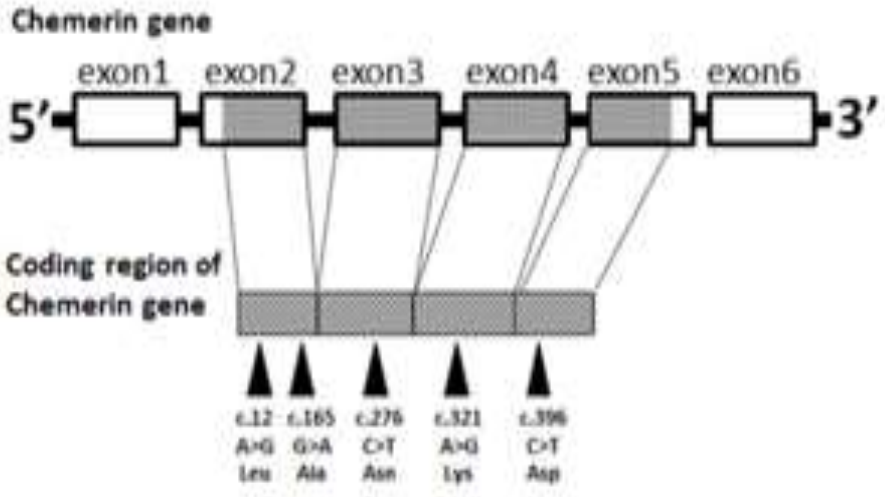

Figure 1: Single nucleotide polymorphisms present in the coding region of the chemerin gene(22).

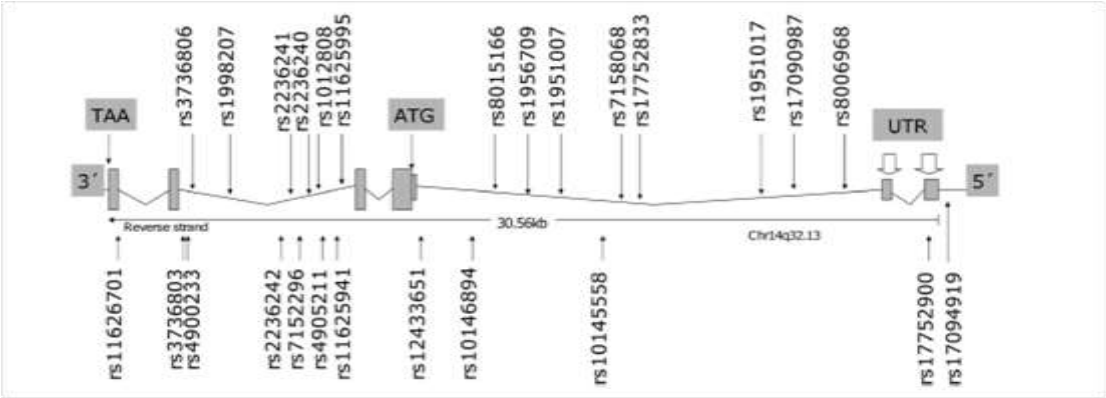

Figure 2. Schematic representation of the vaspin gene and genotype variants (4)

MATERIALS AND METHODS

A case control study on 100 PCOS women recruited from an Al-Yarmouk Teaching hospital, Baghdad Iraq were enrolled in this current study from 2017 until 2018. PCOS diagnosis was based on Rotterdam criteria consensus modified byb other researchers (3).A total of 75 healthy non PCOS females were matched with age and BMI participated in the study. For genotyping analysis a Tetra amplification refractory mutation system and polymerase chain reaction(T-ARMS-PCR) was performed for detection of polymorphisms of vaspin and chemerin in all case and healthy women enrolled in this study according to (11) using ARMS which is more precise than Rapid amplification RAPD technique(20) .The genomic sequences of these adipokines were obtained from the NCBI http://www.ncbi.nlm.nih.gov. These SNP were examined and primers for Tetra amplification refractory mutation system and polymerase chain reaction were designed as observed in table (1).PCR was performed by using commercially available PCR premix (AccuPower PCR Premix; BIONEER ,Korea) according to the manufacturer's instructions. Briefly, 1 µL template DNA (~100 ng/µL),1 µL of each primer (10 pmol/µL), and 15 µL DNase-free water were added to AccuPower PCR Premix. Amplification was done with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 30 s at 95 °C, 15 s at 65 °C for chemerin,30 s at 62 °C for vaspin, respectively, and 30 s at 72 °C with a final step at 72 °C for 10 min . PCR products verified on a 2.0% agarose gel contained 0.5 µg/mL ethidium bromide and photographs were taken as seen in Fig 3 and 4. To certify genotyping quality, all polymorphisms in random samples were regenotyped. The check confirmed the previous genotyping results by 100%.Demographic characteristics and obese PCO associated biochemical parameters were compared for women with and without PCO using the Pearson's chi-square ($\chi 2$).The distribution of genotype distribution and frequency of allele to both SNPs were compared between obese PCO cases and Non PCO women using $\chi 2$ test. For each SNP, ahardy-Weinberg disequilibrium test was conducted in the control group. LSD was used to calculate the odd ratio (OR) and 95% confidence interval .SNP Stats was used to examine the potential associations between Vap. and Chem. gene and risk of PCO. Statistical significance was considered as a *p* value < 0.05**.**

Table 1.The oligoneucleotide sequence SNPs in chemerin and vaspin genes (11)

Gene polymorphism	Primers	Sequence (5′ to 3′)
Chemerin rs17173608	FI (G allele)	ATTGCTATAGTCCAGTGCCCTTCG
	RI (T allele)	CCAGTTCCCTCTGTCGGCTTAA
	FO	GTCAGACCCATGCAGTTTTCAAAC
	RO	GAGTTCCTCTCTCAAGCATCAGGG
Vaspin rs2236242	FI (T allele):	AAGACGCCGCTTCTGTGCACT
	RI (A allele)	CACAGGGACCCAGGATAACTTGCT
	FO	GGAGGCAGACCAGGCACTAGAAA
	RO	ACCATCTCTCTGGCTTCAGGCTTC

FI, forward inner; RI, reverse inner; FO, forward outer; RO, reverse outer.

RESULTS AND DISUSSION

Demographic and clinical characteristics of all women enrolled in the study are shows below in Table 2. Criteria of PCOS, was followed to according to (**3**) for diagnosis PCO between patients enrolled in this current work. According to the criteria, patients are diagnosed with PCOS when they have 2/3 of the following features: oligo- or amenorrhea, clinical or biochemical hyperandrogenism and ultrasonography PCO morphology. The women enrolled in the current work were matched in age and BMI. However, clinically there was significant ($p< 0.05$) elevation in scoring of hirsutism,waist hip ratio (WHR), and there was higher percent of menstrual irregularity and skin inflammation(acne) in the women with obese PCOS compared to the obese women in control group.

Table 2. Clinical and anthropometric characteristics of PCOS and healthy women enrolled in this study

Characteristics	Control Group (No:75)	PCOS group(No=100)	P Value
Age (year)	**28.75±0.72**	**29.12±0.33**	**>0.05**
BMI(Kg/m^2)	**30.94±0.35**	**31.11±0.46**	**>0.05**
WHR	**0.74±0.01**	**0.86±0.02**	**<0.05**
Menstrual irregularity	**11**	**81**	**<0.001**
Hirsutism score	**4.11±0.21**	**8.92±0.36**	**<0.001**
Acne	**10**	**34**	**<0.001**

Chemerin gene polymorphisms

The T-ARMS-PCR electrophoresis pattern for chemerin rs17173608 polymorphism detection in obese PCOS patients and obese non PCOS control women is illustrate in Figures 3. PCR product size fragments for G and T alleles were 262, 332 bp respectively , and 549 bp for control band. Distribution of genotype as well frequency of allele for chemerin rs17173608 SNP (T>)G in obese PCO and healthy obese non PCO Iraqi women were noticed in table 3.The distributions of genotype SNPs were in the Hardy-Weinberg equilibrium in both obese PCO cases and obese non PCO control women ($p > 0.05$). As observed in table 3, a statistically significant variation was found in TT genotype distribution between obese PCO patients and obese non PCO controls. In the dominant effect, TT genotype distribution in obese PCO women were increased significantly in comparison to non obese control women (OR = 2.642,95 percent Cl = 1.386-5.0345,and p value = 0.003 .While GT genotype distribution in PCO women were decreased significantly in comparison to non obese control women (OR = 0.39;95 percent Cl = 0.214-0.735;and p value = 0.003.

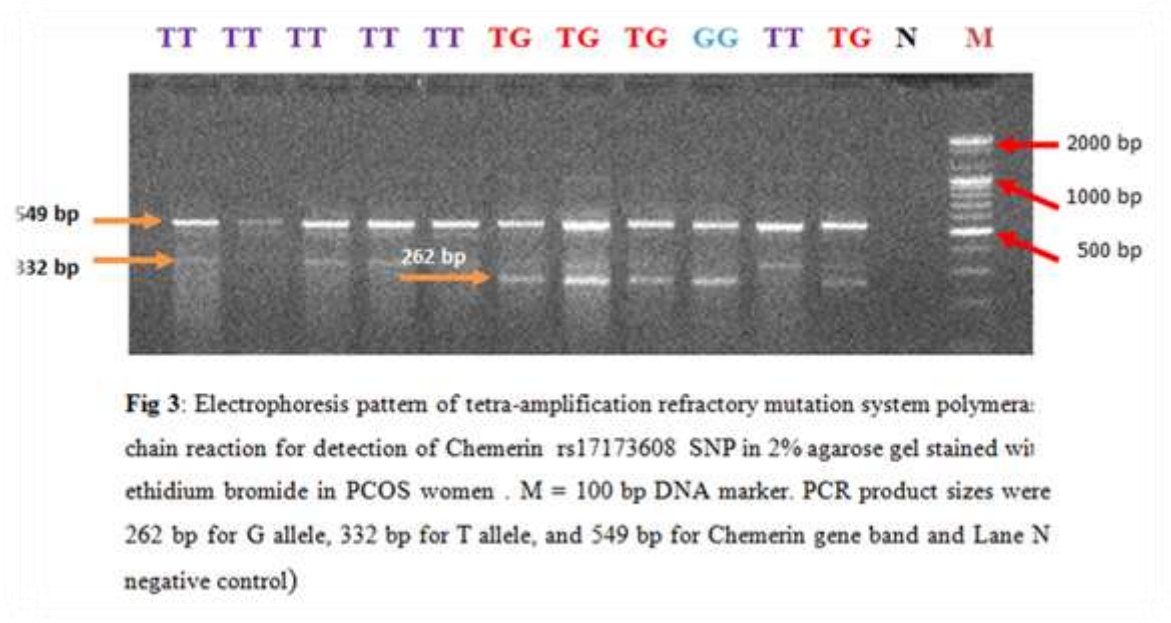

Fig 3: Electrophoresis pattern of tetra-amplification refractory mutation system polymera: chain reaction for detection of Chemerin rs17173608 SNP in 2% agarose gel stained wi ethidium bromide in PCOS women . M = 100 bp DNA marker. PCR product sizes were 262 bp for G allele, 332 bp for T allele, and 549 bp for Chemerin gene band and Lane N negative control)

Table 3. Alleles frequency and genotype distributions of chemerin gene polymorphisms in obese PCOS and non PCOS women

Genotype or Allele	PCOS Patients (No.=100) No.	%	NON-PCOS (No.=75) No.	%	OR*	95 percent Cl	P value*
TT	49	49	20	26	2.6422	1.3866 to 5.0345	0.003
GT	40	40	47	62	0.39	0.2146 to 0.7351	0.003
GG	11	11	8	10	1.0351	0.3946 to 2.7151	0.944
G	62	31	63	42	0.62	0.3990 to 0.9648	0.034
T	138	69	87	58	1.61	1.0365 to 2.5064	0.034

Results from table 3 demonstrated a significant TT genotypes distribution and T allele frequency in obese PCO women in comparison to obese non PCO healthy subjects which suggest a significant association with PCO risk in a sample of obese Iraqi women (OR=2.64;95%Cl= 1.3866-5.034 and p= 0.003 for TT genotype and OR=1.61; 95%Cl =1.0365-2.506 and p=0.034 for T allele) , in contrast to distribution of GT genotypes and G allele frequency which are more abundant in non PCO healthy women. These data proposed a protective role for the G allele in contrast to the role of T allele which seems to be a predisposing factor to PCO in Iraqi population for the first time. On the other hand , this information seem to reinforce the relation between TT genotype with the PCO risk .Minor allele (G allele) frequency of chemerin rs17173608 gene SNP in obese PCO and obese non PCO healthy women was 31 and 42, respectively. A significant cooperative between T allele and PCO risk was demonstrated (OR: 1.61, 95% CI 1.036-2.506, p= 0.034). This work is the first report demonstrated rs17173608 chemerin SNPs contributed to risk to obese PCOS in Iraqi women . Novel data of an correlation between genetic variants chemerin rs17173608 SNPs and risk of PCO were identified. The chemerin rs17173608 SNPs increased risk of PCO in obese Iraqi women. Chemerin is one of important adipocytokines that display elevated in expression of mRNA in adipose tissues of obese animals. In humans, serum chemerin values are correlated with several main attitudes of MS such as BMI, and hypertension (30,31).A potential correlation of chemerin with the pathogenesis of IR, obesity, and MS was investigated (13,18,31). Visceral fat is not considered a main site of chemerin liberation, and increasing the systemic chemerin concentrations in obesity appear to be associated with inflammation rather than BMI ,and exhibits a positive correlation with numerous manner of MS (30). Whatever till now there is no information respecting the role of chemerin or vaspin polymorphism on MS which occurred in PCOS,except one study by (27) which observed association between chemerin rs17173608 polymorphisms and visceral adiposity as well the minor allele of chemerin gene in obese women was associated with lower WHR in comparison with healthy subjects. On the other hand it was also reported that rs17173608 chemerin SNPs increased the hazard of metabolic syndrome(MS) through a statistically significant cooperative between the T allele and hazard to MS (13,19). This SNP is located in intron 2 of the chemerin gene and the introns are considered as a major targets for mutations due to long sequences harboring functional element, including intron splice enhancers, silencers, and cis-acting RNA elements that improve alternative splicing (19). The rs17173608 chemerin SNP in the intronic region of appears to give potential clinical significance, although the precise kinetics through which it utilizes its response on chemerin gene expression remain unclear. The findings of this present work showed that the G allele of chemerin gene increased the exposure to PCOS; regardless, after elimination the differences in BMI and age, the cooperative was still present between chemerin rs17173608 SNP and PCOS risk.

Vaspin gene polymorphisms

Results of T-ARAM-PCR for vaspin genotypes were observed in Figures 4 as well

Table 4 which showed a statistically significant variations in genotype distributions between obese PCO and healthy non PCO group in regard to *vaspin* rs2236242 SNP (OR = 0.59, 95%CI = 0.37–0.95, p < 0.03). The T allele heightened the hazard of PCOS (OR = 2.34,95%CI = 1.22–4.45, p <0.01) in comparison to the A allele which was possess a protective role against PCO (OR = 0.197, 95% CI = 0.08–0.442, p < 0.0001) .There was a significant cooperation between rs2236242 SNP vaspin gene and PCOS risk after adjusting BMI. Findings of this study investigated a significant correlation between *vaspin* rs2236242 SNP and the risk of PCOS in Iraqi obese women and this relationship is not affected by obesity status when examined in obese healthy women. T allele frequency was 0.45 ; 0.68 , and A allele frequency was 0.32 ;0.48 for the control group, and obese PCOS patients, respectively. In the dominant genetic model, A allele showed protective effects for PCOS (OR:0.39, 95%CI: 0.25-0.60, P< 0.0001) . Moreover, TT genotype in vaspin rs2236242 polymorphism are associated with increased hazard of PCOS in women. Changes in vaspin levels and its gene expression has been described that is linked to BMI and biomarkers of insulin sensitivity in MS patients (28), although the information of different researchers are confounding. Youn and his workers have referred that increased in vaspin serum levels were linked with BMI and insulin resistance (30),while other study in obese children showed a negative cooperation with Homostatstis-insulin resistance (HOMA-IR) (26,24) However, there is no correlation between mean serum vaspin levels and HOMA-IR in non diabetic humans was found by (28).On the other hand Seeger and his workers (24) have also recorded that circulatory vaspin is not independently correlated with markers of glucose metabolism, and vaspin levels do not associate with insulin

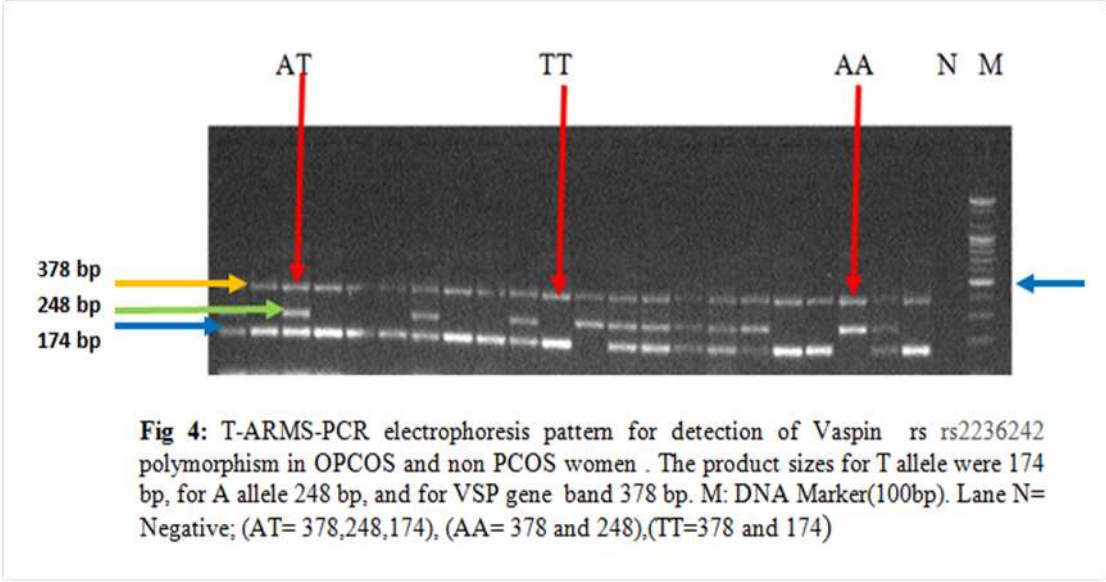

Fig 4: T-ARMS-PCR electrophoresis pattern for detection of Vaspin rs rs2236242 polymorphism in OPCOS and non PCOS women . The product sizes for T allele were 174 bp, for A allele 248 bp, and for VSP gene band 378 bp. M: DNA Marker(100bp). Lane N= Negative; (AT= 378,248,174), (AA= 378 and 248),(TT=378 and 174)

Table 4. Alleles frequency and genotype distributions of Vaspin gene polymorphisms in OPCOS and non PCOS women

Genotype or Allele	PCOS Patients (No.=100)		NON-PCOS (No.=75)		OR*	95 percent Cl	P value*
	No.	%	No.	%			
TT	46	46	20	26.6	2.34	1.22-4.45	< 0.0001
TA	44	44	28	37.3	1.36	0.74-2.528	NS
AA	10	10	27	36.0	0.197	0.08-0.442	< 0.0001
T	136	68	68	45.3	2.56	1.65-3.96	<0.0001
A	64	32	82	54.6	0.39	0.25-0.60	< 0.0001

status in maternal, fetal and neonatal samples(15,17) It has been investigated that serum vaspin concentration are not elevated in morbidly obese non PCO women and do not associate with BMI, markers of glucose or lipid metabolism (28).The outcome of vaspin on insulin sensitivity is uncertain and the correlation between vaspin and BMI is also unclear till now. Data from this study investigated that the AA genotypes decreased

the PCO risk in comparison with the genotype TT,thus a considered as protective against PCO risk. Polymorphisms of the chemerin and vaspin genes could be assigned to play a role in elevating the PCOS risk in a sample of Iraqi women in addition obesity characterization of PCOS cases enrolled in this current work plays a major role in aggravating the hormonal alterations related with PCOS. Adipokine gene polymorphisms may be associated with increased PCOS risk in Iraqi women, Further studies on a large number scale should be done to confirm this association.

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