



# Association of Serum Resistin Level and Resistin (*RETN*) Gene (-420 C>G) Polymorphism in Pakistani Women with Polycystic Ovarian Syndrome

## Polikistik Over Sendromlu Pakistanlı Kadınlarda Resistin (*RETN*) Gen (-420 C>G) Polimorfizmi ve Serum Resistin Düzeyi İlişkisi

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### Abstract

**Objective:** The objective of the present study was to investigate the association of altered serum resistin levels to *RETN* gene (420C>G) polymorphism in women with polycystic ovarian syndrome (PCOS) and in healthy controls. **Material and Methods:** Eighty (40 PCOS cases and 40 healthy controls) individuals were included. Whole blood and serum samples were taken from all participants. Enzyme linked immunosorbent (ELISA) was performed for measuring the levels of serum resistin. Whole blood was used for extracting total genomic DNA by the phenol-chloroform method. Polymerase chain reaction with fragment length polymorphism was performed for detecting single nucleotide polymorphism (SNP) in the promoter region (-420 C>G) of the resistin (*RETN*) gene by amplifying the oligonucleotide sequence of the SNP. The amplified products were first confirmed on 2.0% agarose gel for product size, and then restriction digestion of these products was performed by using the Bpil restriction enzyme. After completion of digestion, the products were resolved on 2.5% agarose gel with a 100 bp DNA ladder, and the bands were inspected to infer genotype. Data analysis was done using SPSS software and the association between serum resistin levels and *RETN* genotypes was analyzed. **Results:** There was no significant difference ( $p=0.125$ ) observed in serum resistin levels between PCOS cases (mean $\pm$ SD=19.33 $\pm$ 3.50) and healthy controls (mean $\pm$ SD=13.48 $\pm$ 1.31). The frequency of the G allele was high in PCOS cases (65%) than in controls (53.7%). The GG genotype frequency of SNP (-420 C>G) was high in PCOS cases (40%) than in controls (20%), but no association was found ( $p=0.148$ ). The high serum resistin levels were significantly associated with the GG genotype in PCOS cases ( $p=0.027$ ). **Conclusion:** High serum resistin levels are not associated with the genotypes of *RETN* (-420 C>G) polymorphism in PCOS women and controls, although women with PCOS had high GG genotype levels of serum resistin. Further studies with large sample size should be conducted to explore the mechanism of genetic factors in complex diseases like PCOS.

**Keywords:** Resistin; polycystic ovarian syndrome; genetic polymorphism; association; Pakistan

### Özet

**Amaç:** Polikistik over sendromu (PKOS) olan kadınlarda ve sağlıklı kontrollerde değişmiş serum resistin düzeylerinin *RETN* geni (-420C>G) polimorfizmi ile ilişkisini araştırmaktır. **Gereç ve Yöntemler:** Çalışmaya, seksen kişi (40 PKOS hastası ve 40 sağlıklı kontrol) dâhil edildi. Tüm katılımcılardan tam kan ve serum örnekleri alındı. Serum resistin düzeylerini ölçmek için enzim bağli immünosorbent (ELISA) yapıldı. Tam kan, fenol-kloroform yöntemiyle total genomik DNA'nın ekstrakte edilmesi için kullanıldı. Tek nükleotit polimorfizminin (SNP) oligonükleotid sekansını amplifiye ederek resistin (*RETN*) geninin promotör bölgesinde (-420 C>G) SNP saptanması için polimeraz zincir reaksiyonu ile parça uzunluk polimorfizmi yapıldı. Amplifiye edilen ürünler önce ürün boyutu için %2,0 agaroz jel üzerinde doğrulandı ve daha sonra bu ürünlerin sindirimi Bpil restriksiyon enzimi kullanılarak gerçekleştirildi. Sindirimin tamamlanmasından sonra ürünler, 100 bp'lik bir DNA merdiveni ile %2,5 agaroz jel üzerinde çözüldürüldü ve bantlar genotip çıkarımı için incelendi. SPSS yazılımı kullanılarak veri analizi yapıldı ve serum resistin düzeyleri ile *RETN* genotipleri arasındaki ilişki analiz edildi. **Bulgular:** PKOS hastaları (ortalama $\pm$ SS=19,33 $\pm$ 3,50) ile sağlıklı kontroller (ortalama $\pm$ SS=13,48 $\pm$ 1,31) arasında serum resistin düzeylerinde anlamlı bir fark izlenmedi ( $p=0,125$ ). G allelinin sıklığı PKOS (%65) hastalarında kontrollerden (%53,7) yüksekti. SNP'nin (-420 C>G) GG genotip frekansı, PKOS (%40) vakalarında kontrollerden (%20) yüksekti, ancak ilişki bulunmadı ( $p=0,148$ ). Yüksek serum resistin düzeyleri PKOS hastalarında GG genotipi ile anlamlı şekilde ilişkili idi ( $p=0,027$ ). **Sonuç:** PKOS'lu kadınların serum resistin GG genotip düzeyleri yüksek olsa da PKOS'lu kadınlarda ve kontrollerde yüksek serum resistin düzeyleri ile *RETN* (-420 C>G) polimorfizmi genotipleri ilişkili değildir. PKOS gibi karmaşık hastalıklarda genetik faktörlerin mekanizmasını araştırmak için büyük ölçekli daha ileri çalışmalar yapılmalıdır.

**Anahtar kelimeler:** Resistin; polikistik over sendromu; genetik polimorfizm; ilişki; Pakistan

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## Introduction

Polycystic ovarian syndrome (PCOS) is an endocrine defect commonly affecting women of reproductive age with a prevalence of 5-13%. The characteristic features of PCOS include infrequent or absent menstruation, polycystic ovaries, and high blood levels of androgen (1,2). PCOS is characterized by oligomenorrhea, hirsutism, infertility, insulin resistance, obesity, and acanthosis nigricans with polycystic ovaries (3). In Pakistan, the frequency of PCOS in fertile women is about 17.6% (4). The syndrome occurs in all races and geographical locations and is the most common disorder and cause of infertility (5). The prevalence of PCOS in infertile Pakistani women is 40.9% (6), and the prevalence of PCOS patients among first-degree relatives is 25-50%, suggesting a high-risk inheritance (7). Published literature has described that genetic factors strongly contribute to the development of PCOS. Although various studies have investigated the variable changes in the genes regarding the complex biological mechanism, the role of genetic predisposition on PCOS pathophysiology is not elucidated remarkably (8). Several candidate genes of metabolic defects have a role in PCOS, although the contributing genes remain to be elucidated (9,10). Some recent studies have identified various single nucleotide polymorphism (SNP) sites to be associated with PCOS in different populations (11-13).

Resistin is a cysteine-rich hormone belonging to the resistin-like molecule, and it acts as a macrophage in multiple inflammatory disorders (14). Resistin is involved in various metabolic defects like metabolic syndrome and diabetes (15), atherosclerosis and coronary artery diseases (16), and osteoarthritis (17).

Various SNPs have been reported in the resistin (*RETN*) gene and an important promoter region SNP (-420 C>G; rs1862513) is associated with various diseases and with variable serum resistin levels (18,19). Previous genetic association studies have demonstrated a link between *RETN* polymorphism and metabolic syndrome (20). However, some studies did not establish the role, while in other studies the disease susceptibility to the *RETN* gene was heteroge-

neous and conflicting. Several SNPs of the *RETN* gene have been reported for the association with variable serum or plasma levels of resistin in different pathologies like the development of insulin resistance and dyslipidemia (21).

The genetic association of *RETN* polymorphisms has been not determined in PCOS women. We aimed to assess the role of SNPs (-420 C>G; rs1862513) in PCOS predisposition in Pakistani women. The association between serum resistin levels and PCOS was also explored. According to our knowledge, this is the first study ever to investigate the relation between serum resistin levels and *RETN* genetic variants in PCOS susceptibility.

## Material and Methods

Ethical permission was obtained from Advance Studies & Research Board (AS&RB) of the University of Health Sciences, Lahore, Pakistan. Written informed consent was obtained from all the participants and 2013 modified Helsinki guidelines were followed for human subjects. This was a case-control study and PCOS cases were retrieved from a teaching hospital of Lahore (Jinnah Hospital) and age- and sex-matched healthy controls of similar ethnicity were recruited. Family history was obtained and clinical examination of PCOS women was performed and demographic data were recorded. The diagnosed cases of PCOS according to the criteria were included. Patients with diabetes and other metabolic defects were excluded. The Rotterdam diagnostic criterion (22) was used to establish the PCOS cases (presence of at least two contributing factors from the following; **a.** oligo/anovulation, **b.** hyperandrogenism [clinical: hirsutism; biochemical: raised androgen levels], **c.** polycystic ovaries on ultrasound). The controls were also screened for these criteria, and some control participants carried a single feature at the time of recruitment like irregular menstruation, hirsutism, acne without raised androgen levels and absence of other factors.

After obtaining the written informed consent, about 5 mL of venous blood was drawn from the participants under aseptic conditions, which was divided into two different vacutainers: 2 mL in a serum-separating

tube for serum resistin hormone analysis; after clot formation, the sample was centrifuged at 3000 rpm for 10 min to separate serum and stored at -20 °C until resistin assay was performed. About 3 mL of blood was collected in an EDTA tube for genomic DNA extraction and stored at 4 °C until further process.

Serum resistin levels were determined using a commercially available ELISA kit, which was based on the sandwich principle (Glory Science Co. Ltd, USA), according to the kit manual. The absorbance of samples was taken by reading the micro-plate on a semi-automated micro-plate reader (Bio-Rad, Germany) at a wavelength of 450 nm. Resistin standards were also tested on the plate along with the samples and a standard curve was generated to measure serum resistin levels.

Genomic DNA extraction was performed by the phenol-chloroform standard method (23). The primer sequence of the *RETN* gene (-420C>G) was used as described previously (24). The oligonucleotide sequences of SNP (forward primer 5'-TGTCATTCTCAC-CCAGAGACA-3' and reverse primer 5'-TGGGCTCAGCTAACCAAATC-3') were amplified by PCR. The reaction was performed in a 25- $\mu$ L reaction tube containing 14  $\mu$ L deionized water, 8  $\mu$ L PCR Master Mix (2X GreenTaq), 0.5  $\mu$ L forward and reverse primers (10  $\mu$ M), and 2.0  $\mu$ L DNA template. Thermal cycler conditions were the following: first strand denaturation (one cycle at 95 °C for 5 min), then 35 cycles of denaturation (95 °C), annealing (64 °C), extension (72 °C) for 30 s of each steps, and the final extension at 72 °C for 5 min. Amplification was verified on 2.0% agarose gel electrophoresis with a 100-bp DNA ladder and the amplicon size was 534 bp for the oligonucleotides. The PCR products were digested by restriction endonuclease *Bpil* enzyme, also known as *Bbs1* (Fermentas, USA), and the digested PCR products were digested at 37 °C for 16 h. Enzyme inactivation was performed by incubating at 65 °C for 20 min. The digested PCR products were resolved on 2.5% agarose gel and band resolution was observed on the Gel Doc system (Bio-Rad) to interpret the genotype.

The data were analyzed using SPSS for Windows, version 21. Quantitative variables

such as age and BMI were presented as mean $\pm$ standard deviation. Serum resistin levels were presented as mean $\pm$ standard error of the mean. An independent t-test was used to determine the mean difference in serum resistin levels between the groups. Categorical variables such as polymorphism were calculated in frequencies and percentages. In order to calculate differences in genotypes and allele frequencies, Fisher's exact test was used. The effect of SNP on the risk of developing PCOS was estimated with an odds ratio (OR) by the chi-square test. A p-value of less than 0.05 was considered statistically significant.

## Results

The study included 80 participants: 40 PCOS cases and 40 healthy controls without a history of PCOS. The comparison of different clinical parameters between cases and controls and their demographic data are given in Table 1. There were significant differences in clinical features (irregular menstrual cycle, weight gain, and hirsutism) between PCOS women and healthy controls ( $p<0.05$ ). The quantitative variables are presented in Table 2. In PCOS cases, the mean $\pm$ SD age was 24.20 $\pm$ 4.76 years, while in controls it was 22.30 $\pm$ 3.52 years. The mean BMI was 27.44 $\pm$ 7.855 and 20.69 $\pm$ 3.982 in cases and controls, respectively. The mean serum level of resistin was higher in PCOS women (19.33 $\pm$ 3.50 ng/mL) than in controls (13.48 $\pm$ 1.31 ng/mL), but there was no significant difference ( $p=0.125$ ).

Genotype and allele distribution of *RETN* (-420C>G) SNP showed a single band of 534 bp for CC genotype (wild, homozygous), double bands of 327 and 207 bp for GG genotype (rare, homozygous), and triple bands of 534, 327, and 207 bands for CG (heterozygous) (Figure 1). The frequency of the *RETN* genotype in PCOS cases was 10.0% ( $n=4$ ) for CC, 50.0% ( $n=20$ ) for CG, 40.0% ( $n=16$ ) for GG genotypes, and in controls was 12.5% ( $n=5$ ) for CC, 67.5% ( $n=27$ ) for CG, and 20% ( $n=8$ ) for GG genotypes. The allele distribution frequency shows that the G allele was present in 52 (65%) PCOS cases, which is higher compared with that in controls [43 (53.7%)]. The association determined by using chi-

Table 1. Comparison of the demographic data between the PCOS cases and controls.

Parameters	Cases (n)	Controls (n)	Total (n)	OR (CI)	p-value
Menstrual Cycle Pattern					
Irregular	40	7	47	0.149 (0.4-0.8)	<0.001*
Regular	0	33	33		
Menstrual cycle less than 9 in a year					
Yes	31	4	35	31.000 (0.5-0.9)	<0.001*
No	9	36	45		
History of Recent Weight Gain					
Yes	25	6	31	9.444 (0.4-0.8)	<0.001*
No	15	34	49		
Hirsutism					
Yes	23	2	25	25.706 (0.1-0.12)	<0.001*
No	17	38	55		
Acne					
Yes	11	6	17	2.086 (0.4-0.8)	0.190*
No	29	34	63		

\*P-values by independent t-test.

Table 2. Comparison of quantitative variables in women with PCOS and controls.

Variables	PCOS Cases	Controls	p-value*
Age (years)	24.20±4.762	22.30±3.517	0.046
BMI (kg/m <sup>2</sup> )	27.44±7.855	20.69±3.982	0.001
Resistin (ng/mL)	19.33±3.508	13.48±1.316	0.125

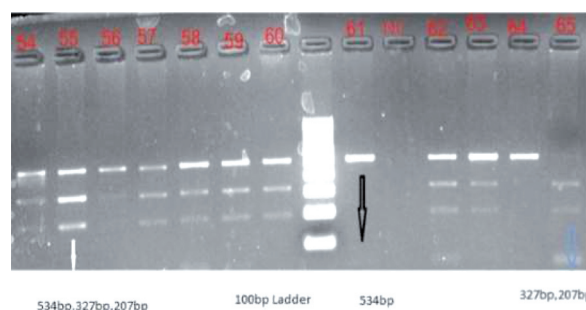
\*P-values were calculated by unpaired t-test.

square ( $\chi^2$ ) test was not significant ( $p=0.148$ ) between genetic polymorphism and disease (Table 3).

The association between serum resistin levels and *RETN* gene polymorphism was analyzed for genotype carriers. GG genotype carriers had the highest resistin levels in PCOS cases than in controls and a significant association was noted ( $p=0.027$ ), while CG and CC genotypes were not associated between cases and controls (Table 4).

### Discussion

The prevalence of PCOS is alarmingly increasing and PCOS is becoming a health issue for women of reproductive age. Several genetic and environmental factors are responsible for PCOS. Resistin, an adipocytokine, is considered a risk factor of metabolic syndrome, PCOS, osteoarthritis, type 2 diabetes, insulin resistance, and obesity (14-17). Several SNPs of the resistin (*RETN*)



**Figure 1:** Restriction fragment analysis for *RETN* (-420C>G) polymorphism. Bpil (Bbs1) did not digest the C allele giving a band with a length of 534 bp (black arrow). G allele was cleaved by Bbs1 and showed two bands with lengths of 327 and 207 bp (blue arrow) while heterozygous from CG gave three bands with fragment sizes of 534, 327, and 207 bp (white arrow).

gene have been reported for complex diseases, but the promoter region SNP -420C>G has a potential influence on circulating resistin levels and *RETN* gene expression (17).



Table 3. Polymorphism of *RETN* Gene promoter region (-420C>G) in the cases and controls.

Genotype/Allele	PCOS Cases	Controls	OR (CI)	p-value
CC	4 (10.0%)	5 (12.5%)		<b>0.148*</b>
CG	20 (50.0%)	27 (67.5%)		
GG	16 (40.0%)	8 (20.0%)		
C	28 (35%)	37 (46.3%)	1.59 (0.846-3.017)	<b>0.148<sup>a</sup></b>
G	<b>52 (65%)</b>	<b>43 (53.7%)</b>		

\*p-value was calculated by Fisher's exact test, <sup>a</sup>calculated by chi-square test.

Table 4. Association of *RETN* (-420C>G) polymorphism and serum resistin levels in PCOS cases and controls.

Genotype	Serum resistin		p-value*
	PCOS Cases	Controls	
CC	11.88±1.008	12.92±2.009	<b>0.659</b>
CG	13.49±2.726	14.72±1.867	<b>0.703</b>
GG	<b>28.48±7.671</b>	<b>9.66±0.527</b>	<b>0.027</b>

\*p-value was calculated by Student's t-test.

In the present study, the allele distribution frequency of the *RETN* gene (-420C>G) SNP was not different in PCOS patients than in controls (p=0.148). The GG genotype distribution of the *RETN* gene was higher in PCOS cases than in controls [16 (40%) and 8 (20%), respectively], but no statistically significant difference was observed. The findings of the present study are consistent with those of the previous studies, in which no association was reported between *RETN* polymorphism and PCOS (25,26). A study of Spanish women with PCOS showed a high frequency of the G allele of the *RETN* gene (420 C>G; rs1862513) polymorphism, but no association was found (27). Similarly, the frequency of the G allele variant of the *RETN* gene is common in Pakistani women. A study of South Indian women reported a high frequency of G allele of the *RETN* promoter region (28), which is consistent with the results of the present study. In contrast to the current study, some previous studies have demonstrated the association of *RETN* polymorphism in PCOS women (29). The exact molecular mechanism of -420C>G polymorphism is still unclear, but the polymorphism may be a disease predisposing factor in the combination of complex genetic and environmental contributors. In the present study, serum resistin levels were higher

in PCOS patients but there was no association in the levels between PCOS cases and controls. Previous studies have described high serum resistin levels in PCOS cases, but the association was not significant (28,30,31). On the other hand, some studies did not find higher serum resistin levels in PCOS women compared to healthy controls (31,32).

The variants of the *RETN* gene affect mRNA expression and circulating serum levels of resistin. Our results showed that women with the GG genotype had higher serum resistin levels compared with control GG carriers. A previous study found elevated levels of resistin mRNA in the adipocyte cells of PCOS patients and reduced adipocyte-resistin mRNA expression in laparoscopic ovarian drilling (33). Many studies on genetic polymorphism have described *RETN* gene involvement in PCOS pathogenicity. These studies were inconsistent and non-conclusive, which may be due to the variabilities in sample size, disease status, and ethnicity (18,24,34,35). PCOS is a complex syndrome that depends on the interaction of genetic and environmental factors. Variants in proteins acting functionally upstream of the resistin gene could modulate the expression of serum resistin levels and affect the disease phenotype.

## Conclusion

The association of *RETN* (-420C>G) polymorphism genotype frequencies and serum resistin levels are not associated with disease susceptibility of PCOS in Pakistani women. Although GG genotype carriers with PCOS had higher serum levels of resistin than control GG phenotype carriers did, there is no exact role of the genotype in disease pathogenicity. Furthermore, future studies including large sample size and various ethnic or language groups are needed to understand the molecular mechanism of disease progression.

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## Ethical Consideration

Ethical approval for this study was obtained from the Ethical Committee and IRB of the University of Health Sciences, Lahore.

## Author Contributions

Idea/Concept: Sumbla Ghazanvi, Shah Jahan; Design: Sumbla Ghazanvi; Control/Supervision: Sumbla Ghazanvi; Data Collection and/or Processing: Yasar Nawaz, Nadia Rasheed; Analysis and/or Interpretation: Shah Jahan, Muhammad Ikram Ullah; Literature Review: Yasar Nawaz, Nadia Rasheed; Writing the Article: Yasar Nawaz, Nadia Rasheed, Muhammad Ikram Ullah; Critical Review: Sumbla Ghazanvi, Shah Jahan, Muhammad Ikram Ullah.

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