



# Resistin -420 C>G Polymorphism in Female Patients of Acne in Pakistan

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## Abstract

Acne vulgaris is an inflammatory skin disorder of pilosebaceous units. The clinical characteristics are seborrhea, sebaceous glands, non inflammatory lesions, inflammatory lesions and severe scarring. Propionibacterium acnes are the main pathophysiological factor influencing the acne development. Resistin gene is a novel proinflammatory cytokine involved in the initiation of inflammatory disorders like cardiovascular diseases, obesity and atherosclerosis. Various SNPs have been discovered in human RETN gene which is involved in different inflammatory disorders. Purpose of this study was to analyze RETN -420 C>G polymorphism in female acne patients of Pakistani population. About 58 control female subjects and 76 acne female patients were recruited for this study. In order to examine the RETN -420 C>G polymorphism in the promoter region, PCR and RFLP techniques were used. Statistically significant P value <0.05 for the RETN -420 C>G polymorphism was obtained in genotype/alleles frequencies between acne patients and control female subjects. Significant association between genotype frequencies, disease severity and acne scars distribution among female patients was also observed. It is concluded that RETN -420 promoter polymorphism was not associated with family history and skin types; however it was significantly associated with acne pathophysiology.

**Keywords:** Acne Vulgaris; Pilosebaceous Units; Pro-Inflammatory Cytokine; Resistin Gene; Case Control Study

**Abbreviations:** RETN: Resistin; C>G: Cytosine to Guanine; SNP: Single Nucleotide Polymorphism; PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism.

## Introduction

Acne is an epidermal cutaneous chronic inflammatory disorder of sebaceous glands involving altered keratinization, androgen-induced sebum overproduction, bacterial colonization, and inflammation of the pilosebaceous units located at the base of hair follicles of face, neck, chest, and back [1]. The sequence of events leading to the development

of different acne lesions is not fully understood [2]./The detection of genetic factors involved in the development of acne may provide an insight into the biological pathways affecting disease initiation or progression and may aid for new therapies [3].

Resistin gene ('resistance to insulin') was originally named for its ability to resist insulin action [4]. Resistin is a 12.5kD Cysteine-rich protein that consists of 108 amino acids in human [5]. The human resistin gene is located on chromosome 19 and is expressed mainly in monocytes and macrophages, and its expression in human adipose tissue is predominantly owing to non-adipocyte resident

inflammatory cells [6]. The levels of resistin and their gene polymorphism may play a role in many inflammatory diseases such as inflammatory bowel disease, chronic pancreatitis and atherosclerosis [7]. During hyperkeratinization the epithelial cells of the follicular wall accumulates in the lumen of pilosebaceous follicles. Adrenal androgen and gonadal hormones stimulate sebum production by sebaceous glands. Microcomedones formation takes place and becomes larger in size due to accumulation of sebum [8]. The pressure develop and converts the micro comedones into visible comedones results in the rupturing of follicular wall and release of comedones contents in to the surrounding tissue, give signal to the formation of inflammatory lesions [9]. Formation of inflammatory lesions occurs by the process called comedogenesis. It was reported that Bacterial population called *Propionibacterium acnes* (*P.acnes*) are gram positive anaerobic bacteria in sebaceous follicles. When ligands such as *P.acnes* come in contact with TLR's located at surface of monocytes it regulates NF $\kappa$ B [10] through intracellular domain and activates the expression of genes which mediates the immune response against *P.acnes* [11]. Through TLR-dependent pathway regulates high sebum production and hyperkeratinization of follicular wall [12]. Recent reports suggested that acne vulgaris is inflammatory process rather than infection in which bacteria worsen the acne lesion [8]. Acne vulgaris is classified on the base of disease severity into mild acne to moderate- severe acne. During development of acne there is formation of black heads, white heads, papules, pustules, cysts and nodules [13].

There are also some other risk factors of acne vulgaris (socioeconomic impact). Reports showed that 80-90% population are affected by acne in developed countries while in non-industrialized and non-modernized countries the ratio was less due to simple life style [8]. (Diet) Previous studies have been suggested that in acne patients due to excessive use of sweet foods and carbohydrates affected the blood glucose level and shows resistance toward carbohydrates metabolism [14]. One study tells that stress activates specific regions in neuro-peptides which stimulates the pathogenic step of acne pathogenesis and undergoes in the formation of acne lesions [15]. Study reported that in acne vulgaris an endogenous hormones play a significant role includes androgen, estrogen, progesterone, glucocorticoid, insulin like growth factors (IGF), progesterone and testosterone [16]. It was reported that in westernized countries the prevalence of acne in teenagers is approximately 47% to 90% [17]. One survey study claimed that 3% cases held in 7 to 9 years, 30% cases in 10 to 12 years, 78% cases in 13 to 15 years, 93% in 16 to 18 years old individuals, 47% of acne was reported in women aged 20-29 years and 30% was reported in women aged 30-39 years [18]. In Pakistan the individuals aged between 13 to 35 years had acne problems and about 1/5<sup>th</sup>

of them were concerned with dermatologist [19].

As reviewed resistin is a mediator of inflammation. It was suggested that in endothelial cells resistin mediates the expression of adhesion molecules by activating monocytes [20,21]. The study suggested that SNP-420 C>G (rs1862513) is widely studied promoter variant of resistin located in exon2 and 420 nucleotide upstream from translatory region. Binding of transcription factor to the promoter region become altered due to polymorphism [22] the stimulating proteins Sp-1 and Sp- 3 bind with G alleles and increase the promoter activity [23]. The SNP -420 C>G is most significant polymorphism in promoter region of resistin gene [24]. The present study aimed to investigate the relation of different clinical variants (disease severity, acne scars, skin type, family history) and association of resistin gene polymorphisms (RETN -420C>G) in a sample of Pakistani population with acne vulgaris as well as controls.

## Subjects and Methods

### Subjects

A case control study was conducted on female patients having acne vulgaris. The study population comprised 134 individuals. 58 healthy female subjects (age  $21.78 \pm 2.10$ ) were enrolled from Quaid e Azam University Islamabad considered as group A-control and 76 female were considered as group-B case (age  $22.59 \pm 4.34$ ) recruited from Benazir Bhutto Hospital, Rawalpindi. Inclusion criteria for Group-A control were normal female without acne scars and no past history of acne lasting more than 1 week, Group-B female patients having clinically diagnosed acne vulgaris (comedones, papules, nodules, greasy skin, and scarring mainly on the face and minor extent on back and chest) were recruited for this study. Acne was classified as mild, moderate and severe in group B female patients. From each participant written informed consent was obtained. The information was on family history of acne, sex, current treatment, type of lesion, acne scars, type of skin and acne severity. The research was reviewed and approved by the Institutional Review Board, Quaid-i-Azam University.

### Collection of Blood Specimens

The blood specimen's collection was done from output department of Benazir Bhutto hospital (BBH) Rawalpindi Pakistan. The 5mL blood was collected with sterile syringes (Becton Dickinson Pakistan). Initially blood was transfer to ethylene diamine-tetra acetic acid (EDTA) tubes (BD, USA) and gently inverted to mix as anti-coagulant with blood. Blood samples were kept in ice box for transportation to laboratory. The samples were kept at 4°C for further analysis.

### DNA Extraction from Blood

For genomic DNA extraction, Standard Phenol-Chloroform method was followed [25]. The result of genomic DNA was checked on 1% agarose gel.

### PCR Assay

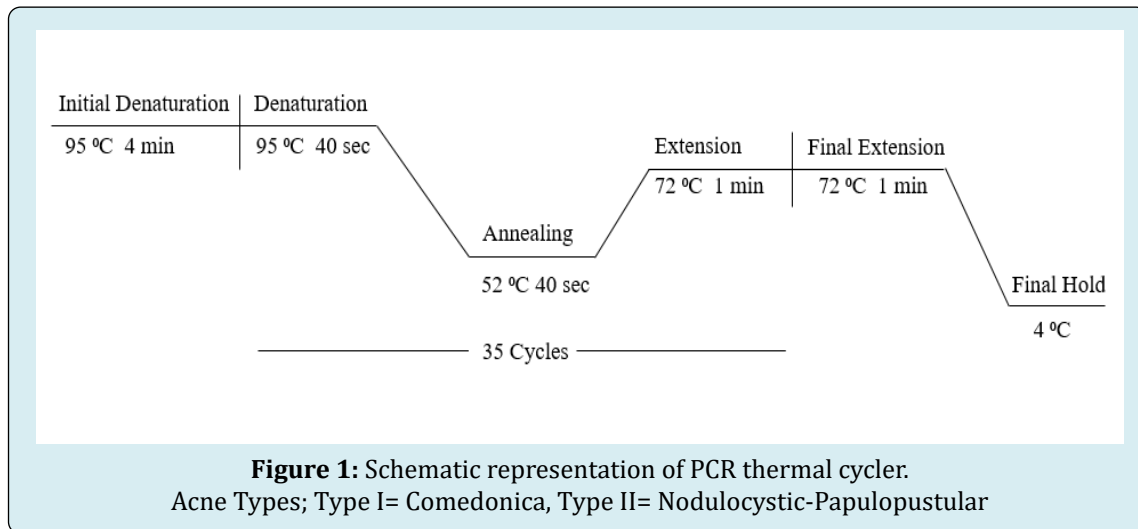
The region of resistin gene containing -420 C>G polymorphism (rs1862513) was amplified by polymerase chain reaction using the following primer

F: 5'TGTCATTCTCACCCAGAGAC 3'

R: 5'TGGGCTCAGCTAACCAAATC 3'

Polymerase chain reaction (PCR) was performed in 0.2mL

PCR tubes (Axygen USA) containing 25 $\mu$ L reaction mixture. The each reaction was consisting of 2.5 $\mu$ L of Taq Buffer, 0.5 $\mu$ L dNTP's 2 $\mu$ L MgCl<sub>2</sub>, F-primer 1.25 $\mu$ L, R-primer 1.25 $\mu$ L, Taq DNA Polymerase 0.25, Genomic DNA 3 $\mu$ L and PCR water 14.25 $\mu$ L. PCR assays were performed in thermal cycler Applied Biosystems Gene Amp<sup>®</sup> PCR system 9700 (Life Technologies, USA). The cycling condition applied was as follows: the initial denaturation was at 95°C for 4 min, then 35 cycles of denaturation at 95°C for 40 s, annealing at 52°C for 40 s, elongation at 72°C for 1 min and the final elongation at 72°C for 1 min and hold at 25°C for 15 min. Amplified PCR products of DNA samples were analyzed on two percent (2%) agarose gel electrophoresis shown in figure 1.



### Design for Restriction Fragment Length Polymorphism (RFLP) Analysis

The sequence of targeted (Resistin at position -420 C>G) 533 bp amplicon was retrieved from the NCBI databank. An online software NEBcutter version 2.0 (<http://tools.neb.com/NEBcutter>) was used to select the suitable restriction endonucleases specific for the PCR amplicon in order to confirm distinctive RFLP patterns for individual target.

### Enzymatic Digestion and RFLP Analysis of PCR Products (Restriction Fragment Length Polymorphism)

The PCR product (533bp) was digested with restriction endonuclease BphI enzyme. The total reaction volume of each digestion was 20 $\mu$ L consisting of 4.5 $\mu$ L of PCR product, 1 $\times$  digestion buffer was 3.5 $\mu$ L and 12 $\mu$ L BphI enzyme, and an adjusted amount of sterilized water. After gentle mixing, the reaction mixtures were spun down and incubated at 37°C

for 16 hrs. 3% agarose gel was used to analyze the digested PCR fragments.

### Statistical Analysis

Student t test was applied to compare the mean value  $\pm$  standard deviation between control and patients. The data was articulated in numeral values and percentages. Fisher exact test and Chi square test were used to evaluate the genotype and alleles frequencies between control and female acne patients. Statistical significance was recognized at  $P < 0.05$ . Odd ratio (OR) was used to calculate the strength association and risk of acne. For 2 $\times$ 2 contingency table 95% CI (Confidence interval) was obtained. Graph Pad Prism was used for statistical tests.

### Results

For genetic analysis the study population comprised of 76 female acne patients from Benazir Bhutto Hospital Rawalpindi and 58 healthy females from Quid-e-Azam

University. PCR-RFLP technique was used for genotyping. The PCR amplified product (533 bps) figure (6) was digested with enzyme endonuclease BpiI to detect C>G polymorphism at -40C>G promoter site. This study proved that acne vulgaris shows potential association with diseases severity related to skin types of different age groups and RETN gene polymorphisms -420 C /G.

### Baseline and Clinical Characteristics of Patients and Controls

Age of study population ranged from 16 years to 35 years

Age group	Control n (%)	Patient's n (%)	OR (95% CI)	P value
	58 (100)	76 (100)		
16-20 years	07 (10)	36 (47)	0.15 (0.06-0.37)	<0.0001
21-35 years	52 (90)	40 (53)		

**Table 1:** Baseline characteristics of female control subjects and female acne patients.

Fischer exact-test was applied for calculation of *P* value; OR, odd ratio; CI, Confidence interval

Characteristic	Control(n=58 ) Mean ± SD	Patients (n=76) Mean ± SD	t value	P value
Age (years)	21.78 ± 2.10	22.59 ± 4.34	1.3	0.1929

**Table 2:** Clinical characteristics of controls and acne patients.

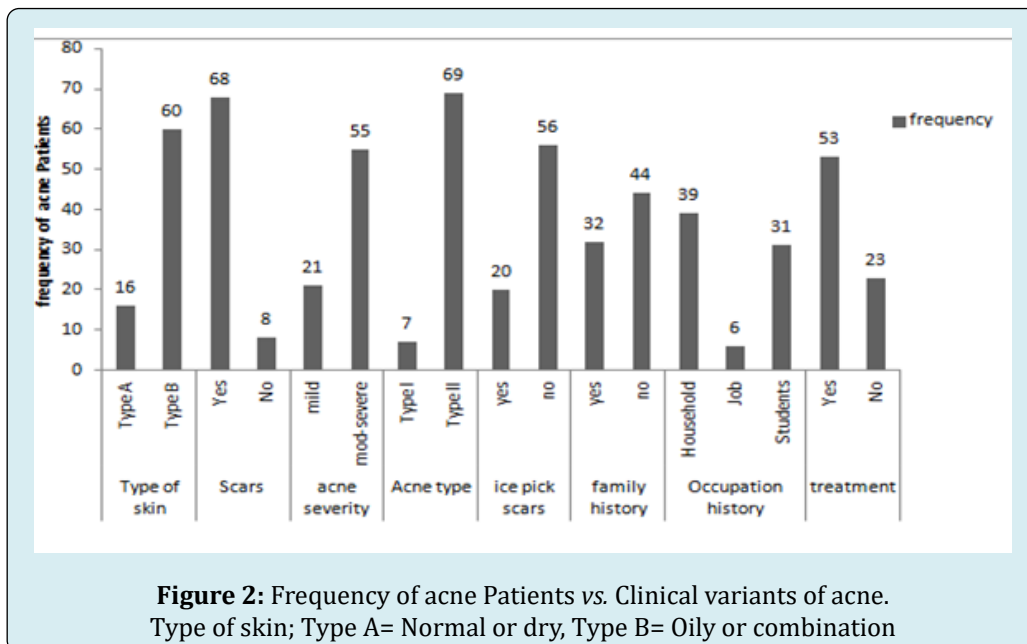
Values are represented as mean ± SD

Student *t* test was applied for calculation of *P* value; SD= standard deviation

### Correlation of Various Clinical Variant Groups in Acne Vulgaris Patients

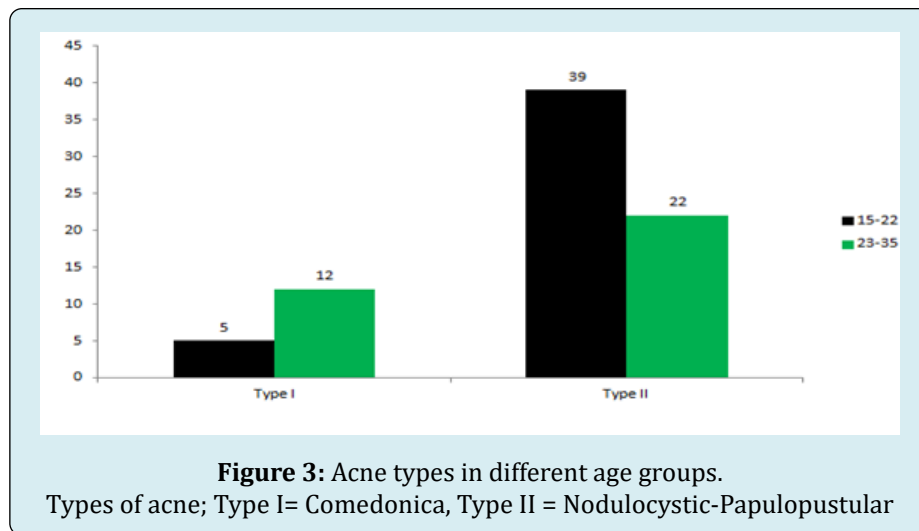
represented (figure 2).

The clinical variables of acne vulgaris patients were



From 76 acne patients 16 (21%) patients had Type A (normal or dry) skin type and 60 (79%) patients had Type B (oily combination or sensitive) skin type. 68 (89%) patients had scars on their skin while rest of patients had no skin scars. On the basis of acne severity 21 (28%) patients had mild level of acne while 55 (72%) patients had moderate to severe level of acne. In 76 acne patients 20 (26%) patients had ice pick scars on their skin while rest of patients had skin without ice pick scars. In 76 acne patients, 32 (42%) reported a positive family history (i.e. either parents or siblings having acne), while 39 cases reported a negative family history of acne. It was stated that during treatment about 38(50%) patient had facial acne and 32(42%) cases reported had acne

breakout on other parts of body (i.e. back shoulder, arms, neck, chest and leg) and 6(8%) cases reported had acne throughout their body. Non-significant P-value was obtained by comparison of family history in different severity groups mild, moderate-severe ( $P>0.05$ ,  $OR=1.47$ ,  $95\%CI=0.53-4.06$ ) while significant P value was obtained by comparison of severity groups in different skin types ( $P<0.05$ ,  $OR=3.67$ ,  $95\%CI=1.24-10.85$ ) and also association is found between acne types and different age groups ( $P<0.05$ ,  $OR=0.2$ ,  $95\%CI=0.07-0.74$ ). Drug response was no significant associated with duration of disease ( $P>0.05$ ,  $OR=1.43$ ,  $95\%CI=0.48-4.25$ ) result shown in table 3.



Clinical Variables	Family history		Odd ratio	OR (95% CI)	P -value
	Positive	Negative			
Disease severity	Positive 21 (100%)	Negative 55 (100%)	1.47	(0.53- 4.06)	0.602
Mild	10 (48%)	21 (38%)			
Moderate-Severe	11 (52%)	34 (62%)			
Disease severity	Skin types		3.67	(1.24-10.85)	0.022
	Type A 22 (100%)	Type B 54 (100%)			
Mild	10 (45.5%)	10 (18.5%)			
Moderate-severe	12 (54.5%)	44 (81.5%)			
Age groups	Acne types		0.23	(0.07-0.74)	0.01
	Type I 17 (100)	Type II 61 (100)			
15-22	5(29.42%)	39(63.94%)			
23-25	12 (70.58%)	22(36.06%)			
Duration of disease (Years)	Drug response		1.43	(0.48-4.25)	0.59
	Yes 30 (100)	No 23 (100)			
0.5-3	17 (57%)	11 (48%)			
04-13	13 (43%)	12 (52%)			

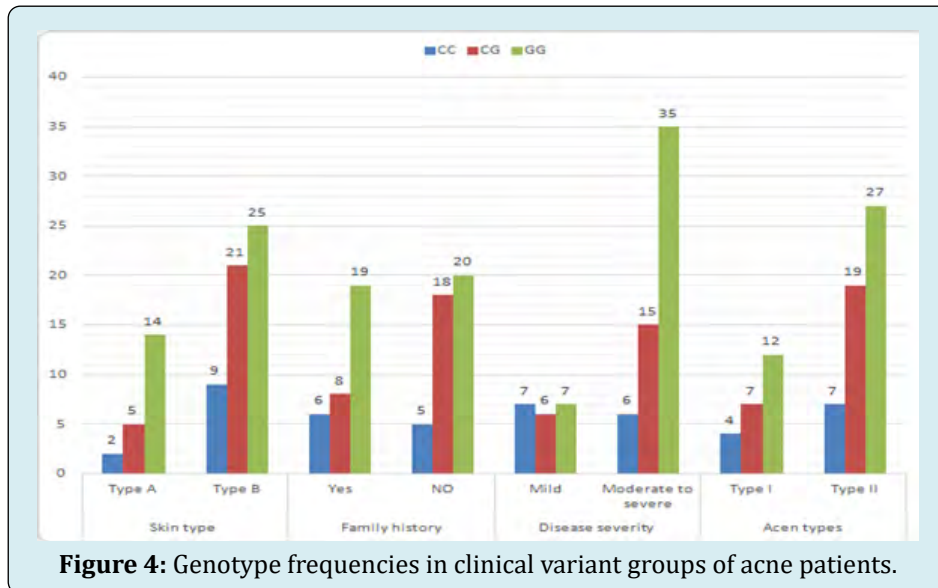
**Table 3:** Comparison of Clinical variant groups in Acne vulgaris Patients.

Skin type A= Normal or Dry, Skin type B=Oily, Combination or Sensitive. Acne Type I= Comedonica (non-inflammatory lesions), Acne Type II= Nodulocystic-Papulopustular (inflammatory lesions). Fischer exact test and chi square test was applied for calculation of P value.  $P<0.005$  statistically significant = Skin type vs. Disease severity, Acne type vs. Age groups.

### Association of RETN Polymorphism in Various Clinical Variant Groups of Acne Vulgaris Patients

To illuminate the association of RETN -420C>G single nucleotide polymorphism (SNP) with the pathogenesis of acne, genotype frequencies were compared with variables like severity of acne, skin type, family history, scars and acne types figure.4, figure.5. The results of significance tests interpreted that the severity of acne was associated with resistin (RETN) genotypes ( $P < 0.05$ ;  $\chi^2 = 7.15$ ). There has been non-significant association of RETN polymorphism in the

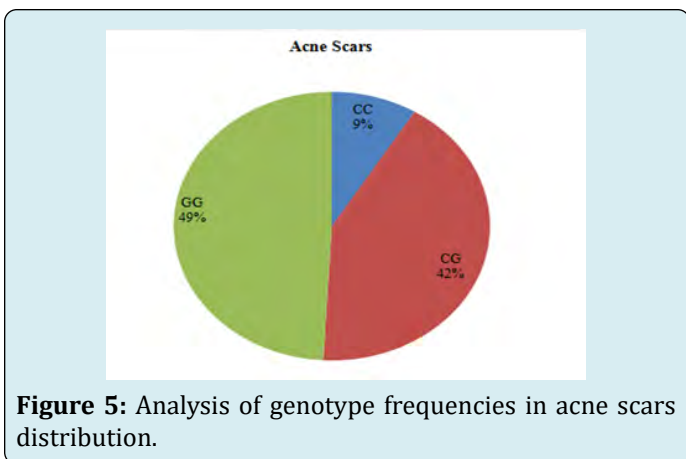
distribution of different acne types and skin types ( $P > 0.05$ ,  $\chi^2 = 0.34$ ;  $P > 0.05$ ,  $\chi^2 = 2.29$ ). The genotype frequencies in the patients having a positive family history was not significantly different as compared with patients having a negative family history ( $P > 0.05$ ,  $\chi^2 = 2.69$ ). The results showing the relationship between RETN polymorphism and acne scars was significance ( $P < 0.05$ ,  $\chi^2 = 9.48$ ). Association of RETN polymorphism in different variables and test statistics is shown in Table 4.



Variables	Total	Genotypes CC CG GG			$\chi^2$	P value
<b>Disease severity</b>						
Mild	20 (100)	7 (35%)	6 (30%)	7 (35%)	6.33	0.04
Mild-severe	56 (100)	6 (11%)	15 (27%)	32 (62%)		
<b>Skin types</b>						
Type A	21 (100)	2 (9%)	5 (24%)	14 (67%)	2.74	0.14 (NS)
Type B	55 (100)	9 (16.5%)	21 (38%)	25 (45.5%)		
<b>Scars</b>						
Presence	57 (100)	5 (9%)	24 (42%)	28 (49%)	9.48	0.008
Absence	19 (100)	6 (32%)	2 (10%)	11 (58%)		
<b>Family History</b>						
Positive	33 (100)	6 (18%)	8 (24%)	19 (28%)	2.69	0.26 (NS)
Negative	43 (100)	5 (12%)	18 (42%)	20 (46%)		
<b>Acne types</b>						
Type I	23 (100)	4 (17.4)	7 (30.4%)	12 (52.2%)	0.34	0.84 (NS)
Type II	53 (100)	7 (13%)	19 (36%)	27 (51%)		

**Table 4:** Comparison of genotype frequencies of RETN C>G-420 among Clinical variant groups of acne patients.

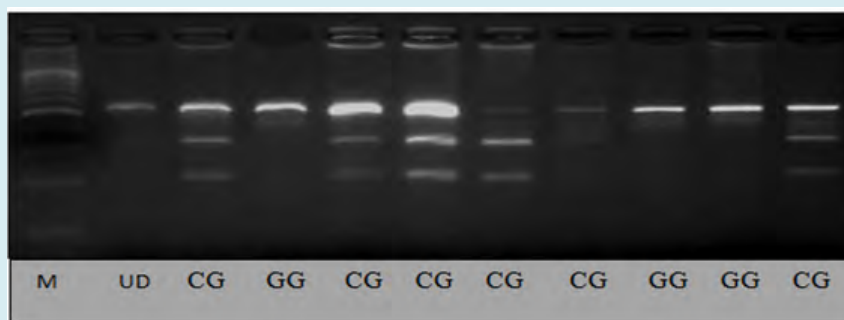
Skin type A= Normal or dry Skin, type B= oily combination or sensitive, Acne type I= Comedonica (non-inflammatory lesions), Acne type II= Nodulocystic-Papulopustular (Inflammatory lesions). P value was calculated by Fischer's exact test, NS= non-significant, OR= odd ratio. Statistically significant  $P < 0.05$



**Figure 5:** Analysis of genotype frequencies in acne scars distribution.

### RETN Polymorphism: Genotype and Alleles Distribution among Study Population

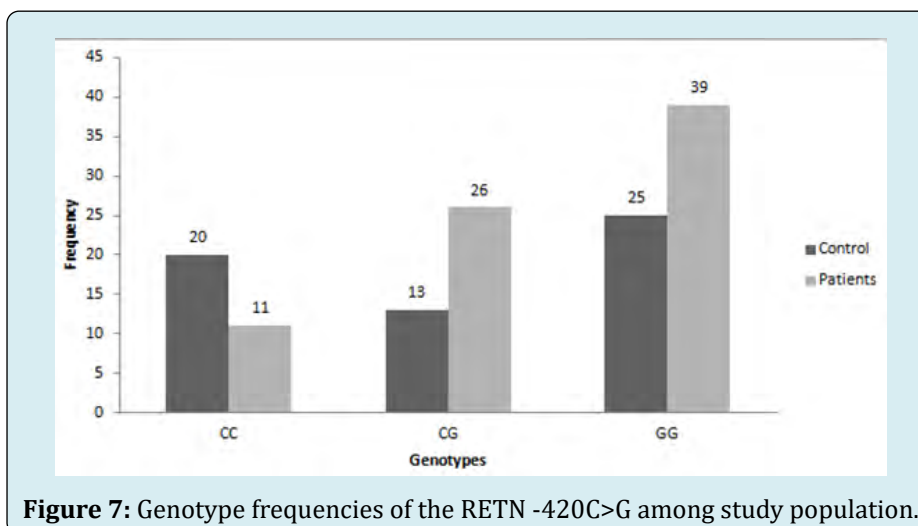
Susceptibility of disease was analyzed to associate the genotype pattern of acne patients with SNP of -420 resistin gene. Genotyping was carried out by PCR-RFLP method using Bpil restriction enzyme. Bpil recognized 5'...GAAGAC(N<sub>2</sub>)↓... 3' sequence, and digested 533bp amplified PCR product into different bands depending upon genotype of sample. Band sizes were CC (326, 207 bp), CG (533, 326, 207bp), GG (533bp) respectively (Figure 6). Association of RETN polymorphism among study population has been represented in Table 5.



**Figure 6:** Electropherogram 3% Agarose gel showing banding pattern of genotype obtained with Bpil restriction enzyme digest at -420 C>G site of RETN gene. Lane M represents 100 bp DNA Marker while lane UD refers to the undigested PCR amplified (533bp) fragments. Genotypes are characterized as CG (533, 326 and 207bp) CC (326, 207bp) and GG (533bp).

A significant P value was obtained when genotypes (CC, CG and GG) of between patient and control groups were compared ( $P < 0.05$ ;  $\chi^2 = 4.04$ ). The genotype distribution in patients and controls was following CC = 11 (15%), CG = 26 (34%) and GG = 39 (51%); CC = 20 (35%), CG = 13 (22%) and GG = 25 (43%) respectively (figure 7). Comparison of wild type CC and dominant model (CG+GG) for resistin -420

C>G polymorphism showed that variant genotype was more common in patients (85%) as compared to the control group (63%) ( $P < 0.05$ ; OR = 4.98; 95% CI = 2.18-11.35: table 5). Similarly, allele frequencies were compared between the two groups and a significant P value was obtained ( $P < 0.05$ ; OR = 1.82; 95% CI = 1.10-3.00) and frequency of G allele is high in acne patients as compare to control subjects.



**Figure 7:** Genotype frequencies of the RETN -420C>G among study population.

Genotypes	Total(134)	Control (58)	Patient (76)	$\chi^2$	OR (95% CI)	P- value
CC	31 (23%)	20 (35%)	11 (15%)	4.04	-	0.02
CG	39 (29%)	13 (22%)	26 (34%)			
GG	64 (48%)	25 (43%)	39 (51%)			
CC	31 (23%)	20 (35%)	11 (15%)	3.11	(1.30-6.90)	0.007
CG+GG	103 (77%)	38 (65%)	65 (85%)			
Alleles	Total (268)	Controls (116)	Patients (152)	1.82	(1.12-3.00)	0.022
C	101 (38%)	53 (46%)	48 (32%)			
G	167 (62%)	63 (54%)	104 (68%)			

**Table 5:** Comparison of resistin SNP genotypes and allele's frequencies of studied polymorphism in control and patients.

For P value Chi square test and Fischer exact test was applied

OR= Odd Ratio, CI= confidence interval. Statistically significant  $P < 0.005$

## Discussion and Conclusion

Acne vulgaris is a chronic inflammatory disorder of pilosebaceous units. As compared to other diseases the incidence ratio of acne vulgaris is high [26]. Prevalence of acne is high in adolescence and in young adults. Acne vulgaris have complex etiology and pathophysiology. Hormonal factors such as androgens and immunogenic cytokines increases the production of sebum, favors the conversion from comedones to inflammatory lesions [16].

It was concluded from different studies that main risk factors of acne vulgaris are deficiency in sleep, stress, family history, socioeconomic impact and use of low quality cosmetics and skin type [13]. Studies show that proinflammatory cytokines are present in pilosebaceous units which mediate the formation of acne. Resistin is a proinflammatory cytokine [27]. Officially Steppan and his co-workers first discovered resistin in 2001 [3]. One study reported that resistin mediates the expression of adhesion molecules through monocytes; results in the up-regulation of ICAM-I, VCAM-I and MCP-1 expression by p38 MAPK dependent pathway cause atherosclerosis which is inflammatory process [20,28]. Monocytes are main source of resistin hormone which is mainly concerned with up-regulation of inflammatory response [29]. The current case control study was designed to investigate the association of resistin RETN -420C>G polymorphism with acne in female patients of a Pakistani population. The results showed that there is a significant association between the RETN -420C>G polymorphism and the studied female acne patients. These findings are similar to those described in previous studies showing an association of the -420C>G polymorphism in chronic and inflammatory diseases such as cardiovascular diseases, atherosclerosis and obesity [30-32]. In the present study there is significant association of the RETN -420C>G with diseases severity and acne scars while no relationship was found with family history and skin type. This study did

not found any significant association among duration of disease, family history of acne in female acne patients while Bataille and coworkers in 2002 demonstrated a significant association between high risk of acne development and family history of acne [33]. This study shows a significant association between acne types in different age groups and with skin types among acne patients. These results are agree with previous studies demonstrating women with comedonal acne were older than women with inflammatory lesions and age matched peers had high incidence of acne as compared to postmenopausal women [34]; Wei and coworkers suggested that the oil skin may be main risk factor of acne [35-42].

In conclusion the pathophysiology of acne vulgaris is multifactorial and complex that may be due to a number of gene. The study should be replicated in other to determine the role of said SNP in acne and its functional aspect.

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