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2016 A.D. **1438 A.H.**

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بسم الله الرحمن الرحيم

((إِنَّمَآ إِلَٰهُكُمُ اللَّهُ الَذِي لَا إِلَهَ إِلَّا هُوَ وَسِعَ كُلَّ

شَيْءٍ عِلْماً))

صَدَقَ اللهُ العَلِيُ العَظِيْم

طه/98

Dedication

To the Prophet of mercy, ***Mohamed and his household*** *(peace be upon him and his household)…*

To the dears, ***my family especially my parents and my wife…***

To our protectors, ***the Iraqi army and the Popular army …***

Mohammed Jaafar
2016

Acknowledgements

First of all, thanks to **Allah** for giving me the power and the insistence to complete this work, and next to his prophet, **Mohammed** peace be upon him and his household,.

I would like to express my deep gratitude to my supervisor Professor **Dr. Hammadi A. Al –Hilali** College of Medicine, Al-Qadisiyah University for his guidance, kindness and continuous support throughout this work.

I am in debt to all of the members of the Department of Medical Microbiology, College of Medicine / Al-Qadisiyah University.

Also I produce my thanks to the Dermatologist **Dr. Mohsen AL-Dalimy** College of Medicine / Kufa University for their support and help during the period of this study.

Special thanks for **Dr. Mohammed Al-zayadi & Dr. Mohammed Emad** College of Science / Kufa University and **Dr. Nabil al-Sharifi** College of Veterinary Medicine / Kufa University for their help in performing this work.

My deepest thanks to all member of Dermatology Center of AL-Sadder Teaching Hospital and to physicians for their support and help during the period of this study.

I must express my hearts full gratitude to each patients and non-patients members for their great assistance and cooperation in carrying out the research.

Mohammed Jaafar

2016

Summary

Acne vulgaris is a multifactorial inflammatory skin disease and the inflammation play an important role in its pathogenesis, many pro-inflammatory adipokines and cytokines contribute to this pathogenesis, from these IL-8 and resistin which act as pro-inflammatory mediators and encoded by different genes.

Forty nine Iraqi patients (21 male and 28 female) who were suffered from acne vulgaris have enrolled at dermatology center of Al-Sader medical City and other dermatology Clinic in Al-Najaf city, with ages ranged between 13 to 35 years old during the period from November/2015 to March/2016, compared with 25 healthy controls (9 males and 16 females) at age range of 14-40 years old. Blood samples were collected from both group member to measure the levels of resistin and IL-8 in their serum. Their DNA was extracted from whole blood for further molecular study; RETN -420 gene polymorphisms. The restriction fragment length polymorphisms-polymerase chain reaction (RFLP-PCR) was performed for that purpose by using BpiI restriction enzyme to cut the fragment where the polymorphisms will occur.

This study showed that 59.2% of the patients in the age range of (16-20) years old (52% of them was male and 48% of them was female), while 8.2% of the patients with clinical manifestations after 25 years old, all of them are females. This study showed that the patients who have mild (38.78%) and moderate (55.1%) acne vulgaris were more than those with severe cases(6.12%), also showed that males have high tendency to have severe acne than females. The family history of the disease was found in 43% of the patients. This result shows that the presence of such history was non-significantly correlated with the severity of the disease (*P-value*=0.52). Also, this study shows that there is no correlation

between the body mass index (BMI) and the severity of acne. While the BMI is correlated with the duration of disease that acne persist in obese patients more than non-obese. Also, there is no correlation between the mean duration of acne and severity of the disease.

This study shows that there is no significant correlation between the level of serum IL-8 and the development of acne vulgaris(*p-value*=0.174). Also, it shows that there is no correlation between the mean serum level of resistin and the development of acne vulgaris (*P-value*=0.082). But in contrast to the male the female shows a significance different between the case and control.(P value=0.29 for male and 0.03 for female).

The genotyping of RETN gene shows three genotypes; the homozygous CC type, the heterozygous CG & the homozygous GG type, the frequency of these types in acne patients were (6%, 14% and 80%) respectively, while in control group they were (8%, 24% and 68%) respectively. The frequency of all genotypes did not find any significant differences between acne patients and the healthy controls (P=0.53). No significant difference in genotype frequency regarding the gender between patients and the healthy group can be noted also between the same gender of patients and healthy group, as well as there was no association detected between this type of polymorphism and the degree of severity of acne (P value=0.74).

In conclusion, this study suggests that the levels of IL-8 in blood and RETN-420G>C single nucleotide polymorphism do not contribute with the pathogenesis of acne vulgaris and there was no significant association between different alleles and the severity of the disease in two gender. But it shows that the levels of resistin in blood is correlated with the presence of acne in female rather than in male.

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List of Abbreviations

Code	Meaning
AP	Activator protien
ADSF	Adipocyte-secreted factor
ACTH	Adrenocorticotropic hormone
α	Alpha
ADA	American Diabetes Association
Ab	Antibody
Ag	Antigen
Bp	Base Pair
BMI	Body Mass Index
JNK	c-Jun N-terminal kinase
cAMP	Cyclic adenosine monophosphate
DHES	Dehydroepiandrosterone sulphate
δ	Delta
DHT	Dihydrotestosterone
EBP	Enhancer binding protein
ELISA	Enzyme-Linked Immunosorbent Assay
EGFR	Epidermal growth factor receptor
EFAs	Essential fatty acids
EDTA	Ethelen Diamen Tetracetic Acid
FIZZ3	Found in inflammatory zone 3
γ	Gamma
GH	Growth hormone
GM-CSF	Granulocyte-macrophage colony-stimulating
HAT	Histone acetyl transferase
hBD2	Human b-defensin-2
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IFN-γ	Interferon gamma
IL	Interleukin
IL-8	Interleukin-8
KDa	Kilo Dalton

Kg	**Kilo Gram**
MMP	**Matrix metalloproteinase**
MSH-α	**Melanocyte stimulating hormone alpha**
mRNAs	**Messenger Ribo Nucleic Acid**
μl	**Microliter**
ml	**Milliliter**
MAPK	**Mitogen-activated protein kinase**
ng	**Nano Gram**
NAFLD	**Non-alcoholic fatty liver Disease**
NF	**Nuclear factor**
NFKB	**Nuclear factor that binds to the enhancer element**
No.	**Number**
OCT	**Octamer**
OR	**Odd Ratio**
ω	**Omega**
O.D	**Optical Density**
PBMCs	**Peripheral blood mononuclear cells**
PPAR	**Peroxisome proliferative-activated receptors**
pg	**Pico Gram**
PSU	**Pilosebaceous unit**
PCR	**Polymerase Chine Reaction**
ROS	**Reactive oxygen species**
RETN	**Resistin gene in human**
RFLP	**Restriction Fragment Length Polymorphism**
SNPs	**Single nucleotide polymorphisms**
SBP	**Systolic Blood Pressure**
TLR2	**Toll-like receptor 2**
TFs	**Transcription factors**
TBE	**Tris-Borate-EDTA**
TNF-α	**Tumor necrosis factor alpha**
T2DM	**Type 2 diabetes mellitus**
UV	**Ultraviolet**
UTR	**Untranslated Region**
WHO	**World Health Organization**

Chapter One

Introduction

&

Literature Review

1. Introduction and literature review:

1.1. Introduction

Acne vulgaris is one of the most common chronic skin diseases affecting adolescents, 90% of an adolescent being affected by some degree (Al-Salih, 2008), however, acne can remain throughout adulthood in 8 % of the patients (Grange *et al*., 2010).Clinically, adolescent acne consists of a combination of non-inflammatory (open and closed comedons) and inflammatory(papules , pustules, and nodules) lesions(Webster, 2005). Although acne is not a life-threatening disease, but still can lead to serious physical (permanent scarring and hyperpigmentation) and psychosocial difficulties (depression, anxiety, anger, impairment in self-image) (Fried, 2006). The pathogenesis of acne is currently attributed to multiple factors such as increased sebum production and alteration in its quality , increased androgen activity, follicular hyperkeratinization , proliferation of *Propionibacterium acnes(P. acnes)* and exhibition of pro- and anti-inflammatory properties (Kurokawa *et al.,* 2009). The affected persons' genetic background may also play an important role in acne predisposition, some studies showed that 81% of the variance of the disease was attributable to additive genetic effects and the remaining 19% was attributable to environmental factors(emotional stress, drugs, food , menstruation, smoking) (He *et al*., 2006).

Resistin, also called adipocyte-secreted factor (ADSF), found in inflammatory zone 3 (FIZZ3), is a novel hormone secreted by adipocytes (Holcomb *et al*., 2000). Resistin belongs to a family of cysteine-rich C-terminal proteins, termed RELMs (Resistin- Like Molecule) (Gerstmayer *et al*., 2003). Many studies was found that human resistin is mainly synthesized by non-adipocytes inflammatory resident cells such as

peripheral blood mononuclear cells (PBMCs), bone marrow cells, monocytes and most predominantly by macrophages (Kunnari *et al*., 2009). The resistin acts as an inflammatogenic cytokine have potent proinflammatory properties as it triggers the release of TNF-α, IL-1, and IL-6 (Bokarewa *et al*., 2005). In effect, mounting evidence has identified neutrophils as dominant sources of resistin at the site of inflammation and even systemically during severe bacterial infections (Kunnari *et al*., 2009). It promote inflammation (Bokarewa *et al*., 2005). The human resistin gene (RETN) is sited on chromosome 19p13 (Menzaghi *et al*., 2006). Several single nucleotide polymorphisms (SNPs) have been associated with resistin levels (Osawa *et al*., 2007). The levels of resistin and their gene polymorphism may contribute to many inflammatory diseases such as inflammatory bowel disease (Konrad *et al*., 2007), chronic pancreatitis (Adrych *et al*., 2009), systemic lupus erythematosus (Almehed *et al*., 2008), acne vulgaris (Al-Shobaili *et al*, 2012) and in other disease.

Interleukin (IL-8) is one of the main proinflammatory cytokines that plays an important role in initiating and regulating the cytokines cascades in the inflammatory process (Palmqvist *et al*., 2007). IL-8 is produced by leukocytes and other granulocytes, T cells, fibroblasts, some smooth muscle cells, epithelial and endothelial cells (Russo *et al*., 2014).The gene that encoding for IL-8 is located on chromosome 4q (Modi *et al*.,1990). There is many functional single nucleotide polymorphism (SNP) in the IL-8 gene have been identified. One of them at position -251 T to A substitution, and has a putative effect on the expression IL-8 and associate with many diseases such as increased risk of nephritis in cutaneous vasculitis (Amoli *et al*., 2002) therefore affecting the overall immune response. The association of -251T>A polymorphism with

susceptibility to inflammatory diseases have been widely reported. Many studies show that the levels of IL-8 were associated with many inflammatory diseases as a pro-inflammatory mediator such as in gingivitis and psoriasis (Vlahopoulos *et al*., 1999), and in few studies with acne vulgaris (Younis *et al*., 2016).

The relation between these markers (resistin and IL-8) and the acne vulgaris remain subject of controversy and to the best of our knowledge, there is no similar studies in Iraq or even, in the regions around. Thus this study was designed to evaluate the association of circulating resistin and IL-8 levels and RETN -420 C>G polymorphism with acne vulgaris in Al-Najaf population.

1.1.2. Aim of the study:

The aim of the present study is to investigate whether RETN-420 C>G gene polymorphism as a predisposing factor, and to get an idea about the serum levels of resistin and IL-8 as two important mediators during the course of Acne vulgaris among patients in Al-Najaf city. To achieve this goal, the following methodology has proposed on group of patients compared with apparently healthy individuals;

1. RFLP-PCR to detect RETN gene rs1862513.
2. ELISA, to detect the level of serum resistin.
3. ELISA, to detect the level of serum IL-8.
4. The statistical analysis to reveal the etiology/protection of polymorphism, associations between different variables.

1.2. Literature review:

1.2.1. Acne vulgaris

1.2.1.1. Historical review.

The first one that use the term 'acne' was the Emperor Justinian's physician, Aetius Amidenus in the sixth century AD. The term was translated from Greek into Latin when confusion arose regarding its original meaning. The debate continues as to whether its origin is from the Greek *acme,* meaning peak, or whether acne was actually the original term. Its use became obsolete until the I800s when 'acne' regained medical dictionaries.(Goolamali and andison, 1977 ; Tilles, 2014).

1.2.1.2. Anatomy of the pilosebaceous unit.

The hair follicle along with the associated sebaceous gland form the pilosebaceous unit (PSU), figure (1-1), the hair follicle size ranges from 10-70µm depending on the hair type and it's composed from three layers, outermost layer which is a keratinized layer continuous with the epidermis and indistinguishable from it and surrounding the inner root sheath (the second layer) and the follicle as a whole is surrounded by a cellular basement membrane known as glassy membrane. Sebaceous gland is collaborated to the hair follicle by a duct (Strauss *et al*., 1983), the common excretory duct which is supplied by smaller ducts that originate in the acini of the gland. As the flattened cuboidal peripheral sebaceous cells move toward the center of the gland, lipid synthesis within the cell increases (Singh *et al*., 2000).

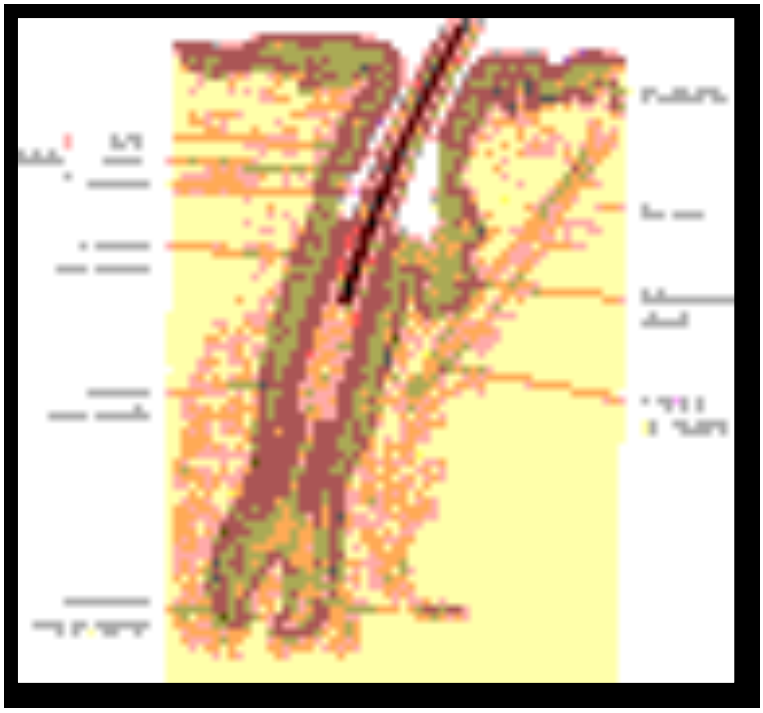


Figure (1-1): The anatomy of the pilosebaceous unit (Meidan *et al.*, 2005)

1.2.1.3. Definition of acne vulgaris:

Acne vulgaris is an inflammatory disorder of the pilosebaceous unit affecting up to 85% of the population. (Zouboulis *et al*., 2005 ; Dawson and Dellavalle, 2013) that characterized by seborrhea, formation of comedones, erythematous papules and pustules, less frequently by nodules, deep pustules, or pseudocysts on the face, neck, upper trunk and upper arm (Arura *et al*.,2011; Sharquie *et al.*,2012). The acne vulgaris is considered from the most common inflammatory skin condition, although often comprehension as a self-limited disease of adolescence, but also remain with high prevalence in adulthood. It is a multifactorial inflammatory skin disease of the component of the sebaceous gland hair follicle, causing scar and severe psychological effect on patients (Williams *et al*., 2012).

1.2.1.4. Epidemiology of the disease:

Acne is the eighth most prevalent disease globally affecting 79–95 % of the population (Murray *et al*., 2012). About 90% of teenagers in the united states have acne, and half of them continue to experience symptoms as adults (Yentzer *et al*., 2010 ; Thiboutot *et al*., 2009 ; Purdy. *et al., 2*006). An increasing prevalence of acne in children showed by many recent analyses , perhaps because of pubertal onset (Friedlander *et al*., 2011). So that acne may persist for decades and long-term therapy was required, there has been a recent effort to reclassify acne as a chronic disease (Thiboutot *et al*., 2009 ; Gollnick *et al*.,2008). Acne has clear detrimental psychosocial effects and may lead to permanent scarring (Barnes *et al*.,2012).

1.2.1.5. Clinical characteristics of acne vulgaris:

Acne vulgaris is a polymorphic inflammatory disease result in a physical symptom such as soreness, itching, and pain. The clinical features of acne include seborrhoea (excess grease), non-inflammatory lesions (open and closed comedones), inflammatory lesions (papules and pustules), and various degrees of scarring (Layton, 2010). The distribution of acne corresponds to the highest density of pilosebaceous unit (PSU) (face, neck, upper chest, shoulders, and back). Nodules and cysts comprise severe nodulocystic acne (Williams *et al*., 2012) as in figure (1-2).

1.2.1.5.1. Non-inflammatory acne.

Non-inflammatory lesions are the earliest lesions to develop in young patients (Lucky *et al.*, 1994) and include either:

a. Open (blackheads) comedon which represent dome-shaped papules in which there are dilated follicular opening filled with keratin, the black

color is thought due to melanin deposit and lipid oxidation within the cellular debris (Burke and Cunliffe, 1984).

b. Closed (white head) comedon are typically small-approximately 1mm-skin colored papules with no apparent follicular opening or associated erythema, better detected by stretching the skin(Pochi *et al*.,1991).

1.2.1.5.2. Inflammatory acne.

The inflammatory lesions of acne originate with comedon formation but then expand to form papules, pustules, nodules and cysts of varying severity as in figure (1-3) (Adityan, Kumari and Thappa, 2009). Erythematous papules range from 1 to 5mm in diameter. Pustules tend to be approximately equal in size and are filled with sterile, white pus, as the severity of lesions progresses, nodules form and become markedly inflamed, indurated and tender. The cysts of acne are deeper and filled with a combination of pus and serosanguineous fluid. In patients with severe nodulocystic acne, these lesions frequently coalesce to form massively inflamed complex plaques that can include sinus tract (Williams, Dellavalle and Garner, 2012).

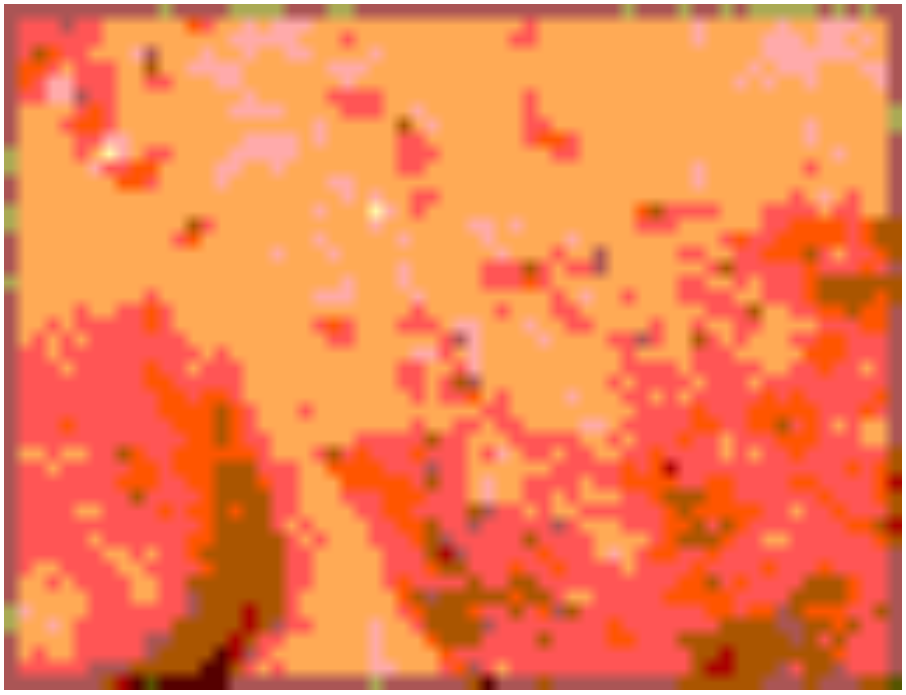


Figure (1-2): comedonal acne with black head comedon predominate (Lynn, 2007)

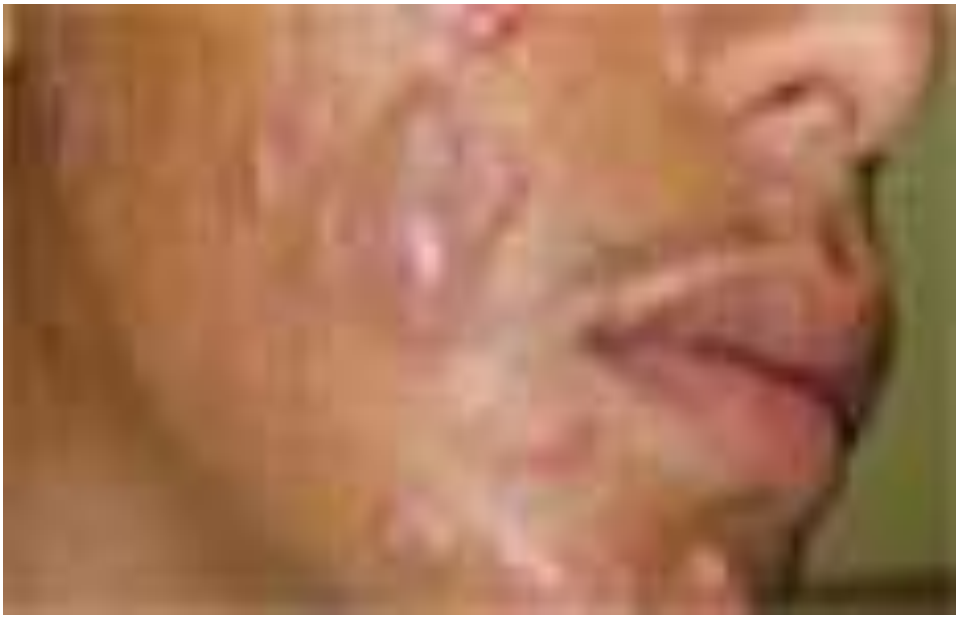


Figure (1-3) Case of nodulocystic acne (Lynn, 2007).

The comedone formation is intrinsic to the diagnosis of acne vulgaris when not clinically apparent, considers alternative diagnoses. Diseases that mimic acne include rosacea, folliculitis, angiofibromas, perioral dermatitis, and keratosis pilaris (Haider and Shalita, 2004 ; Friedlander *et al*., 2011 ; Archer *et al*., 2012). The patient's age may also help to distinguish these disorders from acne. Keratosis pilaris and perioral dermatitis, for example, tend to present in childhood, whereas rosacea tends to affect older adults. In cases of diagnostic uncertainty, referral to specialist care is warranted. Several groups have proposed standardized measures for classifying acne, although none has been universally accepted (Haider and Shalita, 2004 ; James *et al*., 2005 ; Nast A. *et al*., 2012). Classification is important because it helps to inform treatment strategies (Purdy *et al*., 2006 ; Strauss *et al*., 2007). Acne is classify into mild, moderate and severe acne groups, according to system developed for acne grading by Lehmann *et al* (Lehmann *et al.*, *2*002). Mild acne is typically limited to the face and is characterized by the presence of non-inflammatory closed and open comedones with few inflammatory lesions. Moderate acne is characterized by an increased number of inflammatory papules and pustules on the face and often mild truncal disease. Finally, acne is considered to be severe when nodules and cysts are present (Pochi, Shalita, Strauss and Webster, 1991).

1.2.1.6. Complication of acne vulgaris lesions.

a. Scarring, acne lesions have the potential to resolve with sequelae, almost all acne lesions leave a transient macular erythema after resolution, but in darker skin types, post-inflammatory hyperpigmentation may persist months after resolution of acne lesions(Zaenglein *et al.*,2008). The most disfiguring sequelae are acne scars, Scarring is a consequence of abnormal resolution or healing after the damage that

occurs in the sebaceous follicle during acne inflammation. Cell-mediated immune response not only contributes to the clearances of antigen but also to the tissue damage that leads to scar formation (Holland and Jeremy, 2005).

b. Psychological impact of acne vulgaris which depend on many factors including the personality, perceptions, age, and social and cultural factors, as well as disease characteristics (duration, severity, scarring) (Fried *et al.,* 2010). Acne negatively affects quality of life, in one of the Iraqi studies on the effects of acne on quality of life shows that females were more affected than males, age group 21–25 years more affected than the other age groups and the greater the grade "severity" of acne, the greater the level of impairment of quality of life (Ismail and Mohammed-Ali, 2012).

1.2.1.7. Etiology and Pathogenesis:

Acne is an inflammatory disease of the pilosebaceous duct that results from many primary pathophysiologic processes include: abnormal in keratinocyte proliferation and desquamation that leads to ductal obstruction, androgen driven lead to an increase in sebum production, proliferation of *Propionibacterium acnes* and Inflammation. (Gollnick *et al*., 2003 ; Haider and Shalita, 2004 ; Thiboutot *et al*., 2009 ; Abad-Casintahan *et al.*,2011 ; Williams *et al.*,2012). Other causes also include delayed-type immune reaction, external factors, and genetics (Gollnick, 2003 ; Zouboulis *et al.,* 2005).

1.2.1.7.1. Sebum secretions

The sebaceous gland in human is a multiacinar, holocrine-secreting tissue found in all areas of the skin except the soles and palms (and only sparsely on the dorsal surfaces of the hand and foot) (Zouboulis, 2004). The most noticeable function of the sebaceous gland is to excrete sebum.

Human sebum consists of squalene, esters of glycerol, wax, and cholesterol, as well as free cholesterol and fatty acids (Picardo *et al.*, 2009). Degradation of sebum occurs as it passes through the sebaceous duct by bacterial hydrolysis convert some of the triglycerides to free fatty acids (Zouboulis, 2001) which are comedogenic and cause inflammation in and around hair follicles(Zouboulis, 2004).

Hormonal Control of Sebaceous Gland: Acne vulgaris first develops at the onset of puberty as a result of hormonal changes, Several hormones implicated in the regulation of sebaceous gland activity and hence linked to acne (Melnik and Schmitz,2008),which include :

Androgens: With the onset of puberty, androgen (from testes, ovaries, and adrenals) mediates stimulation of the sebaceous gland results in increased sebum production in both sexes. They are without a doubt the most important hormones controlling sebaceous gland activity (George *et al*,2008).Acne starts at the time of adrenarche when adrenals and gonads being to produce dehydroepiandrosterone sulphate (DHES) which is the precursor of testosterone (Zouboulis *et al.*, 2005),testosterone is then converted by the iso-enzyme 5α-reductase to dihydrotestosterone (DHT) , which is (5-10) times more potent than testosterone, (DHT) is then bound to specific cytosol receptor of the sebaceous gland to stimulate the genes involved in the production of sebum(Auffret, 2003), and the targets are sebaceous glands of the face, back and chest armored with highly sensitive androgen receptors which are localized at the basal layer of the sebaceous glands and the outer root sheath of hair follicles (Thiboutot *et al.*, 2004). The increased androgen production causes abnormal epithelial desquamation and follicular obstruction, which lead to the primary precursor lesion in acne which called micro comedones. Microcomedones are pathological structures not visible to the naked eye that evolve into visible lesions (Thielitz and Gollnick, 2008). An increase

in circulating androgens also promotes sebum production, causing these obstructed follicles to fill with lipid-rich material and form visible open and closed comedones (Brown *et al*., 1998 ; Chen *et al.,* 2002).

Role of Estrogen hormone in acne vulgaris: Unlike male hormone androgens, female hormone estrogens have beneficial effects on acne, that is why some physicians recommend birth control pills for women who have acne(Arura *et al.,* 2011).It has been hypothesized that estrogens may act by reducing endogenous androgen production in acne vulgaris by one of several mechanisms, including (**1**) direct opposition of androgens within the sebaceous gland (Lucky, 2004 ; Jerzy *et al*., 2005) (**2**) inhibition of androgen production by the gonads through a negative feedback loop on gonadotrophin release, or (**3**) regulation of genes involved in sebaceous gland growth or lipid production (George *et al*, 2008 ; Thiboutot *et al*, 2004).

Role of progesterone on acne vulgaris: The effect of progesterone on sebaceous glands has been a matter of dispute. The fluctuation of sebum production in women during menstrual cycle has been blamed on progesterone and progesterone administration can produce acne (Simpson *et al.,* 2004)possible mechanism by which progesterone aggravates acne lesions is by increasing sebum secretion and stimulation of proliferation of keratinocytes (Kanda *et al*., 2005).

d. The role of glucocorticoid on acne vulgaris: Although corticosteroids themselves exert immunosuppressive or anti inflammatory effects, it is well known clinically that systemic or topical glucocorticoid treatment enhance Toll-like Receptor 2 (TLR2) gene expression in human keratinocytes provoking an acneiform eruption(Marta *et al*., 2006 ; Shibata *et al*., 2009 ; Arura *et al.*, 2011).

1.2.1.7.2. Hyperkeratinization of the pilosebaceous duct.

One of the most crucial initial events in the development of acne lesions is hyperkeratinization in the follicular infundibulum resulting in microcomedon, which develop into full comedon that is either open or closed head comedon (Ferrar and Ingham, 2004). During comedogenesis, two changes in the normal pattern of keratinization occur: (1) hyperproliferation of keratinocytes lining the follicle wall and (2) reduced desquamation due to increased cohesion between keratinocytes. These changes lead to an accumulation of cornified keratinocytes and blockage of the follicular opening (Holland andJermey, 2005).

1.2.1.7.3. Propionibacterium *acnes* (*P. acnes*) hyperproliferation.

Acne is not an infectious disease and, therefore not contagious (Jappe, 2003). *Propionibacteria* are Gram-positive, anaerobic, non-motile, pleomorphic rod-shaped cells that ferment sugars to yield propionic acid as one of the end products in this metabolic process. it is the predominant resident microorganism on sebaceous gland rich areas of skin in adults (Jappe, 2003), but may also inhabit conjunctiva, respiratory tract, gastrointestinal tract and genitourinary tract of human (Alexeyev *et al.*, 2007).The pathogenicity of *propionibacteria* is thought to be due to, first, releasing various enzymes which lead to rupture of follicular walls and tissue injuries such as lipases, proteases, and hyaluronidases. , Second, on the microorganism's interaction with the immune system. *P. acnes* has been shown to stimulate the production of the antimicrobial peptide human b-defensin-2 (hBD2), the chemokine CXCL8 (IL-8), and the cytokine TNF-α in skin cells such as sebocytes (Nagy *et al*., 2006). In keratinocytes, the activation of hBD2, TNF-α, GM-CSF, and IL-1α, IL-1β, and IL-8 has been observed upon contact with the bacterium .*P. acnes* can induce the production of proinflammatory cytokines IL-1β, IL-8, IL-12, and TNF-α (Chen *et al*., 2002; Nagy *et al*., 2005) from monocytes.

1.2.1.7.4. Inflammation in acne vulgaris.

The pilary canal is plugged by the mechanical effect. As the hormone-driven keratinocytes multiply, they are propelled towards the center of the duct, which expands to accommodate the increasing bulk of the microcomedon until a point is reached beyond which the inelastic 'glassy membrane' that encloses the pilosebaceous duct can expand no further. Further production of keratinocytes into this closed system causes an increase in intraluminal pressure and this causes hypoxia centrally in the duct. This produces an anoxic environment that favors the development of intraductal *P. acnes* colonies, leads to rupture of the duct walls, release of the luminal antigens to the deeper layer of the skin and worsening of the condition during these events both acquired and innate immune system participate in its pathophysiology (Kurokawa *et al.*, 2009).

A. **Innate Immunity**

Polynuclear neutrophil chemotaxis: *P.acnes* produces many substances that diffuse into the pilosebaceous follicle, (15%) of these have low molecular weight and can easily cross the comedon wall, they have the chemotactic potential to attract polynuclear neutrophils (Pawin *et al.*, 2004).

Proinflammatory cytokines: Interleukin-1α(IL1α) , TNFα and IL8 secreted by keratinocyte, amplify the inflammatory reaction in the pilosebaceous follicle and polynuclear neutrophil chemotaxis, IL-1α encourage the formation of comedon, interferon gamma and TNF-α , the diffusion of the inflammatory reaction (Downi *et al.,* 2004) as in figure (1-4) that show the normal sebaceous follicle develops into a micro comedones and a comedone through keratinocyte hyperproliferation and reduced sloughing. This process is associated with an increase in levels of IL-1. *P. acnes* population density increases. Initiation of inflammation

may occur through either a specific CD4_ T-cell–mediated pathway or a nonspecific pathway involving increased production of proinflammatory cytokines by keratinocytes. Inflammation is augmented by activation of macrophages and neutrophils. Finally, the inflammatory response is down-regulated, allowing the lesion to heal (Farrar and Ingham, 2004).

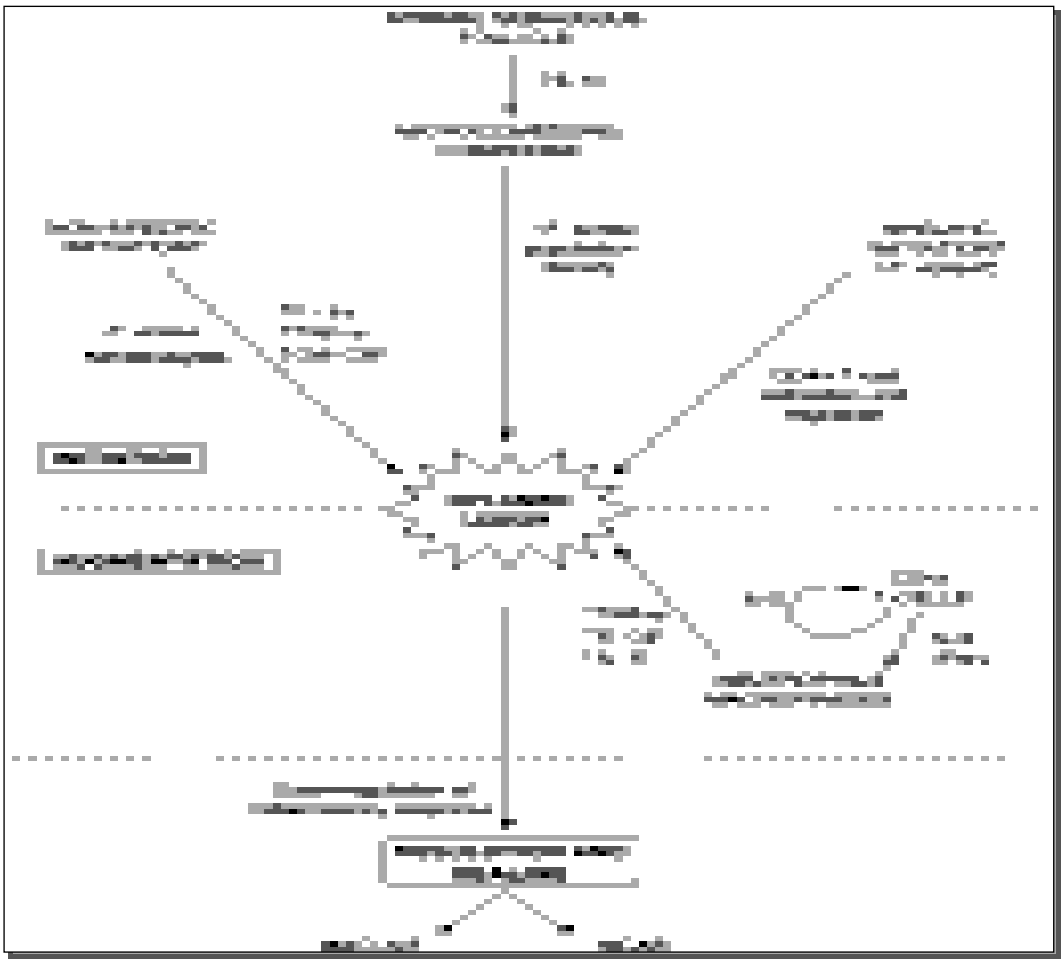


Figure (1-4): Events in the evolution of an inflammatory acne lesion (Farrar and Ingham, 2004).

Toll-like receptors (TLRs): Toll-like receptors expressed on the cell surface of macrophages surrounding pilosebaceous follicles (Kim *et al.*, 2002). TLR regulate the production of proinflammatory cytokines by activated it, in particular IL-1α, IL-8, TNFα and metalloprotease (Kawai *et al.*, 2002; Simpson *et al.*, 2004). The expression of active Toll-like receptors 2 and 4 and CD14 in human keratinocytes (Pivarcsi *et al*., 2003)

have implicated *P. acnes* and Toll-like receptors in acne inflammation. However, *P. acnes* were unable to induce IL-1α expression in human keratinocytes in vitro (Ingham *et al*., 1998), therefore, *P. acnes* seems to induce later events not being involved in the initiation of acne lesions.

B-defensins -1 and -2: B-defensin are antimicrobial peptides produced by keratinocytes (Ali *et al.*, 2004), they act by making target membrane permeable (Jones, 2005).

Matrix metalloproteinases (MMPs): MMP-9 are produced by keratinocytes and polynuclear neutrophils mainly, these endopeptidases have a role in the destruction of the pilosebaceous wall and diffusion of inflammation to deep layers of the skin and may participate in scar formation (Pawin *et al.*, 2004; Trivedi *et al*., 2006).

Peroxisome proliferative-activated receptors (PPAR): induce the production of IL-1 and TNF-α and therefore involved in acne formation (Pawin *et al.*, 2004).

Melanocortin 1 receptors: expression was demonstrated in sebocytes of normal human skin; receptor activation by Melanocyte stimulating hormone alpha MSH-α (a neuromediator) may decrease the production of (IL-8) and thus act as a modulator of the inflammatory response (Bohm *et al.*, 2002 ; Pawin *et al.*, 2004).

Complement deposition: is stimulated by extracellular products of *P.acnes* which is a strong promoter of both, the classical pathway (which requires the presence of anti-bacterial antibodies) and the alternative pathway (which is triggered by cell wall carbohydrates) (Burkhart *et al.*, 1999).

B. Acquired Immunity:

Cellular Immunity: The peri-lesional cell infiltrate in the first four hours of inflammation of an acne lesion is essentially composed of CD4+ lymphocyte (Jermy *et al.,* 2003). CD8+ cells occur perivascularly and periductally and CD1+ cells are present in low numbers in these locations (Jappe *et al.,* 2002), indicating that keratinocytes and sebocytes function as antigen-presenting cell and activate natural killer T cells by presenting lipids antigens with the help of CD1+ molecules (Koreck *et al.,* 2003).

Humoral Immunity: Antibodies to *P.acnes* are present in all humans and the correlation to disease severity is unclear, it has been shown that the level of IgG1 and IgG3 was significantly higher in patients with severe acne compared to those with moderate acne (Ashbee *et al.,* 1997; Pawin *et al.,* 2004). *P.acnes* has superantigenic activity by some membrane fraction of it causing amplification of inflammatory reaction; this phenomenon may perhaps play a role in the development of acne fulminans after the first week of treatment with isotretinoin (Pawin *et al.,* 2004).

1.2.1.8. Environmental factors influence acne.

1.2.1.8.1. Nutrition:

Acne is driven by hormones and growth factors [particularlyIGF-1] acting on the sebaceous glands and the keratinocytes lining the pilary canal (Kurokawa *et al.,* 2009). Milk and dairy products contain 5α-reduced steroid hormones and other steroid precursors of DHT that drive sebaceous gland function (and likely pilar keratinocyte) (Kurokawa *et al.,* 2009). Drinking milk causes a direct rise in IGF-1 through a disproportionate elevation in blood sugar and serum insulin levels (Hoyt *et al.,* 2005), these hormones stimulate adrenal and gonadal androgen production leading to increase sebum production and perhaps, to acne.

High glycemic load foods also cause IGF-1-mediated elevations in DHT (Charakida *et al.*, 2007). IGF-1 levels during teenage years closely parallel acne activity and are likely synergistic with the steroid hormones. Linoleic acid likewise has an ambivalent role in acne (Namazi, *2004*), Foods containing ɷ-3 essential fatty acids (EFAs) and EFA supplements may help to control inflammation (Treloar *et al.*, 2008) and may reduce acne risk by decreasing IGF-1 levels and preventing hyperkeratinization of sebaceous follicles (Zouboulis *et al.*, 2005). Danby (2008) found in his study that Acne can be improved by controlling hormones and inflammation, both of which are influenced by diet; he mentioned that full acne control requires dietary control concurrent with standard anti-acne therapy, all dairy products, and all high glycemic foods should be stopped for at least 6 months to plugging of pores in deficient individuals.

1.2.1.8.2. Premenstrual flare of acne vulgaris:

In female 60%-70% complain from flare of acne premenstrualy, the increase in acne comes about 7-10 days before the onset of menstruation, and often subsides when menstruation begins (Dreno *et al.*, 2003)

1.2.1.8.3. Sweating:

Up to 15% of acne patients notice that sweating causes deterioration of their acne especially if they live or work in a hot humid environment (Simpson and Cunliffe , 2004).

1.2.1.8.4. Smoking and Acne:

Previous investigation studies revealed smoking to be a clinically important contributor to acne vulgaris prevalence and severity (Capitanio, 2009), in fact, even data exists on possible smoking protective effects due to the anti-inflammatory properties of nicotine (Klaz *et al.*, 2006). Also another study revealed the association between acne and smoke shows an increased risk. Moreover, people ≥ 18 years of age have twice the risk compared to persons < 18 years of age (Mannocci *et al.*, 2012)

1.2.1.8.5. Effect of the sun-light on acne:

Although there was some evidence to suggest that various spectra of artificial light may be beneficial this could not be generalized to natural sunlight (Parker *et al.,* 2005*)*. Other important considerations for not advocating natural sunlight as a treatment for acne relates to the potential long-term sequelae of sun exposure including an increased risk of melanoma and non-melanoma skin cancer, an increased risk of photosensitivity whilst the patient on some acne medications, particularly tetracycline and isotretinoin (Layton, 2010).

1.2.1.8.6. Stress and Acne vulgaris:

Acne flares have been seen during periods of stress. Stress is known to increase the output of adrenal steroids , corticotropin-releasing hormone, melanocortins, beta-endorphin, substance P (Levenson, 2008) ,while acne itself induce stress (Thibotout *et al.*,2009).

1.2.1.8.7. Drug induce acne:

Many drugs have been implicated as possible aggravators or inducers of acne. Acneiform eruptions account for about 1% of all drug-induced skin eruptions that shownin Table (1-1). Clinically acne presents as monomorphic inflammatory papules and pustules with little evidence of comedones, contrasting with the heterogeneous morphology usually seen in normal acne (Melink *and* Schmitz, 2008). The face and upper trunk are most frequently affected). Recently, a new class of therapeutic agents; epidermal growth factor receptor (EGFR) antagonists (gefitinib, erlotinib, cetuximab) – have been recognized as a trigger of acneiform eruptions (Degitz *et al.*,2007).

Table (1-1): **Drugs exacerbating acne or causing acneiform eruptions (William and Layton, 2006).**

Corticosteroids	Systemic And Topical Steroids, ACTH
Anabolic Steroids	Danazol, Stanozolol, Synthetic Androgens
Antiepileptic	Carbamazepine, Phenytoin, Gabapentin, Topiramate
Antidepressants	Lithium, Sertraline, Amitryptiline, Barbiturates, Chloral Hydrate
Antipsychotic	Pimozide, Risperidone
Antitubercular	Isoniazid, Rifampicin, Pyrazinamide
Antineoplastic	Dactinomycin, Pentostatin
Antiviral	Ritonavir, Ganciclovir
Immunosuppressive	Ciclosporin
Halogens	Potassium Iodide, Sodium Flouride, Halothane
Vitamins	Cyanocobalamin (B12), Vitamin B6

1.2.1.9 Genetic predisposing factors in pathogenesis of acne vulgaris:

Genetic studies have a 100-year history, classic twin and population-based studies provided unambiguous evidence concerning the role of inherited factors in the pathogenesis of acne(Cordian *et al.*, 2002 ; Ghodsi *et al.*, 2009) that can lead to an enhanced sensitivity to various external stimuli, which enhances the proneness to the development of chronic inflammatory diseases (Melnik and Schmitz ,2009 ;Duvnjak and Duvnjak ,2009). In this state, small changes in the levels of various pro-inflammatory cytokines or in the metabolism of hormones and other bioactive small molecules will lead to chronic inflammatory diseases.

Such small changes can arise from genetic polymorphisms that affect the expression and/or function of key genes(Szabo and Kemeny ;2011). The strong involvement of familial inheritance is noted in patients who display severe inflammatory symptoms (Goulden *et al.,* 1997; Dreno *et al.,* 2003; Evans *et al.,* 2005 *;* Ghodsi *et al.,*2009).This is especially true for families in which the affected individuals are found on both the maternal and the paternal side, although the maternal influence is more important (Ghodsi *et al.,*2009).

1.2.1.10. Treatment of acne vulgaris:

In acne, the therapeutic approach should begin with a careful assessment of the patient's history and education, because there are many myths surrounding this disease. It is important to emphasize that acne is not infectious, not caused by poor hygiene, female patients should be informed that acne may worsen during the week before menses and patients of both sexes should be advised against picking at lesions. In addition, patients should receive instructions about proper skin care and application of topical medications also they need to understand that, although cases of acne can be cleared with existing medications, therapy requires time, and improvement may not be very apparent until after four to six weeks of therapy (sometimes longer).(Gollnick *et al.,* 2003).

Topical therapies are the standard of care for mild to moderate acne. Retinoids and antimicrobials such as benzoyl peroxide and antibiotics are the mainstays of topical acne therapy. Such treatments are active at application sites, and they can prevent new lesions (Kraft and Frieman, 2011).

Systemic therapy: Patients with mild acne can be treated with topical therapies; however, those with moderate to severe acne and in those with scarring acne will require systemic therapy regardless of disease severity.

Hormonal therapies, and isotreretinoin are the mainstay systemic therapies for acne (Eichenfield *et al*., 2010).

Physical treatments for acne include comedon extraction, chemical peels (Strauss *et al.,* 2007) microdermabrasion, intralesional cortico-steroid injection for acne cysts, and high-intensity, narrow band blue light photodynamic therapy as well as injectable fillers and laser resurfacing for acne scarring (Kraft and Frieman, 2011).

1.2.2. Resistin:

1.2.2.1. Definition of resistin:

Resistin is a member of a secretory protein family, known as resistin-like molecules (RELMs) (Steppan and Lazar, 2004). Resistin is a macrophage-derived signaling polypeptide hormone secreted by adipocytes with molecular weight 12.5 kDa and its length is 108 amino acids in humans (Nogueiras *et al*., 2010). It was originally named for its resistance to insulin (Steppan *et al*., 2001). Resistin is expressed in white adipose tissue with the highest levels in female gonadal adipose tissue (Steppan and Lazar, 2002), which is highly expressed in omental and abdominal subcutaneous white fat than in adipose tissue from the thigh or breast (McTernan *et al*., 2002). It plays a principal role in the regulation of insulin sensitivity (Steppan *et al*., 2001). However, the blood circulating levels of resistin have been shown to be upregulated in subjects with insulin resistance, T2DM, metabolic syndrome and cardiovascular disease (Chen *et al*, 2009; Momiyama *et al*., 2011).

1.2.2.2. Resistin as inflammatory adipokine:

Several recent human studies have supported the concept of inflammatory cytokine mediation of resistin (Mattevi *et al*., 2004), however, resistin associations with inflammatory markers appear to be independent of

BMI, suggesting that resistin may have a direct proinflammatory role or mediate its effects via yet to be discovered obesity-independent mechanisms(Greeshma *et al*., 2004)

More importantly, resistin may participate in the inflammatory cascade reaction as a proinflammatory mediator (Tilg and Moschen, 2006). Interleukins and microbial antigens can enhance the activity of the resistin gene in basal sebocytes(Kaser *et al*., 2003 ; Lu. *et al*., 2002). Resistin, via its receptor, activates NFkB and JNK pathways to promote transcription of TLR-2, IL-1, IL-6, IL-12, and TNFa (Lee *et al*., 2014 ; Tilg and Moschen, 2006 ; Bokarewa *et al*., 2005 ; Silswal *et al*., 2005). In addition, resistin up-regulates chemotactic proteins responsible for leukocyte recruitment to sites of infection and inflammation (Verma *et al*., 2003; Kang *et al*., 2013). Another study also suggested that resistin has a proinflammatory action in hematopoietic stem cells (HSCs), thereby participating in liver fibrogenesis (Bertolani *et al*., 2006).

1.2.2.3. Resistin (RETN) gene:

A recently published investigation (Yannakoulia *et al*., 2003) demonstrated that serum resistin levels are positively correlated with the amount of body fat mass in humans. The human resistin gene (RETN) is located on chromosome 19p13.3 (Ghosh *et al*., 2003). Several single-nucleotide polymorphisms (SNPs) have already been identified in the RETN gene, all mapped to non-coding regions, but only a few have minor allele frequencies of 5%, and thus could provide useful information in association studies. Several studies investigated the association of these gene variants such as RETN-420C/G and+62G/A are associated with type 2 diabetes mellitus (T2DM) and hypertension (Wang *et al., 2*002; Tan *et al., 2*003 ; Tsukahara *et al*., 2009) , with few indications of positive

results (Ma *et al.*, *2*002; Tan *et al.*, *2*003). Genetic factors, as well as environmental factors, are important for the development of Non-alcoholic fatty liver disease (NAFLD) (Merriman *et al*., 2006 ; Gambino *et al*., 2007). However, the correlation between RETN-420 G/C polymorphism and acne vulgaris is not known.

Functional polymorphisms in the promoter and intron region of cytokine and adipocytokine genes affect transcriptional activity defining the level of proteins in biological fluids. Specifically, several polymorphisms in the resistin gene have been described to determine plasma resistin concentrations (Cao and Hegele. 2001 ; Cho *et al*., 2004). Among these, the C<G polymorphism at -420 site of resistin gene is functional and it increases transcriptional activity by promoting binding of Sp1/3 transcription factors (Osawa *et al*., 2004). Moreover, the G allele at -420 has been found to be associated with higher resistin mRNA and serum levels(Cho *et al*., 2004 ; Osawa *et al*., 2005).

1.2.2.4. Resistin and acne vulgaris:

Several single nucleotide polymorphisms in pro-inflammatory cytokine genes have been associated with the pathogenesis of acne (Al-Shobaili *et al*., 201). Among the cytokines, resistin (RETN) gene is relatively novel and little known about the role of resistin in the pathophysiology of *Acne vulgaris*. Therefore, it could be remarkable to study the link of a functional polymorphism of the RETN gene at -420 with *Acne vulgaris*.

1.2.3. Interleukin-8 (IL-8):

1.2.3.1. Definition:

Inflammatory chemokines (chemotactic cytokines) are a family of small (8-15 kDa) proteins sharing common structural and functional motifs which traffic leukocytes to areas of injury(Zlotnik and Yoshie, 2000)). Chemokines are divided into four subfamilies: CXC, CC, CX3C

and XC based on the number and position of four conserved cysteine residues in the N-terminal end of the protein (Pease and Williams, 2006 ; Russo *et al*., 2014). To date, around 50 chemokines and 18 chemokine receptors have been identified (Steinke and Borish, 2006, Colobran *et al*., 2007 ; Balkwill, 2004). The majority of chemokines perform their functions via binding of their N-terminal region (Deshmane *et al*., 2009) to G-protein coupled receptors present on different cells including leukocytes and endothelial cells (Murphy, 1994 ; Mélik-Parsadaniantz and Rostène, 2008). This reaction initiates various intracellular processes activating different signaling pathways and corresponding physiological effects. An additional 28 complexity is achieved as cells express receptors for several chemokines and are a target for several mediators with overlapping effects (Murdoch and Finn, 2000 ; Viola and Luster, 2008). Chemokines play a pivotal role in the immune response due to their ability to sample antigen and recruit/direct leukocytes to the site of injury or infection by trans-endothelial migration (Van Coillie *et al*., 1999 ; Zlotnik and Yoshie, 2000 ; Speyer and Ward, 2011). Chemokines also play a role in host immune responses, homeostasis, T cell development, angiogenesis, wound healing, and immune surveillance (Zlotnik and Yoshie, 2000 ; Steinke and Borish, 2006 ; Speyer and Ward, 2011). Chemokines are classified as inducible (inflammatory) or constitutive (homeostatic). Inducible chemokines are induced by bacterial products, growth factors such as TGF-ß, pro-inflammatory mediators such as IL-1 and several pathophysiologic conditions both independently and in cooperation with other stimuli (Brat *et al*., 2005). In contrast, constitutive chemokines are expressed in the absence of infection or damage (Colobran *et al*., 2007).

IL-8, or CXCL8 based on the latest nomenclature, represents the prototypical chemokine of the CXC subfamily (Remick, 2005). IL-8 is

actively secreted into the extracellular space as a result of a variety of cellular stimuli. IL-8 is a small protein; its mature, fully active form has only 72 amino acids. Transcription of the IL-8 gene encodes for a protein of 99 amino acids that is proteolytically cleaved to a biologically active peptide of either 77 amino acids in non-immune cells or 72 amino acids in monocytes and macrophages (Waugh and Wilson, 2008). IL-8 is secreted by leukocytes and other granulocytes, T cells, fibroblasts, airway smooth muscle cells, endothelial and epithelial cells (Brat *et al*., 2005). It is induced by TNF-α, IFN-γ, other chemokines including IL-1, bacterial flagella and the lipopolysaccharide (LPS) component of the bacterial wall, and viruses (Hoffmann *et al*., 2002 ; Shi *et al*., 2004 ; Venza *et al*., 2009). IL8 acts on two heterotrimeric G protein-coupled surface receptors, CXCR1 and CXCR2 (Nasser *et al*., 2009) expressed on the surfaces of leukocytes (mostly granulocytes) and endothelial cells. IL-8 receptors share 78% homology, but differences in the N-terminal domains result in different binding peculiarities (Russo *et al*., 2014). Whereas CXCR1 binds IL-6 and IL-8, IL-1, 2, 3, 5, 6, 7 and 8 have a higher affinity towards CXCR2 (Balkwill, 2004).

IL-8(CXCL8) functions: IL-8 has a range of biological functions including promotion of directed chemotaxis in target cells and their migration to the site of inflammation (Qazi *et al*., 2011). The sequence of physiological reactions prerequisite for migration and phagocytosis includes an increase in intracellular calcium (Ca^{2+}) levels, exocytosis, release of a variety of lysosomal enzymes from activated neutrophils, and the respiratory burst (Brat *et al*., 2005). The latter is vital in allowing phagocytes to degrade bacteria through the swift release of Reactive oxygen species (ROS) from immune cells including neutrophils and monocytes coming into contact with bacterial particles. IL-8 can also promote neutrophil adhesion to endothelial cells and their trans-

endothelial migration (Mukaida, 2003 ; Qazi *et al*., 2011) as well as neutrophil activation (Qazi *et al*., 2011) and histamine liberation from human basophils (Brat *et al*., 2005). IL-8 is also involved in the regulation of ion transport, activation and proliferation of cells including epithelial cells, phagocytosis, angiogenesis and tumorigenesis (Rossi and Zlotnik, 2000 ; Brat *et al*., 2005). Collectively, all these properties and functions indicate that IL-8 is a key component of the inflammatory response.

IL-8 transcription and regulation: IL-8 transcription and regulation Gene expression are tightly regulated by well-established mechanisms resulting in the transcription of target genes in response to stimulation by specific signal transduction pathways that can either activate or silence gene expression (Venters and Pugh, 2009). Gene expression is mediated via a coordinated binding of different TFs rather than by sole presence or absence of a single TF (Hoffmann *et al*., 2002). The IL-8 expression is also regulated post-transcriptionally by stabilization of mRNA transcripts (via the p38 mitogen-activated protein kinase (MAPK) pathway); stationary mRNA levels are usually comparative to IL-8 secretion (Hoffmann *et al*., 2002 ; Li *et al*., 2002 ; Shi *et al*., 2004). Sequencing analysis of the IL-8 promoter region has shown that the 5'-flanking region encompassing an area from -425 to -70 (Hoffmann *et al*., 2002 ; Mukaida, 2003) relative to the transcription start site comprises binding sites for various TFs including CCAAT/enhancer binding protein (C/EBP)β, nuclear factor (NF)-$_{KB,}$ activator protein (AP)-1, and octamer (Oct)-1 binding proteins (Campbell *et al*., 2013 ; Brat *et al*., 2005 ; John *et al*., 2010) as in figure (1-5). Synchronized binding of NF-$_{k}$B, AP-1, and C/EBPβ is required for the integrated effect and ultimate activation of IL-8 transcription upon induction by inflammatory stimuli (John *et al*., 2009, Verhaeghe *et al*., 2007b, Hoffmann *et al*., 2002). Though all three factors

are involved in the regulation and transcription of IL-8, studies using transient transfections in cancer cell lines have demonstrated that IL-8 expression is NF-kB-driven. Although different members of the kB/RelA family have a different DNA-binding affinity, it is RelA that influences IL-8 gene transcription (John *et al*., 2009 ; Chen *et al*., 2002). NF-kB then cause recruitment of a large co-activator complex incorporating histone acetyltransferase (HAT) proteins such as cAMP response element binding protein (CREB) binding protein (CBP) and p300/CBP (PCAF), transcriptional intermediary factor-2 (TIF-2), p160 family members and steroid receptor coactivator-1 (SRC-1) (Jenkins *et al*., 2001 ; Adcock *et al*., 2006). Histone acetylation and/or DNA methylation can also influence IL-8 transcription (Muselet-Charlier *et al*., 2007 ; Bartling and Drumm, 2009).

Figure (1-5): Schematic representation of the human CXCL8 promoter region. The CXCL8 gene promoter contains binding sites for C/EBPβ, NF-kB, and AP-1 located in close proximity to each other (Richmond, 2002).

1.2.3.2. IL-8 and acne vulgaris:

More recently, additional single nucleotide polymorphisms (SNPs) in IL-8 that can be linked to cellular metabolic processes have been reported (Sobjanek *et al*, 2013). Little is known about the role of IL-8 in the pathophysiology of acne vulgaris. Studies have shown that IL-8 is an inflammatory chemokine, and is involved in various clinical conditions

including acne, which is mediated by the migration of cells to the site of inflammation (Oka *et al*., 2000 ; Sobjanek *et al*., 2013). The increased expression of IL-8 in skin biopsies showed a significant association with epidermal hyperplasia and follicular hyperkeratosis in inflammatory acne vulgaris. (Abd El All *et al.*, 2007). Gene encoding IL-8 is located on chromosome 4q12-q21 (Baggiolini *et al*., 1995). Several functional SNP in the IL-8 gene have been identified, one of them represents T to A substitution at position -251, and has a putative effect on IL-8 expression. (Andia *et al.*, 2011). The association of -251T>A polymorphism with susceptibility to inflammatory diseases have been widely reported. However, the association between IL-8-251T>A polymorphism and acne vulgaris is not very clear and recommended to be studied comprehensively (Sobjanek *et al* ,2013).; therefore, it could be remarkable to study the functional relationship of IL-8 gene polymorphism and IL-8 levels with acne vulgaris.

1.2.4. Previous studies about acne vulgaris:

There are many previous studies showed that acne development depends on the activity of sebocytes and the subsequent activation of inflammatory cytokines. Twins studies proved genetic relation with acne (Bakry *et al*., 2014). Other reported that single nucleotide polymorphisms (SNPs) in promoters of IL-6, IL-1a and TNFa cytokines genes are associated with risk of acne development in Pakistani and other populations(Aisha *et al*., 2015 ; Hamadi and Ethar, 2013 ; Szabo and Kemeny, 2011 ; Younis and Javed, 2014). Although the information about the mechanisms of formation and progression of acne is available, the main regulators that can be targeted to heal the disease remain unidentified. Other studies showed the polymorphism in the IL-8 Gene Promoter (Hussain, Iqbal, Sadiq, Feroz, and Satti. 2015) and Resistin gene polymorphisms (Hussain, Faraz and Iqbal 2015; Younis,

Blumenberg and Javed, 2016) predispose the risk of Acne Vulgaris. While other studies showed that there is no correlation between IL-8 and acne vulgaris. (Sobjanek, Gleń and Nedoszytko, 2015)

So we hypothesized that resistin, an adipocytokine protein found in follicles, and may participate in key processes of acne pathogenesis, also hypothesized that IL-8, a tissue-derived endogenous chemotactic polypeptides (chemokines) the key mediators in the migration of leukocytes to sites of inflammation, injury, and infection, may participate with the same disease.

Chapter Two

Materials &Methods

2.Materials and Methods:

2.1.Materials:

2.1.1.Instruments and Equipments:

The equipments and Instruments that used in this study are listed in table (2-1).

Table(2-1): The instruments and equipments.

	Instruments and equipment	Manufacture's (State)
1	Syringe	MEDECO (Germany)
2	Cotton	China
3	Alcohol	IOSEPT (Turkey)
4	EDTA tube (anticoagulant tube)	Sun (H.K.J)
5	Gel tube	AFCO (Jordan)
6	Centrifuge	Jolabo (Germany)
7	Plain tube	SUN (H.K.J)
8	Rack	SUN (H.K.J)
9	Refrigerator	Hetticl (Germany)
10	Deep freezer	GFL (Germany)
11	Beakers	AMSCO (Germany)
12	Cylinders	GFL (Germany)
13	Flasks	AMSCO (Germany)
14	Ependrof tubes	Sterline Ltd. (UK)

		bioneer (Korea)
15	Incubator	Jarad (Syria)
16	Water bath	Kottermann (Germany)
17	Micropipette (different sizes)	Eppendorf (Germany)
18	Multichannel micropipette	Eppendorf (Germany)
19	Micro-plate washer.	Bio TeK (U.S.A)
20	Vortex	Stuart (UK)
21	Micro-titer plate reader	Bio TeK (U.S.A)
22	PCR tubes	Sterilin Ltd. (UK)
23	Tips	Sterellin Ltd. (UK)
24	Nanodrop device	Biodrop (UK)
25	Thermo cycler	Sprint-IP20 (USA)
26	Gel electrophoresis apparatus	Bioneer (Korea)
27	Microwave	Samsung (Malaysia)
28	Digital camera	Sanyo (Japan)
29	Micro centrifuge	Lab Tech (Korea)
30	Water distillatory	Lab Tech (korea)
31	Autoclave	Sturdy (Taiwan)

2.1.2. Chemicals and Biological Materials

The chemicals and biological substances used in this study are listed below.

Table (2-2): Chemicals and biological materials with their remarks

Chemicals & biological materials	Manufacture's (Company)
Agarose	Promega (USA)
Bromophenol blue dye	BDH (UK)
Deionized water	Bioneer (korea)
Distal water	AL-Rafidain (Iraq)
Ethanol (96%)	Lobachemie (India)
Ethidium bromide dye	BDH (UK)
Protinase- K	Favorgene (Taiwan)
PCR water	Promega(USA)
Tri-Borate EDTA Buffer (TBE buffer)10X	Promega (USA)
loading Dye	Promega (USA)

2.1.3. ELISA (Enzyme –Linked ImmunoSorbent Assay) Kits:

Human IL-8 and Resistin ELISA Kit:

These ELISA kit are used for determination the serum concentration of IL-8 and resistin respectively, there component shown in table (2-3).

Table (2-3) : The component of IL-8 and resistin ELISA kit each one contain the following components.

Reagents and materials provided	Quantity	Remark
1- Pre-coted, ready to use 96-well strip plate.	1	Cloud-Clone Corp (USA)
2- Standard.	2	
3- Detection reagent A.	1x120 µl	
4- Detection reagent B.	1x120 µl	
5- TMB substrate.	1x9 ml	
6- Wash buffer (30 x concentrate).	1x20 ml	
7- Plate sealer for 96 wells.	4	
8- Standard diluent.	1x20 ml	
9- Assay diluent A.	1x12 ml	
10- Assay diluent B.	1x12 ml	
11- Stop solution.	1x6 ml	
12- Instruction manual.	1	

2.1.4. Moleculer Kits:

The RFLP-PCR kits used in this study are clarify in table below.

Table (2-4): REFLP-PCR kits with their remarks:

<table>
<tr><th>Type of kit</th><th>Manufacturer(State)</th></tr>
<tr><td>Blood genomic DNA extraction mini kit.
This kit contain the followings:
1- FABG mini column.
2- FABG buffer.
3- Elution Buffer.
4- W1 Buffer.
5- Wash Buffer.
6- Protenase K.
7- Elution tube
8- collection tube.</td><td>Favorgen (Taiwan)</td></tr>
<tr><td>Master mix
This kit contains the followings:
1- dNTPs(dATP, dCTP, dGTP, and dTTP) 250µM
2- KCl (30 µM)
3- MgCI2 (1.5 µM)
4- Top DNA polymerase (1U)
5- Tris-HCl -pH 9.0 (10 µM)
6- Stabilizer and Tracking dye</td><td>Bioneer (Korea)</td></tr>
<tr><td colspan="2">Primers with their sequence.
<table>
<tr><th>Primer</th><th colspan="2">Sequence</th></tr>
<tr><td rowspan="2">RETN gene promoter SNP-420</td><td>F</td><td>5’-TGT CAT TCT CAC CCA GAG ACA-3’</td></tr>
<tr><td>R</td><td>5’-TGG GCT CAG CTA ACC AAA TC-3’</td></tr>
</table>
</td></tr>
</table>

To be continued…

Restriction enzyms: • NE Buffer 2.1 (10X concentrate) 1.25 ml • BpiI (BpsI) restriction enzyme (10,000 U/ml) 0.03 ml (300 units)	Bioworld (USA)

2.1.5.Molecular Weight Markers:

The molecular weight marker used in this work are clarify in table below

Table (2-5): Molecular weight marker:

DNA Ladder	Description	Source
Ladder (KAPA Universal Ladder)	This ladder kit is designed to determine the approximate size of dsDNA on agarose gel. It contain many DNA fragments (in base pairs) these include : 100, 150, 200, 300, 400, **500**, 600, 800, **1000**, 1200, **1600**, 2000, 3000, **4000**, 5000, 6000, 8000, and 10000. This Ladder contains four reference bands for orientation. (500, 1000, 1600, and 4000) .	KAPA/ Biosystems

2.2. Methods:

2.2.1. Sample collection:

A five ml of blood is withdrawn vein puncture from acne vulgaris patients and healthy control and divided into two aliquotes, the 1^{st} (2 ml) put in EDTA tube for molecular studies and the 2^{nd} (3 ml) put in gel tube for separate the serum by centrifuge and then transfer it into plain tube and storage at -20 ºC until use ELISA test.

2.2.2. Patients and Control Groups:

The subjects enrolled in this study was comprised of 49 patients (28 female and 21 male) who were suffering from acne vulgaris and there ages are between 13 and 35 years, and the healthy control individuals of 25 (16 female and 9 male) with ages ranges of 14-40 years old, thy were attended at Dermatology Center of Al-Sader medical City and private clinic in the period from November/2015 to March/2016. The acne vulgaris was diagnosed by consultant physicians. The data of patients were acquired from their own records and from direct questionnaire of patients which is involved name, sex, age, height, weight, severity of disease, duration of disease, family history and treatment.

Note: Patients suffered from acne that result from steroid drugs were excluded from the current study.

2.2.3. Body Mass Index (BMI):

It is a direct calculation that describes relative body weight for height, is not gender specific, and is significantly correlated with total body fat content, calculated according to standard equation:

Body mass index (BMI) = Body weight (BW)(in kilogram) \[body height (BH)(in meter)]^2

Participant will be categorized according to their BMIs according to the WHO classification.

- Individuals with BMI <18.5 kg\m^2 were considered to be underweight.
- 18.6 to 24.9 as being of normal weight.
- 25 to 29.9 as being overweight.
- 30 and 34.9 as being obese class I.
- 35 – 39.9 as being obese class II
- ≥ 40 as being obesity class III (morbid obesity).

(Keys *et al.*, 1972 ; Guyton & Hall, 2006).

2.2.4. Enzyme Linking Immunosorbant Assay (ELISA).

ELISA kits were used to measure the IL-8 and resistin levels in serum (Cloud-Clone Corp/USA)

2.2.4.1. Human IL-8 ELISA Kit:

A. Test principle:

The micro titer plate provided in this kit has been pre-coated with an antibody specific to IL-8. Standards or samples are then added to the appropriate micro titer plate wells with a biotin-conjugated antibody specific to IL-8. Next, Avidin conjugated to horseradish peroxidase (HRP) is added to each micro plate well and incubated. After TMB substrate solution is added, only those wells that contain IL-8, biotin-conjugated antibody and enzyme conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured

spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of IL-8 in the samples is the determined by comparing the O.D. of the samples to the standard curve.

B. Procedure:

1. The standards reagents and samples are prepared by bring them to the room temperature.
2. One hundred µl from standard or sample was added to each well of micro titer plate, then Incubated it for 2 hours at 37ºC.
3. Then 100µl from prepared detection reagent A was aspirated and then added to the micro titer plate and incubated for 1 hours at 37ºC.
4. Then it was aspirated and washed 3 times.
5. After that 100µl from prepared detection reagent B was added and incubated it for 30 minutes at 37ºC.
6. Then it was aspirated and washed 5 times.
7. After that 90 µl from substrate solution was added and then incubated for 10-20 minutes at 37ºC.
8. At the end 50 µl from stop solution was added, and then the absorbance was read at 450nm immediately.

C. Calculations of the Data:

The standard curve of IL-8 was plotted in Figure (2-1) and the level of IL-8 in each sample was determined.

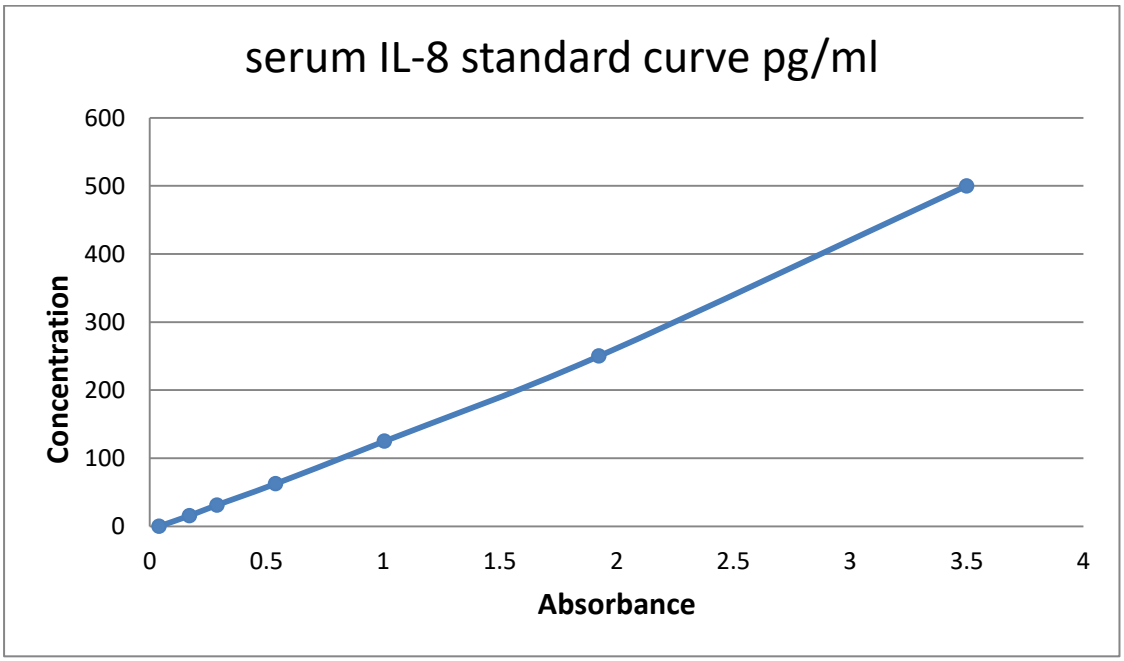


Figure (2-1): The standard curve of IL-8. They are graphed by plotting known standard **c**oncentration against their correspondent optical densities (O.D).

2.2.4.2. Human Resistin ELISA Kit:

A. Test principle:

The micro titer plate provided in this kit has been pre-coated with an antibody specific to resistin. Standards or samples are then added to the appropriate micro titer plate wells with a biotin-conjugated antibody specific to resistin. Next, Avidin conjugated to horseradish peroxidase (HRP) is added to each micro plate well and incubated. After TMB substrate solution is added, only those wells that contain resistin, biotin-conjugated antibody and enzyme conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of

sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of resistin in the samples is the determined by comparing the O.D. of the samples to the standard curve.

B. Procedure:

1. The standards reagents and samples are prepared by bring them to the room temperature.
2. One hundred µl from standard or sample was added to each well of micro titer plate, then Incubated it for 2 hours at 37ºC.
3. Then 100µl from prepared detection reagent A was aspirated and then added to the micro titer plate and incubated for 1 hours at 37ºC.
4. Then it was aspirated and washed 3 times.
5. After that 100µl from prepared detection reagent B was added and incubated it for 30 minutes at 37ºC.
6. Then it was aspirated and washed 5 times.
7. After that 90 µl from substrate solution was added and then incubated for 10-20 minutes at 37ºC.
8. At the end 50 µl from stop solution was added, and then the absorbance was read at 450nm immediately.

C. Calculations of the Data:

The standard curve of resistin was plotted in Figure (2-2) and the level of it in each sample was determined.

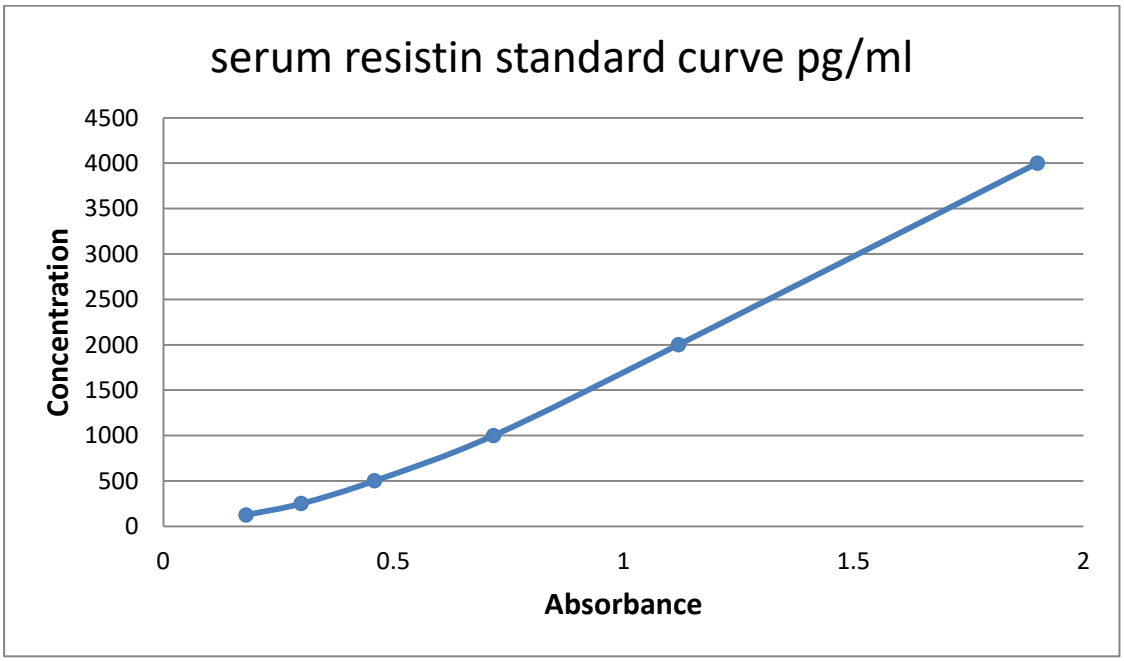


Figure (2-2): The standard curve of resistin. They are graphed by plotting known standard **c**oncentration against their correspondent optical densities (O.D).

2.2.5. Molecular (RFLP-PCR) Technique:

This Technique was carried out to detect the resistin gene polymorphism SNP -420 (G>C) in samples of both patients with acne vulgaris and as well as the control group; in this technique after DNA was extracted and the gene was amplified by PCR, the RETN gene was cut by using "restriction endonuclease" (BpiI) and this process was performed as follow.

2.2.5.1. Extraction of the Genomic DNA:

The genomic DNA was extracted from human blood by using genomic DNA extraction kit (Blood genomic DNA extraction mini kit. Favorgene/Taiwan) and done according to company instruction as following:

1. About 200 µl from sample (whole blood) was added into a microcentrifuge tube.
2. Twenty µl from Proteinase K and 200 µl from FABG Buffer was added to the sample and mix thoroughly by pulse-vortexing.
3. Incubated under 60 ºC for 15 minutes to lyse the sample with vortex the sample each 3-5 minutes.
4. Then the tube was spin briefly to remove drops from the inside of the lid.
5. Ehanol 96%(200µl) was added and mix thoroughly by vortex for 10 sec.
6. The tube was spin briefly to remove any drop from the inside of the Iid.
7. A FABG Mini Column was put in a collection Tube and the mixture was transfered carefully to the FABG Mini Column.
8. The mixture then centrifuged for 1 min at 6,000 x g and transferred the FABG Mini Column to a new Collection Tube.
9. Four hundred µl from W1 Buffer was added to the FABG Mini Column and centrifuge at full speed (18,000 x g) for 30 sec then discard the flow-through.
10. Then 750µl from the Wash Buffer was added to the Mini Column and centrifuged at full speed for 30 sec and then the flow-through was discarded.
11. Then Centrifuged at full speed for 3 minutes to dry the column.
12. Then the FABG Mini Column was placed in the Elution Tube.

13. One hundred and fifty µl from Elution Buffer was added to the membrane center of FABG Mini Column. Stand FABG Mini Column for 3 minutes.
14. It was Centrifuged at full speed for 1 minutes to elute total DNA.
15. The extracted DNA was Stored at 4°C.

2.2.5.2. Genomic DNA Profiling:

The DNA that extracted from blood sample was checkup by using Nanodrop device (Biodrop. UK) measure the purity of DNA and the concentration of DNA in the sample as following steps:

1. After turn on the device, an appropriate program is selected (nucleic acid DNA).
2. The surface of the lower measurement pedestal by is cleaned dry cotton several times.
3. One µl from ddH2O is taken and it is put onto the surface of the lower measurement pedestal.
4. The Nano drop is initialized by press OK, then the ddH2O is cleaned.
5. Blanking solution(which is same to elution buffer of RNA samples) is added as black solution and press trace.
6. After that, the pedestals is cleaned and 1µl from the DNA sample is pipetted for measurement.
7. The purity of DNA was measured by reading the absorbance in Nano drop device at 260 nm and 280 nm, so the DNA has its absorption maximum at 260 nm and the ratio of absorbance at 260 nm to 280 nm is used to evaluate the purity of DNA. A ratio ~1.8 is generally accepted as “pure” for DNA ;If the ratio is noticeably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

2.2.5.3. Primer Preparation:

The primer preparation was occur according to manufacturer instructions by liquefying the lyophilized primers with deionized distill water (ddH2O) to produce stock solution (100 pmol/µl concentration), primer working solution was prepared by dilution the stock solution with deionized water, according to this equation "C1V1=C2 V2".

2.2.5.4. Master Mix Preparation of PCR:

The PCR mix reaction was prepared by using Accu Power PCR Premix kit (Bioneer .Korea), and achieved according to company instruction as in table (2-7).

Table (2-6): PCR Master Mix Reaction

PCR master mix reaction	Volume
Forward primer(10pmol)	5ul
Reveres primer(10pmol)	5ul
PCR premix (Lyophilized)	5ul
DNA template	10ul
Total volume	25ul

Then the PCR tube was mixed by vortex until the lyophilized pellet dissolved.

2.2.5.5. The Conditions of Thermo Cycler (PCR):

Thermocycler conditions for amplification of RETN gene of human were carried out by useing conventional PCR device. The condition of this reaction was identified by Hussain S. *et al*., (2015) with some modifications as in table (2-8).

Table (2-7): The conditions thermocycling of for resistin (RETN) gene detection.

PCR step	Time (minute)	Temp. (°C)	No. of cycle
Preparation step	**5**	**94**	**1**
Denaturation	**1**	**94**	**40**
Annealing	**1.5**	**57**	
Elongation	**1.5**	**72**	
End cycle	**7**	**72**	**1**
Storage	∞	**4**	**_**
The final product of PCR 533-bp was stored at -20°C until used later.			

2.2.5.6. PCR Product Analysis:

A. TBE(1X) buffer preparation:

TBE 1X was prepared by adding 10ml from TBE(10X) to 90ml distil water.

B. Agarose gel preparation: The agarose gel was prepared in concentration 1.5% for PCR product analysis, this preparation has been proceeded as follow (Sambrook and Rusell, 2001).

- 1.5gm Agarose was added to 100ml from TBE(1X) then transferred in to microwave at 95 °C for 1.5min., then the solution was let at room temperature for 10 min to reach 50°C to add about 4µl from ethidium bromide.
- The solution was transmitted into tray after the comb fixed in appropriate site and insure don't form any bubbles in the solution then wait 20 min for solidifying, after that comb was removed from the tray to form wells to wich the PCR product will loaded.

C. Sample loading and electrophoresis:

- 5µl from PCR product was loaded into well and 5ul from Ladder was loaded into another well.
- The chamber of electrophoresis device was filled with TBE buffer and then transfer the gel gently into it. The devise was justified under 75 v and 400 AM for 2 hour.
- Then the amplified PCR product (533-bp) is saw, which is specific for resistin -420 gene by useing UV Transilluminator device.

2.2.5.7. Master Mix Preparation of Restriction Enzyme:

The master mix for RFLP-PCR was prepared for detection RETN -420 (C>G) polymorphism in blood samples of patients and healthy control group by using special restriction endonuclease (BpiI) that digest PCR product of RETN gene that revealed a two-allele polymorphism produced three bands of different sizes: a 533-bp fragment analogous to the absence of restriction site which is a homozygous mutant type, a set of three band (533, 323 and 210 bp) analogous to the presence of the restriction site in one allele which is the heterozygous mutant form, and a set of two band (323 and 210 bp) analogous to the presence of the restriction site in two allele which is the homozygous normal form, and this master mix done according to company instructions showed in table (2-13). After that, this master mix placed in vortex centrifuge at 3000rpm for 2 minutes, then incubate it at 37°C for 18houres. After that, the product was analysis by agarose gel electrophoresis methods that comment in PCR product analysis.

Table (2-8): Digestion mixture of PCR products by BpiI restriction enzyme.

RFLP-PCR Master mix	Volume
PCR product mixture	8 µl
Restriction enzyme 1X	4µl(3 unit)
NE buffer 1X	3 µl
Deionized distil water	5 µl
Then complete the size to 200µl by ddH2O	

Note: NE buffer 1X = 1 µl from NE buffer 10X + 9 µl ddH2O.

Restriction enzyme 1X(3Unit) = 1 µl R.E 10X (9unit) + 9 µl ddH2O.

2.2.5.8 Gel Electrophoresis for Detection of RETN Genotypes.

After incubation 3µl of 6X blue/ orange loading dye added to the previous mixture and electrophoresis of the digested PCR products were performed, 7µl of the digested PCR product was loaded on 1.5% agarose gel that was stained with ethidium bromide previously, the genotyping of RETN was determined by fragments separation at 75 volt for 90 minutes with the use of 100 bp ladder as a standard for each gel lane and these fragments were visualized by UV transilluminator system.

2.2.6. Statistical Analysis

The data were translated into a computerized database structure. An expert statistical advice was sought for. Statistical analyses were done using SPSS version 20 computer software (Statistical Package for Social Sciences) in association with Microsoft Excel 2010.

Independent sample T test was used to test the statistical significance of difference in average of acne severity between 2 groups. The $\chi 2$ Pearson Chi-square test was used to assess the statistical significance of

association between 2 categorical variables. We assumed the level of statistical significance at P <0.05. All analyzed tests were bilateral.

LSD and one way ANOVA was used to measure the statistical significance of difference in the mean of resistin and interlukin-8 between the 4 groups.

To measure the strength of association between 2 categorical variables, such as the presence of certain genotype and disease status the odds ratio (OR) was used The statistical significance of difference in mean of a continuous response variable which is known or assumed to be normally distributed (like age and duration of illness) between more than 2 groups was assessed by ANOVA. Associations between 2 categorical variables was explored by cross-tabulation. The statistical significance of such associations was assessed by Chi-square ($\chi 2$) test.

An estimate was considered statistically significant if its P value was less than an α level of significance of 0.05.

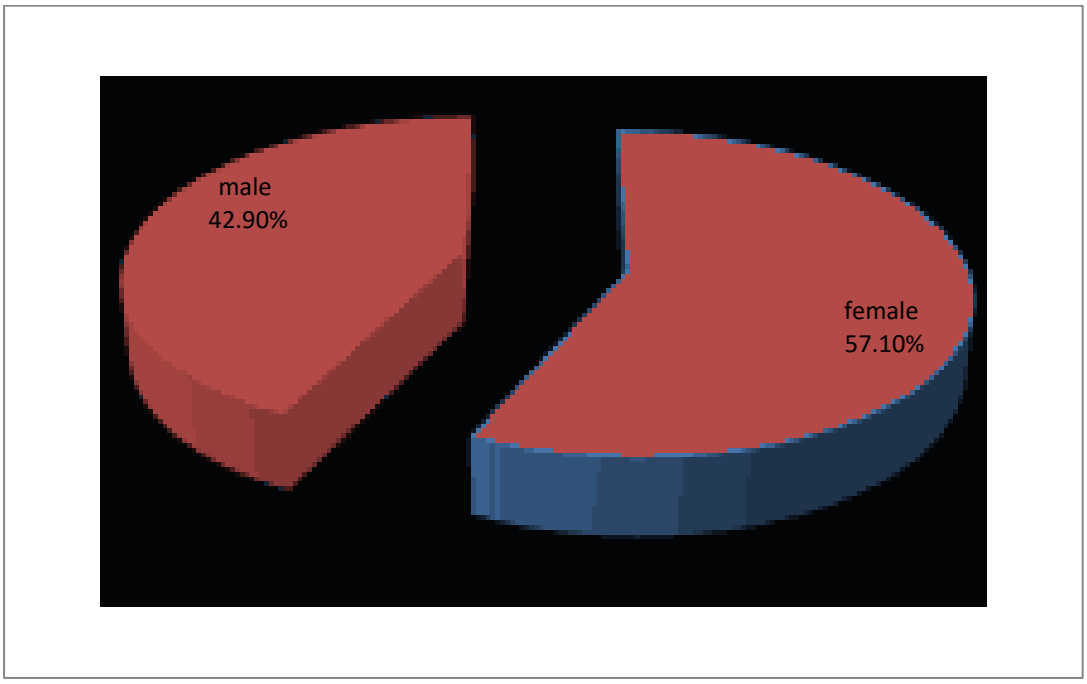
Chapter Three

Results & Discussion

3. Results and Discussion:

3.1. Subjects Characteristics.

In this case-control study, 49 patients with acne vulgaris were collected in AL-Sader Teaching Hospital/ Dermatology clinic(21 male (48.2%) and 28 female (57.2%)) and their ages are between 13 and 35 years with a mean age of 19.3 years (SD± 4.53), as in figure (3-1), compared with 25 healthy control (16 female and 9 male) and there ages range are between 14 and 40 years and mean age of (26.1).



Figure(3.1): The distribution of acne according to gender

3.2. The Distribution of Patients with Acne Vulgaris According to Gender and Age group.

Table (3-1) demonstrate the age and gender characteristic of acne vulgaris patients enrolled in this study, the age group of 16-20 years old was the highest frequency among patients (59.2%)followed by the age group of 21-25 years old (16.3%), and age group 11-15 years old (16.3%), this result agreed with Adityan and Thappa (2009) which

showed that the age group of 16-20 years was the most prevalent, this result, may support the concept that acne is a disease of young adults and adolescence and in the puberty, increased androgen production and it mediate the stimulation of the sebaceous gland to increase sebum production in both sexes, a matter might contributes to acne vulgaris. Usually it start through the adolescence and begin to heal during mid-twenties, also the acne vulgaris can be shown in the last life of the adult as shown in this study in which 4.1 % of the patients had persistence of the disease after the age of 25 years, similar result have been mentioned to by Goulden *et al.*, (1997), who found that 7-17% of clinical acne persist after the age of 25 years. The more severe cases of acne often take longer period to resolve, and they may persist into adults years (Arura *et al.*, 2011), about 90 % of these patients with persistent acne were females, as in one community-based UK study estimated the prevalence of acne in adult women aged between twenty-six and forty-four years to be 14%.The reasons for persistent acne are not fully understood, the post-adolescent acne in females can be divided into 'persistent acne', which represents a continuous acne from adolescence into adult, and 'late-onset' acne, which represents significant acne occurring for the first time after the 25 years old ,this may be due to endocrine abnormalities, the ingestion of certain drugs or the use of certain cosmetics (William and Layton, 2006).

Table (3-1) : Distribution of patients with acne vulgaris over age groups and gender.

Age (years) Groups	Males No.	Females No.	Total No.(%)
11 – 15	3	5	8(16.3%)
16-20	15	14	29(59.2%)
21-25	3	5	8(16.3%)
26-30	0	2	2(4.1%)
31-35	0	2	2(4.1%)
Total	21	28	49(100%)

The predominance of female among our patients (M:F ratio =1:1.4), as shown in previous table, is just mimic the results of Al-Ameer and Al-Akloby (2002)M:F ratio=1:1.8 and the results of Tallab (2004) who found that women were affected more frequently than men (M:F ratio = 1: 2.5). However, other studies showed that the prevalence of the disease did not show any preference for male or female (Jappe, 2003; Hanisah *et al.*, 2009). Although the gender frequencies doesn't matter in such condition, the difference of the results may be due to the size of sample in which the female is predominate than males, in addition to the fact that this research did not study the prevalence of acne in a defined population but it is based on selective profile of clinic attendees, in addition, the environmental, ethnic, nutritional, and even weather factors, may play a vital role in such difference.

3.3. The Relationship Between Severity of the Acne Vulgaris among Acne Patients and Gender.

The mild and moderate degrees of acne vulgaris were found with higher prevalence (38.8%, 55.1% respectively) among the patient's group in this study, while 6.1% percent suffer from severe acne as shown in figure (3-3). The study of Bizwas *et al.* (2010) in Iran has shown that Grade II "moderate" is the most frequent (45%) of the patients, also the findings of Akyazi *et al*. (2011) in Turkey have proven that moderate degree of acne was the most common (55.4%).

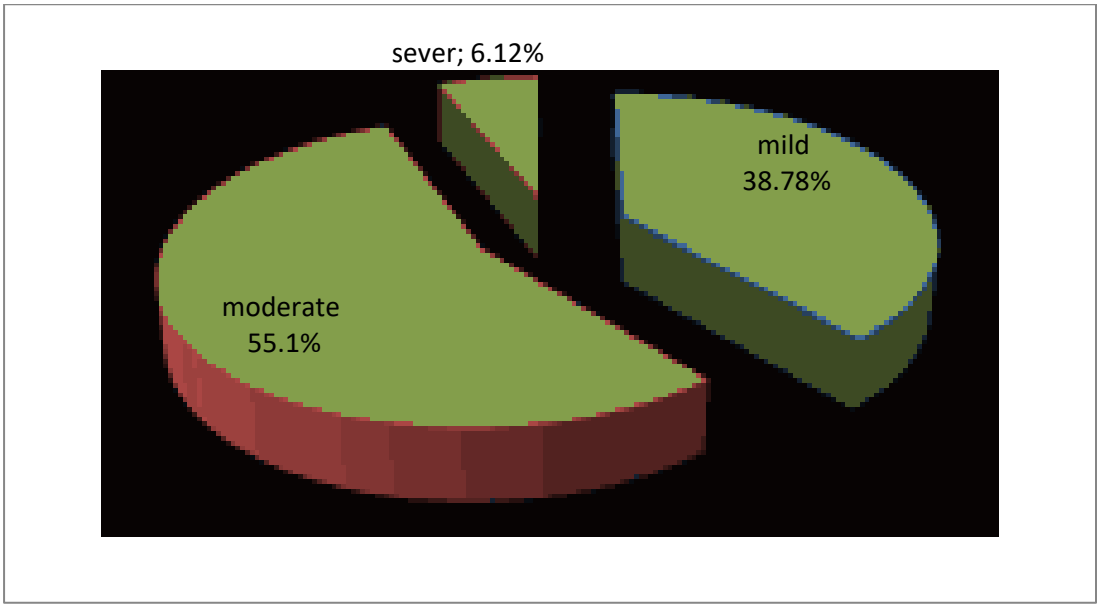


Figure (3-2): The severity of acne vulgaris in the patients.

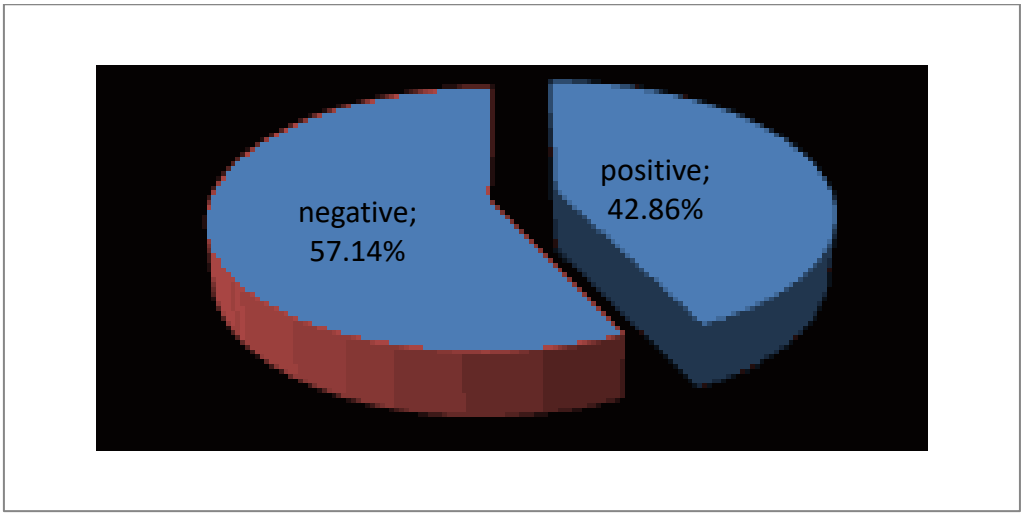
Our finding shows a difference between the severity of acne and gender, in which the female patients had mild acne(42.8%) more than males (33.3%), while the males show high tendency to those having moderate(57.2%) and severe acne (9.5%) compared with (53.6 %) and (3.6%), respectively of the females as shown in table (3-2), but this difference was statistically non-significant (P value=0.61), this may be due to few number of cases, the males tend to have more severe acne than females because they have oilier complexion and higher in their androgen levels (Hanisah *et al*, 2009).

Table (3-2): The association between acne severity and gender

Gender	Severity[no.(%)]			Total	P value
	Mild	Moderate	Severe		
Female	12(42.8%)	15(53.6%)	1(3.6%)	28(100%)	0.61 (non-significant)
Male	7(33.3%)	12(57.2%)	2(9.5%)	21(100%)	

3.4. The Effect of Family History on Patients with Acne Vulgaris.

This study showed that there is no significant correlation between the family history and the presence of acne as shown in figure (3-5) where 42.86% of patients have family history of the disease while 57.14% of them will not, this result contrast to the Xu *et al.*, (2007), they found that the risk of acne vulgaris occurring in a relative of a patient was significantly greater than for the relative of an unaffected individual.



Figure(3-3): The correlation between the family history and the presence of acne vulgaris.

In addition, this results showed that the prevalence of severe acne was higher in patients with negative family history (57.14%) than in those with positive family history (42.86%) as shown in table (3-3) but this was statistically not significant(P value=0.24), these results were in contrast with those of (Evans *et al.*, 2005; Ballanger *et al.*, 2006; DiLandro*et al.*, 2012).

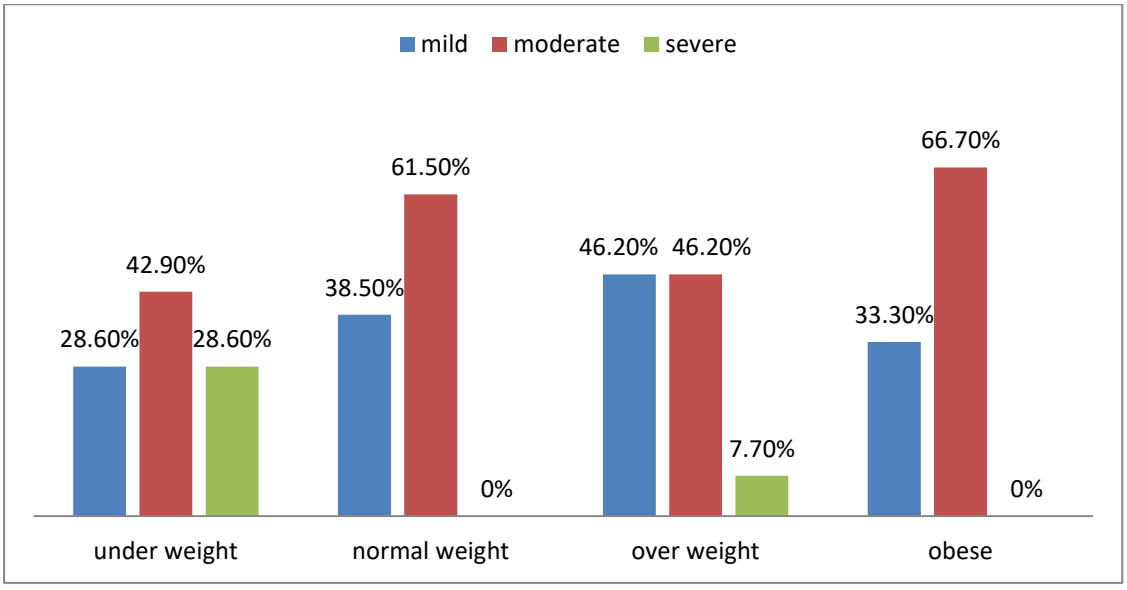
On the other hand, family history of acne was less common in patients with moderate to severe acne (42.8% and 4.8% respectively) than in patients with mild disease (52.4%) as shown in table (3-3), a finding that contrasted with earlier reports of Evans *et al*. (2005). Ballanger *et al.* (2006) have reported increased rates of retentional lesions and therapeutic difficulties, indicating more severe grades of acne in patients with a positive family history.

Table (3-3):The relation between family history of acne vulgaris and the severity of the disease.

	Mild acne N(%)	**Moderate acne** N(%)	**Severe acne** N(%)	**Total** N(%)	**P value**
Family history +ve	11(52.4%)	9(42.8%)	1(4.8%)	21(100%)	0.24
Family history -ve	8(28.6%)	18(64.3%)	2(7.1%)	28(100%)	

3.5. The Relationship between the Severity of Acne Vulgaris and Body Mass Index(BMI).

The results of this study as in figure(3-7)show that the moderate acne is predominant in obese patients (66.7%) rather than in overweight patients 46.2 percent, but the ratio of moderate acne also is high in normal weight (61.5%). Also, the severe acne in overweight patients is(7.7%) predominant than normal weight obese patients(0% for each) while in underweight is the predominant one (28.6%) this was lead to conclude that there is no clear relationship between severity of acne and the BMI this result was compatible with study conducted in the United Kingdom within the Glasgow Alumni Cohort that does not document any association between acne and BMI. Galobardes *et al*. (2005). Also, it contrasts with other studies such as Di Landro *et al*., (2012).



Figure(3-4):The relationship between the severity of acne vulgaris and BMI.

3.6. The Duration of Acne Vulgaris in Relation to the Gender.

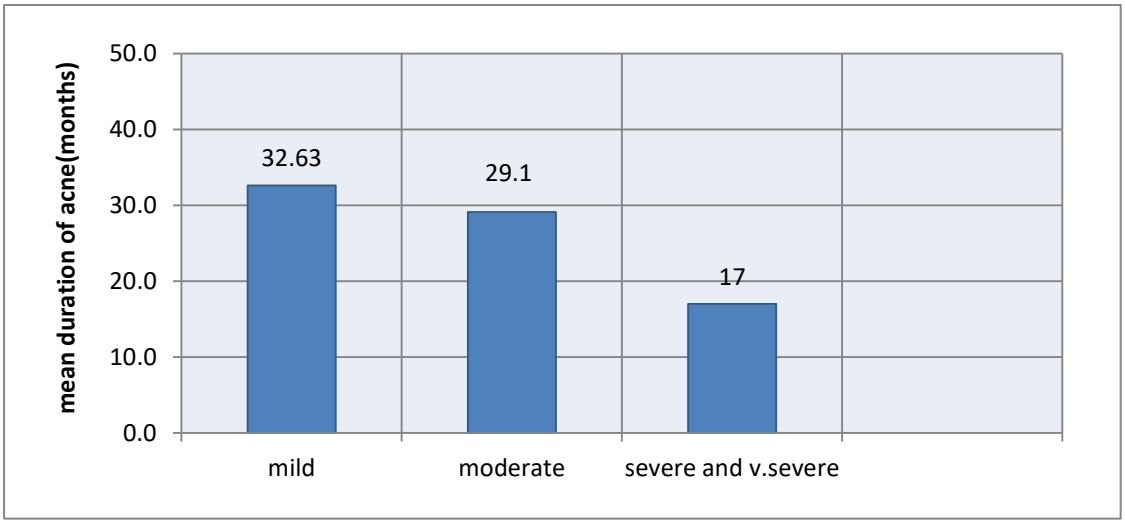
The duration of the disease in the patients collected in this study was range between 1 month and 15 years that agreed with the current study that shows the duration of the disease was range between 1 month and 25 years (Adityan andThappa, 2009). More than one-half of the patients (57.1%) had the lesions remain for more than 1year as shown in the table (3-4). This result was compatible with the result of a Canadian study by Jerry *et al.*(2001), they found that 64% of the patients had acne between 2-5 years. Also, Talleb (2004) and Al-Robaee (2005) was showed that 76% and 47.9% of their patients respectively had the disease for more than 1 year. Acne may persist in women for 30 years or even later (Zaenglein *et al.*,2008), that compared with this study as shown in table (3-4) that the female patients had prolonged duration of acne compared to males but this difference was statistically non-significant (P value =0.33), and it consistent with the results of Talleb (2004).

Table (3-4) The duration of acne vulgaris according to gender.

Duration of acne vulgaris(years)	Male No.	Female No.	Total No. (%)
<1	5	11	16(32.7%)
1-5	13	15	28(57.1%)
6-10	3	1	4(8.2%)
>10	0	1	1(2%)
Total No.	21	28	49(100%)

3.7. Duration of Acne Vulgaris in Relation to Severity of the Disease.

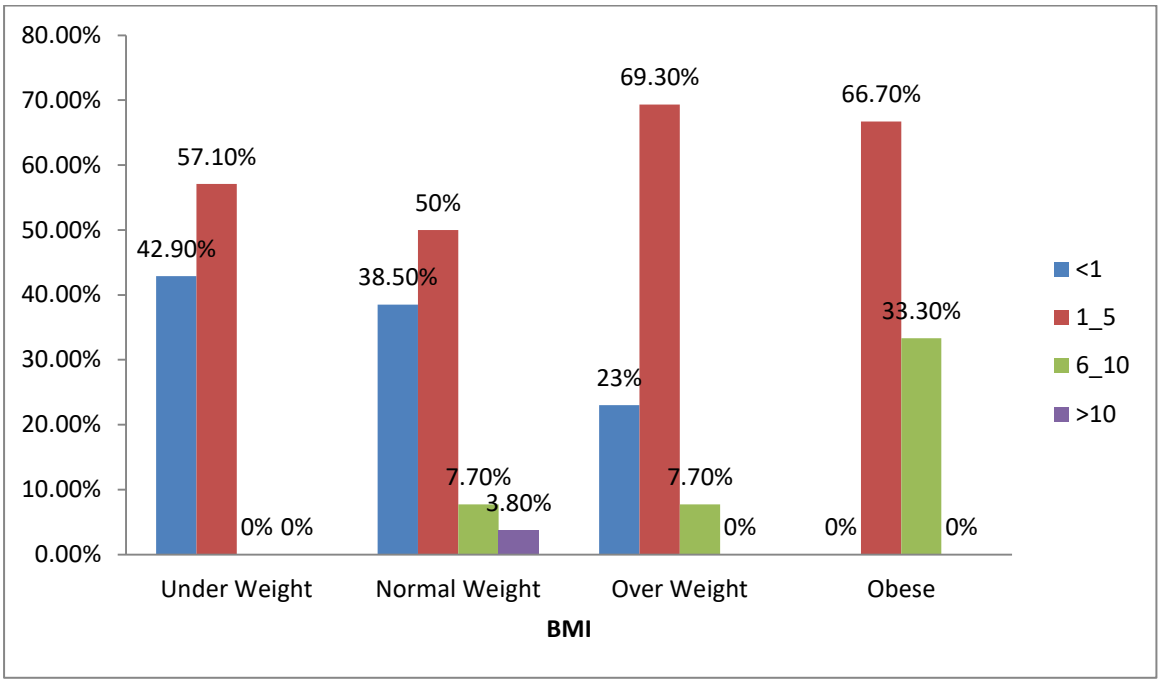
This disease is a chronic disease and may persist for many years but many of those with mild inflammatory acne diminish or disappears in a large proportion of those with acne in their teens (William *et al*, 2012). This study showed that the mean duration of mild acne as (32.63) months, lower than mild is the moderate (29.1 months) while the severe record the lowest value (17) months, figure (3-9), in contrast with Adityan and Thappa (2009) who stated "patients with longer duration of disease had more severe degree of acne vulgaris", this difference may cause by the different in the ethnicity or environmental factors that influence on the severity of acne, as well as there is no international agreed uniform system for classification of acne which considers as a disturbing factor in different studies. Although most patients pure by their early twenties, some patients extending well into the 3rd or 4th decades. The degree of involvement varies, and spontaneous variations in the degree of involvement are the rule rather than the exception(Zaenglein *et al.*, 2008).



Figure(3-5):The duration of disease according to acne severity categories.

3.8. The Duration of Acne Vulgaris in Relation to the Body Mass Index(BMI).

This study show that the disease in obese patients will persist in obese patients more than in non-obese as shown in figure(3-10) the period 6-10 years was more in obese patients(33.3%) than in over and normal weight(each one is 7.7%), while the period less than one year <1y was in under and normal weight is (42.9%, 38.5% respectively) higher than overweight (23%) and not found in obese patients. This was postulated that obesity may be accompanied by peripheral hyperandrogenism (Tsai *et al*., 2006) that lead to increasing sebum production which may be associated with delayed in the healing of acne. To our knowledge, there is no research associate the duration of acne with BMI.



Figure(3-6): The duration of disease according to the BMI.

3.9.The Relationship between the Level of Serum Interleukin-8 (IL-8) and Acne Vulgaris.

The records shown in Table (3-5) stated that there is no significant different in the mean of serum IL-8 between the patients and controls (P-value=0.187), also when compare between the male of patients (G1) and the male of control (G3), when compare the female of patients (G2)with the female of control (G4) show that there is no any significant difference between them(p-value= 0.103 and 0.446 respectively) as shown in table (3-6). This result indicates that there is no significant association between IL-8 and acne vulgaris. The study of Hussain S. *et al*.,(2015 B) found that the single nucleotide polymorphism(SNP) in The IL-8-251T>A (rs4073) lead to increase in the IL-8 that lead to increase in the acne development. Moreover, another study shows that the polymorphism in interleukin 1A but not in interleukin 8 gene predisposes to acne vulgaris (Sobjanek *et al*., 2013) that exclude the association of IL-8 with acne vulgaris.

Table (3-5): The difference in mean serum IL-8 between the patients and control.

	Study group	N	Mean ng/ml	P-value
IL8 ng/ml	patients	49	5.64 ± 0.34	0.187 (Non-significant)
	controls	25	5.98 ± 0.35	

Table (3-6): The difference in mean serum IL-8 between the groups of gender in patients and control.

Study group	N	Mean of IL8 ng/ml	P-value
Male of patients (G1)	21	6.29 ± 0.52	0.103 (Non-significant)
Male of controls(G3)	9	6.4 ± 0.3	
Study group	N	Mean	P-value
Female of patients (G2)	28	5.23 ± 0.43	0.446 (Non-significant)
Female of controls (G4)	16	5.73 ± 0.52	

3.10. The Relationship Between the Mean of Serum Interleukin-8 (IL-8) and the Severity of Acne Vulgaris.

There were no significant association between the IL-8 levels and the severity of acne vulgaris (P-value=0.27) as shown in the table(3-7).

Table(3-7): IL-8 levels according to the severity of the disease.

	N.	Mean of IL-8 (pg/ml)	P-value
Mild	19	5.59	0.27 (Non-significant)
Moderate	27	5.31	
Severe	3	6.9	

3.11. The Relationship between the Level of Serum Resistin and Acne Vulgaris.

When compare the mean of serum resistin level of all patients with different genders as a group with all healthy controls group as shown in the table (3-8), we found that there is no significant difference between them (P-value=0.08).

Table (3-8): The difference in mean serum resistin between the patients and control.

	Study group	N	Mean	P-value
Resistin ng/ml	patients	49	3.03 ± 0.39	0.08 (Non-significant)
	controls	25	2.21 ± 0.44	

Also, when compare the mean of serum resistin between the male of patients (G2) and the male of control show that there is no significant difference between them (P-value=0.291) as shown in figure (3-9). While when to compare between the female of patients and controls as shown in figure (3-9) we found a significant difference between them (P-value=0.033).

This result was referred to that the association between the level of serum resistin and acne vulgaris occur in female in contrast to male this may be due to the difference in the hormones between the genders and ethnic characters and this result was incompatible with other studies such as Younis *et al*., (2016), that refer to the elevation of serum resistin level that result from the polymorphism in RETN-420 gene lead to the development of acne vulgaris.

Table (3-9): The difference in mean serum resistin between the groups of gender in patients and control.

Study group	N	Mean of resistin ng/ml	P-value
Male of patients (G1)	21	3.5 ± 0.66	0.291 (Non-significant)
Male of controls(G3)	9	3.9 ± 0.87	
Study group	N	Mean	P-value
Female of patients (G2)	28	2.69 ± 0.49	0.033 (Significant)
Female of controls (G4)	16	1.49 ± 0.38	

3.12. The Relationship between the Mean of Serum Resistin and the Severity of Acne Vulgaris.

This study showed that there is no significant association between the resistin levels and the severity of acne vulgaris (P-value=0.67) as shown in the table (3-10).

Table(3-10): Resistin levels according to the severity of the disease.

	N.	Mean of resistin (pg/ml)	P-value
Mild	19	4.39	0.67 (Non-significant)
Moderate	27	3.26	
Severe	3	3.1	

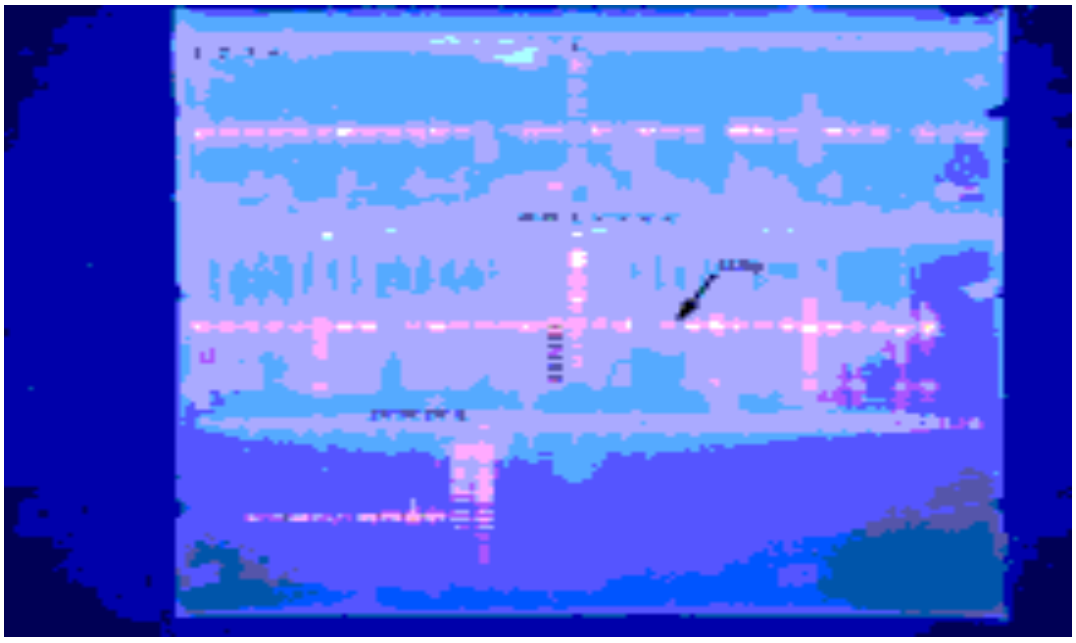
3.13. Molecular Study

3.13.1. DNA Concentration Measurement

After the DNA was extracted, the concentration of DNA was measured by nanodrop device and the concentration of the DNA in samples was rate about 20±5(ng/µl).

3.13.2. DNA Appearance:

The products of successful attachment between the extracted DNA and particular primers for Resistin-420 gene promoter site were showed by gel electrophoresis analysis using DNA marker "(KAPA Universal Ladder)" and the final product was 533bp length for both studied group, Figure (3-11).

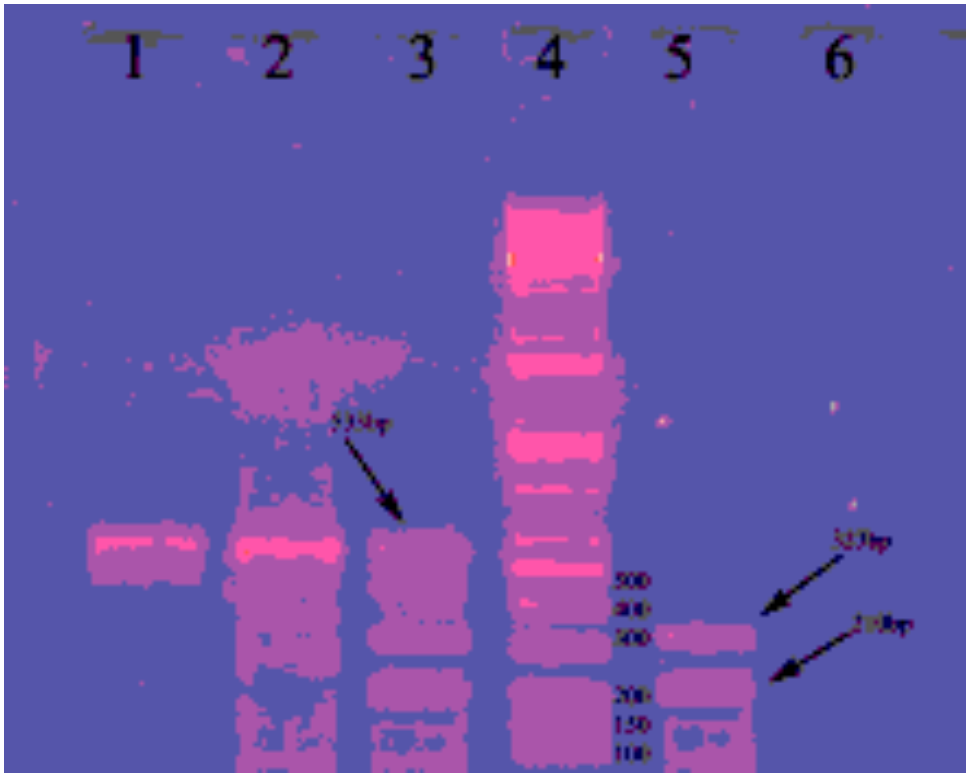


Figure(3-7): Ethidium bromide-stained agarose gel of PCR amplified RETN-420 gene(533bp) show:

- **Lane (L):DNA molecular size marker (100 bp ladder).**
- **Lane 1- 49 for acne patients.**
- **Lane 1C-25C for the control group.**

3.13.3. Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR) Product Appearance:

The product of RFLP-PCR technique showed the single nucleotide polymorphism (SNP) that occur in RETN-420 (rs1862513) and show three bands in size 533bp, 323bp and 210bp this means there is three genotype polymorphism; CC homozygous when two band 323bp and 210bp will appear, GG homozygous when one band 533bp will appear and CG heterozygous when the three band will appear as shown in figure(3-12), and this result was agreed with the earlier report of Kumar, *et al*. (2014), that this locus had three genotypes only.

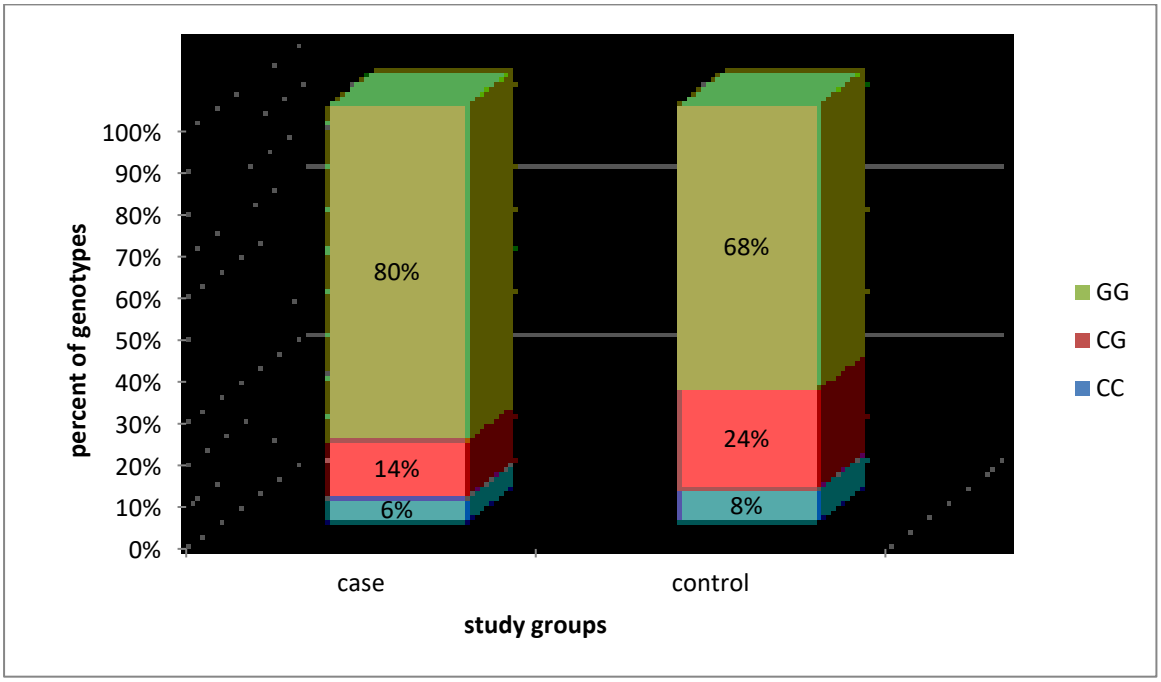


Figure(3-8): Ethidium bromide-stained agarose gel of RFLP-PCR product after digested by BpiI restriction enzyme show:

- **Lane 1 and 2: Homozygous mutant GG (533bp).**
- **Lane 3: Heterozygous mutant CG (533bp, 323bp and 210bp).**
- **Lane 5: Homozygous wild CC (323bp and 210bp).**
- **Lane 4: DNA molecular size marker (KAPA Universal Ladder).**

3.13.4.Genotype Distributions in Subjects Under Study:

In the current study, a direct comparison between resistin genotypes and the clinical features of acne vulgaris (susceptibility and severity) was investigated focusing on the two alleles single nucleotide polymorphism in RETN gene in the context of the promoter region. The frequency of genotypes in acne patients were as follow: CC(6%), CG(14%) and GG(80%) ; while in the healthy control subjects: CC(8%), CG(24%)and GG(68%), figure(3-13). The genotype distribution had no deviation from Hardy-Weinberg equilibrium (HWE) in all groups.



Figure(3-9): Case-control comparison in the relative frequency of the three selected genotypes (CC, CG and GG).

The GG genotype frequency was higher in acne patients (80%) when compare with healthy controls (68%), but the difference in the distribution of RETN genotypes between patient and control groups was statistically non-significant, (Chi=1.27 and P-value=0.53), as shown in the table (3-11).This result was contrast with the results of Hussain S., *et*

al. (2015 B) on acne vulgaris in Pakistani population (Chi=8.5 and p-value=0.014), this support the null hypothesis that there is no association between the RETN-240 C>G SNP polymorphism and the susceptibility to the disease , these differences may be explained by the difference in the geographically different population studied

Table (3-11): Genotype distribution of resistin RETN-420 gene polymorphism in patients and controls

Genotypes	Controls N(%)	Patients N(%)	OR	P	Chi
CC	2 (8%)	3 (6%)	0.75	0.53	1.27
CG	6 (24%)	7 (14%)	0.59		
GG	17 (68%)	39 (80%)	1.83		
Total	25 (100%)	49(100%)			

OR=odd ratio, p= p-value

3.13.5. The Distribution of the Genotypes in Acne and Healthy Control Subjects According to Gender .

The results of this study according to the three genotype distributions in acne patients show that there is no gender-specific differences were detected between male and females in acne patients , the P value=0.15 for all genotypes as shown in table (3-12), according to our knowledge there is no study demonstrate this association.

Table (3-12): Genotypes distribution of RETN-420 gene polymorphism among acne patients according to the gender.

Genotypes	Female N(%)	Male N(%)	OR	Chi	P value
CC	1 (3.6%)	2 (9.5%)	2.83	3.799	0.15
CG	2 (7.1%)	5 (23.8%)	4.06		
GG	25 (89.2%)	14 (66.7%)	0.24		
Total	28 (100%)	21 (100%)			

When Compare between the study groups regarding the same gender, the data showed that there was no association between the RETN-420 C>G polymorphism and the two genders of acne patients (P value for female and male =0.22 and 0.9 respectively) as shown in figure (3-14) for female and (3-15) for male, These results was not completely accordant with the results of Younis *et al*. (2016), that show that the variant genotype is associated with acne in female patients (p = 0.008) whereas, in male patients the variant genotype frequency, while higher, did not reach statistical significance.

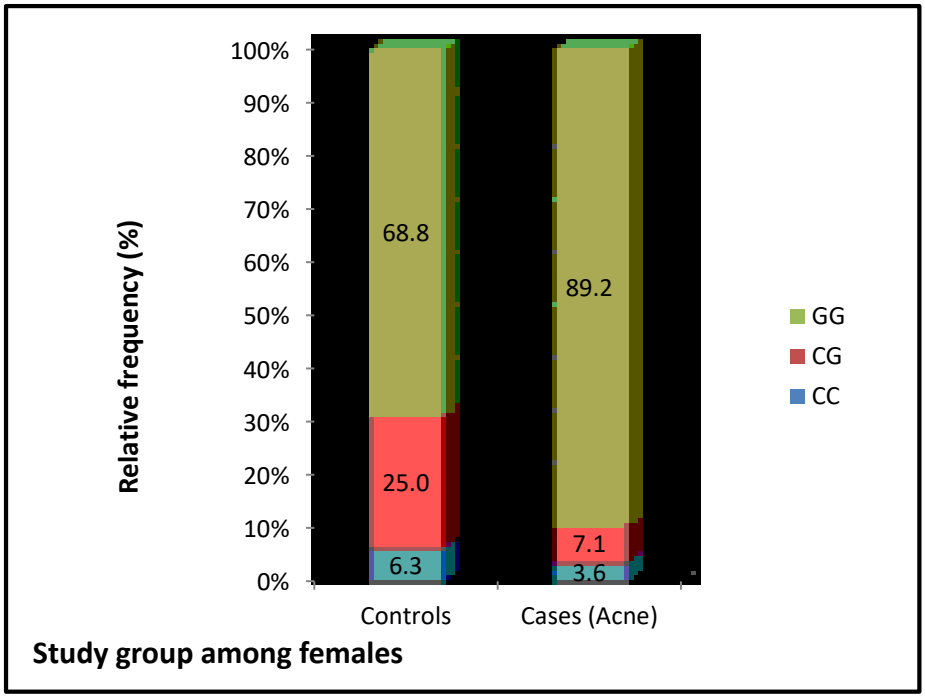


Figure (3-10): Case –control comparison in the relative frequency of the three selected genotypes in females.

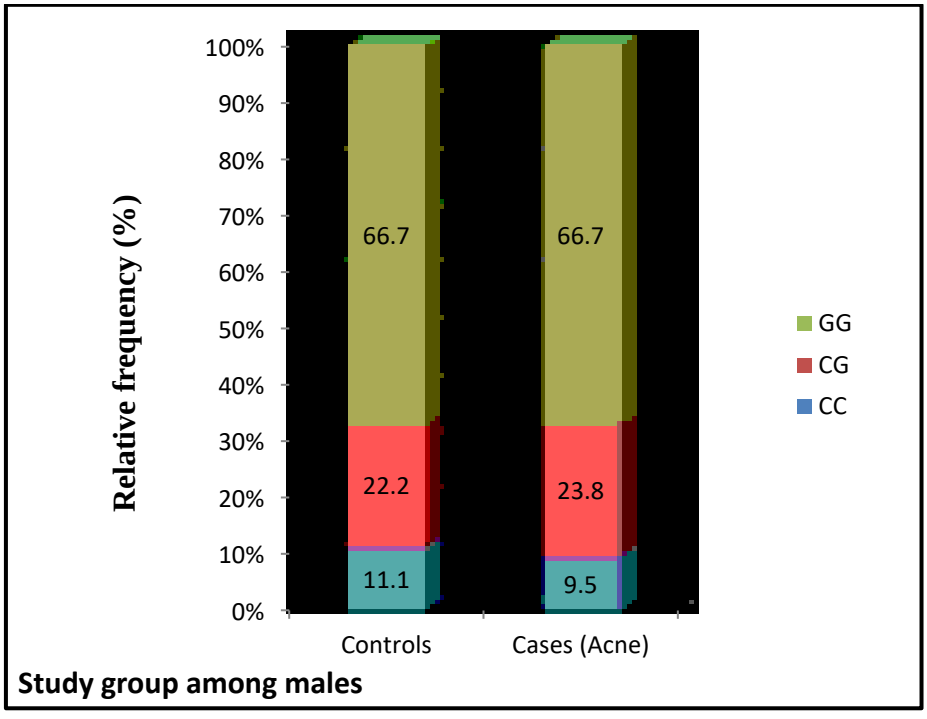


Figure (3-11): Case –control comparison in the relative frequency of three selected genotypes in males.

3.13.6. The Association between RETN-420 C>G Polymorphism and the Severity of Acne.

In this study there is no association was detected between the severity of acne and the distribution of the frequency of the three genotypes in acne patients, in which the *P* value=0.74) as shown in the table (3-13). This result contrast with Younis S. *et al*.(2016), show that both G/G genotype and G allele frequencies in -420C>G polymorphism are associated with severity of acne symptoms (G/G: p = 0.027; G: p = 0.020). In view of acne is a multifactorial disease ,it is possible that the severity of acne may be readily influenced by other factors such as environmental and other genetic elements(Cordian *et al*., 2002 ; Szabo and Kemeny, 2011).

Table (3-13): The association between RETN-420 C>G polymorphism and degree of severity of acne patients

Genotype	Mild N (%)	Moderate N (%)	Severe N (%)	P-value
CC	1 (5.3%)	2 (7.15%)	0 (0%)	0.74 (Non-significant)
CG	4 (21%)	3 (10.7%)	0 (0%)	
GG	14 (73.6%)	23 (82.15%)	2 (100%)	
Total	19 (100%)	28 (100%)	2 (100%)	

3.13.7. Geographic Differences of the Observed Genotypes and Allele Frequencies of RETN-420 G>C SNP.

In this study, a comparison of observed allele frequencies of the RETN-420 G>C SNP, this study show no association of this polymorphism and acne vulgaris while the two other published studies investigate the association of this polymorphism and acne vulgaris, the present study found that the frequency of the G allele containing CG and GG genotypes in acne patients were different in the three studies (First study 20.9%, second study 56%, This study 86.7%) as shown in table (3-14)

Table (3-14): Observed RETN-420 G>C genotype frequencies of acne patients in various populations

Population	No. of samples	Genotypes no.(%)			P value	Alleles		P value	Reference
		CC N.(%)	CG N.(%)	GG N.(%)		C N.(%)	G N.(%)		
Pakistan	Cases 180	117 (65)	51 (28.3)	12 (6.7)	0.014	285 (79.1)	75 (20.9)	0.002	**Hussain S. *et al.*, 2015 A.**
	Controls 180	141 (78.3)	34 (18.9)	5 (2.8)		316 (87.8)	44 (12.2)		
Pakistan	Cases 530	97 (18)	274 (52)	159 (30)	0.027	468 (44)	592 (56)	0.013	**Younis S. *et al.*, 2016.**
	Controls 550	121 (22)	302 (55)	127 (23)		544 (49)	556 (51)		
Iraqi population	Cases 49	3 (6)	7 (14)	39 (80)	0.53	13 (13.3)	85 (86.7)	0.285	**Current study 2016.**
	Controls 25	2 (8)	6 (24)	17 (68)		10 (20)	40 (80)		

Conclusions
&
Recommendations

Conclusions:

1. There is no any association between the levels of interleukin-8 (IL-8) and the presence or development of acne vulgaris in two genders.
2. There is no association between the levels of resistin and the presence, development of acne vulgaris in male, while that association is found in the female.
3. There is no prospect association between RETN-420(C>G) single nucleotide polymorphism and the presence or development of acne vulgaris in two genders.
4. There is no association between the presence of different genotypes or alleles in the population may predict the probability of developing the disease.
5. The studied differences between populations could be due to environmental factors and /or genetic elements.

Recommendations:

1. Larger sample size is recommended.
2. Study of other possible genetic predisposing factors.
3. The study of other cytokines that effect on the development of acne vulgaris.
4. Using of more advanced technique such as sequencing to read the accurate sequences of genes might play roles in acne vulgaris.
5. Investigation of other inflammatory mediators polymorphisms reflected the disease.