TNF-alpha Gene Polymorphism and its Association with Osteoarthritis in Pakistani Patients

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CERTIFICATE

A thesis submitted in the partial fulfillment of the requirements for the degree of the Master of Philosophy. We accept this dissertation as conforming to the required standard.

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DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree. All the sources have been quoted and acknowledged by means of complete references.

Maria Bibi

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

Dedicated to My Mother

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TABLE OF CONTENTS

	Contents	Pages
	List of Abbreviations	I
	List of Figures	II
	List of Tables	III
	Abstract	IV
Chapter 1	**INTRODUCTION**	**1**
1.1	Epidemiology of Osteoarthritis	4
1.2	Diagnosis and assessment of OA	5
1.3	Grading of OA by Kellgren-Lawrence Grading System	7
1.3.1	Grade 0	7
1.3.2	Grade 1	7
1.3.3	Grade 2	7
1.3.4	Grade 3	8
1.3.5	Grade 4	8
1.4	Risk Factors for OA	10
1.4.1	Age	10
1.4.2	Sex	11
1.4.3	Obesity and metabolic disease	11
1.4.4	Smoking	14
1.4.5	Genetics	14
1.5	Molecular mechanisms involved in the pathogenesis of osteoarthritis	18
1.5.1	Articular cartilage degeneration and extracellular matrix in OA pathogenesis	20

1.5.2	Role of Chondrocytes in the destruction of articular cartilage and OA pathogenesis	22
1.5.3	Role of growth factors in OA pathogenesis	22
a	Insulin-like growth-factor-I	22
b	Transforming growth factor-β superfamily	23
1.5.4	Role of proteinases in pathogenesis of osteoarthritis	23
a	MMPs	24
b	ADAMTS	25
1.6	Inflammation in OA	26
1.6.1	Role of Proinflammatory Cytokines	26
1.7	Tumor Necrosis Factor-alpha (TNF-α)	27
1.7.1	TNF-α Gene Location and its Structure	27
1.7.2	TNF-α Protein Structure and synthesis	27
1.7.3	TNF-α Gene Expression	28
	TNF-α Gene regulation on various levels	28
	Genetic regulation	28
	Biological regulation	29
	Pathophysiological regulation	29
1.7.4	Biological Function of TNF-α	30
1.7.5	TNF-α Signaling Pathway	32
a	TNFR1; p55 and signal transduction	32
b	TNFR2; p75 and signal transduction	33
1.7.6	Single Nucleotide Polymorphisms (SNPs) in Promoter Region of TNF-α	35

1.8	Aims of the Study	36
Chapter 2	**MATERIALS AND METHODS**	**37**
2.1	Study Design	37
2.2	Study Subjects and their Inclusion Criterion	37
2.3	Exclusion Criterion of Patients	37
2.4	Questionnaire Filling	38
2.5	Blood Sample Collection and Storage	38
2.6	Molecular Analysis	38
2.6.1	Preparation of Stock Solutions	38
2.6.2	Preparation of Gel Electrophoresis Solutions	39
2.6.3	Genomic DNA Extraction	39
	Step 1: Cell Lysis	40
	Step 2: Protein Precipitation	40
	Step 3: DNA Precipitation	40
	Step 4: DNA Hydration	41
2.6.4	Confirmation of DNA	41
	One Percent (1%) Agarose Gel Electrophoresis	41
2.6.5	Polymerase Chain Reaction	41
	PCR Cycles	42
2.6.6	Two Percent (2%) Agarose Gel Electrophoresis	42
2.6.7	Genotyping of TNF-α Gene at Position -308G>A	43
2.6.8	Agarose Gel Electrophoresis	43
2.7	Statistical Analysis	44

Chapter 3	**RESULTS**	**46**
3.1	Baseline characteristics and clinical parameters of the study subjects	46
3.2	Genotype distribution in the study population	54
3.2.1	Genotype and allele frequencies of -308G>A SNP in OA study subjects	54
Chapter 4	**DISCUSSION**	**60**
	Future directions	63
Chapter 5	**REFERENCES**	**64**

LIST OF ABBREVIATIONS

%	Percentage
(NH4)2SO4	Ammonium sulphate
µL	Microliter
ADAMTS	A Disintegrin and Metalloproteinase with
Ala	Alanine
AP	Activator protein
APC	Antigen Presentation Cells
BMP	Bone Morphogenetic Protein
bp	Base pair
CI	Confidence interval
COPCORD	Community-Oriented Program for the Control of Rheumatic Diseases
COX-2	Cyclooxygenase-2
DCs	Dendritic Cells
dH2O	Distilled water
DISC	Death Inducing Signaling Complex
dNTPs	Deoxynucleotide triphosphates
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Regulated Kinase
EtBr	Ethidium bromide
FADD	Fas-associated protein with death domain
g	Gram
HCl	Hydrochloric acid
HRT	Hormone Replacement Therapy
IFN-γ	Interferon Gamma
IGF-1	Insulin-like Growth Factor 1
IKK	IκB kinase
IL(s)	Interleukin(s)
iNOS	Inducible Nitric Oxide Synthase
IκBα	Inhibitor Of NF-Kbα
JNK	C-Jun N-Terminal kinase

JSN	Joint Space Narrowing
KDa	Kilo-Dalton
M	Molar
MAPK	Mitogen Activated Protein Kinase
MEKK1	MAP/MEK kinase kinase 1
MgCl2	Magnesium Chloride
MHC	Major Histocompatibility Complex
MKK	MAP kinase kinase,
mL	Milli liter
mM	Milli molar
MMP	Matrix Metalloproteinase
N	Number
NaOH	Sodium hydroxide
NF-κB	Nuclear Factor Kappa-B
NHANES	National Health and Nutrition Examination Survey
EDTA	Ethylene Diamine Tetra-Acetic Acid
NK	Natural killer cells
NO	Nitric Oxide
OA	Osteoarthritis
OPD	Out Patient Department
OR	Odd ratio
P	Probability value
PA	Plasminogen Activator
PCR	Polymerase chain reaction
PG	Prostaglandin
RBC	Red Blood Cells
RE	Restriction enzyme
RFLP	Restriction Fragment Length Polymorphism
RIP	Receptor Interacting Protein
ROS	Reactive Oxygen Species
rpm	Revolution per minute
SD	Standard deviation
SNPs	Single Nucleotide Polymorphisms
sTNF-α	Soluble TNF-A Protein

T cell	Thymus Derived Cell
TAK1	TGF-B–Activated Kinase 1
TBE	Tris borate EDTA
TGF-β	Transforming Growth Factor-Beta
TIMP	Tissue Inhibitor of MMP
TLR	Toll Like Receptor
TMB	Tetramethylbenzidine
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TNF-α	Tumor Necrosis Factor-Alpha
TRADD	TNFR-Associated Death Domain
TRAF-2	TNF-Receptor Associated Factor-2
TTP	Tristetraprolin
UV	Ultraviolet
V	Volts
w/v	Weight/Volume

LIST OF FIGURES

Figure No.	Title	Page No.
1.1	Comparisons between healthy knee joint and OA affected knee joint	2
1.2	Osteoarthritic joint radiograph showing osteophytes at margins, loss of articular cartilage and subchondral bone cysts. (A) represents the normal femoral head, while (B) represents the femoral head of the OA patient (Dieppe & Lohmander, 2005)	3
1.3	Common clinical features that can lead to the diagnosis of the OA in patients (Dieppe & Lohmander, 2005)	6
1.4	Radiographic changes during the progression of OA from mild to severe and advanced stages (Ryu *et al*., 2012)	9
1.5	Risk factors of OA and disabilities related to the disease (Suri *et al*., 2012)	13
1.6	Role of overall genetic variations as a trigger of vulnerability and progression of OA (Valdes & Spector, 2010)	15
1.7	Joint showing the loss of articular cartilage, formation of osteophytes, subchondral bone remodeling and sclerosis, and synovial membrane activation in OA (Thomas Aigner & Schmitz, 2011)	19
1.8	Articular cartilage is mainly composed of extracellular matrix. Chondrocytes are the living cells embedded in between the matrix that make up the articular cartilage tissue (Aigner *et al*., 2006)	21
1.9	Molecular mechanisms involved in the progression of OA, including the role of matrix metalloproteinase (MMP), plasminogen activator (PA), tumor necrosis factor (TNF), nitric oxide (NO), transforming growth factor (TGF), prostaglandin (PG), bone morphogenetic protein (BMP), tissue inhibitor of MMP (TIMP) (Abramson *et al*., 2006)	25

1.10	TNF-α gene expression and its biological activities (Neurath, 2014)	29
1.11	Proinflammatory cytokine TNF-α dual role. MHC, Major histocompatibility complex; iNOS, Inducible nitric oxide synthase; TGF-β, Transforming growth factor-beta; APC, Antigen presentation cells; T cell, Thymus derived cell; IL, Interleukin; DCs, Dendritic cells (O'Shea *et al*., 2002)	31
1.12	TNF-α downstream expression showing the activation of AP-1, apoptosis initiation, and activation of NF-κB. TNF-α, Tumor necrosis factor-alpha; MEKK1, MAP/MEK kinase kinase 1; JNK, c-Jun N-terminal kinase; TRADD, TNFR-associated death domain; RIP, Receptor interacting protein; AP-1, Activator protein-1; MKK, MAP kinase kinase; NF-κB, Nuclear factor-kappa B; FADD, Fas associated death domain; ROS, Reactive oxygen species; ERK, Extracellular signal regulated kinase; TAK1, TGF-β activated kinase 1; TRAF2, TNFR-associated factor; IKK, IκB kinase; IκBα, Inhibitor of NF-κB alpha (Aggarwal *et al*., 2012)	34
1.13	TNF-α gene location within MHC. The arrows represents the orientation of transcription in TNF-α gene. +1 represents the transcription initiation site. The upstream positions of SNPs are shown (Verweij, 1999)	35
3.1	Comparisons between frequencies of male and female genders among OA patients (A). The line shows the increased susceptibility in females as compared to males (B). Gender based comparison among OA patients and healthy controls (C)	49
3.2	Comparison of frequencies of two sets of age groups between control individuals and OA patients (A). Comparison of ages among OA patients and healthy control individuals (B & C)	52
3.3	Comparisons of BMI frequencies between OA patients and healthy control groups	53

3.4	Electropherogram of ethidium bromide stained 4% agarose gel showing genotype pattern of NcoI restriction digest at TNF-α -308G>A site. The wells are identified as: M, nucleotide fragment size marker; UD, PCR product before restriction enzyme processing; and 1-16, different genotypes after restriction digestion. Genotypes are illustrated as GG (114 bp and 20 bp fragments), GA (134 bp, 114 bp and 20 bp fragments) and AA (134 bp). 20 bp fragment could not be visualized on gel	56
3.5	Comparison of genotype frequencies of TNF-α -308 G>A polymorphism between the patient and control groups	57
3.6	Comparison of wild type and variant genotypes among control and patient groups (A). Comparison of allele frequencies among control and patient groups (B)	59

LIST OF TABLES

Table No.	Title	Page No.
1.1	Genes involved in the onset and progression of the OA	16
1.2	Proteinases involved in the degradation of cartilage matrix	24
2.1	SNP Marker for TNF-α Gene, its Primer Sequence and Product Size	45
2.2	Programming of PCR Cycles	45
2.3	Summary of RFLP Analysis	45
3.1	Baseline characteristics of OA patients and control subjects	48
3.2	Comparison of age and BMI between OA patients and control subjects	50
3.3	Comparison of genotype frequencies of TNF-α -308 G>A polymorphism between the patient and control groups	57
3.4	Comparison of wild type and variant genotype and allele frequencies among control and patient groups	58

ABSTRACT

Osteoarthritis (OA) is musculoskeletal degenerative synovial joint disorder, including knee, hand, spine, hip and foot, characterized by loss of joint function and synovial proliferation due to changes in the joints, which include formation of osteophytes, joint space narrowing, subchondral sclerosis, bone marrow lesions and loss of articular cartilage; consequently, causing pain in the joints. The damage in the joint is due to the contributions of certain risk factors, including female gender, increased age, obesity, any related trauma, and genetics. Many Single Nucleotide Polymorphisms (SNPs) in the TNF-α gene are reported to be involved in the onset and progression of different diseases. So, the present case-control study was designed to explore the association of -308G>A SNP in TNF- α region with the OA disease susceptibility in a Pakistani population. PCR-RFLP method was used for the genotyping of -308G>A polymorphism in 70 OA patients and 38 healthy controls. Homozygous mutant genotype AA was found more prevalent in the control group as compared to the patients, and wild type genotype GG was found more prevalent among OA patients. Statistically significant difference in the allele frequencies of patients and control individuals was observed and *P* value was calculated to be significant ($P = 0.0001$). Baseline characteristics were also studied for validating the relationship with the disease, including age, sex and BMI. In result, OA was found more prevalent in women, individuals with increased age and increased BMI values. The genotyping analysis at molecular level explains the negative association of variant allele and concludes that -308G>A polymorphism is not associated with the OA susceptibility in Pakistani population. This study was performed for the first time, so, further analysis with larger sample size is required to validate the results for this SNP in Pakistani as well as other populations.

INTRODUCTION

Osteoarthritis (OA) is a musculoskeletal degenerative joint disorder, primarily related to synovial joints including knee, hand, spine, hip and foot (Dieppe & Lohmander, 2005), characterized by the loss of joint function and synovial proliferation that give rise to pain due to the progressive loss of articular cartilage along with the formation of new bone at the joint margins (osteophytosis) (Abramson & Attur, 2009), thickening of joint capsule and mild synovitis (Figure 1.1 and 1.2) (Pritzker, 2003). OA is the whole joint disease, which onset takes place due to the complex changes at cell, matrix and tissue levels, and their complex interactions at the tissue level (Abramson & Attur, 2009). The disease severity of knee and hip is related to various risk factors that cause the physical disability in the joints and give rise to high levels of pain, ultimately, increasing the need of joint replacement in severe conditions (Katz, 2006). In the advanced stages of OA, radiographs can show visible narrowing of joint space, subchondral bone changes and the appearance of osteophytes (Watt & Doherty, 2003) in the affected joints; commonly knee, spine and hand (Petersson, 1996). These radiographic and pathological changes in the joints are also related to the development of clinical problems including the cracking of joints called crepitus, stiffness and pain in the joints and their inactivity. In old age, the localized pain level is high among the patients of OA (Linaker *et al*., 1999; Peat *et al*., 2001).

The injury in the joints is due to the contributions of certain risk factors, including, genetics, obesity, sex, age, and any related trauma, which trigger the abnormal biochemical and pathophysiologic processes in the affected areas involving the synovium, bone, cartilage and its surrounding tissues; resulting in the pharmacologic and behavioral intervention, and development of the characteristic features of the OA, *i.e*., osteophytes formation in the joints, articular cartilage and meniscal degradation, subchondral sclerosis, synovial proliferation and bone marrow lesions (Abramson & Attur, 2009).

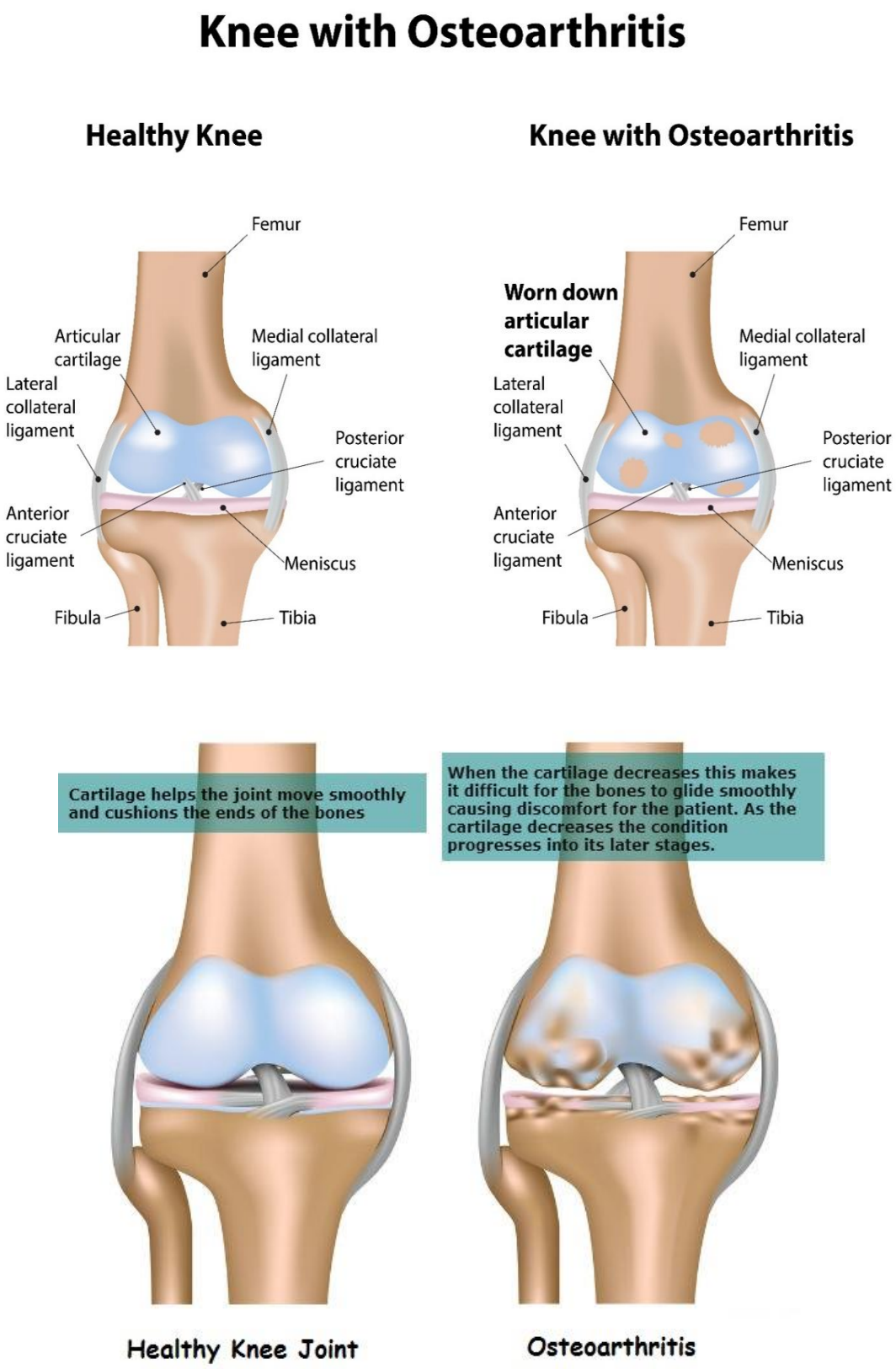


Figure 1.1: Comparisons between healthy knee joint and OA affected knee joint.

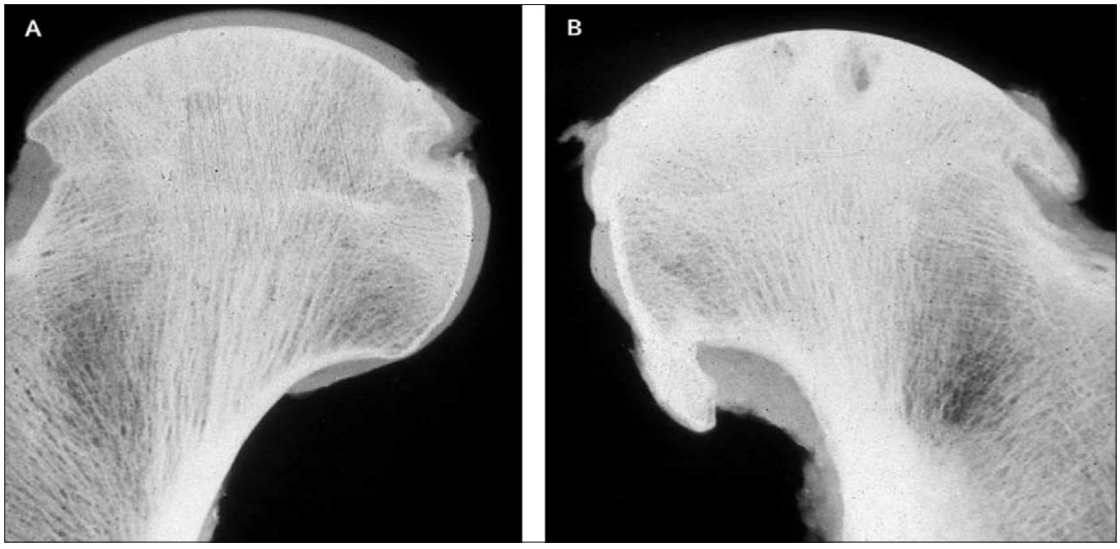


Figure 1.2: Osteoarthritic joint radiograph showing osteophytes at margins, loss of articular cartilage and subchondral bone cysts. (**A)** represents the normal femoral head, while (**B)** represents the femoral head of the OA patient (Dieppe & Lohmander, 2005).

1.1. Epidemiology of Osteoarthritis

Genetic epidemiology is the study of the impact of the overall aspects of the diseases in populations. Osteoarthritis (OA) majorly attributes to the disability in the joints of hips and knees, and is considered as the fourth leading cause of disability worldwide. In Asia, the ageing process is very rapid and OA is strongly related to aging. Moreover, the disease has close association with the heavy physical activities (Fransen *et al*., 2011). There are a variety of diagnostic classifications for the accurate diagnosis of the disease, including the radiographic case definition, which is the most reliable method, giving the highest estimates of the levels of damage occurred in the joints of patients suffering from OA, and symptomatic and self-reported definitions almost giving the similar estimates overall. The prevalence and occurrence of the disease estimates is variable in different populations due to the selection of different diagnostic method (Litwic *et al*., 2013). The prevalence of hip OA is low, while osteoarthritis in knee joints is considered as highly prevalent in the older populations in the urban and rural areas of Asia, and is also related to obesity. Moreover, it is also considered that if this population molds its living and activities to certain extent by avoiding kneeling, heavy activities, squatting, *etc*., this practice would give them relief from their chronic pains in the knees, thus reducing the progress of disease to further advanced stages (Fransen *et al*., 2011).

According to the World Health Organization's Scientific Group on Rheumatic Diseases, clinically diagnosed OA is estimated to occur in 10% of the world population with 60 years of age or above (Litwic *et al*., 2013). Moreover, the estimates shows that the percentage increases to 40% in the people who are 70 years old or above (Dieppe & Lohmander, 2005). So, OA is more prevalent than any other form of arthritis in the world's population (Lawrence *et al*., 2008). According to the estimates, where the percentage of OA in the people aged 65 years or above was 6.8% in 2008, it would upsurge up to 16.2% by the year 2040 (Kinsella & He., 2009).

The disease prevalence in the hip and knee OA patients has been studied by a few large population-based surveys by relating the changes in the radiographic findings to the pain level, however, the true symptomatic disease prevalence could not be related to the radiograph as the early stage of disease has no structural changes in the joints, hence

could not be detected radiographically. Whereas, the estimates based on the self-reported joint pain are not significant as the pain and stiffness may not attribute to OA for all cases (Fransen *et al*., 2011). Community-Oriented Program for the Control of Rheumatic Diseases (COPCORD) conducted a study on the evaluation of association of pain level with the disease prevalence in Asia, particularly in the developing countries and its rural communities. The survey majorly included the hip and knee joint OA cases, but is more focused on the systematic rheumatic and inflammatory diseases (Haq *et al*., 2008).

1.2. Diagnosis and assessment of OA

Severity of OA can be assessed and diagnosed on the basis of presence of a few clinical signs initially, and the disease presence can then be confirmed from the radiographs to support the clinical diagnosis. Although radiography can cause harm, so, these are only performed as a last resort to diagnose the disease stage accurately to treat the ailment by mechanical interventions, *i.e*., joint replacement, or osteotomy (Dieppe & Lohmander, 2005). In the knee OA patients, both the knee joints are assessed carefully to mark the disease severity and decide a treatment accordingly and its evaluation in terms of the effectiveness of the response to the disease condition (Cooper *et al*., 2000). Figure 1.3 explains the clinical features for the accurate diagnosis of OA.

Diagnosis of the OA via common clinical features	
Increased age **Patient's age should likely be 40 or above for the development of OA.**	**Reduced movement** The joints are painful during the movements and patient can only perform restricted movements.
Pain **Pain can be experienced when the joints are being used, and the pain is relieved if the patient is resting. The disease is also related to the pain during the patient is resting or at night in bed in severe conditions or the advanced states.**	**Swelling** Swelling can be developed on the margins of the joints due to the appearance of bony outgrowths (osteophytes) or minor swellings of soft tissue can be developed due to secondary synovitis.
Stiffness **Joints can experience stiffness in symptomatic OA.**	**Crepitus** Affected joints crack or creak during the performance of daily routine activities and movements.

Figure 1.3: Common clinical features that can lead to the diagnosis of the OA in patients (Dieppe & Lohmander, 2005).

1.3. Grading of OA by Kellgren-Lawrence Grading System (Kellgren & Lawrence, 1957)

OA is described and diagnosed, based on many of the definitions among which the classification into systematic OA and radiographic OA are most common and accepted worldwide. Kellgren-Lawrence (K-L) grading system is the most accurate and commonly used grading system to describe the exact stage of the disease by determining the disease severity via radiograph on the basis of the presence and absence of osteophytes, reduction of joint space, tibiofemoral joint deformity and sclerosis (Kellgren & Lawrence, 1957). K-L grading system is used for all types of OA including hip and knee joints, and radiograph can determine K-L grade 2 or more specifically in the patients of knee OA (Suri *et al*., 2012). Kellgren and Lawrence system explains the following five severity levels of the disease (Figure 1.4).

1.3.1. Grade 0

This stage indicates the absence of any radiographic changes that can refer to the disease, but the patient is clinically diagnosed to be suffering from OA.

1.3.2. Grade 1

This stage of OA indicates the osteophytic lipping and doubtful joint space narrowing (JSN); *i.e.*, the space between the bones might be reduced due to the cartilage cover reduction.

1.3.3. Grade 2

This stage of OA indicates the presence of definite osteophytes (small bony projections around the bone margins) in the affected joints along with the definite joint space narrowing (JSN). The osteophytes cause the pain in the joints and restrict their locomotory activities too.

1.3.4. Grade 3

This stage of OA indicates the advance phase of the disease with the presence of multiple osteophytes, sclerosis, definite joint space narrowing, and expected bone contour deformity.

1.3.5. Grade 4

This stage of OA indicates multiple and large osteophytes, definite bone contour deformity and joint space narrowing with severe sclerosis. Figure 1.4 shows the radiographic changes occurring during the disease progression from mild to advanced stages.

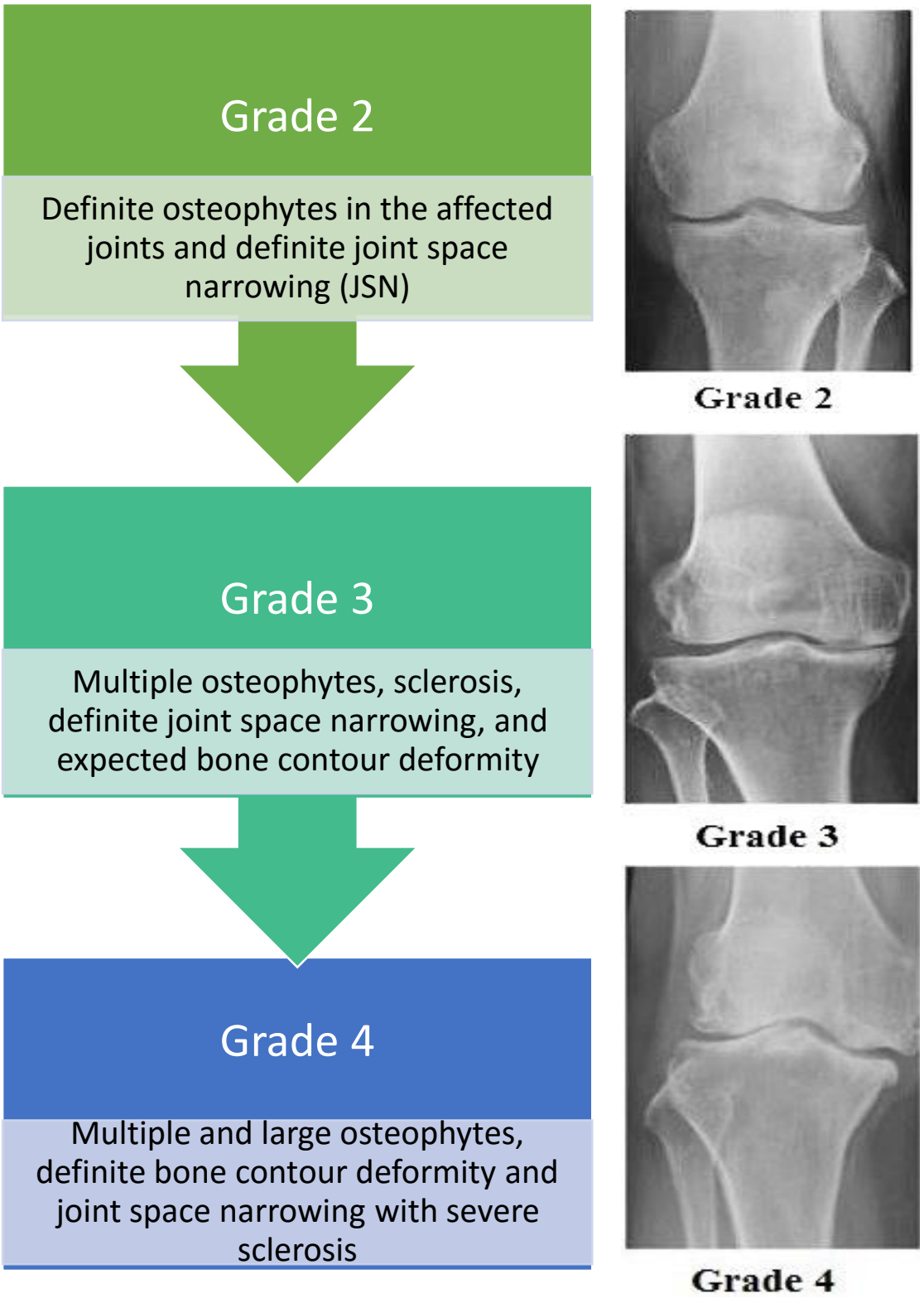


Figure 1.4: Radiographic changes during the progression of OA from mild to severe and advanced stages (Ryu *et al*., 2012).

1.4. Risk Factors for OA

A number of risk factors are involved in the occurrence of osteoarthritis, which also have close association with the type of activities performed in common life routines.

1.4.1. Age

A few cohort studies conducted in large populations from China, Japan, Pakistan and Korea have related the increased risk of incidence of OA with the risk factors including older age, obesity and female gender (Gibson *et al*., 1996; Du *et al*., 2005; Zeng *et al*., 2005; Zeng *et al*., 2006; Sudo *et al*., 2008; Muraki *et al*., 2009; Kim *et al*., 2010). It is reported that the risk of occurrence of the disease significantly increase with age (Felson *et al*., 1995; Grotle *et al*., 2008), but some of the studies support the view of the positive association of the progression of the disease with increasing age, while others conclude that there are no associations between disease progression and aging (Lievense *et al*., 2002; Belo *et al*., 2007). A few of the underlying factors are involved in the incidence of the disease and its relationship with the increasing age, *i.e*., weakening of the muscle, oxidative damage, reduction in proprioception and thinning of the cartilage. Moreover, the mechanisms of maintenance of tissue homeostasis is subjected to decline with increasing age, which is one of the basic cellular mechanisms that saves the body by responding appropriately to joint injury or stress, so, destruction of the joint tissue and its loss is initiated. All these factors contribute towards the incidence of radiographic and symptomatic OA with aging, making age to be the first important risk factor in its occurrence (Litwic *et al*., 2013). A study concluded that the changes occurring in the joint tissues with aging is a cause of development of OA. These changes, along with all others, also include cell senescence, in which, senescent secretory phenotype is developed, and also, advanced glycation end-products formation occurs in the matrix, which in turn affect the mechanical functioning of the joint (Anderson & Loeser, 2010). The prevalence is linked positively to the increasing age as in the aging process, the muscle strength is reduced and wear and tear of the subchondral bone and articular cartilage takes place (Grimby & Saltin, 1983; Fiatarone *et al*., 1990). So, the decrease in the age related muscle function is associated with the increase in the occurrence of OA (Hurley, 1999).

1.4.2. Sex

OA is more prevalent in women as compared to men, and the risk is likely to increase around the time of menopause (Srikanth *et al*., 2005). The increased risk of the incidence of the disease is related to the contribution of the hormonal factors or changes in the hormones during or around the menopause, but this is only a hypothesis as it is not supported by the proof of epidemiologic and clinical studies (Cirillo *et al*., 2006; de Klerk *et al*., 2009). A few of the studies have concluded that hormone replacement therapy (HRT) or estrogen therapy is likely to show protective effect on radiographic knee and hip OA (Spector *et al*., 1997), and advancement towards joint replacement (Cirillo *et al*., 2006). A population based study concluded in a meta-analysis that the association of the disease incidence can be linked to female gender in a site-specific manner, showing that male gender is less prone to develop radiographic knee OA by showing the risk ratio of 0.63 and 95% confidence interval (95% CI) 0.53-0.75, and incidence rate ratio of 0.55. 95% CI 0.32-0.94 (Srikanth *et al*., 2005). It is evident that symptomatic knee OA is highly prevalent in female gender (Jordan *et al*., 2007), but evidence for the radiographic disease progression positive association with female gender is still a conflicting point as some of the studies hypothesize it to have positive link and others hypothesize the association to be negative (Belo *et al*., 2007; Bierma-Zeinstra & Koes, 2007), but some studies have termed the relationship to be too complex (de Klerk *et al*., 2009).

1.4.3. Obesity and metabolic disease

There is high risk of developing OA due to obesity as it is the best-established and strongest risk factor that contribute to the occurrence of disease. The association between obesity and the development of knee OA is stronger as compared to the incidence of hip OA, showing the confidence interval as 2.81; 95% CI 1.32-5.96 (Grotle *et al*., 2008). It is reported that there might be a common pathogenic mechanism that give rise to systemic inflammation and metabolic abnormalities because of the association of OA with metabolic syndrome. According to the study conducted using the NHANES III data, it is concluded that the risk of development of metabolic syndrome increases in the individuals at the age of 43.8 years (average age of the population). Strong associations are also reported in-between OA and the cardiovascular risk factors, including cholesterol and hypertension (Puenpatom &

Victor, 2009). However, several studies have suggested that there is no significant clinical association between OA and diabetes (Frey *et al*., 1996). Obese individual is at three times higher risk to develop OA as compared to a person with normal weight and body mass index as obesity is known to have local mechanical, as well as systemic effects on OA (Blagojevic *et al*., 2010). Obesity is a modifiable risk factor that can be controlled by lowering the BMI from more than 30 to fall in the optimum range; *i.e*., lower than 25, which means that 29% of the knee OA is estimated to reduce in a population, thus decreasing the overall impact (Zhang, 2010). Triggering the smaller changes in the BMI values can also produce significant results. According to The Framingham study, the risk of development of symptomatic knee OA was decreased up to 50% by only decreasing the BMI by ≥2 units; *i.e*., ~11 lb. (Felson *et al*., 1992). Moreover, according to Johnston County study, it is concluded that there is only a 30% of the lifetime risk for an individual to develop the symptomatic knee OA who has BMI 1<25, while the risk increases to 47% for individuals falling in the BMI range of 25-30, and even more elevated to 61% for obese individuals, *i.e*., with the BMI >30 (Murphy *et al*., 2008). Furthermore, in a study based on cohort of 60 to 64 years old adults in U.S., it is estimated that during the period of 10 years, the risk of progression from early symptomatic knee OA; *i.e*., K-L grade 2, to advanced knee OA; *i.e*., K-L grade 3, is only 37% in individuals with BMI falling in normal range and is as high as 63% in obese individuals (Holt *et al*., 2011). It is estimated in the meta-regression techniques that the weight reduction up to 10% can resultantly produce a great amount of clinical improvement in the disability due to OA (Christensen *et al*., 2007). Moreover, moderate exercise along with modest weight loss is also reported to improve the physical performance in the people of old age along with the reduction of pain levels (Messier *et al*., 2004). So, most of the evidences are in the favor of the positive and robust associations of obesity with knee OA, while a few negate the notion (Belo *et al*., 2007; Reijman *et al*., 2007). While, for progressive hip OA, its relationship with obesity is inconsistent and unclear, and the association is positive for the hand OA, explaining the role of systemic factors involved in the onset of disease (Carman *et al*., 1994; Oliveria *et al*., 1999; Karlson *et al*., 2003; Reijman *et al*., 2007; Grotle *et al*., 2008). Figure 1.5 shows the overall risk factors that contribute to the disease progression.

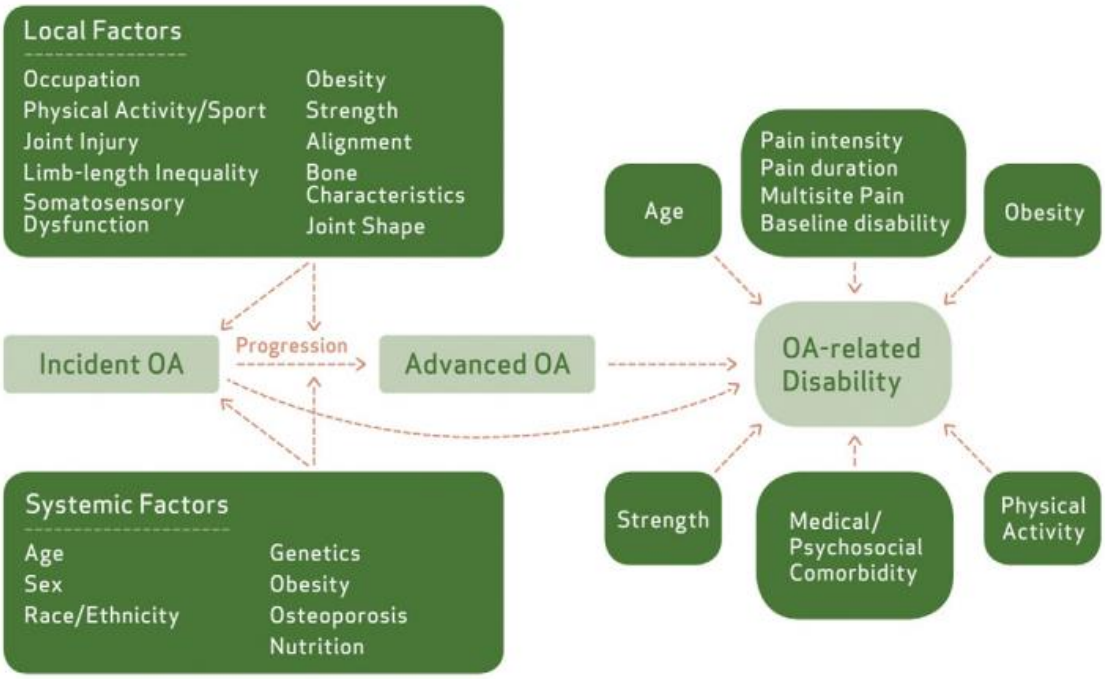


Figure 1.5: Risk factors of OA and disabilities related to the disease (Suri *et al*., 2012).

1.4.4. Smoking

Previous studies shows a conflicting role of smoking in the incidence of OA, a few showing its positive link, while others showing its negative associations with the disease condition, but a meta-analysis in 2011 has proved its positive association to be false and termed the study to be biased at the selection of the control group individuals (Hui *et al*., 2011).

1.4.5. Genetics

Studies revealed that genetics play a crucial role in the onset and progression of OA in humans. Molecules crucial to the pathology of joint components involved in certain signaling pathways are identified, which include apoptotic-related molecules, wingless-type signaling, bone morphogenetic protein (BMP) signaling, and thyroid pathway. In the susceptibility of OA, some of the factors held responsible are production of cytokines, arachidonic acid metabolism and prostaglandin, and all these pathways might be targeted by pharmacological intervention. Current therapeutic approaches are not able to prevent the initiation and progression of OA to cure the musculoskeletal disability (Valdes & Spector, 2010). Susceptibility of OA can be mediated through diverse pathways and various stages (Figure 1.6). Genes and the pathways involved in the progression of OA; specifically knee OA, are mentioned briefly in the Table 1.1.

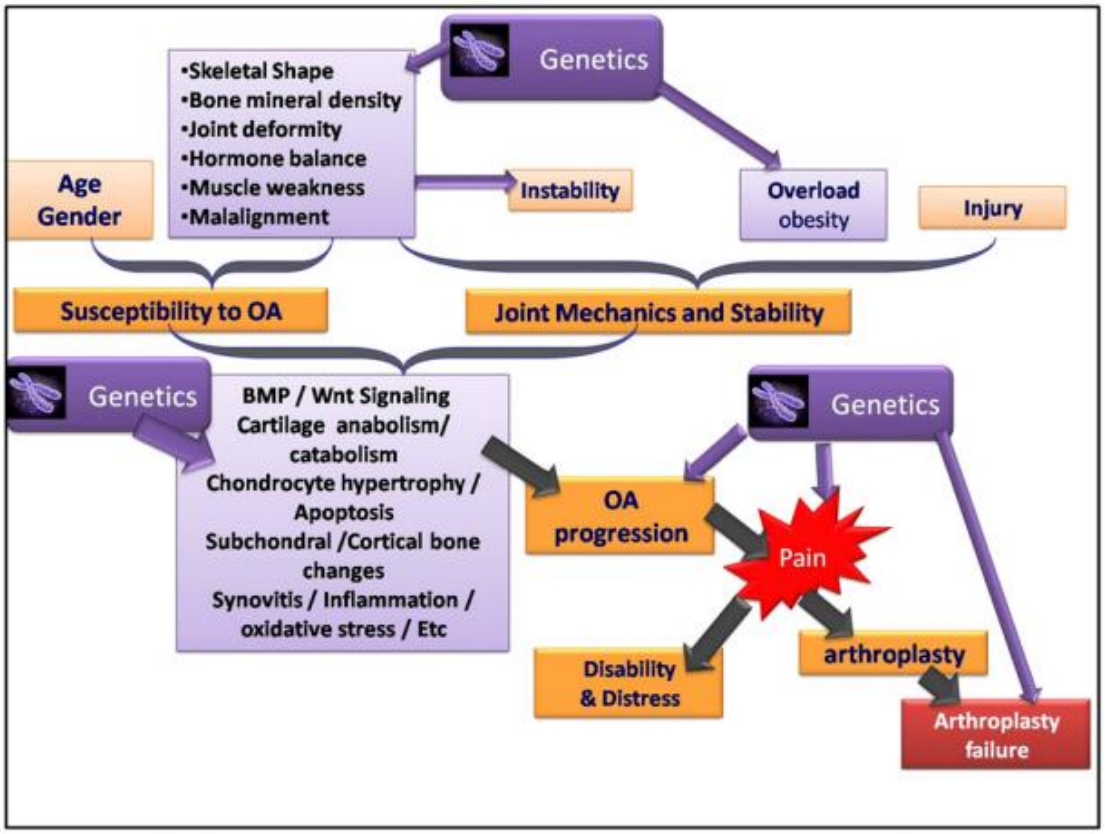


Figure 1.6: Role of overall genetic variations as a trigger of vulnerability and progression of OA (Valdes & Spector, 2010).

Table 1.1: Genes involved in the onset and progression of the OA.

S.No.*	Gene	Symbol	Pathway	Putative or known Function
a	Bone morphogenetic protein 2	*BMP2*	BMP	Growth factor involved in chondrogenesis and osteogenesis
b	Asporin	ASPN	BMP	Cartilage extracellular protein that regulates the activity of TGFb
c	Cartilage intermediate layer protein	CILP	BMP	Inhibits TGFß1–mediated induction of cartilage matrix genes
d	Osteoprotegerin	OPG	BMP/Wnt	Regulation of osteoclastogenesis
e	Cytosolic phospholipase A2	PLA2G4A	Inflammation	Catalyzes the rate-limiting step in the production of pro-inflammatory eicosanoids and free radicals
f	Cyclooxygenase 2	PTGS2	Inflammation	COX-2-produced prostaglandin-E2 modulates cartilage proteoglycan degradation in OA
g	Interleukin (IL-)1 alpha, IL- beta and IL-1 receptor antagonist	IL1 gene cluster	Inflammation	Regulation of metalloproteinase gene expression in synovial cells and chondrocytes
h	Interleukin 6	IL6	Inflammation	Pro-inflammatory cytokine, involved in the cartilage degradation but also induces ILRa
i	Interleukin 10	IL10	Inflammation	Anti-inflammatory cytokine inhibits the synthesis of IL-1
j	Vitamin D receptor	VDR1	Other	Nuclear receptor, mediates effects of vitamin D whose serum levels affect incidence severity and progression of OA
k	A disintegrin and metalloproteinase domain 12	ADAM12	Other	Metalloprotease involved in osteoclast formation and cell-cell fusion
l	Iodothyronine-deiodinase enzyme type 2	DIO2	thyroxin	Thyroxin signaling: Regulates intracellular levels of active thyroid hormones in target tissues
m	Secreted frizzled-related protein 3	FRZB	Wnt	Wnt antagonist and modulator of chondrocyte maturation

n	Low- Density Lipoprotein Receptor-Related Protein 5	LRP5	Wnt	Receptor involved in Wnt signaling via the canonical beta-catenin pathway

*****a (Valdes *et al*., 2004; Valdes *et al*., 2006), b (Kaliakatsos *et al*., 2006; Rodriguez-Lopez *et al.*, 2006; Valdes *et al*., 2007), c (Valdes *et al*., 2004; Valdes *et al*., 2007), d (Valdes *et al*., 2004; Valdes *et al*., 2006), e (Valdes *et al*., 2008), f (Valdes *et al*., 2004; Valdes *et al*., 2005; Valdes *et al*., 2006; Valdes *et al*., 2008), g (Meulenbelt *et al*., 2004; Smith *et al*., 2004; Moxley *et al*., 2007; Kanoh *et al*., 2008), h (Nicklas *et al*., 2005; Pola *et al*., 2005; Kamarainen *et al*., 2008;), i (Fytili *et al*., 2005; Riyazi *et al*., 2005), j (Valdes *et al*., 2004; Valdes *et al*., 2007), k (Valdes *et al*., 2004; Valdes *et al*., 2006), l (Meulenbelt *et al*., 2008), m (Lane *et al*., 2006; Valdes *et al*., 2007; Evangelou *et al*., 2009;), n (Smith *et al*., 2005).

1.5. Molecular mechanisms involved in the pathogenesis of osteoarthritis

A combination of changes and events together makes the pathology of any disease, and for OA, the changes in the bone and loss of focal and progressive hyaline cartilage associated with it are the major events that leads to the progression of the disease. These changes majorly involves the events including the formation of osteophytes along with the thickening of subchondral bone along the course of occurrence of the disease and its progression. Marginal outgrowths are particularly observed and the soft tissue structures associated with the joint is affected, which include synovium, bridging muscles and ligaments. Bridging muscles becomes weak in the patients suffering from OA, synovium show inflammatory infiltrates, while ligaments are regularly lax (Hedbom & Häuselmann, 2002). Figure 1.7 shows the pathology of the joint of patient suffering from OA.

By the onset of OA, activation and increased synthesis of extracellular proteinases also starts, which trigger many morphological changes later. These extracellular proteinases also include metalloproteinase. Furthermore, according to the studies, it is also concluded that due to the deficient stimulation by the growth factors, minimum or insufficient production of new matrix macromolecules occurs that becomes the underlying cause of morphological changes. The interleukin-1 proinflammatory cytokine production elevates during this condition, due to which, the chondrocytes also show response by increasing the production of prostaglandin E_2 and nitric oxide; that are responsible for the changes related to OA on cellular level (Hedbom & Häuselmann, 2002). All these events are responsible for the onset of morphological changes including the cleft formation, cartilage surface fibrillation and loss of cartilage volume afterwards (Dieppe & Lohmander, 2005).

Cartilage has a dominant role in the pathogenesis of the joint damage, but scientists has not linked it with the pain generated in the OA affected joint directly. The studies revealed that the nerve endings associated with the synovium, subchondral bone, periosteum, joint capsule and ligaments associated with the affected joint may serve to generate pain stimuli in the localized affected region in OA (Kidd *et al*., 2004). It is concluded from the imaging study of the knee joint that there is a positive connection

and association of synovitis and subchondral bone with the generation of pain in OA (McCrae *et al*., 1992; Creamer *et al*., 1996; Felson *et al*., 2003). In these patients, there is no clear link of the generation of pain in the joint from the normal joint tissues, but it is characterized by the peripheral pain sensitization (Farrell *et al*., 2000; Bajaj *et al*., 2001; Bradley *et al*., 2004) that could be triggered by the cytokines or nerve growth factors (Kidd *et al*., 2003). Evidence also reveals that central pain sensitization at the cortical or spinal level may occur in OA affected joints (Melzack *et al*., 2001).

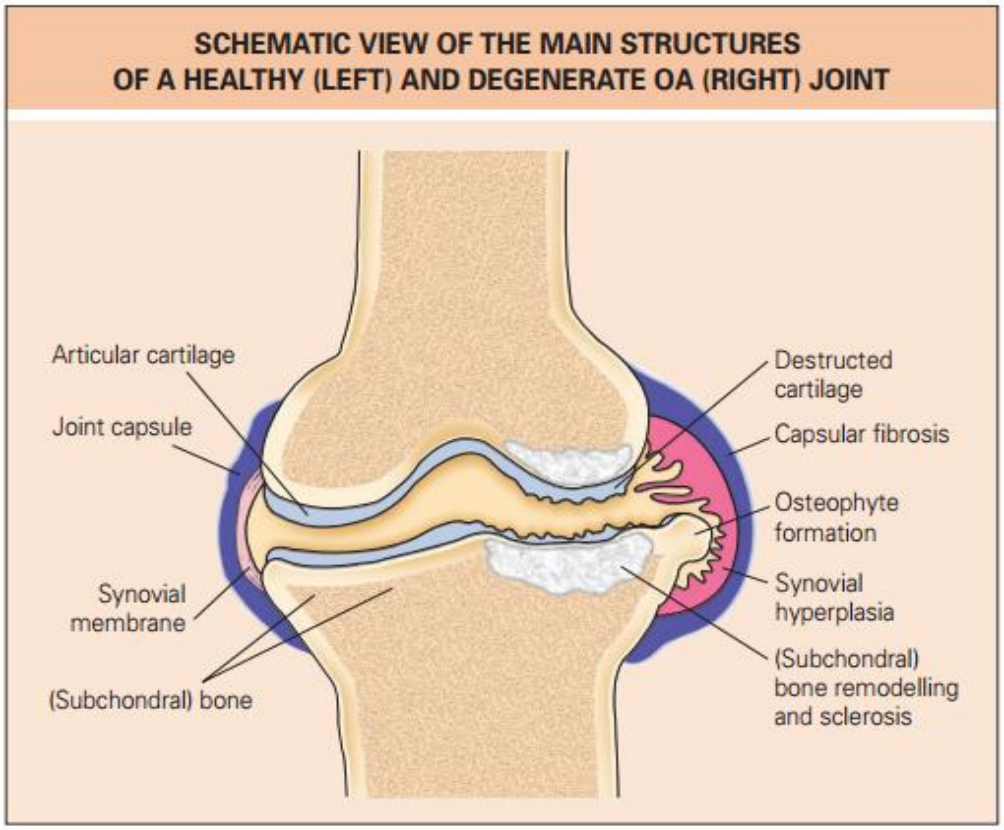


Figure 1.7: Joint showing the loss of articular cartilage, formation of osteophytes, subchondral bone remodeling and sclerosis, and synovial membrane activation in OA (Thomas Aigner & Schmitz, 2011).

Osteoarthritis is a heterogeneous condition and many of the hypotheses are developed by the scientists to explain the onset of the disease. A few of the possible hypotheses for the initiation and progression of OA are explained below.

1.5.1. Articular cartilage degeneration and extracellular matrix in OA pathogenesis

Human articular cartilage is a uniquely designed and highly specialized biomaterial that can be remodeled by anabolic and catabolic processes (Hedbom & Häuselmann, 2002; Aigner & Schmitz, 2011). In advanced OA, most of the changes occur in the articular cartilage. Articular cartilage is the tissue that covers the joints in the form of thin layer; resting on subchondral bone. The bone contain the blood vessels and nerves, while cartilage lack them. It is meant to decrease the friction in the joints and distributes the static and dynamic joint load. The cartilage cells are intended to maintain the collagen and proteoglycans rich cartilage matrix, which in turn maintains the functional properties of the cartilage. With the occurrence of OA, gradual proteolytic degradation of this matrix starts. Moreover, the chondrocytes trigger the increased synthesis of this matrix or matrix with a slightly different composition (Aigner *et al*., 2001; Heinegard, 2003; Sandy & Lark, 2003), while in normal adult cartilage, chondrocytes are responsible to maintain a proper balance in between the formation of new components of extracellular matrix and their degradation. Consequently, the chondrocytes metabolic activity is shifted towards the synthesis of the new matrix, which trigger the gradual articular cartilage degeneration (Hedbom & Häuselmann, 2002). Figure 1.8 shows the articular cartilage and its composition which depicts that 95% of the tissue volume of the articular cartilage consist of extracellular matrix and chondrocytes are the reactive cells interspersed between the matrix.

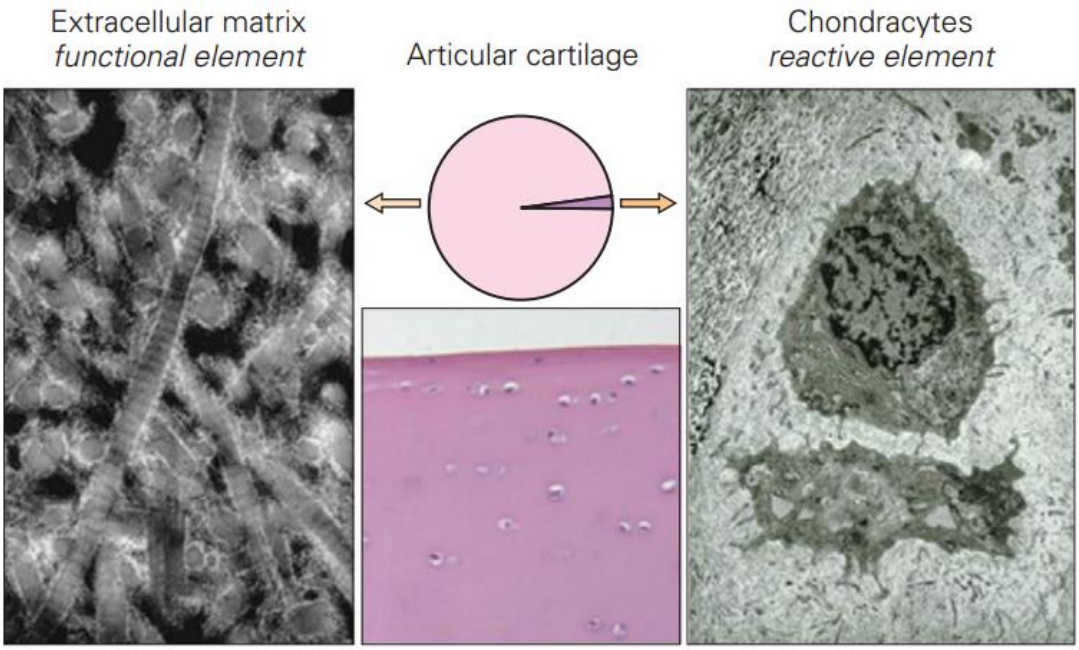


Figure 1.8: Articular cartilage is mainly composed of extracellular matrix. Chondrocytes are the living cells embedded in between the matrix that make up the articular cartilage tissue (Aigner *et al*., 2006).

1.5.2. Role of Chondrocytes in the destruction of articular cartilage and OA pathogenesis

Chondrocytes are the cells that make up the hyaline cartilage and play a major role in the growth and development by the formation of extracellular matrix. Moreover, it also play important role in adult life by maintaining the tissue homeostasis to the optimum. In mature articular cartilage, chondrocyte lack mitotic activity and the rate of new matrix formation and degradation is very low. In early OA, extracellular matrix undergo structural changes, which in turn, trigger the chondrocyte proliferation or clonal growth, PG biosynthesis, increased collagen biosynthesis and elevated production of matrix-degrading proteinases and catabolic cytokines (Hedbom & Häuselmann, 2002).

1.5.3. Role of growth factors in OA pathogenesis

Soluble growth factors are responsible to upsurge the anabolic activity of chondrocytes (Hascall *et al*., 1983). Here two important growth factors and their role in the pathogenesis of OA is discussed briefly.

a. Insulin-like growth-factor-I

One of the growth factors responsible for the stimulation of PG biosynthesis is Insulin-like growth factor (IGF)-I (McQUILLAN *et al*., 1986); hence, it is prominent anabolic factor. IGF-I; in presence of IL-I and TNF-α, is known to enhance the PG biosynthesis. Moreover, it is also known to reduce the degradation of cartilage that is normally enhanced and stimulated by these two cytokines (Tyler, 1989). Furthermore, IGF-I is also known as a differentiation factor. It is also responsible for the triggering of reexpression of dedifferentiated aggrecan and cartilage-specific collagen II (Yaeger *et al*., 1997). In OA, the expression of IGF-I is higher and the high expression is shown by the cells in the areas where more advanced OA lesions are present (Middleton & Tyler, 1992). So, the cartilage of patients of OA show an increased expression of IGF-I and OA chondrocytes possess more IGF receptors than normal (Doré *et al*., 1994; Middleton *et al*., 1996); but are hyporesponsive to exogenous IGF-I (Doré *et al*., 1994; Loeser *et al*., 2000). A few distinct changes occur in the cartilage when a patient is suffering from the disease OA and these changes or events might be triggered by the loss of IGF-I effects on the articular chondrocytes where the mechanism is the

upregulation of IGF-binding proteins (Chevalier & Tyler, 1996; Olney *et al*., 1996; Tardif *et al*., 1996).

b. Transforming growth factor-β superfamily

Chondrocyte biosynthesis is also triggered by the members of transforming growth factor (TGF)-β family and other associated and related bone morphogenetic proteins (BMP) family. Articular chondrocytes express three isoforms of TGF-β in mammals (Frazer *et al*., 1991). Undifferentiated mesenchymal cells expression is induced by TGF-β and chondrocyte phenotype is expressed (Seyedin *et al*.,1985). Cartilage of patients suffering from OA is more sensitive to TGF-β in response for the stimulation of PG synthesis in vitro as compared to the normal cartilage. The studies conclude that impaired function of TGF-β within joints can be the potential cause of OA (Lafeber *et al*., 1997).

1.5.4. Role of proteinases in pathogenesis of osteoarthritis

Several proteinases are involved in the degradation of the cartilage matrix and its constituents. Chondrocytes are responsible for the synthesis and secretion of these proteinases and its capability of degradation is directly dependent on the rate of synthesis and secretion of these proteinases (Table 1.2). For the appropriate and optimum morphogenesis and tissue remodeling in normal conditions, these enzymes are synthesized and their activity is controlled on optimum levels. Proteinases activity is regulated on three levels; its synthesis and secretion by chondrocyte cells, activation, and finally inactivation by the inhibitors for the confirmation of optimum expression. In OA, the expression of the proteinases is up-regulated (Billinghurst *et al*., 1997; Shlopov *et al*., 1997; Tetlow *et al*., 2001).

Table 1.2: Proteinases involved in the degradation of cartilage matrix

Metalloproteinases	**Collagenases**	**MMP-1, MMP-8, MMP-13**
	Gelatinases	MMP-2, MMP-9
	Stromelysins	MMP-3, MMP-7, MMP-10, MMP-11
	Membrane type	MMP-14
Aggrecanases	Disintegrin and metalloproteinase type	ADAM-TS4, ADAM-TS5
Other Proteinases	Elastase	
	Cathepsin	Cathepsin B, D , G, L
	Proteinases of the coagulation system	tPa, uPA, plasmin

a. MMPs

MMPs are proteinases that hold definite and prominent role in the degradation of cartilage in OA (Billinghurst *et al*., 1997; Shlopov *et al*., 1997; Tetlow *et al*., 2001). Studies conclude that synovial fluid of the patients of OA has higher concentrations of MMPs compared to healthy controls (Clark *et al*., 1993; Ishiguro *et al*., 1999). TIMP-1 levels are also elevated in the synovial fluid during the disease condition in OA patients which are the tissue inhibitors of metalloproteinases. TIMP-1 levels increase can possibly be linked to the elevated levels of MMPs and can be considered as the response of chondrocytes to balance the proteinases activity overall. Interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and MMP13 can cleave the collagen if present in native states and this step is considered as the rate-determining step in the process of degradation of collagen. The remaining fragments from the initial degradation are subjected to other enzymes including MMP-2, MMP-3, MMP-9 and cathepsin B for further degradation. Among all these proteinases, MMP-13 is of the prime importance in OA as it is responsible for the degradation of type II collagen (Knäuper *et al*., 1996) and is highly expressed in the disease condition of OA (Reboul *et al*., 1996; Shlopov *et al*., 1997; Tetlow *et al*., 2001).

b. ADAMTS

ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) is a protein family that contain aggrecanase enzymes including aggrecanase 1 and 2. Aggrecanase can cleave the amino acids Glu373 and Ala374 and take part in the aggrecan degradation, thus play a role in the pathogenesis of OA (Lark *et al*., 1997; Abbaszade *et al*., 1999; Tortorella *et al*., 1999). Figure 1.9 shows the overall mechanisms involved in the progression of OA.

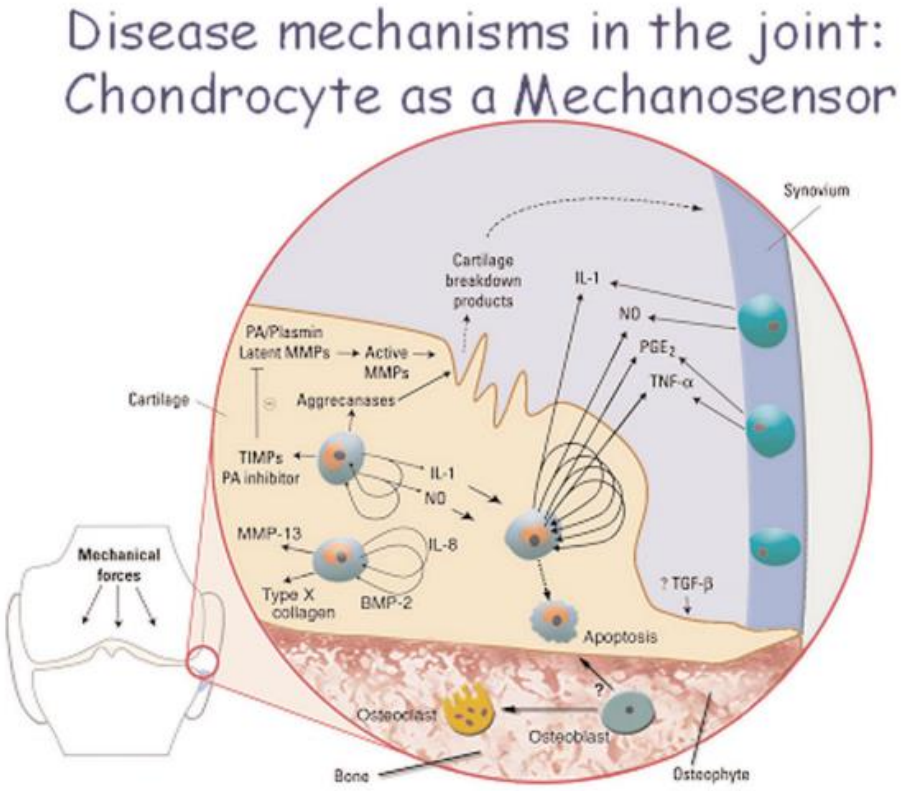


Figure 1.9: Molecular mechanisms involved in the progression of OA, including the role of matrix metalloproteinase (MMP), plasminogen activator (PA), tumor necrosis factor (TNF), nitric oxide (NO), transforming growth factor (TGF), prostaglandin (PG), bone morphogenetic protein (BMP), tissue inhibitor of MMP (TIMP) (Abramson *et al*., 2006).

1.6. Inflammation in OA

In the diseases like rheumatoid arthritis, cartilage destruction is triggered by the inflammatory mediators including cytokines, NO (Nitric oxide) and prostanoids (Häuselmann, 1997; Arend, 2001). In this case, inflammation occurs in the synovial membrane, and later on, cartilage destruction starts. OA is among non-inflammatory arthropathies, and this condition can be developed when cartilage release components in the synovial fluid and synovial cells reacts towards these components (Pelletier *et al*.,1995; Smith *et al*., 1997), but, the chief pathogenic events occurs within the cartilage (Amin *et al*., 1999; Moos *et al*., 1999). Overall, the studies conclude that the proinflammatory cytokines and inflammation mediators are the principal cause of the development of OA when these express in autocrine/paracrine fashion in the cartilage (Towle *et al*., 1997; Amin *et al*., 1999).

1.6.1. Role of Proinflammatory Cytokines

Immune cells secrete proinflammatory cytokines, including TNFs, interferons, ILs, and colony stimulating factors, and these are responsible to control the function of other cells of the immune system. Except for the effect on the immune cells, many of these proinflammatory cytokines affect the non-immune cells too, including chondrocytes and fibroblasts. In the pathogenesis of OA, TNF-α and IL-1 β play a major role in its development and progression (Westacott & Sharif, 1996). The overall effect of contribution of TNF-α and IL-1 β in the pathogenesis of OA is dependent upon their absolute concentrations and this effect may vary by the influence of other modulating cytokines, including IL-6, IL-8 and LIF (Leukemia Inhibitory Factor). Although the IL-6, IL-8 and LIF synthesis is triggered to influential values when chondrocytes are stimulated with TNF-α and IL-1 β (Lotz *et al*., 1995; Henrotin *et al*., 1996; Westacott & Sharif; 1996). IL-17 and IL-18 are known to induce the expression of TNF-α, IL-1 β, MMP-3 and iNOS in the articular chondrocytes, which may be the contributors towards the disease condition (Shalom-Barak *et al*., 1998, Olee *et al*., 1999). While cytokines including IL-4, IL-10 and IL-13 are known to perform antinflammatory role by inhibiting the activity of proinflammatory cytokines (Alaaeddine *et al*., 1999), thus, metabolic state of chondrocytes is influenced and controlled by the complex network of all these cytokines. Studies reveal that IL-1 plays major role in the cartilage

destruction, while TNF-α plays role in the onset of OA (Goldring, 1999; Van den Berg, 1997).

1.7. Tumor Necrosis Factor-alpha (TNF-α)

Tumor Necrosis Factor-alpha (TNF-α) is a cytokine that has major role in instigating inflammation, holds a prominent part in immune system, controls the cell apoptosis, differentiation and proliferation (Baud & Karin, 2001). TNF-α; identified originally 3 decades ago, is a member of TNF ligand Family, which is a large cytokine family. It is able to produce tumor necrosis in animal model (Wajant *et al*., 2003). Initially, it was identified as cachectin protein that was involved in cachexia development (Beutler & Cerami, 1985).

1.7.1. TNF-α Gene Location and its Structure

The TNF-α gene in humans is located on the short arm of chromosome 6 (6p21.3) within the major histocompatibility complex (MHC) class III region (Carroll *et al*., 1987; Arnett & Reveille, 1992). It comprises of 3 introns and 4 exons; while fourth exon codes for 80% sequence of the cytokine. The gene has binding sites for the transcription factors including NF-κB, AP-1 and 2 and cAMP responsive element that are sensitive to signals triggered by TNF-α itself and lipopolysaccharide (Spriggs *et al*., 1991, Ruuls & Sedgwick, 1999, Idriss & Naismith, 2000). The binding of tristetraprolin (TTP) to 3' UTR mediates the post transcriptional regulation of the gene transcript (Deleault *et al*., 2008).

1.7.2. TNF-α Protein Structure and synthesis

TNF-α exists in 26kDa longer isoform, *i.e*., transmembrane protein (tmTNF), and a soluble protein (sTNF) of 17kDa as a shorter form, overall, making two isoforms (Kriegler *et al*., 1988; Luettig *et al*., 1989), and is available for the extracellular signals to bind (Eissner *et al*., 2004). Macrophages and T lymphocytes activates, and in turn, triggers the production of TNF-α 26kDa protein, which is localized in plasma membrane. Metalloproteinases cleaves the extracellular domain of TNF-α and produce the 17kDa soluble isoform of the protein. The transmembrane protein isoform is the more active form among both its isoforms, and both the isoforms are characteristic cone

shape homotrimers (Eck & Sprang, 1989; Black *et al*., 1997). This homotrimeric molecule is the hallmark of TNF super family and is biologically active (Jones *et al*., 1989). Initially, TNF-α transcript is expressed as a larger membrane bound or cell surface type II peptide. In this state, it is an important stable form, containing 233 amino acid residues along with the hydrophobic and hydrophilic domains at N-terminal sequence (Luettig *et al*., 1989). Then, this translated protein is arranged into 26-kDa and inserted into membrane (Grell *et al*., 1995). Now, at this state, matrix metalloproteinase TNF-α converting enzyme can cleave the transmembrane 26-kDa protein into 17-kDa trimeric soluble TNF-α protein (sTNF-α) (Jones *et al*., 1989; Black *et al*., 1997; Idriss & Naismith, 2000).

1.7.3. TNF-α Gene Expression

TNF-α gene expression is not only triggered by macrophages and T-cells, but other cells including neutrophils, NK cells, endothelial cells, B cells, smooth muscle cells, osteoclasts, osteoblasts, fibroblasts, mast cells, keratinocytes, astrocytes, adipocytes, microglial cells, dendritic cells, adipocytes, adrenocortical cells and glomerular mesangial cells also triggers its expression (Figure 1.10) (Tracey *et al*., 1989; Lin *et al*., 2000; Bradley, 2008). Moreover, the expression is also influenced by histone acetylation and chromatin modifications (Lee *et al*., 2003), while attenuation of TNF-α production is triggered by corticosteroid and prostanoids (Beutler *et al*., 1986; Camussi *et al*., 1991; Sullivan, 2003).

TNF-α Gene regulation on various levels

Genetic regulation

Polymorphisms like microsatellite markers and single nucleotide polymorphisms (SNPs) control the level of TNF-α, and low levels of TNF-α were found with microsatellite allele a2, a6 and a10 expression (Derkx *et al*., 1995). The single nucleotide polymorphism at -308GA, -238GA and at 3'end of TNF-α transcript are known to express increased level of TNF-α and unstable transcripts (Jacob *et al*., 1996).

Biological regulation

TNF-α has an appropriate feedback inhibition to control its expression and this process is aided by TNFR1 and TNFR2 (Peschon *et al*., 1998). Interferons and numerous cytokines are involved in the upregulation of the gene (Idriss & Naismith, 2000).

Pathophysiological regulation

TNF-α gene expression is also stimulated by other factors including lipopolysaccharides (bacterial endotoxin), C5a anaphylotoxin, immune complexes, GM-CSF, viral, parasitical and mycotic enterotoxins, IL-1, TGF-β and IFN-γ. Furthermore, inflammation, heart failure, apoplexy, trauma, unstable angina pectoris, asthma, brain injury and burns are known to upsurge its expression many folds (Cairns *et al*., 2000).

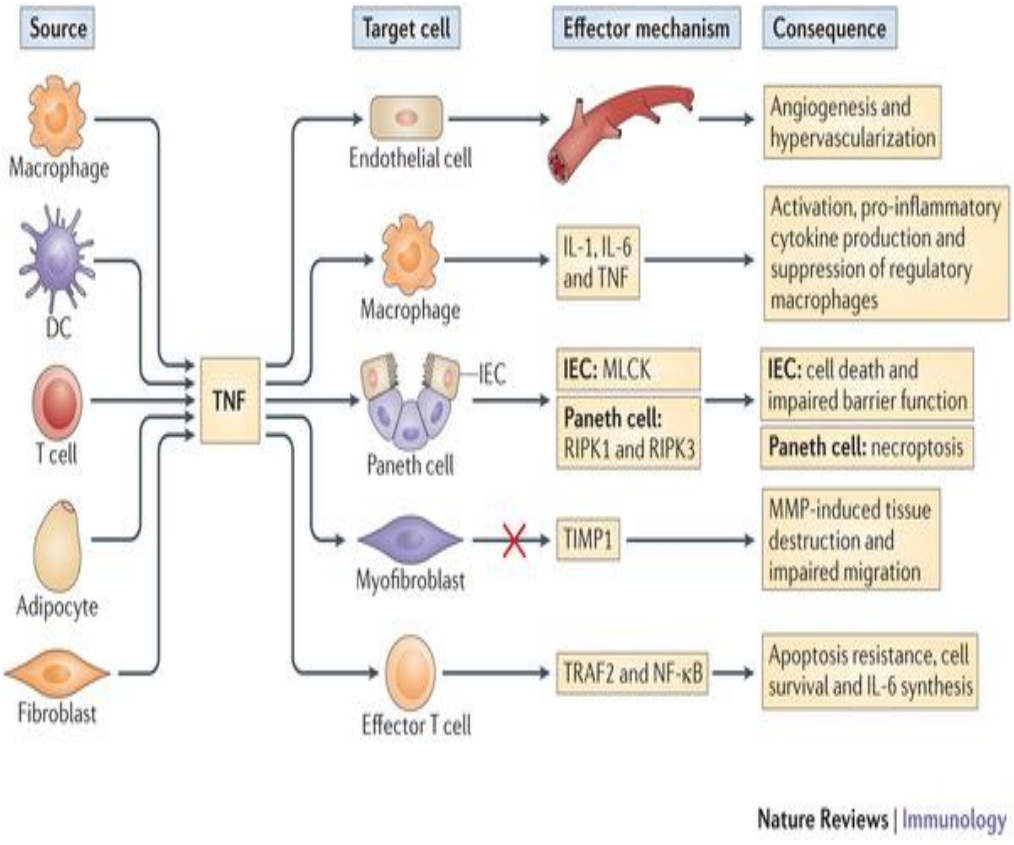


Figure 1.10: TNF-α gene expression and its biological activities (Neurath, 2014).

1.7.4. Biological Function of TNF-α

TNF-α has the ability to perform its role at all levels. It can lead to survival and apoptosis of cells at cellular level, activates the inflammatory and immune components at multi-cellular levels, while it lead to fever and loss of appetite at organism level (Eigler *et al*., 1997). The cytotoxic activity and memory response of macrophages, lymphocytes, NK cells and monocytes as well as antibody production is due to constant expression of tmTNF-α (Agostini *et al*., 1995; Cowley *et al*., 2007). TNF-α produce IL-1, IL-6 and IL-10, which result in the sleepiness and high body temperature (Tracey *et al*., 1986). It also has a role in the inhibition of differentiation of adipocytes, thus playing a role in the insulin resistance (Xu *et al*., 2002). It upsurge the inducible nitric oxide synthase (iNOS) expression from the normal levels in phagocytes, which release abundant amount of NO (nitric oxide) to destroy pathogen (Sanders *et al*., 2001). In a study, mice with TNF-α gene knocked out showed defected brain development (Bruce *et al*., 1996). Figure 1.11 explains the role of various factors associated with the TNF-α function.

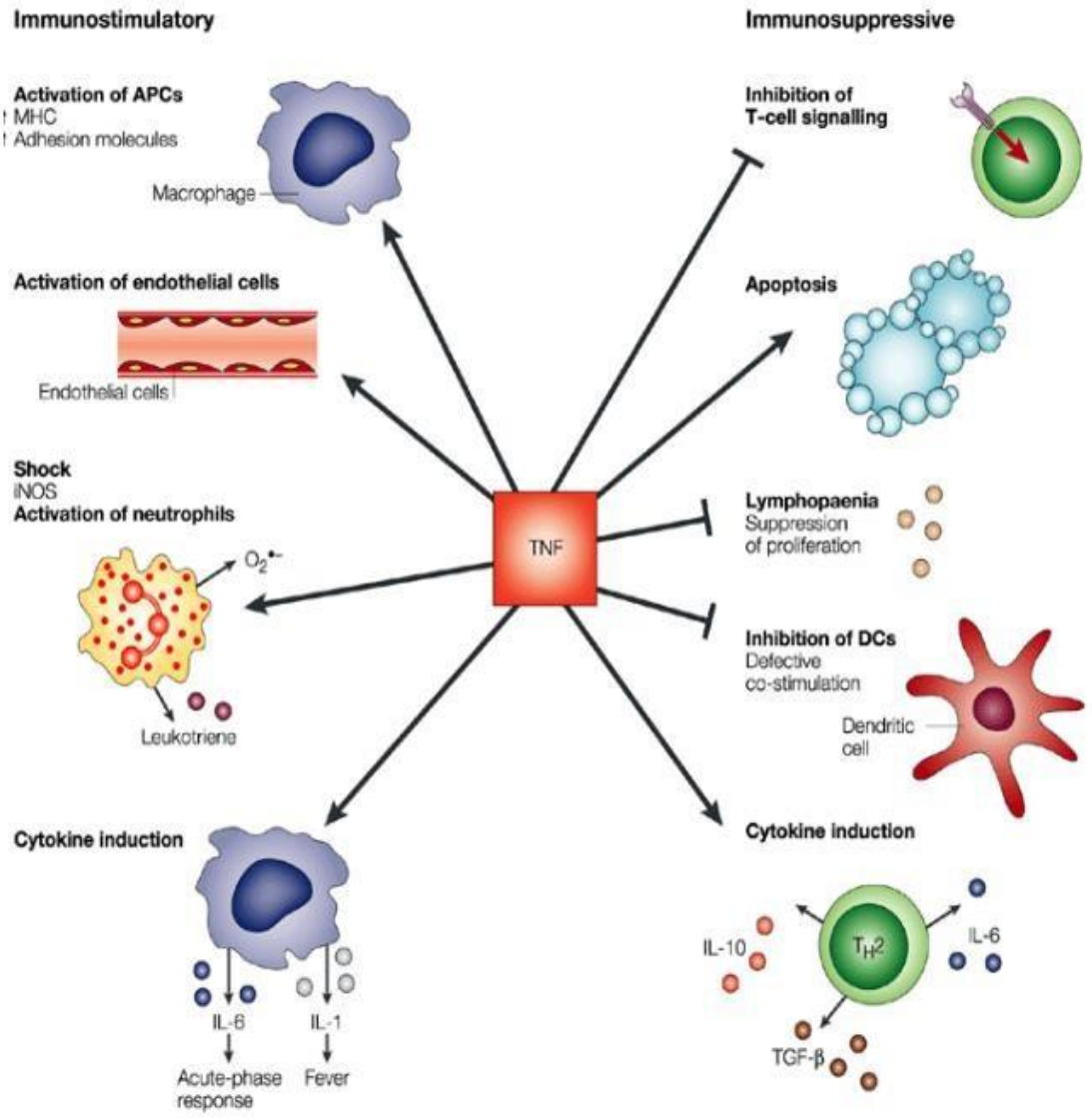


Figure 1.11: Proinflammatory cytokine TNF-α dual role. MHC, Major histocompatibility complex; iNOS, Inducible nitric oxide synthase; TGF-β, Transforming growth factor-beta; APC, Antigen presentation cells; T cell, Thymus derived cell; IL, Interleukin; DCs, Dendritic cells (O'Shea *et al*., 2002).

1.7.5. TNF-α Signaling Pathway

TNF-α downstream effect induce several pathways including induction of apoptosis, p38 mitogen-activated protein kinase (p38 MAPK) pathway, c-Jun N-terminal kinase (JNK) pathway, activation of NF-κB and extracellular signal regulated kinase (ERK) pathway (Figure 1.12) (Aggarwal *et al*., 2012). In the biological response of TNF-α, two types of receptors are involved; tumor necrosis factor receptor type 1 (TNFR1, p55) and tumor necrosis factor receptor type 2 (TNFR2, p75) (Aggarwal, 2003).

a. TNFR1; p55 and signal transduction

Production of proinflammatory cytokines, cell survival, and cell death are modulated by the activation of TNFR1 by TNF-α binding (Zhang *et al*., 2003).

- **Formation of signaling complex I (TRADD-RIP1-TRAF2)**

Formation of signaling complex I activates the cell survival process. TNFR1 activates and it consents the binding of TNFR type 1 associated death domain (TRADD) protein via its death domain. In this process, two other proteins called TNF-receptor associated factor-2 (TRAF-2) and receptor interacting protein-1 (RIP-1) are also landed at the site (Bradley, 2008). A complex forms in which RIP-1 triggers the activation of MAP3K (Mitogen Activated Protein Kinase) and its members including JNKs (C-Jun N-Terminal kinase), Erk (Extracellular-signal-regulated kinase) and p38 to phosphorylate inhibitors of NF-κB and AP-1. The transcriptional factors then migrate to the nucleus for the transcription of genes involved in the process of cell proliferation (Eder, 1997; Hsu *et al*., 1996).

- **TNF-α and NF-κB Pathway**

TNF-α has a role in the processes of cell survival and proliferation, which is best explained by the NF-κB signaling pathway. NF-κB is activated by TNF-α by the sequential recruitment of TNFR1, TRADD, TNFR-associated factor 2 (TRAF2/TRAF5), receptor interacting protein (RIP), TGF-β–activated kinase 1 (TAK1), IκB kinase (IKK) complex, and inhibitor of NF-κBα (IκBα). Degradation occurs through phosphorylation and ubiquitination and ultimately nuclear translocation of p50 and p65 followed by DNA binding takes place (Devin *et al*., 2000). TNF-α proinflammatory expression is upsurged through the proteins regulated by NF-κB, including IL-6, IL-8, IL-18, inducible nitric oxide synthase (iNOS), chemokines,

cyclooxygenase-2 (COX-2), 5-LOX and also itself, which are responsible for inflammation (Aggarwal, 2003).

- **TNF-α and Fas Associated Pathway**

A ligand binds to TNFRI, and TRADD is recruited (Hsu *et al*., 1995), which recruits FADD (Fas-associated protein with death domain), thus activating the caspase 3 and caspase 8 to induce apoptosis (Hsu *et al*., 1996). Moreover, TNF-α binding can facilitate the release of Bax, cytochrome C and ROS, which in turn activates the caspase 3 and caspase 9; resulting in apoptosis (Morgan & Liu, 2010).

- **Formation of signaling complex II (TRADD-RIP1-TRAFF2-FADD-procaspase 8)**

This complex help the cell in the apoptosis process. When the activation of NF-κB halts, the signaling complex 1 dissociates (Ihnatko & Kubes, 2007), and it binds with Fas-associated death domain protein (FADD) and procaspase 8 to form another complex called as death inducing signaling complex (DISC). DISC activates the caspases including procaspase 8 in the cytosol, triggering the cell death (Muzio *et al*., 1998).

b. TNFR2; p75 and signal transduction

TNFR2 does not have the potential to bind with TRADD, but it forms a complex after binding effectively with TRAF2-RIP1 (Naude *et al*., 2011). This complex is known to activate NF-κB gradually, but for the long term purposes, the activation occurs via JNK and p38 pathway to increase inflammatory response, improve cell adhesion, migration and cell survival (Zhang *et al*., 2003).

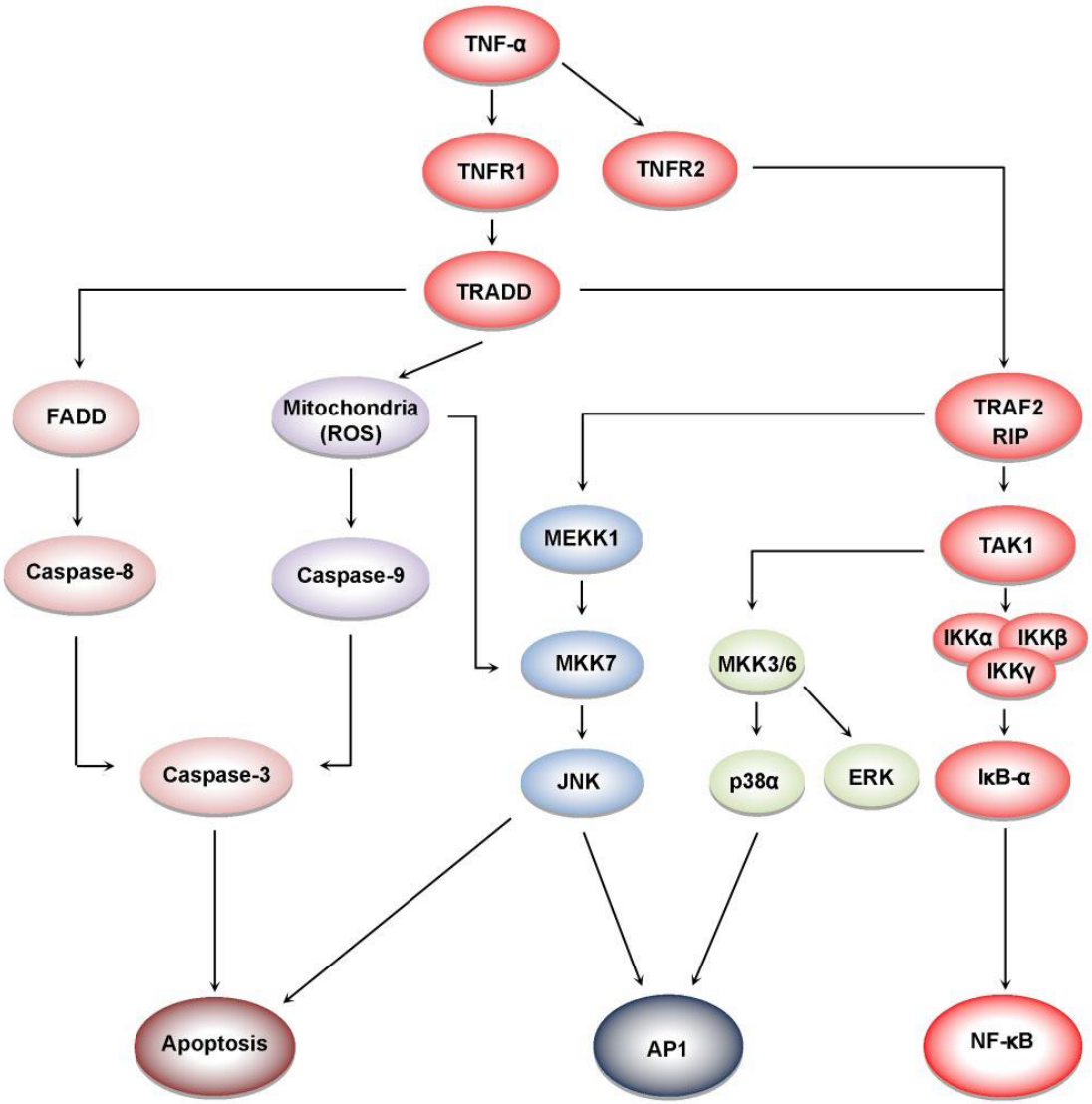


Figure 1.12: TNF-α downstream expression showing the activation of AP-1, apoptosis initiation, and activation of NF-κB. TNF-α, Tumor necrosis factor-alpha; MEKK1, MAP/MEK kinase kinase 1; JNK, c-Jun N-terminal kinase; TRADD, TNFR-associated death domain; RIP, Receptor interacting protein; AP-1, Activator protein-1; MKK, MAP kinase kinase; NF-κB, Nuclear factor-kappa B; FADD, Fas associated death domain; ROS, Reactive oxygen species; ERK, Extracellular signal regulated kinase; TAK1, TGF-β activated kinase 1; TRAF2, TNFR-associated factor; IKK, IκB kinase; IκBα, Inhibitor of NF-κB alpha (Aggarwal *et al*., 2012).

1.7.6. Single Nucleotide Polymorphisms (SNPs) in Promoter Region of TNF-α

TNF-α is located in the region of major histocompatibility complex (MHC) and these regions are considered the most prominent regions for the occurrence of single nucleotide polymorphisms throughout the genome in all vertebrates (Allen, 1999). SNPs or single nucleotide polymorphisms arise due to the change in the single base pair, and most of the SNPs do not affect the biological function of the genes, but some are known to play protective role or contribute in the disease onset and severity (Collins *et al*., 1997). The SNPs present at the promoter region contributes in the allele specific gene expression regulation, which might be useful for the individual, or leave the individual in various conditions including infection, inflammation and cancers (Knight, 2005; Smith & Humphries, 2009). Single nucleotide polymorphisms can occur throughout the gene length, but these are highly conserved if present in the 3' UTR and the coding region (Waldron-Lynch *et al*., 1999). In Caucasian populations, the most frequent SNP's occurring in promoter region of TNF-α gene are located at -238 (D'Alfonso & Richiardi, 1994; Wilson *et al*., 1997), -308 (Wilson *et al*., 1997), -857 (Herrmann *et al*., 1998), -863 and -1031 (Higuchi *et al*., 1998) position with respect to the transcription start site, and SNPs at the promoter regions of TNF-α in other populations are also reported including -162, -237, -274, -375, -575, -850, and -1037 locations (Figure 1.13)(Allen, 1999).

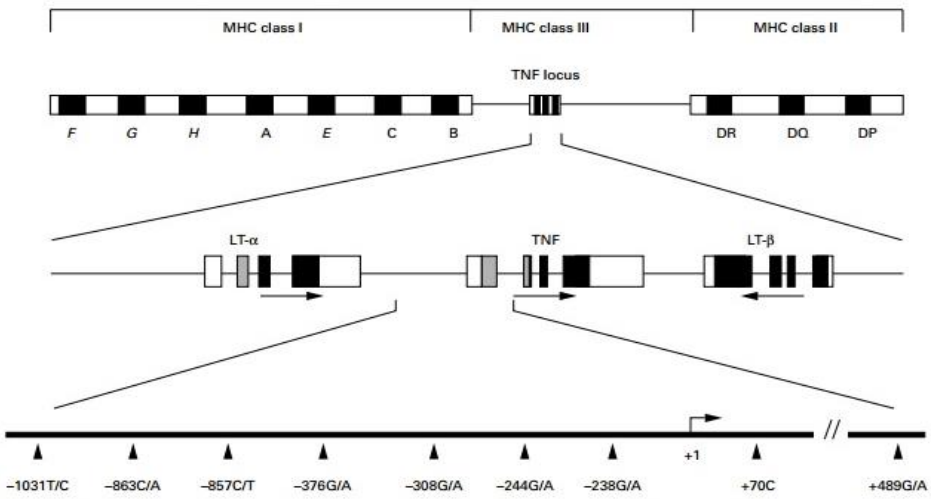


Figure 1.13: TNF-α gene location within MHC. The arrows represents the orientation of transcription in TNF-α gene. +1 represents the transcription initiation site. The upstream positions of SNPs are shown (Verweij, 1999).

1.8. Aims of the Study

Polymorphisms have shown a clear role in the disease onset and progression, and there are many evidences of the SNPs' association with TNF-α gene, so the study is designed with the following objectives.

- To investigate the variant genotypes of TNF-α -308G>A in Pakistani osteoarthritis patients and identify if this natural sequence variation (polymorphism) in the promoter region modulate the risk of OA.
- To find out the relationship of this polymorphism with the occurrence of OA.

MATERIALS AND METHODS

2.1. Study Design

The Blood samples from patients of osteoarthritis were subjected to basic, clinical and genetic analysis in this study. All the samples were collected from the Orthopedics Department of PIMS (Pakistan Institute of Medical Sciences) Hospital Islamabad, Pakistan, with due permission of the Ethical committee and Head of the department; and patients were initially diagnosed by the physician expert in the disease. Maximum of the patients' age was 40 years and above, and they belong to different socio-cultural origins in Pakistan.

2.2. Study Subjects and their Inclusion Criterion

Seventy patients with osteoarthritis were registered in this study. Patients were diagnosed thoroughly in the Out-patient department (OPD) of Orthopedics department, both by clinical symptoms and radiographs, and the patients who showed confirmed diagnosis of the disease were selected for the study. The grade of the disease was confirmed from the radiographs of the patients according to the Kellgren-Lawrence Grading System (Kellgren and Lawrence, 1957). According to this system, patients were categorized into four different severity levels on the basis of changes in the radiographs. Fresh radiographs were considered for the study.

2.3. Exclusion Criterion of Patients

Patients with the history of any of the following diseases were excluded from the study.

- Secondary OA and any other arthritis
- Intraoperative, clinical, or pathological indication of trauma
- Inflammatory arthropathy
- Avascular necrosis
- Coronary Artery Disease (CAD)
- Myocardial Infarction (MI)
- Diabetes Mellitus (DM)
- Any allergy

- Recent trauma and infection.

2.4. Questionnaire Filling

Consent form was duly signed by each patient that permitted their blood sample for biochemical and genetic analyses. The information from the OA patients were collected with doctors' involvement through appropriate procedure of questionnaire and their private interviews. Clinical data including family history of the disease, its duration, the levels of pain in various activities, *etc*., was collected along with the personal data including the name, age, sex, ethnicity, fertility status in women, height and weight, *etc*.

2.5. Blood Sample Collection and Storage

Blood samples were collected from the out-patient department (OPD) of orthopedics, Pakistan Institute of Medical Sciences (PIMS) Islamabad. Venous blood was drawn from the patients in 3ml sterile syringes (Becton Dickinson) via aseptic vein puncture technique. Blood was transferred to ethylene diamine tetra-acetic acid (EDTA) tubes (BD, USA), followed by gentle inversion for thorough mixing. Blood samples were stored at 4°C for molecular analysis.

2.6. Molecular Analysis

2.6.1. Preparation of Stock Solutions

Chemicals	Method
EDTA (0.1 M, pH 8)	• 29.2g of EDTA was dissolved in 800mL of dH_2O with continuous stirring on a magnetic hot plate. • For pH adjustment, pastilles of NaOH were added and it was adjusted to 8. • 1000mL final volume was made by adding dH_2O.
TBE Buffer (10X):	• 108g of Tris-base, 55g of boric acid and 200mL of EDTA (0.1M, pH 8) were dissolved one after the other in 700mL of dH_2O.

- dH_2O was added to make the final volume 1000mL.

Stock solutions were autoclaved and stored at 4°C and used for the preparation of working solutions according to the protocol.

2.6.2. Preparation of Gel Electrophoresis Solutions

1X TBE Buffer (Working Solution)

Chemicals	Quantity	Method
TBE buffer (10X stock)	100mL	• 100mL of 10X TBE buffer was mixed with 900mL of dH_2O to prepare the working solution. • Final volume of 1000mL was adjusted with dH_2O.
Distilled water	900mL	

Gel Loading Dye (Bromophenol Blue)

Chemicals	Quantity	Method
Sucrose	40g	• 40g sucrose followed by 0.25g bromophenol blue was dissolved in 80mL of dH_2O. • The final volume was adjusted to 100mL by adding dH_2O.
Bromophenol blue	0.25g	

Ethidium Bromide (10mg/mL)

Chemicals	Quantity	Method
Ethidium bromide (EtBr)	1.0g	• 1g of EtBr was dissolved in 95mL of dH_2O. • By using dH_2O, final volume was adjusted to 100mL.
Distilled water	95mL	

2.6.3. Genomic DNA Extraction

A commercial blood DNA preparation kit (Jena Bioscience, Germany) was used for genomic DNA extraction from whole blood. The protocol comprised of four steps;

Step 1: Cell Lysis

- Whole blood (300µL) from each EDTA vacutainer containing the patient's blood sample was transferred into an autoclaved 1.5mL microtube.
- 900µL RBC lysis solution was added to each tube followed by ten times gentle inversion.
- Room temperature was provided to the tubes for 3 minutes to ensure complete RBC lysis.
- After incubation at room temperature, centrifugation was performed at 15,000rpm for 30 seconds.
- With the use of a pipette, supernatant was decanted, leaving behind 10-20µL of residual liquid and visible white cell pellet.
- Microtubes were then vigorously vortexed (Vortex-T, Gene 2, USA) for 10 seconds to completely resuspend the white cells in the residual liquid.
- For proper cell lysis, 300µL of cell lysis solution was added to the tube followed by up and down pipetting until clumps disappeared.

Step 2: Protein Precipitation

- Protein precipitation solution of 100µL was added to each microtube containing cell lysate.
- The tubes were thoroughly mixed by vortexing, and then centrifuged for 1 minute at 15,000rpm.
- A tight, dark brown pellet of precipitated protein was formed.

Step 3: DNA Precipitation

- Supernatant was transferred into a new 1.5mL microtube, 300µL of isopropanol >99% was added to the new microtube and mixed by gentle inversion for 1 minute.
- Microtubes were centrifuged at 15,000rpm for 1 minute and DNA was visible as a small white pellet.
- Tubes were drained after discarding supernatant on a clean absorbent paper to ensure its complete removal.
- Washing buffer (99% ethanol) of 500µL was added to each tube and inverted a number of times for DNA pellet washing.
- It was followed by spinning at 15,000rpm for 1 minute.

- Ethanol was carefully removed and pellet was dried at room temperature.

Step 4: DNA Hydration

- DNA pellet was dissolved in a suitable volume (50-100µL) of DNA hydration solution and vortexed at medium speed for 5 seconds.
- After proper mixing, samples were incubated for 30 minutes at 65°C to accelerate rehydration and finally stored at 4°C.

2.6.4. Confirmation of DNA

To determine the yield of DNA extraction, one percent (1%) agarose gel electrophoresis was performed.

One Percent (1%) Agarose Gel Electrophoresis

Preparation of 1% agarose gel	One percent (w/v) agarose gel was prepared by addition of 0.5g agarose in 50mL of 1X TBE buffer in a conical flask. The mixture was heated in a microwave oven until agarose was dissolved properly and allowed to cool for a few seconds. For staining purpose, 5µL of EtBr (10mg/mL) was added in the gel mixture and poured into a gel casting apparatus, which was then allowed to polymerize at room temperature for 40 minutes.
Sample loading	Loading dye mixed with 4µL of DNA was loaded into the well.
Electrophoresis	Electrophoresis was performed at 100V for 30 minutes in a gel tank (Cleaver Scientific Ltd, UK) containing 1X TBE buffer.
Visualizing the bands	UV transilluminator-gel documentation system (Wealtec-Dolphin Doc, USA) was used for visualizing the DNA bands.

2.6.5. Polymerase Chain Reaction

TNF-α gene promoter region polymorphism at position -308G>A was investigated to determine the allelic variant. The specific gene fragment of interest was amplified by

Polymerase Chain Reaction for determining the alleles present in each OA patient. The primers specific to polymorphism are given in the Table 2.1.

PCR amplification was carried out in a 200µL PCR tube (Axygen, USA) for SNP of TNF-α gene. A PCR reaction mixture of 25µL was prepared by adding following components;

- Each sample DNA 2.0µL
- 10X *Taq* buffer (750mM Tris-HCl (pH 8.8) at 25°C), 200mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20) 2.5µL
- $MgCl_2$ (25mM, Fermentas, UK) 2.0µL
- dNTPs (10mM, Fermentas, UK) 0.5µL
- Forward primer (0.1µM, Fermentas, UK) 1.25µL
- Reverse primer (0.1µM, Fermentas, UK) 1.25µL
- *Taq* DNA polymerase (1.25U, Fermentas, UK) 0.25µL
- PCR water 15.25µL

PCR tubes were then centrifuged for a few seconds to thoroughly mix the reaction mixture and placed in the heating block of automated thermal cycler GeneAmp® PCR system 9700 (Applied Biosystems Inc, Life Technologies, USA) for amplification.

PCR Cycles

The cycling conditions programmed for PCR are listed in Table 2.2. Initial incubation was performed to denature the DNA for improved primer access. During the annealing step, primers bind to their specific sequence sites. At extension phase, temperature was optimized for *Taq* DNA polymerase that allows DNA elongation. Repeated amplification cycles performed at temperatures and durations mentioned in Table 2.2., ensures sufficient product yield for the specific region of polymorphism. PCR for -308G>A SNP was ended with a final extension step and samples were stored at 4°C.

2.6.6. Two Percent (2%) Agarose Gel Electrophoresis

To confirm the accurate amplified product, amplified DNA products were analyzed on 2% agarose gel.

Two percent (w/v) agarose gel preparation	Agarose (2.0g) was melted in 100mL of 1X TBE buffer and 14µL of EtBr was added. Gel casting apparatus was assembled and mixture was poured in it.
Loading of sample	After polymerization of the gel was complete, 3µL of amplified PCR product was mixed with loading dye, and then loaded into the wells.
Electrophoresis	Electrophoresis was performed in 1X TBE buffer at 101V for 35 minutes.
Analyzing the bands	Amplified PCR products were analyzed using UV-transilluminator-gel documentation system.

2.6.7. Genotyping of TNF-α Gene at Position -308G>A

Genotyping of TNF-α gene at position -308G>A in OA patients was performed by using Restriction Fragment Length Polymorphism (RFLP) method. Restriction enzyme specific to the polymorphism site was used to digest the amplified gene product. The enzyme cleaved in only one of the two allelic variants because the restriction digestion was specific to it. So, DNA cleavage pattern easily helped in the determination of the allelic variants. The resulting genotypes and the enzyme used for the restriction digestion is given in Table 2.3.

The restriction mix of 20µL volume was prepared in a PCR tube using 12µL of PCR product, 0.1µL of restriction enzyme (1U, Fermentas, UK), 3µL of 10X Buffer (10mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 50mM NaCl, 0.1mg/ml BSA), and 4.9µL of nuclease free water. These PCR tubes were incubated overnight at 37°C.

2.6.8. Agarose Gel Electrophoresis

The genotype pattern of TNF-α gene polymorphism at position -308G>A was analyzed by separation of *Nco*I digested fragments on 4% (w/v) agarose gel. An appropriate DNA ladder (100bp plus DNA Ladder, Fermentas, UK) was used as a size standard for each gel lane to point out the accurate fragments.

Preparation of gel	Agarose of about 4.0g for 4% (w/v) gel was first melted in 100mL of 1X TBE buffer and 10µL of stain (EtBr) was added. The solution was dispensed into a fully equipped gel casting apparatus. Polymerization was allowed for 30 minutes at room temperature.
Sample loading	PCR digested product (20µL) mixed with loading dye was weighed down the wells accordingly.
Electrophoresis	It was operated in a horizontal gel electrophoresis apparatus having 1X TBE buffer at 120V for 10 minutes and later at 90V for 35 minutes.
Analyzing the bands	Gel electrophoresis visualizing system was used to visualize the bands obtained on the gel.

2.7. Statistical Analysis

The baseline characteristics (gender and age) of OA patients and healthy control individuals were compared using Fisher's exact test and data was represented as n (number) and % (percentage). The genotype and allele frequencies of patients and control group were compared by using the Pearson's chi-square ($\chi 2$) test. *P* value < 0.05 was considered significant. For the strength of association and risk of OA, Odds ratio (OR) and their 95% confidence interval (CI) were calculated from 2 x 2 contingency table data. GraphPad Prism version 6.05 (GraphPad Software, San Diego California USA) was used to carry out all statistical tests.

Table 2.1: SNP Marker for TNF-α Gene, its Primer Sequence and Product Size

SNP (TNF-α)	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplified Product Size (bp)
-308G>A	AGGCAATAGGTTTTGAGGGCCAT	CATCAAGGATACCCCTCACACTC	134

SNP, Single nucleotide polymorphism; TNF-α, Tumor necrosis factor-alpha; bp, Base pair.

Table 2.2: Programming of PCR Cycles

SNP (TNF-α)	Initial Incubation	Denaturation	Annealing	Extension	Final Extension
-308G>A	94°C for 12 minutes	**35 cycles**			72°C for 2 minutes
		94°C for 30 seconds	60°C for 1 minute	72°C for 2 minutes	

SNP, Single nucleotide polymorphism; TNF-α, Tumor necrosis factor-alpha.

Table 2.3: Summary of RFLP Analysis

SNP	Product size (bp)	Incubation Temperature	Endonuclease	Alleles (bp)	Reference
TNF-α -308 G>A	134	37ºC	*Nco*I	A (134) G (114, 20)	Hussain et al. (2015)

SNP, Single nucleotide polymorphism; TNF-α, Tumor necrosis factor-alpha; bp, Base pairs

RESULTS

The aim of the study was to investigate the association of TNF-alpha gene -308G>A polymorphism with osteoarthritis in a Pakistani population comprising of 70 OA cases and 38 healthy controls (sporadic case control study). Genotyping of -308G>A was carried out by PCR-RFLP method. The descriptive (baseline and clinical) characteristics of OA patients and healthy control subjects are listed in Table 3.1-3.4.

3.1. Baseline characteristics and clinical parameters of the study subjects

Baseline characteristics including age and gender of OA patients and control subjects were compared by using Fisher's exact test. The study population included a total of 108 subjects including 70 OA patients and 38 healthy control subjects. Both males and females were enrolled in the study. The patient group comprised of 30% males and 70% females, whereas control group contained 76.32% males and 23.68% females (OR = 0.1330; 95% CI = 0.05375-0.3291; *P* = 0.0001; Table 3.1). The analysis showed a significant positive association of the female gender with the disease vulnerability as it was more common in females among the patients.

As age is one of the important risk factors of OA disease, the study subjects were also grouped into two main age groups *i.e*., 50 to 80 years, and 25 to 49 years, and Fisher's exact test was applied on the data sets. Majority of the patients belonged to the age group 50 to 80 years, making a total of 61.43%, while the patients whose ages were 25 to 49 years were only 38.57%. In the control group, maximum individuals were selected whose age was above 50 years for a robust analysis as increased age is one of the strongest risk factors for OA disease. 94.74% individuals from control group belonged to the 50 to 80 years age group, while only 5.26% were below this age (OR = 11.30; 95% CI = 2.513-50.83; *P* = 0.0001; Table 3.1). The analysis showed a significant positive relationship with the OA susceptibility and increasing age (Fig 3.1A-C).

Student's t-test (unpaired) was used for analysis of age between the patient and control groups and the mean age of patient (53.64 ± 11.464) in comparison with mean age of control group (54.11 ± 9.307) was not statistically significant (t = 0.2114; *P* = 0.8330; Table 3.2)(Fig 3.2A-C).

As obesity is one of the strongest risk factors of OA, so BMI values of control group were compared with the OA patients' group by applying Student's t test (unpaired). Mean value of patients' group (26.38 ± 4.295) was compared with the mean value of control group (24.56 ± 3.301), and significant *p* value was obtained (t = 2.253; *P* = 0.0263; Table 3.2). In the present set of data, maximum of the patients belonged to the overweight and class I obese group (55.7%), while 44.28% patients had normal BMI values. The significant *p* value shows the positive association of the disease with the increased BMI (Fig 3.3A & B).

Table 3.1: Baseline characteristics of OA patients and control subjects

Characteristics	**Patient n (%)**	**Controls n (%)**	**OR**	**95% CI**	***P* value**
	70 (100)	38 (100)			
Gender					
Male	21 (30)	29 (76.32)	0.1330	0.05375-0.329	**0.0001**
Female	49 (70)	9 (23.68)			
Age groups					
25 to 49 Years	27 (38.57)	2 (5.26)	11.30	2.513-50.83	**0.0001**
50 to 80 Years	43 (61.43)	36 (94.74)			

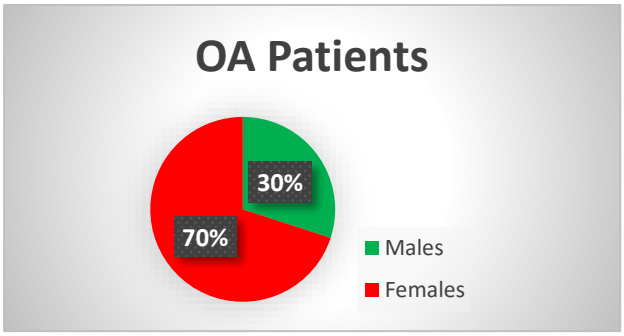
Table shows comparison of gender and age between OA patients and control subjects.

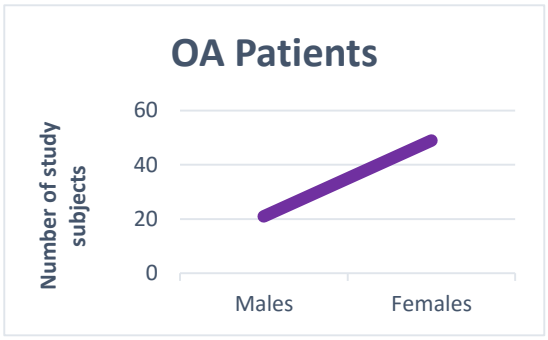
P value was calculated by using Fisher's exact test

OR, Odds ratio; CI, Confidence interval

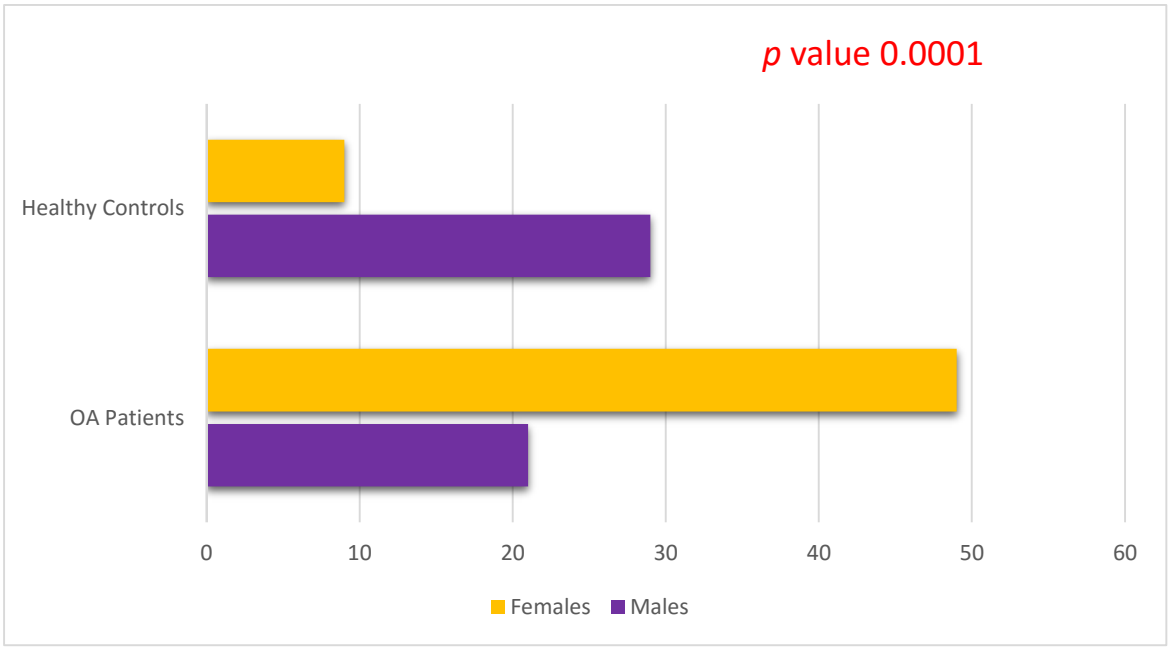
n, number; %, percentage

P value < 0.05 was considered significant





(A) **(B)**



(C)

Figure 3.1. Comparisons between frequencies of male and female genders among OA patients (**A**). The line shows the increased susceptibility in females as compared to males (**B**). Gender based comparison among OA patients and healthy controls (**C**).

Table 3.2: Comparison of age and BMI between OA patients and control subjects

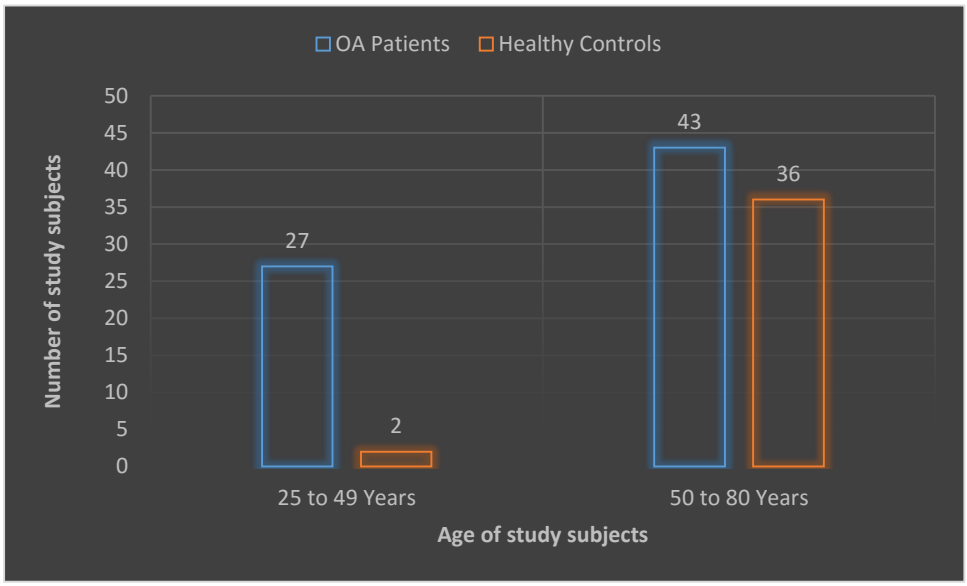
Characteristics	Patient n = 70	Controls n = 38	*t* value	*P* value
	Mean ± SD	Mean ± SD		
Age in Years	53.64 ± 11.464	54.11 ± 9.307	0.2114	0.8330
BMI	26.38 ± 4.295	24.56 ± 3.301	2.253	0.0263

Table represents comparison of patients and control individuals age.

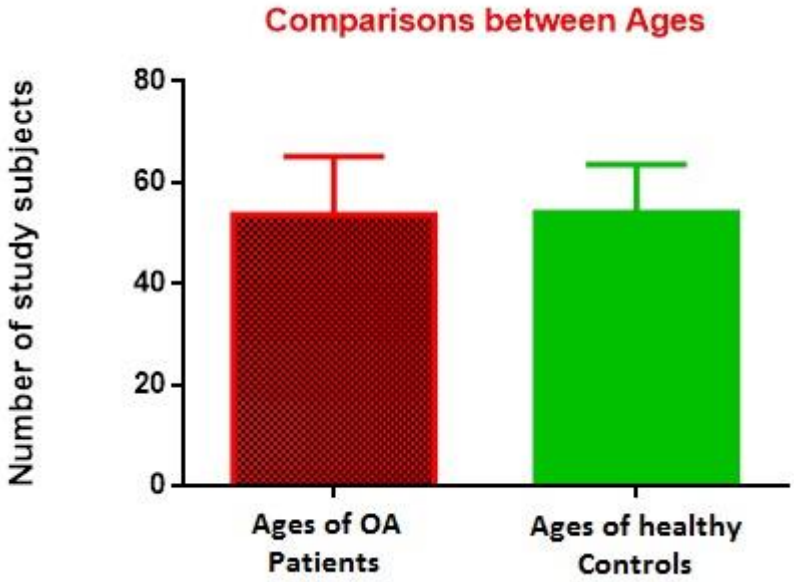
P value was calculated by student's t-test (unpaired)

SD, Standard deviation; n, number

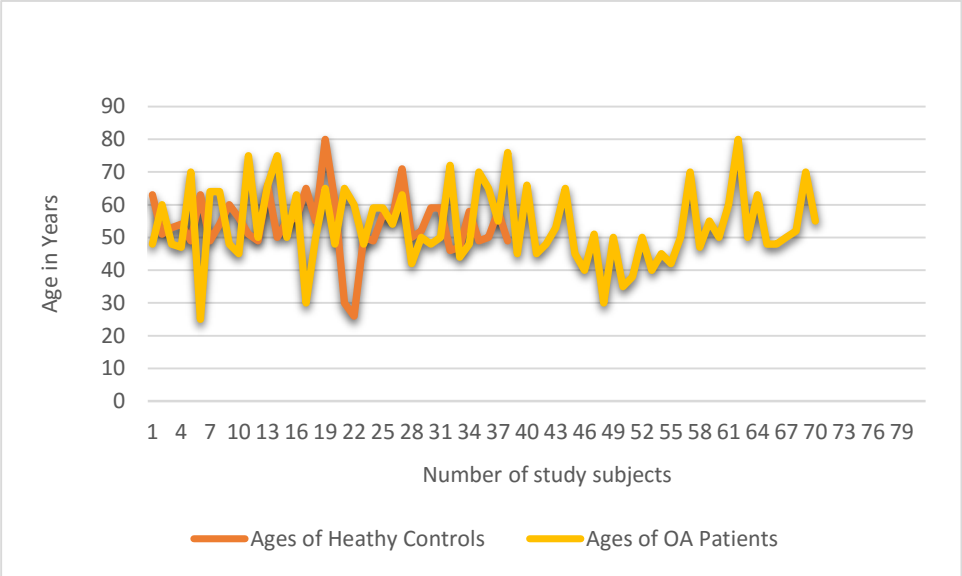
P value < 0.05 was considered significant


OA Patients
Healthy Controls
Number of study subjects
50
45
40
35
30
25
20
15
10
5
0
27
2
43
36
25 to 49 Years
50 to 80 Years
Age of study subjects

(A)

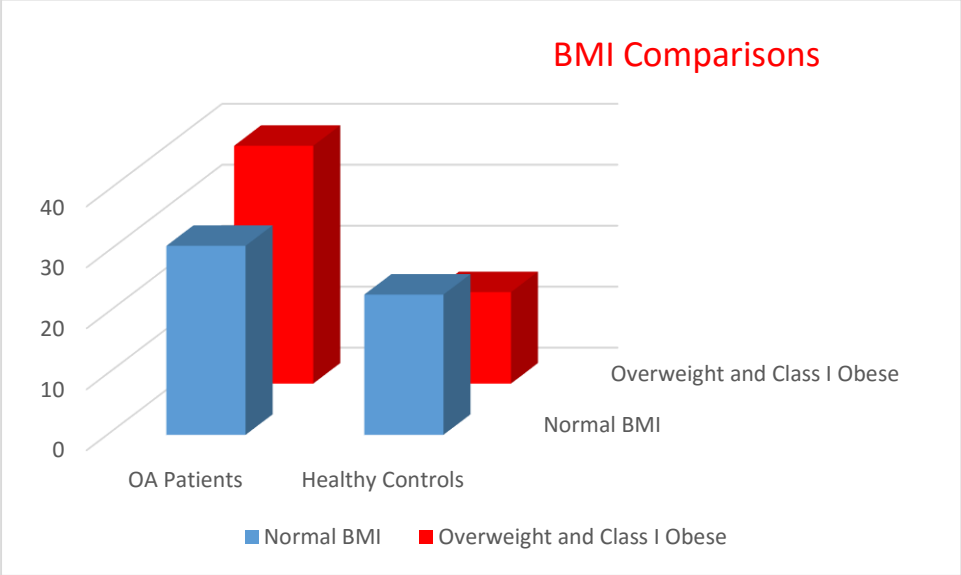

Comparisons between Ages
Number of study subjects
80
60
40
20
0
Ages of OA Patients
Ages of healthy Controls

(B)

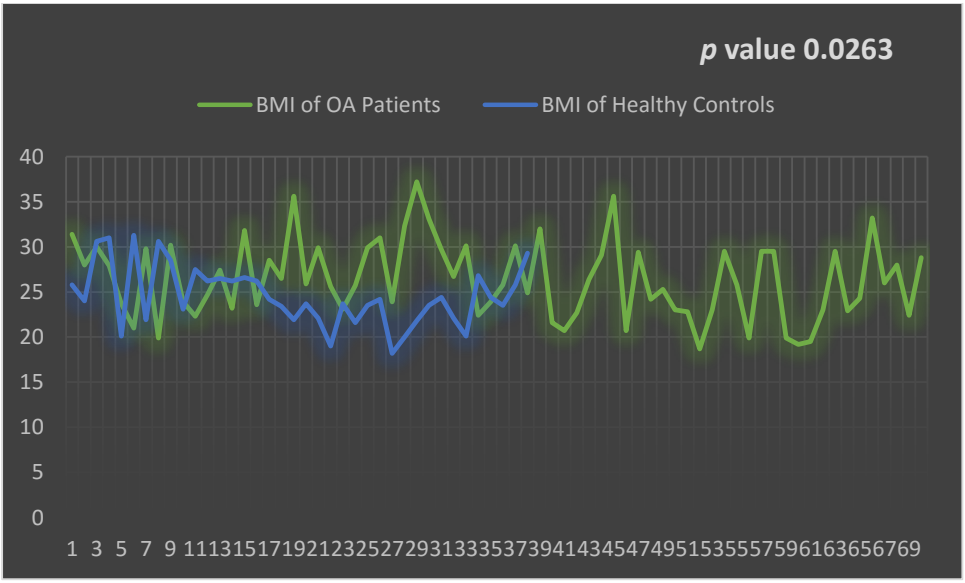


(C)

Figure 3.2. Comparison of frequencies of two sets of age groups between control individuals and OA patients (**A**). Comparison of ages among OA patients and healthy control individuals (**B** & **C**).



(A)



(B)

Figure 3.3. Comparisons of BMI frequencies between OA patients and healthy control groups.

3.2. Genotype distribution in the study population

Genotyping of OA patients was carried out to find the association between the disease susceptibility and the TNF-alpha -308G>A SNP. The study population included total of 108 study subjects including 70 OA patients and 38 healthy controls. Genotype analysis was performed by using PCR-RFLP method, first by amplifying the specific gene fragments and then digesting it by using *Nco*I restriction enzyme. The product was then resolved on 4% agarose gel to visualize the fragments and evaluate the results. Three different genotypes were observed on the gel, *i.e.*, GG, GA and AA; representing the wild type, heterozygous and homozygous variant respectively. The size of amplified fragment was 134 bp after PCR amplification. After restriction digestion, wild type GG genotype revealed 114 bp and 20 bp fragments, heterozygous GA genotype gave 134 bp, 114 bp and 20 bp fragments, whereas the amplified product was left undigested in case of homozygous variant genotype AA with showing only uncleaved 134 bp single fragment (Fig. 3.4). The smallest fragment of 20 bp could not be visualized on the gels.

3.2.1. Genotype and allele frequencies of -308G>A SNP in OA study subjects

Genotype frequencies of OA patients and healthy control individuals are mentioned in the Table 3.3. In OA patients, wild type GG genotype frequency was higher as compared to the variant heterozygous GA and homozygous AA, while in control group, frequency of homozygous AA was greater (Fig. 3.5). Genotype frequencies of TNF-α -308 G>A polymorphism were compared among cases *vs.* controls using chi-square test, a significant *P* value was observed ($\chi^2 = 25.22$; $P = 0.0001$; Table 3.3). In Pakistani population, -308 SNP mutant in TNF- alpha gene is negatively associated with the disease onset and susceptibility.

The frequency of variant genotypes (GA+AA) was lower in OA patients, *i.e.*, 21.43% as compared to control individuals *i.e.*, 68.42%. Wild type genotypes of OA study subjects were compared with variant genotypes of OA patients and significant *P* value was obtained (OR = 7.944; 95% CI = 3.259 - 19.37; $P = 0.0001$; Table 3.4; Fig. 3.6A). Wild type G allele frequency was 82.86% in patients, while 38.16% in control individuals. Similarly, the frequency of mutant A allele was 17.14% in patients and 61.84% in control individuals. Significant difference was observed among the values

and significant *P* value was obtained (OR = 7.833; 95% CI = 4.137 - 14.83; *P* = 0.0001; Table 3.4; Fig.3.6B).

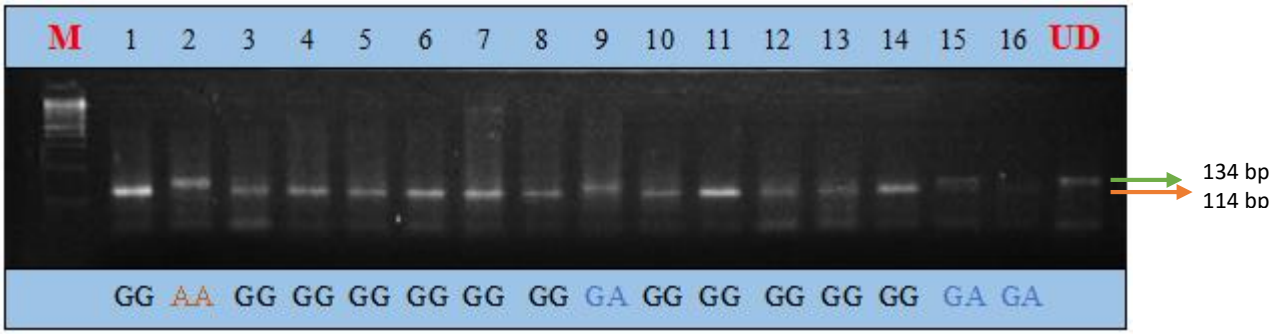


Figure 3.4: Electropherogram of ethidium bromide stained 4% agarose gel showing genotype pattern of *Nco*I restriction digest at TNF-α -308G>A site. The wells are identified as: M, nucleotide fragment size marker; UD, PCR product before restriction enzyme processing; and 1-16, different genotypes after restriction digestion. Genotypes are illustrated as GG (114 bp and 20 bp fragments), GA (134 bp, 114 bp and 20 bp fragments) and AA (134 bp). 20 bp fragment could not be visualized on gel.

Table 3.3: Comparison of genotype frequencies of TNF-α -308 G>A polymorphism between the patient and control groups

Genotype	**Patients n (%)**	**Controls n (%)**	**χ^2 value**	***P* value**
	70 (100)	38 (100)		
GG	55 (78.57)	12(31.58)	25.22	**0.0001**
GA	6 (8.57)	5 (13.15)		
AA	9 (12.85)	21 (55.26)		

Table shows comparison of patient and control group genotype (GG, GA, AA) frequencies.

P value was calculated by chi-square test

P value < 0.05 was considered significant

N, number; %, percentage

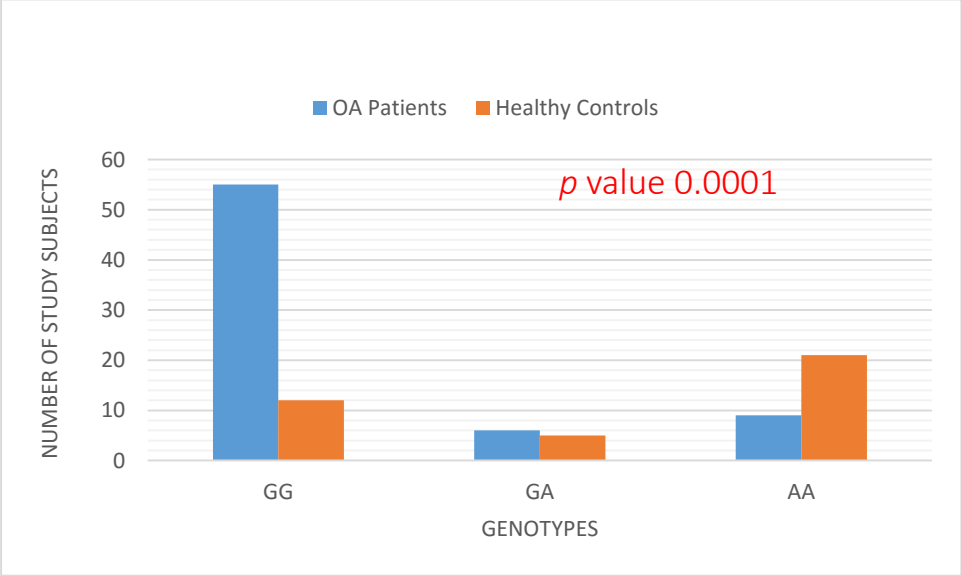


Figure 3.5: Comparison of genotype frequencies of TNF-α -308 G>A polymorphism between the patient and control groups.

Table 3.4: Comparison of wild type and variant genotype and allele frequencies among control and patient groups

Characteristics	**Patients n (%)**	**Controls n (%)**	**OR**	**95% CI**	***P* value**
	70 (100)	**38 (100)**			
Genotype					
GG	55 (78.57)	12 (31.58)	7.944	3.259 - 19.37	**0.0001**
GA + AA	15 (21.43)	26 (68.42)			
Alleles					
G	116(82.86)	29 (38.16)	7.833	4.137 - 14.83	**0.0001**
A	24 (17.14)	47 (61.84)			

P value was calculated by Fisher's exact test

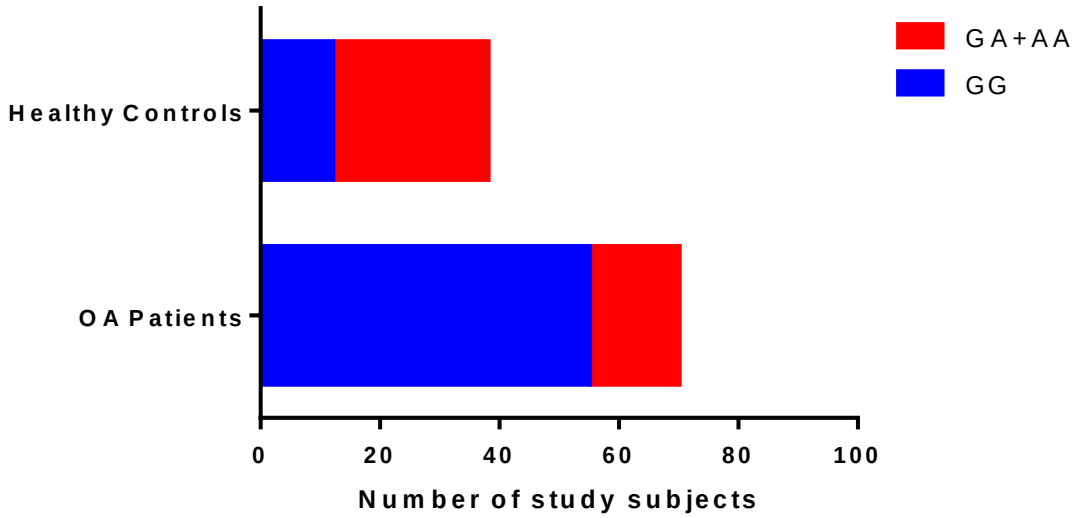
Wild type (GG), Variant genotype (GA + AA)

OR, Odds ratio

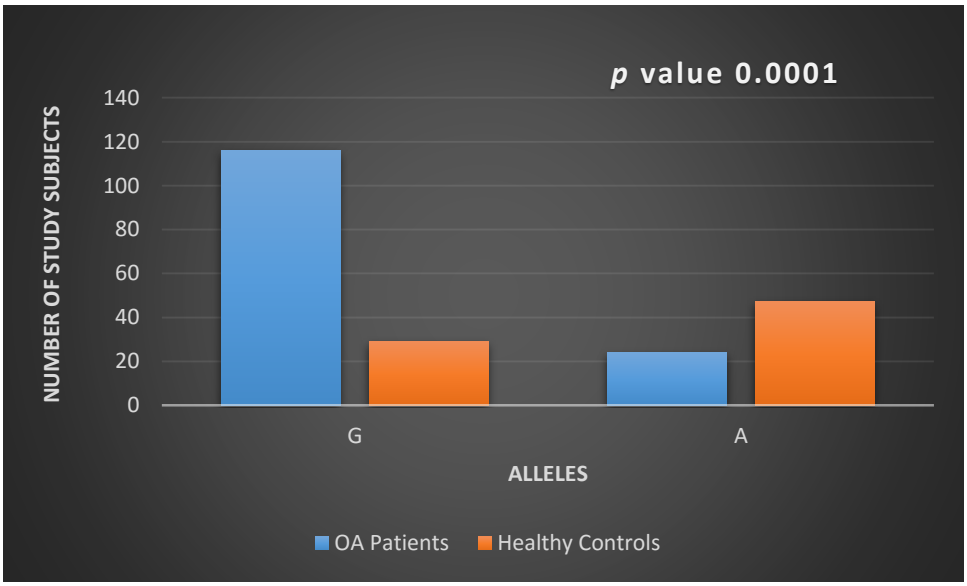
CI, Confidence interval

n, number; %, percentage

P value < 0.05 was considered significant



(A)



(B)

Figure 3.6: Comparison of wild type and variant genotypes among control and patient groups (**A**). Comparison of allele frequencies among control and patient groups (**B**).

DISCUSSION

Osteoarthritis is a joint disorder of synovial joints including knee, hand, spine, hip and foot (Dieppe & Lohmander, 2005), characterized by loss of articular cartilage along with the formation of osteophytes at the joint margins; consequently, loss of joint function. The disease onset and progression is linked to various risk factors including genetics, obesity, sex, age, and any related trauma (Abramson & Attur, 2009). Moreover, the disease has been linked to the work status of individuals, showing more susceptibilty in people performing heavy physical activities in daily routine. This study was conducted specifically for knee OA in a Pakistani population. Its prevalance is variable in different populations according to the studies, and knee OA is highly prevalent in older age population in the rural and urban areas of Asia (Fransen *et al*., 2011). Among all the forms of arthritis, OA is more prevalent in the world's population (Lawrence *et al*., 2008).

Cytokines are important mediators in the development of proper immune response. This response is the determinant of the vulnerability of autoimmune disorders and their severity levels (Laddha *et al*., 2012). TNF-α is a proinflammatory cytokine, produced by macrophages and is responsible for the immune homeostasis, inflammation and host defense (Balkwill, 2009). TNF-α gene expression is regulated at two levels; transcriptional and post-transcriptional. Functional polymorphisms in promoter region of TNF-α may have effect on the transcriptional regulation *via* transcription factors binding sites modification (Skoog *et al*., 1999).

Many of the studies have been conducted to find the association between diseases and -308G>A polymorphism in TNF-α gene, whereas association of this SNP with osteoarthritis is not studied in any population to the best of my knowledge. The present case-control study was designed to observe the role of TNF-α -308 G>A polymorphism in OA susceptibility in a Pakistani population. Comparison of genotype frequencies of -308 G>A SNP in patients (GG = 78.57%, GA = 8.57% and AA = 12.85%) and controls (GG = 31.58%, GA = 13.15% and AA = 55.26%) showed a significant difference and significant *P* value (*P* = 0.0001). Although, *P* value is significant in case of genotype distribution between patient and control individuals, wild type GG frequency is much higher in patient group as compared to control individuals and homozygous mutant genotype AA is higher in controls than patients. The variant genotypes (GA+AA)

frequency is much higher in control individuals (68.42%) as compared to the individuals of patient group (21.43%). The analysis shows negative association between -308 G>A SNP and OA susceptibility. No positive association was found between the minor allele A and OA as its frequency was low in the patient group (17.14%) as compared to control individuals (61.84%), while *P* value was significant (0.0001). These findings are in accordance with (Li *et al*., 2007), where reduced risk was found between the TNF-α -308 G>A polymorphism and the pathogenesis of psoriasis vulgaris disease. The findings are also similar for asthma disease in children (Aytekin *et al*., 2008), clozapine response in schizophrenia (Tsai *et al*., 2003), prostate, lung and colorectal cancer (Wang *et al*., 2011), primary open-angle glaucoma (POAG) in Caucasian population (Mossböck *et al*., 2006), ischemic stroke in Asian population (Gu *et al*., 2013), women with preeclampsia (Stonek *et al*., 2008), pseudoexfoliation glaucoma (Tekeli *et al*., 2008), inflammatory bowel disease (Cantor *et al*., 2005), multiple sclerosis in Han nationality of southern China (Dong *et al*., 2006) and many more related studies. However, in contrast, the SNP shows strong positive association in certain other diseases in different populations including coronary artery disease (CAD) in Pakistani population (Hussain *et al*., 2015), pediatric crohn's disease (Levine *et al*., 2005), sickle cell anemia (Cajado *et al*., 2011), subacute cutaneous lupus erythematosus (Werth *et al*., 2000), breast cancer (Wang *et al*., 2011) and many related studies.

Moreover, characteristics including gender, age and BMI were studies to find the association of each of them with OA susceptibility, and all the three parameters showed a strong positive association with the disease onset. The findings concluded that females are more prone to develop the disease (OR = 0.1330; 95% CI = 0.05375-0.3291; *P* = 0.0001), which is consistent with the findings of Srikanth et al. (2005) and Jordan et al. (2007), while a few of the studies have termed the relationship to be negative or too complex (Belo *et al*., 2007; Bierma-Zeinstra & Koes, 2007; de Klerk *et al*., 2009). Furthermore, the study also revealed that increasing age is one of the strongest determinant of the onset of OA. The Fisher's exact test was applied for the comparison of age data among control and patient groups and a significant *P* value was obtained (OR = 11.30; 95% CI = 2.513-50.83; *P* = 0.0001). The results were consistent with the findings of Felson *et al*. (1995); Gibson *et al*. (1996); Du *et al*. (2005); Zeng *et al*.

(2005); Grotle *et al*. (2008); Sudo *et al*. (2008); Muraki *et al*. (2009); Kim *et al*. (2010); Zeng *et al*. (2006); Litwic *et al*. (2013).

Obesity being one of the strongest risk factors of the disease, was also studied in Pakistani population and a strong association was seen in-between OA disease and overweight and class I obese people. BMI values of the patient group were compared with the individuals of control group and a significant *P* value was obtained (t = 2.253; *P* = 0.0263). Thus, the study revealed one another strong evidence of the relationship of OA with the increasing weight. The results were consistent with the findings of Grotle *et al*. (2008) and Holt *et al*. (2011).

In a nut shell, TNF-α -308 G>A SNP is negatively associated with OA susceptibility and progression as the variant genotype AA was more frequent and prevalent in the control group individuals as compared to the patient group, and wild type GG genotype was more frequent in the study population of patients as compared to the control group, thus, are more prevalent for the disease. The frequency of mutant A allele was 61.84% in control individuals and 17.14% in OA patients, confirming no association of -308 G>A polymorphism in TNF-α region in the disease susceptibility as significant *P* value was obtained for the analysis (OR = 7.833; 95% CI = 4.137 - 14.83; *P* = 0.0001). The study also showed negative association between the variant genotypes of TNF-α -308 G>A polymorphism and the disease onset.

Future directions

Osteoarthritis is the most common form of arthritis worldwide. There is a need to conduct further studies with increased sample size in a Pakistani population as well as in other populations to confirm the results of this study, and also to explore the role of other polymorphisms of the TNF-α in OA susceptibility. It is also expected that the disease age grounds can fluctuate in the larger sample size in Pakistani population. In addition, further investigations on the molecular level could be helpful to understand the susceptibility of OA in detail. Multi-centric studies with larger sample size and measurement of TNF-α serum levels are required to ascertain these results. Therefore, it is suggested that further large studies should be conducted with thorough sequential analysis of the cytokines in relation to the disease occurrence and severity.

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QUESTIONAIRE

Date

Patient Number

Patient Name

Fathers Name

Age

Gender (M/F)

Religion

Height (feet/inches)

Weight (Kg)

BMI

Area

Ethnicity

Family History

Smoking Status

Disease duration

Treatment

Work history