Università degli Studi di Milano
Scuola di Dottorato di Ricerca in Scienze Biochimiche, Nutrizionali e Metaboliche
Direttore: Prof. Sandro Sonnino

Dottorato di Ricerca in Scienze Biochimiche
Coordinatore: Prof. Francesco Bonomi

Clinical, genetical and biomolecular finding in Knee Osteoarthritis

PhD Thesis

FRANCESCO PAOLO CAMMARATA
R10213

RELATORE
PROF. SANDRO SONNINO

CO-RELATORE
DOTT. GIUSI I. FORTE

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Preface

The work described in this dissertation was carried out at Institute of Bioimaging and Molecular Physiology - National Research Council IBFM – CNR, support unit sited in Cefalù (PA) between November 2012 and December 2015

The PhD research took place at HSR-Giglio Hospital under the supervision of Doctor Filippo Boniforti, head of Orthopaedic unit and co-supervision of Prof. Sandro Sonnino, Director of the PhD in Biochemical Sciences and Dott. Mariacarla Gilardi, head of IBFM.

This dissertation is based on experimental research conducted in our IBFM laboratory sited in Cefalù with the collaboration of Dott. Forte Giusi, head of Laboratory of Genomic Methodology and Cell Culture, Dott. Minafra Luigi, Dott. Bravatà Valentina and Dott. Saporito Michele, papers published by our group at IBFM-CNR UOS Cefalù and review in international journals and conferences of osteoarthritis.

Abstract

Osteoarthritis (OA) is a multifactorial, inflammatory and disabling degenerative joints disorder characterized by degeneration of articular cartilage, intra-articular inflammation with synovitis, and changes in peri-articular and subchondral bone. OA involves the synovial tissues and articular cartilage, resulting in symptoms that cause a decrease in the quality of life and disability. The non-modifiable risk factors include gender and age whereas the modifiable risk factors include body mass index (BMI), injury/trauma, among others.

Genetic studies have opened new opportunities in the definition and classification of OA etiopathogenesis describing a multifactorial disease that originates from both genetic and environmental factors. The main genes whose mutations are associated with the onset of OA encode proteins involved in some biological processes: bone morphogenesis, thyroid metabolism, apoptosis and mitochondrial damage, inflammation and the immune response and the Wnt signal cascade.

To date, OA is incurable and most treatments, which include physiotherapy, life-style modifications, pharmacotherapy and surgery, aim to provide symptomatic relief rather than targeting the disease processes themselves.

This work represents a multidisciplinary and translational medicine approach to study OA where clinical, radiographic, genetic and biochemical evaluation could contribute to better define the disease grading and progression for the development of new therapies.

Contents

Introduction and background

Osteoarthritis (OA) is a multifactorial, inflammatory and disabling degenerative joints disorder characterized by degeneration of articular cartilage, intra-articular inflammation with synovitis, and changes in peri-articular and subchondral bone [1]. OA involves the synovial tissues and articular cartilage, resulting in symptoms that cause a decrease in the quality of life and disability, representing a widespread and chronic disease that affects up to 80% of the population over 65 years of age [2]. The non-modifiable risk factors include gender and age whereas the modifiable risk factors include body mass index (BMI), injury/trauma, among others.

Among the risk factors, age contributes to a substantially increased risk of knee OA onset and progression [3-6], even if the association of age with the progression of knee OA is sometimes conflicting [7]. Before 50 years of age, the prevalence of OA in most joints is higher in men than in women. After about age 50 years, women are more often affected than men [8]. A recent report indicates that knee OA is likely to become the fourth most common cause of disability in women and the eighth most common cause in men [9].

To date, OA is incurable and most treatments, which include physiotherapy, life-style modifications, pharmacotherapy and surgery, aim to provide symptomatic relief rather than targeting the disease processes themselves.

Epidemiology

OA may develop in any joint, but most commonly affects the knees, hips, hands, facet joints and feet.

The first epidemiological studies on the OA prevalence (number of people affected in the population sample) are of autopsy. In 1926, Heine has documented a number of 1000 cases with an high presence of articular cartilage alterations in people over the age of 65 years. (10) In 2005, it was estimated that over 26 million people in the US had some form of OA (11). The prevalence of OA, however, varies greatly depending on the definition used, age, sex and geographical area studied. For example, in Dutch population, the prevalence of radiographic osteoarthritis show an increment of rate of the hand, and lower of knee and hip Figure 1 (12). The incidence of hand, hip and knee OA increases with age, and women have higher rates than men, especially after the age of 50 years A levelling off or decline occurs at all joint sites around the age of 80 years (13). Another example of age, sex and geographic modification of epidemiology is showed from the Fallon Community Health Plan in Massachusetts (USA), in which incidence rate was highest for knee OA 240/100,000 person-years, with intermediate rates for hand OA (100/100,000 person-years) and lowest observed rates for hip OA (88/100,000 person-years) (Figures 2-3) (13-14). Incidence rates found by the Dutch Institute for Public Health (RIVM) in 2000 were of a similar level. For hip OA, the reported prevalence was 0.9 and 1.6 per 1000 per year in men and women respectively and for knee OA the corresponding figures were 1.18 and 2.8 per 1000 per year in men and women respectively (10).

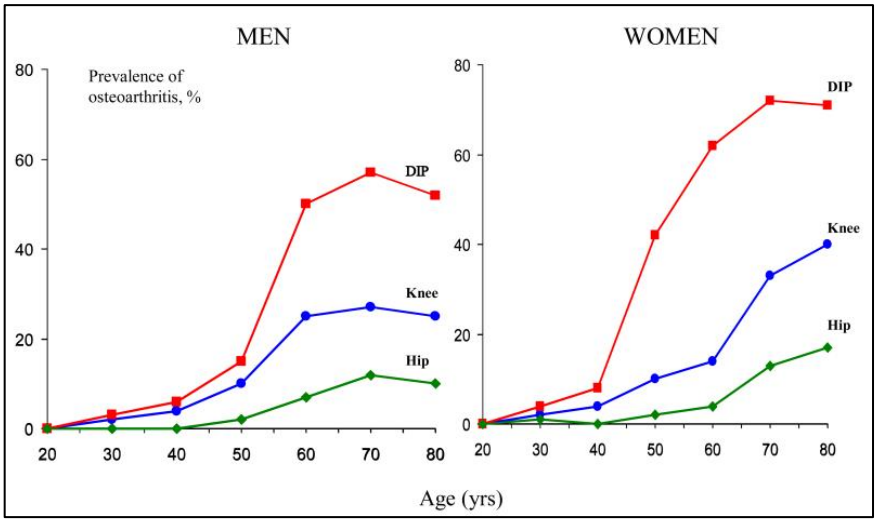

Figure 1: Prevalence of OA in a Dutch population cohort

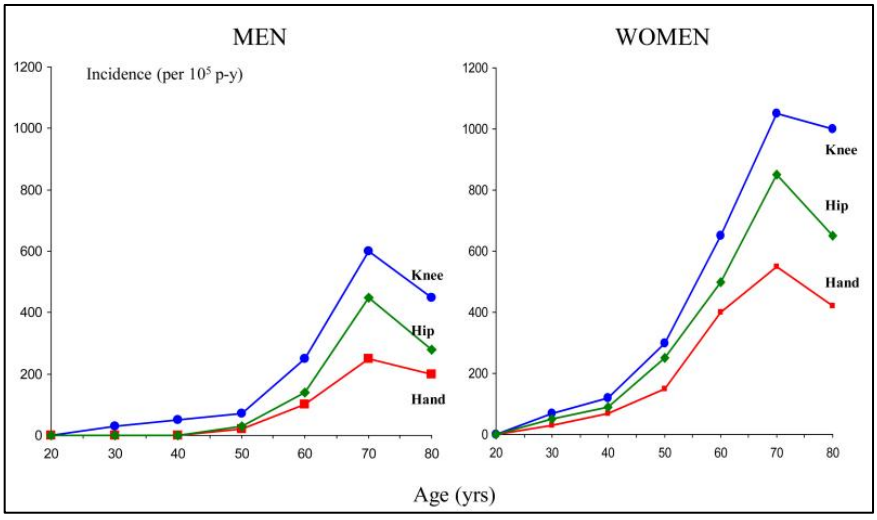

Figure 2: Incidence of Symptomathic OA : Fallon Health plan

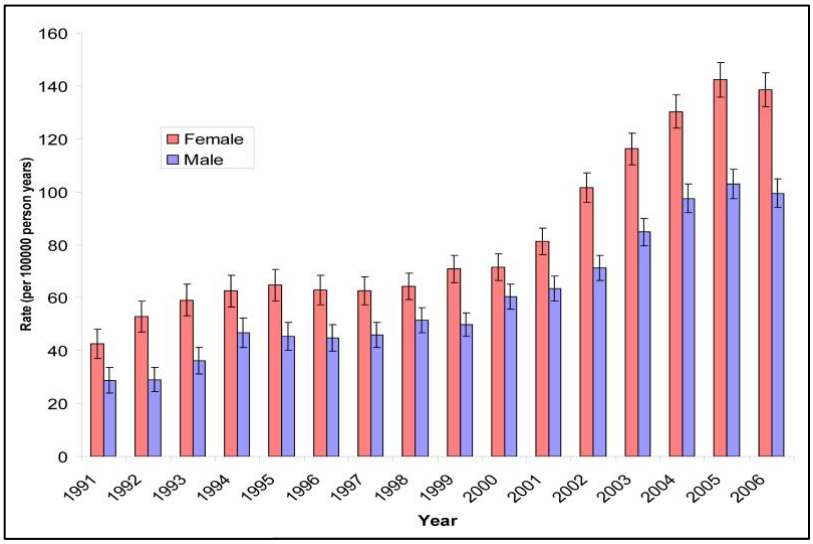

Figure 3:Trend in primary TKA rates from 1991 to 2006 in UK

We can assume that in most epidemiological studies is well established as OA increases with age and in relation to sex: before 50 years of age, the prevalence of the disease is highest in man, on the contrary after 50 years, with interest of hand, foot, knee, spine and hip, is higher in women (TABLE 1) (14-17). Osteoarthritis, along with heart disease and cancer, is ultimately one of the typical ailments of old age and the second cause of incapacity for work after ischemic heart disease (18).

Study	Site	Sex	Incidence rates (per 100000
Wilson et al	Hip OA	M+F	47.3
	Knee OA	M+F	163.8
Kallman et al	Hand OA	M	100
Oliveira et al	Hip OA	M+F	88
	Knee OA	M+F	240
	Hand OA	M+F	100
Coper et al	Knee OA	M+F	250

Table 1: Epidemiological studies of the incidence of OA

Risk Factor

Risk factor for OA development in current knowledge are classified into two fundamental mechanisms related either to the adverse effects of ‘‘abnormal’’ loading on normal cartilage or of ‘‘normal’’ loading on ‘‘abnormal’’ cartilage.

The first category comprises of mechanical factors, such as trauma and microtrauma, overweight, load deviation, periarticular structures alteration (ligaments, menisci), joint incongruity for congenital or acquired pathology (dysplasia, slipped capital femoral epiphysis, osteonecrosis). The second group includes factors such as aging, gender, genetic alterations, metabolic diseases, endocrine (acromegaly) and lifestyle (smoking and alcohol). Aging has been suggested as the primary factor contributing to this ‘‘abnormal’’ state of articular cartilage, although genetic factors causing disruption of chondrocyte differentiation and function and influence the composition and structure of the cartilage matrix also contribute to abnormal biomechanics, independent of the influence of the aging process. Aged articular cartilage presents alterations such as fibrillation and dehydration, that are signs of the altered response of chondrocytes in to the presence of cytokines and other products of matrix degradation, inducing the production of pro-inflammatory mediators (19) (Figure 4)

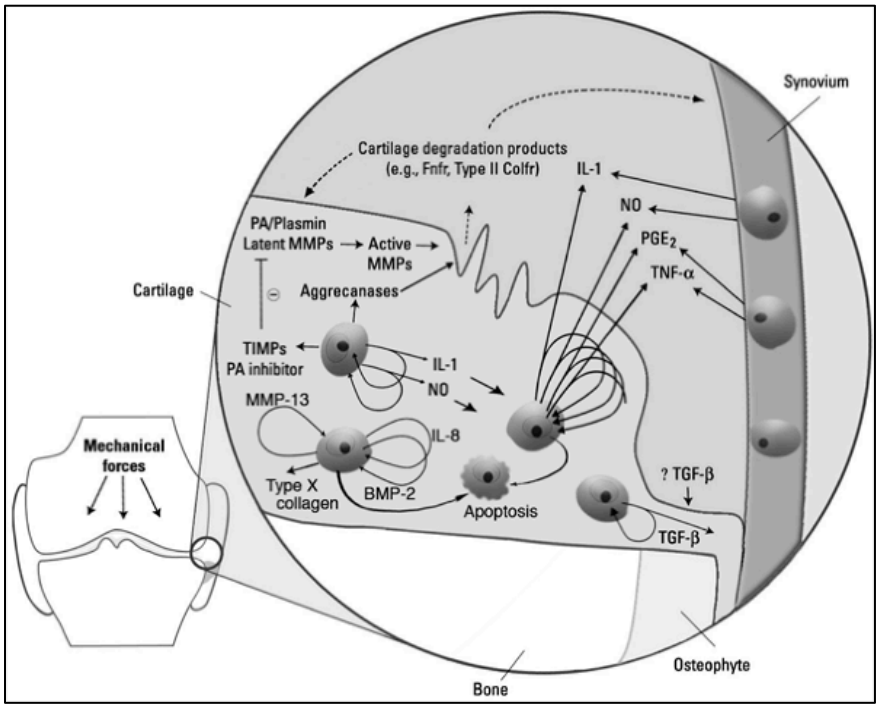

Figure 4: Chondrocytes response to mechanical force, cytokines and matrix degradation product

Different studies have consistently shown a link between overweight or obesity and knee OA. Data from the first National Health and Nutrition Examination Survey (HANES I) indicated that obese women had nearly 4 times the risk of knee OA as compared with non-obese women; for obese men, the risk was nearly 5 times greater. In a study from Framingham MA, overweight individuals in their thirties who did not have knee OA were at greater risk of later developing the disease. Other investigations, which performed repeated x-rays over time also, have found that being overweight significantly increases the risk of developing knee OA. It is estimated that persons in the highest quintile of body weight have up to 10 times the risk of knee OA than those in the lowest quintile. (20-24)

Metabolic diseases such as hemochromatosis, alkaptonuria or ochronosis, Wilson's disease, Gaucher disease are involved in chondrocytes damage and increased deposits in the cartilage matrix with increased risk of secondary OA.

A defect in load axis, valgus or varus knee for example (Figure 5), is an important risk factor that aggravates OA due to the degeneration of the supporting structures of the joint.

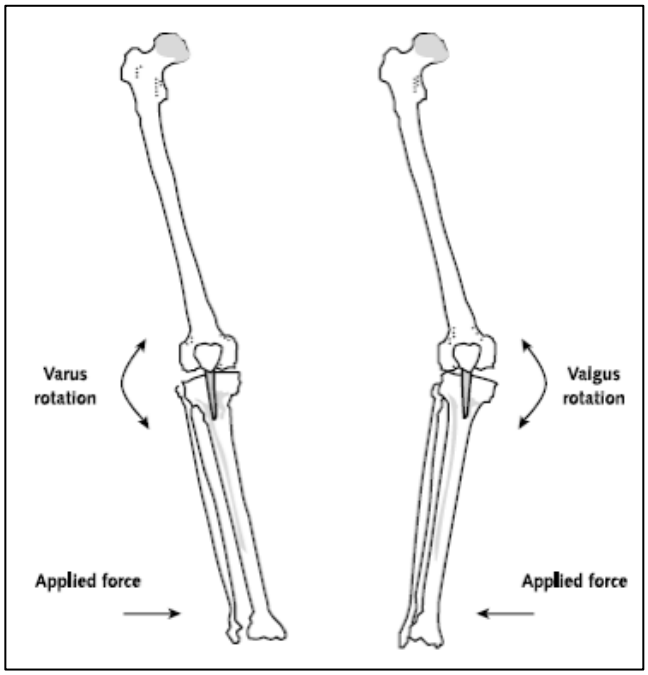

Figure 5. Determination of valgus or varus knee

Recently, genetic studies have opened new opportunities in the definition and classification of OA etiopathogenesis describing a multifactorial disease that originates from both genetic and environmental factors (25-26) . The main genes whose mutations are associated with the onset of OA encode proteins involved in some biological processes: bone morphogenesis (tgf-β, smad, bmp, GDF5), thyroid metabolism (DIO2), apoptosis and mitochondrial damage (anp32A), inflammation and the immune response (IL6, IL1, IL10, PTGS2, pla2GA4, DQB1) and the Wnt signal cascade (frzb, LRP5). Also, have been described mutations associated to the onset of OA of some genes encoding components of the extracellular matrix (COL2A1, col10A1, col6A4, dvwa) and other genes (ESR1, edg2, kl, pitx1, CALM1, CALM2, ace, crush, lep) (27)

Genetic studies of patients with OA can help to unravel the molecular mechanisms responsible for specific disease manifestations, including joint damage, nociception and chronic pain (28)

OA Pathophysiology

The dynamic equilibrium between the on going formation and breakdown of the cartilaginous matrix is regulated by an interplay of anabolic influences (e.g., insulin-like growth factors [IGF] I and II) and catabolic influences (e.g., interleukin-1, tumor necrosis factor [TNF] alpha, and proteinases). To a limited extent, these mechanisms can eliminate or compensate for the harmful influences that cause osteo- arthritis by stimulating and modifying the metabolic activity of chondrocytes. When these harmful influences exceed the system's ability to compensate, however, matrix degradation occurs; this is the first step in the development of osteoarthritis, which can progress to advanced disease (29) (Figure 6).

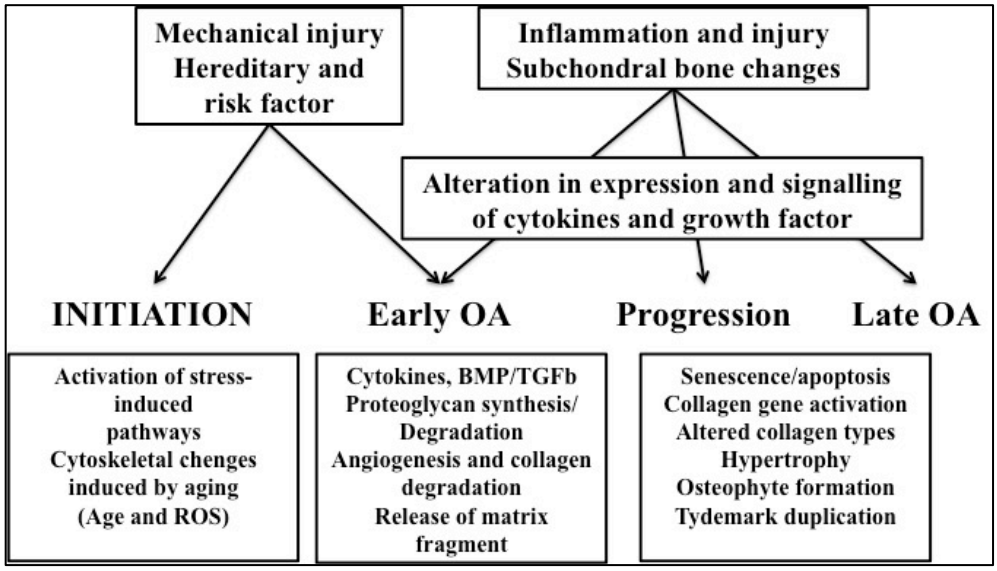

Figure 6: Scheme of events involved in the initiation of OA and progression to late stage OA

Mechanical and enzymatic factors are thought to impair chondrocyte function and damage the matrix

These events are followed by inflammatory phenomena of synovium with increased production of inflammatory cytokines within the joint. Subsequently manifests fibrillation and erosion of the cartilage with involvement of the subchondral bone tissue and consequent sclerosis

Besides the subchondral osteosclerosis, spherical cavities are formed called geodes or subchondral cyst. Geodes are cystic formations that occur around joints in a variety of disorders (including, in addition to OA, rheumatoid arthritis, calcium pyrophosphate dihydrate crystal deposition disease (CPPD) and avascular necrosis). Presumably, one method of geode formation takes place when synovial fluid is forced into the subchondral bone, causing a cystic collection of joint fluid. (Figure 7)

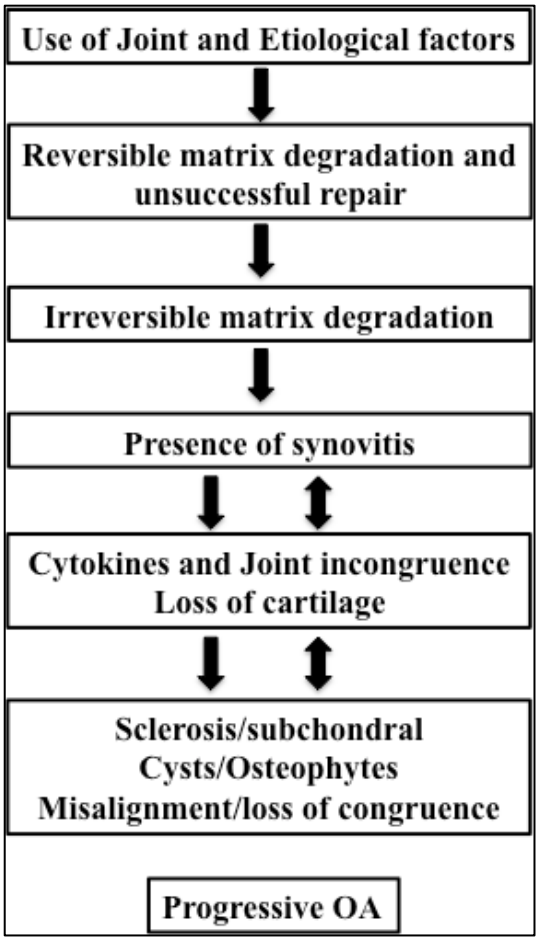

Figure 7: OA Pathogenesis

At the edge of the joint, osteochondral tissue abnormal proliferates with the formation of osteophytes, recognizable during the radiographic examination.

The synovial membrane shows hypertrophic, with different mononuclear cell infiltrates; rarely it is found chondroid metaplasia and calcified areas. The joint capsule and ligaments are often fibrotic, thickened and retracted as a result of metabolic changes and mechanical properties (stiffness) of the degenerative process. (Figure 8 -9)

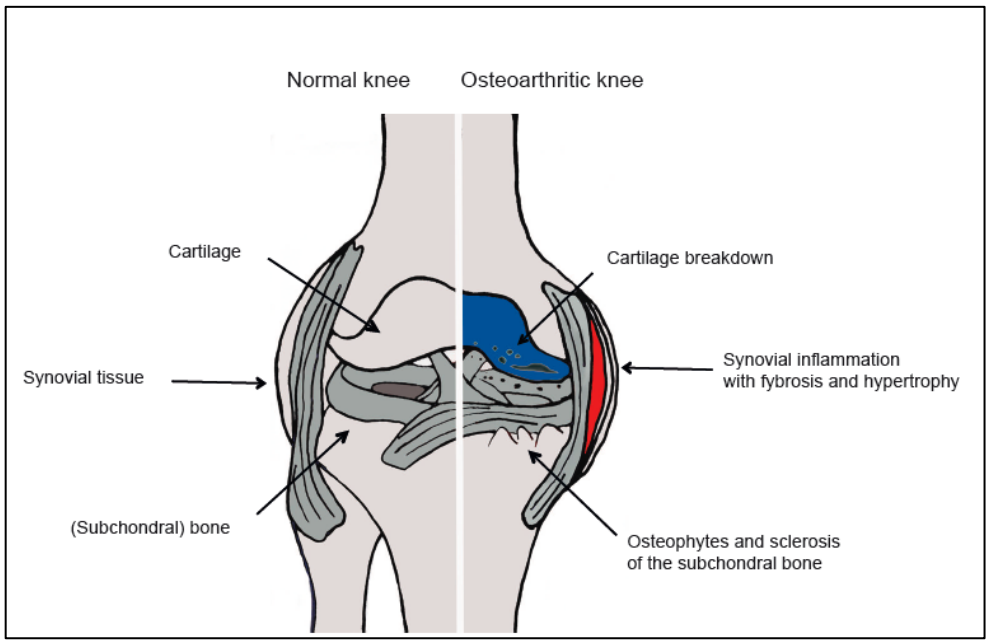

Figure 8 The healthy and osteoarthritic joint

(Hunter et al. Osteoarthritis; BMJ 2011)

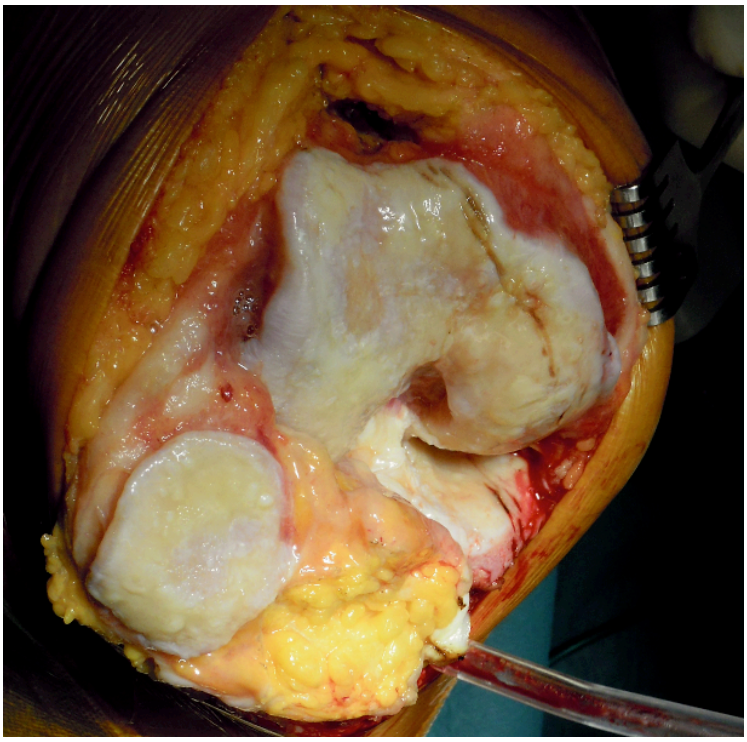

Figure 9: Intraoperative photo of late OA Joint

(Thanks to Dott. Boniforti and Dott. Saporito)

Clinical and radiological assessment of Knee OA

All the three compartments of the knee joint can be affected by OA: the medial femoral tibial, lateral femur tibial and femoral patellar. The medial compartment is most frequently involved (75%) while the lateral is often associated with the femur patellar, is less frequent (25%).

Clinical symptoms and signs can be ascertained by patient history, physical examination and by self-completed questionnaire.

The pain typically occurs during loading and it is particularly accentuated during the walk up the hill, to come down and go up the stairs, in getting up from sitting.

In early OA, pain can relieve with rest, while in late OA pain is continuous and intense and may progress to chronic. Pain is mainly localized in the medial compartment and results in a reduction of the movement, that represent the most frequent symptom of OA

The reduction of the movement is often presented in the morning for about 30 minutes, but it can also appear as a result of activities during the day, making the symptom disabling as much as the pain (30).

During the physical examination are found palpable bone deformity, usually at the level of the medial femoral condyle, and crepitus of joints during passive movement. Swelling and joint effusion may be present without heat or skin rash (31).

The range of motion in the early stages of the disease may be complete but can evolve negatively causing the joint lockout. Moreover, it is frequent the deviation of the load, with deformities in valgus or varus (Figure 10 -11)

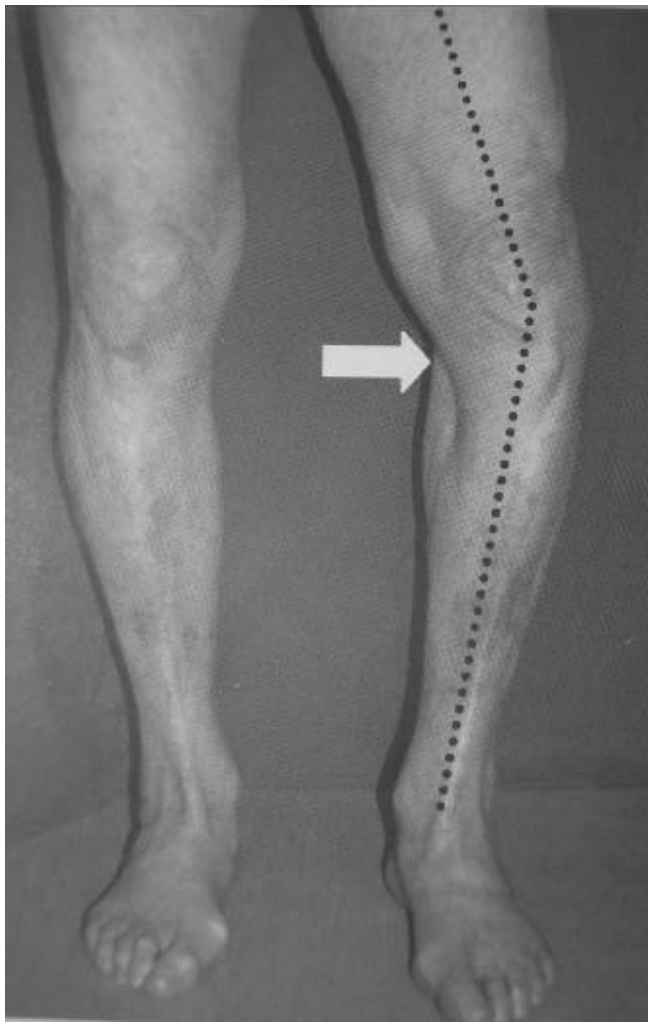

Figure 10 Clinical varus knee OA

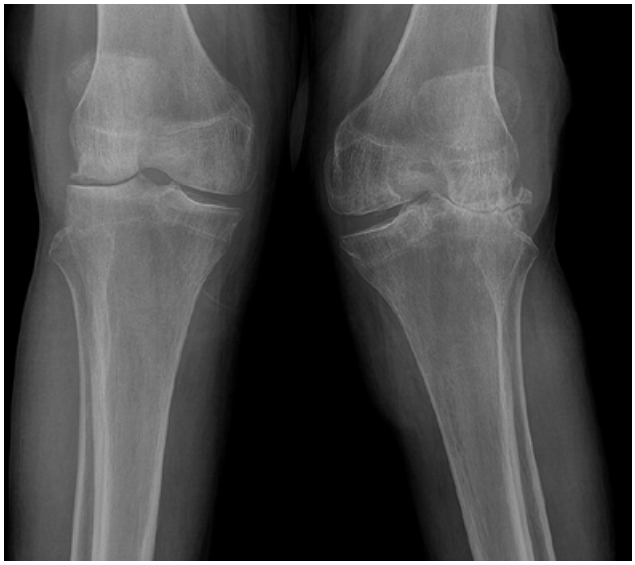

Figure 11 Radiographic valgus knee OA

Resuming, a diagnosis of OA is mainly based on symptoms. A patient that has reached a certain age and has joint pain, limitation of movement, crepitus and, sometimes, effusion in the joint might get the diagnosis of OA. Recommendations for the diagnosis of knee OA were published in 2010. (32) They include three main symptoms: knee pain, short-lived morning stiffness, and functional limitation in combination with three signs on physical examination (crepitus, restricted movement and bony enlargement).

The clinical evaluation may be performed before surgery using the AKSS, which includes two subscores: knee score (KS) and function score (FS). Each subscore ranges from 0 to 100 points. For KS evaluation, pain, range of motion, anterior–posterior and mediolateral stability, flexion contracture, leg extension and varus–valgus alignment were investigated. FS evaluates knee function from a patient's point of view, describing walking ability, climbing stairs ability and the use of walking aids.

For OA radiological X-ray images are the gold standard to confirm the clinical diagnosis and to grade the disease (33-35).

The Kellgren and Lawrence (K&L) classification criteria are the most widely used radiographic classification criteria to identify and grade OA. K&L is performed on anteroposterior and lateral X-ray views of the knee, and includes five grades: grade 0, absence of OA, grade 1, possible narrowing of joint space and possible presence of osteophytes; grade 2, definite narrowing of joint space and definite osteophytes; grade 3, definite narrowing of joint space, multiple osteophytes, sclerosis, cysts and possible deformity of bone contour; and grade 4, marked narrowing of joint space, large osteophytes, severe sclerosis, cysts and definite deformity of bone contour (35-36) (Table 2 Figure 12)

Kellgren - Lawrence classification of Knee Joint OA

Grade	Criteria
0	Normal
I	Doubtfull narrowing of Joint space, possible osteophyte development
II	Definite osteophytes, absent or questionable narrowing of joint space
III	Moderate osteophytes, definite narrowing, some sclerosis, possible joint deformity
IV	Large osteophytes, marked narrowing, severe sclerosis, definite joint deformity

Table 2 Kellgren & Lawrence grading

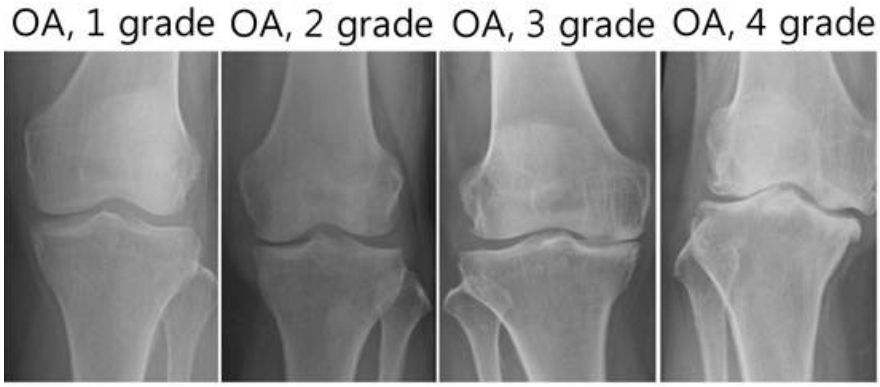

Figure 12. Radiographic representation of K&L scale (37)

A critical point for OA diagnosis is to identify an early onset and an early progression of this disease. Many studies analyse the correlation between knee OA radiographic data and clinical status of the affected joint by using specific clinical scores and radiographic grading scales.

Despite the advent of newer imaging technologies such as MRI, radiological classification will probably remain the diagnostic gold standard for knee OA in large epidemiological studies for many years to come.

OA Single Nucleotide Polymorphisms

Nowadays, molecular genetic investigations have gained an increasingly significant role in the knowledge of OA etiology and have provided evidence for a genetic component to OA (38-40). Single nucleotide polymorphisms (SNPs) are now introduced as risk factors for human disease, thanks to completion of large genome-wide association studies. . Several gene association analyses, either genome wide or a gene candidate approach, identified different genes related to the OA susceptibility, onset and progression. OA may thus be placed into the category of polygenic diseases (41-43). Several association studies between SNPs and OA disease remain unconfirmed or controversial, and it is necessary further research in order to contribute to the etiopathogenesis, to better understand functional influence of specific SNPs on OA.

The main bias that may occur in research projects on OA are in patient enrolling criteria, differences in OA-affected joint sites, in the radiographic evaluation scales used and in subjective differences in patient's pain evaluation scoring, in classification and staging modes. Furthermore, the geographical and ethnic allele distribution become of interest, and is extremely important in fully understanding the SNP variant effects.

In the Online Mendelian Inheritance in Man database – which collects known genetic lesions responsible for human inherited diseases – the following principal loci of osteoarthritis susceptibility (OS) and the associated polymorphisms, SNPs and aspartic acid (D) repeats, are reported: frizzled-related protein (FRZB) rs288326 (OS1A) and rs7775 (OS1B), MATN3 rs77245812 (OS2), ASPN D14 repeats (OS3), parathyroid hormone 2 (PTHR2) rs76758470 (OS4), growth and differentiation factor 5 (GDF5) rs143383 (OS5) and DVWA rs11718863 (OS6).

In particular, the FRZB gene is a member of a family of the soluble Wingless (Wnt) antagonist, codes for "secreted frizzled-related protein 3" (sFRP3). Recent evidence has demonstrated that products

of Wnt and Frizzled play a key role in the development and maintenance of bones and joints. The rs7775 and rs288326 FRZB SNPs variants showed an increased frequency in subjects with generalized radiographic OA, as confirmed by other studies in Caucasian individuals (44 - 45)

Articular cartilage is composed of specialized cells, the chondrocytes, that produce a large amount of extracellular matrix composed of collagen fibres. MATN3 encodes a noncollagenous extracellular matrix protein expressed during the development of the skeletal system and in the cartilage (46). The *matn3* gene codes for matrilin-3. This protein is found in the extracellular matrix, which is an intricate lattice of proteins and other molecules that forms in the spaces between cells. Specifically, matrilin-3 is located in the extracellular matrix surrounding the cells that make up ligaments and tendons, and near cartilage-forming cells (chondrocytes). The polymorphism Thr303Met (rs 77245812) is associated with OA.

Another extracellular matrix component deregulated in the articular cartilage of OA patients is asporin protein, member of the small leucine-rich proteoglycan family, encoded by the ASPN gene and expressed at high levels in knee and hip cartilage of individuals with ASPN D14 repeats (47). The encoded protein may regulate chondrogenesis by inhibiting transforming growth factor-beta 1-induced gene expression in cartilage. This protein also binds collagen and calcium and may induce collagen mineralization. Polymorphisms in the aspartic acid repeat region of this gene are associated with a susceptibility to osteoarthritis. Alternate splicing result in multiple transcript variants.

The pthr2 gene encodes for a member of the G-protein coupled receptor family 2. Its functional role in OA is based on the observation that PTHR2 is expressed in a number of endocrine cell types and regulates pituitary hormone secretion and specifically growth hormone (48). This protein is expressed in different tissues and involved in the regulation of growth hormone secretion,

Ca^{2+}homeostasis and, modulation of growth cartilage in condrocytes. The polimorphism Leu159Phe (rs76758470) is associated to OA.

The *gdf5 gene encodes* for a protein closely related to the bone morphogenetic protein (BMP) family, a member of the TGF-beta superfamily. Growth differentiation factor 5 has a role in skeletal and joint development. Mutations in GDF5 are involved in several disorders of skeletal development and also in hip and knee OA progression (49-51). The polimorphism rs143383 is associated to OA.

Finally, the DVWA gene, which encodes for a protein containing two von Willebrand A domains, was found to harbour the rs11718863 SNP, showing a consistent association with knee OA in Japanese and Chinese OA cohorts (52). The experimental data provided in different studies led to the suggestion of a mechanism for the etiology of the disease, based on an interaction between DVWA protein and beta-tubulin. Two polimorphism, Cys260Tyr (rs7639618) and Tyr169Asn (rs 11718863) are indicated as susceptibility loci for OA.

OA Treatment

OA is incurable and most treatments, which include physiotherapy, life-style modifications, pharmacotherapy and surgery, aim to provide symptomatic relief rather than targeting the disease processes themselves. Mechanisms by which OA arises and progresses are not completely understood (53). The main objective of research is to discover new treatment to alleviate the signs and symptoms of the disease and to slow its progression. The best OA treatment is prevention, as declared in 1966 by Mohing W et al. (54).

There are different therapeutic modalities, from physiotherapy, orthopedic aids and orthoses, pharmacotherapy, and finally surgery and rehabilitation.

According to patient's symptoms, physical and radiological findings surgery is indicated as ultimate treatments.

It's possible classify treatment in three different modality of intervention: conservative, joint preserving surgical treatment and joint replacement surgery (55).

Conservative treatment are indicated depending on the severity and distribution of symptoms as well as any possible accompanying illnesses. The objective are:

1. Pain relief
2. Improved quality of life
3. Improved mobility
4. Improved walking
5. Delayed progression of osteoarthritis

Whitin the conservative treatments there are general measures that include patient education, lifestyle adjustment, and weight loss. Any factors placing excessive and damaging stress on the knee joint should be eliminated. Physiotherapeutic measures for knee osteoarthritis includes exercise therapy and physical measures, as ultrasound application, electrotherapy, muscle stimulation, application of heat and cold, massage, acupuncture, stretching/walking and traction exercises. Orthopedic aids include cushioned heels and wedges correcting the axis to a certain extent and taking mechanical stress off the affected part of the joint.

When signs of inflammation arise, medications are currently used to treat knee osteoarthritis. The most suitable type are analgesics/anti-inflammatory agents, glucocorticoids, opioids, slow-acting drugs for OA, and anti-cytokines.

The next step in the therapeutic scheme is the joint preserving surgical treatment. Surgery is indicated only when all abovementioned measures have been tried without success and considering also patients with advanced osteoarthritis.

The overwhelming majority of intra-articular operations are performed through an arthroscope. It's possible classified joint-preserving surgical option in three classes: Symptomatic, including lavage, shaving and debridement; Bone-stimulating, including drilling, microfracturing and abrasion arthroplasty; Joint surface restoration with Autologous chondrocyte transplantation (ACT) or Autologous osteochondral transplantation (OCT).

The purpose of Arthroscopic lavage is to rid the joint of detritus and inflammatory mediators. Shaving, or chondroplasty, involves removing frayed and fragmented cartilage. Debridement is described as "house-cleaning arthroplasty," serves the same general purposes.

The goal of bone-stimulating techniques is to bring pluripotential stem cells to the joint surface, where they are able to form fiber bundles take advantage of mechanical and biological forces.

In ACT, cartilage cells are taken from the joint, cultured ex vivo and then put back into the joint. In OCT, also termed mosaicplasty, cylinders of cartilage and bone are taken from a part of the non affected joint, and subsequently inserted into the cartilage defect. The reported results of ACT and OCT are very promising.

Last step in therapeutic treatment is joint surgery, that may be carried out by partial joint replacement, one or two compartment, or total replacement of three compartment.

Purpose

The purpose of this study is to highlight possible associations between KL grade, clinical features (AKSS - American Knee Society Score, age) and the abovementioned genetic polymorphisms in order to update the knee OA grading and to improve a personalized treatment program in the future.

Although several studies described the association between these specific polymorphisms and susceptibility to OA, no studies have examined their simultaneous presence in OA patients, especially in the European people groups.

Precisely, Sicilian individuals have a specific genetic background and different allele distribution compared with the rest of Europe and with the rest of Italy (north–south genetic trend), due to distinct gene–environment interactions and, certainly, due to deep human migration movements, which have occurred in Sicily over the centuries as described by several authors (56-58)

Methods

Patients

On admission to hospital, 66 Sicilian patients affected by primary OA, aged 54 to 86 years, candidates for knee surgery of arthroscopy or arthroplasty, were enrolled in this project. The patients were grouped, according to age, into two groups: young (from 54 to 65 years old) and old (from 66 to 86 years old). Among these, 61 patients were selected for genotyping analysis due to availability of a blood sample and for cytokines study. This study (named OA_BIOMOL_1) was approved by the Ethical Committee of the San Raffaele G. Giglio Hospital, Cefalù, Italy (number of protocol: CE 2011/63) and the patients gave their written informed consent according to the Helsinki Declaration (Figure 13).

For each patient, it has designed a data collection sheet in which we considered:

- Number, age and sex of the patient;
- weight and height (to calculate Body Mass Index - BMI)
- Knee examined (right or left);
- Degree of OA of K & L (0 to 4) and the appropriate class (A, B, C),
- Knee score and the related class (1, 2, 3, 4) function score
- Biological samples carried out, executed.

We also enrolled 100 healthy Sicilian subjects as control samples for mutational analysis.

SCHEDA DI RACCOLTA DATI

CODICE PAZIENTE: _____ **ETA'**_____ **SESSO** M F

GINOCCHIO DX SX

K&L 0 1 2 3 4 ***Classe** A B C **Data** ___/___/___
Note_____

KSS (0-100)_____ **†Classe** 1 2 3 4 **Data** ___/___/___
Note_____

Tab. 1 – Tabella classi K&L e KSS

KSS \ K&L	A	B	C
1			
2			
3			
4			

* A per K&L 0-1-2, B per K&L 3, C per K&L 4.
† 1 per KSS 80-100, 2 per KSS 70-79, 3 per KSS 60-69, 4 per KSS <60.

***PRELIEVI*:**

☐ **SANGUE** **Data** ___/___/___ **Note**_____

☐ **URINE** **Data** ___/___/___ **Note**_____

☐ **LIQUIDO SINOVIALE** **Data** ___/___/___
Note_____

☐ **MEMBRANA SINOVIALE** **Data** ___/___/___
Note_____

*Firma del medico*_____

***ANALISI MUTAZIONALE*:**

Tab. 2 – Tabella analisi mutazionale

frzb	*matn3*	*aspn*	*pthr2*	*gdf5*	*dvwa*

Note_____

*Firma del ricercatore*_____

Figure 13 Data collection sheet

Clinical evaluation

The clinical evaluation was performed for each patient before surgery using the AKSS, which includes two subscores: knee score (KS) and function score (FS). Each subscore ranges from 0 to 100 points. For KS evaluation, pain, range of motion, anterior–posterior and mediolateral stability, flexion contracture, leg extension and varus–valgus alignment were investigated. FS evaluates knee function from a patient's point of view, describing walking ability, climbing stairs

ability and the use of walking aids. The AKSS was classified into three levels for both KS and FS: high (HKS, HFS), medium (MKS, MFS) and low (LKS, LFS). The patients with LKS and LFS had scores between 0 and 49 points. The patients with MKS and MFS had scores between 50 and 69 points. The patients with HKS and HFS had scores from 70 to 100.

Radiographic evaluation

The radiographic evaluation was performed on anteroposterior and lateral X-ray views of the knee by a single investigator using the KL, which includes four grades: grade 1, possible narrowing of joint space and possible presence of osteophytes; grade 2, definite narrowing of joint space and definite osteophytes; grade 3, definite narrowing of joint space, multiple osteophytes, sclerosis, cysts and possible deformity of bone contour; and grade 4, marked narrowing of joint space, large osteophytes, severe sclerosis, cysts and definite deformity of bone contour (35, 59). The evaluation was undertaken on an X-ray performed no more than 4 months before surgery. In this study, we grouped grade 1 and grade 2 into a single grade because the radiographic differences in our cohort were considered not relevant compared with those between KL grade 3 and grade 4. The KL classification was therefore summarized into three groups: group A (grades 1 and 2), group B (grade 3), and group C (grade 4).

Genetic analysis

The patients were genotyped by sequencing analysis, for the following genetic polymorphisms associated with OS, SNPs and D repeats: FRZB rs288326 (OS1A) and rs7775 (OS1B), MATN3 rs77245812 (OS2), PTHR2 rs76758470 (OS3), ASPN D14 repeats (OS4), GDF5 rs143383 (OS5) and DVWA rs11718863 (OS6). The Human Gene Mutation Database and the dbSNP Short Genetic

Variations database were used to analyze gene regions containing the selected SNPs (60-61). Genomic DNA was extracted from peripheral blood using the QIAamp DNA blood mini kit, according to the manufacturer's specifications (Qiagen Inc., Valencia, CA, USA). After quality and quantity analysis, DNA was polymerase chain reaction amplified using primers designed by the Primer3 software (62) and listed in Table 3. Polymerase chain reaction reactions were performed with 50 ng genomic DNA in a total volume of 50 µl containing 1°— PCR Gold Buffer, 1.5 mM di-MgCl2, 200 µM dNTPs, 200 nM forward and reverse primer mix and 1.25 U AmpliTaq Gold DNA Polymerase (Life Technologies Monza, MB, Italy). The thermal cycle profile employed a 5-minute denaturing step at 94°C, followed by 35 cycles at 94°C for 45 seconds, 59°C for 45 seconds and 72°C for 45 seconds, and a final extension step of 5 minutes at 72°C. The quality and quantity of polymerase chain reaction products were assessed on the Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA) and were purified using the QIAquick PCR purification kit, according to the manufacturer's specifications (Qiagen Inc., Valencia, CA, USA).

To perform DNA sequencing, purified amplicons were labelled with the BigDye Terminator v3.1 Cycle Sequencing Kit following the manufacturer's standard protocol (Applied Biosystems). The thermal cycle profile employed a 1-minute denaturing step at 96°C, followed by 25 cycles at 96°C for 10 seconds, 54°C for 5 seconds and 60°C for 3 minutes. Labelled samples were purified with the Xterminator purification kit according to the manufacturer's standard protocol and loaded in a 3500-Dx Genetic Analyzer (Applied Biosystems) for separation by capillary electrophoresis. Electropherograms and sequence files were analysed using Sequencing Analysis and SeqScape software (Applied Biosystems).

Primers sequence used for genotyping analysis			
Target gene polymorphism	**Forward primer (5°¨ to 3°¨)**	**Reverse primer (5°¨ to 3°¨)**	**Template size (base pairs)**
FRZB (rs288326; OS1A)	cctcttggcagcaattggaac	gcccctctcccaagaaaaatg	800
FRZB (rs7775; OS1B)	agggcaggaccttgtctgtt	taagagtctgcccccaaacc	884
MATN3 (rs77245812; OS2)	tcacgtcacttcaggctgtg	tggggtctcaccatgttctc	886
ASPN (D14; OS3)	gcacattgctgaattgctttcca	ctttggggtttgctgtactttc	615
PTH2R (rs144641723; OS4)	tctcgaaccagtccctgct	cccatgacagttgctgtgg	602
GDF5 (rs143383; OS5)	gcagatgaattccaggtccag	ccatgaggtggaggtgaaga	818
DVWA (rs11718863; OS6A)	aggctgcctgccattattctt	cccatgctgtttcctttgaaca	924

Table 3 Primers sequence used for genotyping analysis

Synovial fluid sampling and cytokine assay

With the approval of patients, synovial joint fluid samples were collected during knee surgery of arthroscopy or arthroplasty. The samples of synovial fluid were immediately stored at – 80°C until use. Freeze-thaw cycles were avoided. Key biomarkers of inflammation and cytokines quantification was made by Luminex technology.

Before the cytokine assay, SF samples were thawed to room temperature (RT) and clarified at 10000g for 10 min. The supernatant of each sample was then treated with hyaluronidase (HAse). Each sample was prepared and run in duplicates. HAse treatment

significantly improved the number of well with good or excellent bead events for each bead region (63). The samples were tested for a panel of 17 cytokines and chemokines (IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, IFN-c, TNF-a, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1b (MIP-1b), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF)) using Bio-plex kit (BioRad, Milan, Italy) and following the manufacturer's instructions. The assay was carried out using the Luminex system (BioRad, Munchen, Germany), based on the measurement of fluorescent signals released by a suspension of microspheres, bringing immobilized multiplex cytokine specific antibodies in 96-well plates. The combination of a fluorimetric signal of microspheres with that released by a secondary antibody allows us to measure cytokine concentration–related signals converted by a processor. The assay was performed using an eight-point standard curve for every cytokine. Samples were analyzed on a Luminex 100 device (BioRad), and the data were evaluated using the Bio-Plex Manager software (BioRad). Standards, internal controls, and samples were reported as means of duplicate measurements.

Statistical analysis

The association between the clinical data (KS, FS, age) and the radiographic data (KL) and the association between genotypes and KL groups (A, B, C) were analyzed using GraphPad InStat software version 3.05 (64). The Mann–Whitney U test, the chi-square test and Fisher's exact test were performed. Differences in groups were considered significant when $P \leq 0.05$. Hardy–Weinberg equilibrium was evaluated.

The association between DVWA SNPs genotypes and KL groups, was analyzed using GraphPad InStat software version 3.05 (San Diego California USA). Mann Whitney-U test, Pearson's Chi-Square

test and Fisher's exact test were performed. Differences in groups were considered significant when the p-value was less than or equal to 0.05. Hardy-Weinberg Equilibrium (HWE) was calculated. Finally, in order to verify the degree of allelic segregation among the SNPs of our interest, we calculated the Linkage Disequilibrium (LD) coefficients (D' and r2) using Haploview software 3.32.

Results

Clinical and radiographic evaluation

We recruited 66 cases (37 females and 29 males), of which 24 were young (54 to 65 years) and 42 were old (66–86 years). Subsequently, they were divided into three groups (A, B, C) depending on the degree of radiographic knee OA. According to the clinical scores we classified the patients as follows:

- Group A consisted of 24 patients (11 females and 13 males, 14 young and 10 old); KS was poor in 13 cases and fair in 11 cases, and the average FS score was 51 points.
- Group B consisted of 21 patients (15 females and six males, eight young and 13 old); KS was poor in 19 cases and fair in two cases, and the average FS score was 41 points.
- Group C consisted of 21 patients (11 females and 10 males, two young and 19 old); KS was low in all cases, and the average FS was 35 points.

Regarding the treatment, 22 patients of group A underwent arthroscopy and two patients arthroplasty, two patients of group B underwent arthroscopy and 19 patients arthroplasty, and 21 patients of group C underwent arthroplasty (Table 4).

Clinical features and treatment for each radiographic group of patients							
KL group	**Total**	**Females**	**Males**	**Young**	**Old**	**Arthroscopies**	**Arthroplasties**
A	24 (36.5%)	11 (29.7%)	13 (44.8%)	14 (58.3%)	10 (23.8%)	22 (91.7%)	2 (4.8%)
B	21 (31.8%)	15 (40.5%)	6 (20.7%)	8 (33.3%)	13 (30.0%)	2 (8.3%)	19 (45.2%)
C	21 (31.8%)	11 (29.7%)	10 (34.5%)	2 (8.3%)	19 (45.2%)	0	21 (50%)

Table 4 Data presented as number of patients (percentage). KL, Kellgren and Lawrence osteoarthritis grading scale.

According to the KS and FS scores, the patients in the LKS group were in the majority (n = 46), while the 36 patients in the LFS group generally had severe symptoms and high disability. There were 19 patients in the MKS group and 23 patients in the MFS group, and thus one third of patients had moderate to severe symptoms and disability. One patient was in the HKS group and seven patients were in the HFS group, with zero to mild symptoms and disability.
(Table 5).

Patient classification according to knee score and function score					
KS group	**n**	**%**	**FS group**	**n**	**%**
LKS	46	70	LFS	36	55
MKS	19	29	MFS	23	35
HKS	1	2	HFS	7	11

Table 5 FS, function score (LFS, low; MFS, medium; HFS, high); KS, knee score (LKS, low; MKS, medium; HKS, high); n, number of patients.

Association between Kellgren and Lawrence osteoarthritis grading and knee score, function score and age.

Association analyses were performed to verify the possible association between clinical data (KS, FS, age) and radiographic data (KL). A statistical association between the variables analyzed was observed (Table 6).

Association between Kellgren and Lawrence osteoarthritis grading and knee score, function score and age							
	KL score						
	Group A		Group B		Group C		P value
	n	%	n	%	n	%	
Low knee score	8	33.3	17	81	21	100	<0.0001
Medium knee score	15	62.5	4	19	0	0	
High knee score	1	4.2	0	0	0	0	
Low function score	8	33.3	13	62	15	71.4	0.022
Medium function score	10	41.7	7	33.3	6	28.6	
High function score	6	25	1	4.7	0	0	
Age 54 to 65	14	58.3	8	38.1	2	9.5	0.0011
Age 66 to 86	10	41.7	13	61.9	19	90.5	

Table 6 KL, Kellgren and Lawrence osteoarthritis grading scale; n, number of patients. *Chi-squared test.

Kellgren and Lawrence osteoarthritis grading versus knee score

In group A, we observed eight patients (33.3%) with LKS, 15 (62.5%) with MKS and one (4.2%) with HKS. In group B, we observed 17 patients (81%) with LKS, four (19%) with MKS and none with HKS. In group C, we observed all patients (n = 21) with LKS. The highest number of patients with LKS were therefore in groups B and C and the radiographic findings are related to clinical pictures expressed by the KS score (P = 0.0001).

Kellgren and Lawrence osteoarthritis grading versus function score

In group A, we observed eight patients (33.3%) with LKS, 10 (41.7%) with MFS and six (25%) with HFS. In group B, we observed 13 patients (62%) with LFS, seven (33.3%) with MFS and one (4.7%) with HFS. In group C, we observed 15 patients (71.4%) with LFS, six (28.6%) with MFS and none with HFS. These data show that an increase of the OA radiographic severity corresponds to a decrease of the function score (P = 0.022).

Kellgren and Lawrence osteoarthritis grading versus age

In group A, we observed 14 young (58.3%) and 10 old (41.7%) patients. In group B, we observed eight young (38.1%) and 13 old (61.9%) patients. In group C, we observed two young (9.5%) and 19 old (90.5%) patients. So, it is more common to observe a medium to high OA radiographic grade in the population over 65 years old, and a low to medium in adults under the age of 65 years old (P = 0.0011).

Mutational analysis of osteoarthritis susceptibility genes

The OA patients were genotyped for the following polymorphisms associated with OS, such as SNPs and D repeats: FRZB rs288326 and rs7775, MATN3 rs77245812, ASPN D14, PTHR2 rs76758470, GDF5 rs143383, and DVWA rs11718863. Percentages of the wild type, heterozygote and homozygote genotypes for each polymorphism were calculated. We reported genotyping data of the three radiographic groups (A, B, C) and the number of individuals for each genotype (Table 7). In each group, deviations of Hardy–Weinberg equilibrium for all polymorphisms analysed were not observed.

Polymorphism	Genotype	Group A n=20	%	HWe	Group B n=21	%	HWe	Group C n=20	%	HWe
FRZB (rs288326 - OS1A)										
CC	WT	17	85		14	66,7		15	75	
CT	H	2	10	0,05	7	33,3	0,36	4	20	0,33
TT	MUT	1	5		0	0		1	5	
FRZB (rs7775 - OS1B)										
CC	WT	18	90		16	76,2		13	65	
CG	H	2	10	0,03	5	23,8	0,54	7	35	0,34
GG	MUT	0	0		0	0		0	0	
MATN3 (rs77245812 - OS2)										
CC	WT	19	95		20	95,2		18	90	
CT	H	1	5	0,91	1	4,8	0,91	2	10	0,81
TT	MUT	0	0		0	0		0	0	
ASPN (D14 - OS3)										
D13	WT	5	25		3	14,3		4	20	
D13/D14	H	11	55	0,65	14	66,7	0,12	11	55	0,65
D14	MUT	4	20		4	19		5	25	
PTH2R (rs144641723 - OS4)										
GG	WT	19	95		21	100		20	100	
GT	H	1	5	0,91	0	0	NA	0	0	NA
TT	MUT	0	0		0	0		0	0	
GDF5 (rs143383 - OS5)										
TT	WT	3	15		12	57,1		7	35	
TC	H	13	65	0,18	5	23,8	0,04	11	55	0,44
CC	MUT	4	20		4	19		2	10	
DVWA (rs11718863 - OS6)										
TT	WT	15	75		17	81		9	45	
TA	H	4	20	0,33	4	19	0,63	10	50	0,39
AA	MUT	1	5		0	0		1	5	

HWe: Hardy Weinberg equilibrium * *p-value*

Table 7 Mutational analysis of osteoarthritis susceptibility genes

Kellgren and Lawrence osteoarthritis grading and genotype association analysis

To evaluate a potential association between genotypes, wild-type group or mutated (heterozygote and homozygote) group and the KL groups (A, B, C), the Mann– Whitney U test, the chi-square test and Fisher's exact test were performed (Table 6). Analysis showed a statistically significant association between genotype and KL grade for the GDF5 rs143383 and the DVWA rs11718863 polymorphisms ($P = 0.02$ and $P = 0.03$, respectively). These results are in line with the study of Valdes and colleagues where GDF5 rs143383 and DVWA rs11718863 polymorphisms are consistently associated with the risk of knee OA in the Caucasian population (65), but to our knowledge this is the first study that reports the simultaneous presence of these two polymorphisms associated with KL in a European group. Unfortunately, concerning the other four OS SNPs, no genotype showed any significant association with KL data, as revealed by statistical analysis.

Finally, it is possible to note in Table 8 that the DVWA rs11718863 polymorphism (genotype heterozygote + homozygote) is more represented in group C (55%), compared with the other two groups A (25%) and B (19%), suggesting that OS6 can be associated with a more severe OA radiographic grade.

Polymorphism	KL group	WT n	WT %	H+Mut n	H+Mut %	*p-value
FRZB-OS1A (rs288326)	A	17	85	3	15	0,39
	B	14	66,7	7	33,3	
	C	15	75	5	25	
FRZB-OS1B (rs7775)	A	18	90	2	10	0,17
	B	16	76,2	5	23,8	
	C	13	65	7	35	
MATN3-OS2 (rs77245812)	A	19	95	1	5	0,75
	B	20	95,2	1	4,8	
	C	18	90	2	10	
ASPN-OS3 (D14)	A	5	25	15	75	0,69
	B	3	14,3	18	85,7	
	C	4	20	16	80	
PTH2R-OS4 (rs144641723)	A	19	95	1	5	na
	B	21	100	0	0	
	C	20	100	0	0	
GDF5-OS5 (rs143383)	A	3	15	17	85	0,02
	B	12	57,1	9	42,9	
	C	7	35	13	65	
DWVA-OS6 (rs11718863)	A	15	75	5	25	0,03
	B	17	81	4	19	
	C	9	45	11	55	

NA = not available
n = number of patients

*Chi-Squared test

Table 8 Kellgren and Lawrence osteoarthritis grading and genotype association analysis

Linkage disequilibrium analysis

Linkage Disequilibrium (LD) coefficients (D' and r2) was calculated using Haploview software 3.32, in order to verify the degree of allelic segregation among the SNPs of our interest. Based on the HapMap project databases (66-68), this approach was used for rs7639618, rs7639807, rs7651842 by a pairwise tagging mode, because allelic frequencies from different populations are available on this platform for these three SNPs. Instead, no data were available for the other two SNPs, rs17040821 and rs11718863. Then, we replicated the D' and r2 calculation for all the five above-mentioned

SNPs, by using the allelic frequencies from our Sicilian cohort of healthy subjects, with the proper formulas:

D' = D/Dmax,
r2 = D2/ p1p2q1q2

where, D = (x11)(x22) – (x12)(x21); Dmax is the smaller of p1q2 and p2q1, where the haplotype frequencies for the hypothetic loci A and B are defined as described in the Table 9. For analised Sicilian cohort, the pairwise LDs for rs7639618, rs7639807 and rs7651842 perfectly matched with those observed by Haploview software (Table 9).

Finally, similarly to the Haploview LD output form, it was elaborated a r2 LD plot, where the perfect LD between SNPs pair is described by r2 = 1 and is dark marked, whereas the LD absence is described by r2 = 0 and is white marked (Figure 14).

Haplotype	Frequency	Allele	Frequency
A1B1	x11	A1	p1 =x11 +x12
A1B2	x12	A2	p2 =x21 +x22
A2B1	x21	B1	q1 =x11 +x21
A2B2	x22	B2	q2 =x12 +x22

Table 9 Linkage disequilibrium analysis

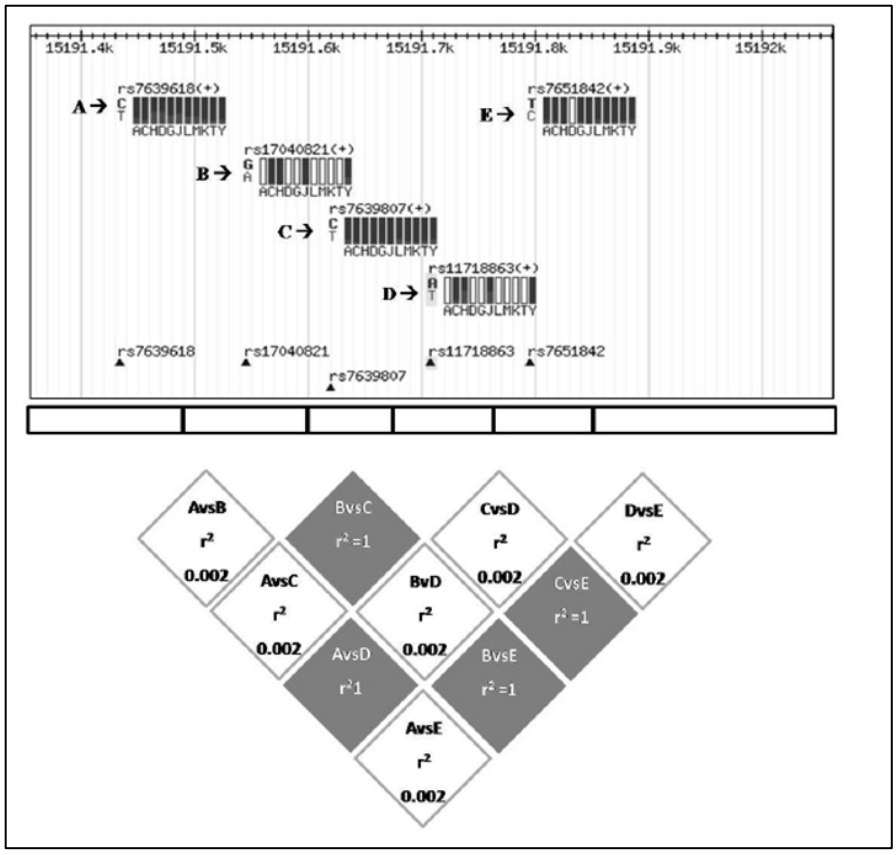

Figure 14 r2 LD plot, where the perfect LD between SNPs pair is (r2 = 1) dark marked, whereas the LD absence (r2 = 0) is white marked

Mutational analysis of OA susceptibility genes

Genotyping analyses were performed by sequencing analysis of amplicons of 924 bp, containing the two DVWA SNPs: rs11718863, rs7639618. Sequencing analysis of the electropherograms revealed the presence of others three less known DVWA SNPs: rs7651842, rs7639807 and rs17040821. Sixty-one osteoarthritis patients and one hundred healthy subjects were genotyped for the abovementioned five DVWA SNPs. Percentages of the wild type (WT), heterozygote (H) and homozygote (MUT) genotypes for each polymorphism were calculated. SNPs, genotype percentages and allele frequencies of 161 individuals investigated in this study are also reported (Table 10). For OA patients, we reported genotyping data of the three radiographic groups (A, B, C) and the number of individuals for each genotype.

Deviations of Hardy-Weinberg equilibrium for all polymorphisms analyzed were not observed
(Table 11).

DVWA polymorhism	Genotype	OA patients n = 61	%	Allele frequencies	%	Controls n = 100	%	Allele frequencies	%
rs11718863									
TT	WT	41	67	*T = 100*	82	72	*72*	T = 100	72
TA	H	18	30	A = 22	18	25	25	A = 56	28
AA	MUT	2	3			3	3		
rs7639618									
GG	WT	41	67	*G = 100*	82	72	*72*	G = 144	72
GA	H	18	30	*A = 22*	18	25	*25*	A = 56	28
AA	MUT	2	3			3	3		
rs7651842									
AA	WT	44	72,1	A = 103	84,4	85	85	A = 170	85
AG	H	15	24,6	*G = 19*	15,6	14	*14*	G = 30	15
GG	MUT	2	3,3			1	*1*		
rs7639807									
GG	WT	44	72,1	A = 103	84,4	85	85	G = 170	85
GA	H	15	24,6	G = 19	15,6	14	14	A = 30	15
AA	MUT	2	3,3			1	*1*		
rs17040821									
CC	WT	44	72,1	C = 103	84,4	85	85	C = 170	85
CT	H	15	24,6	T = 19	15,6	14	14	T = 30	15
TT	MUT	2	3,3			1	1		
n = number of patients.									

Table 10 Genetic analysis result

DVWA Polymorphism	Genotype	A group n=20	%	HWE	B group n=21	%	HWE	C group n=20	%	HWE
rs11718863										
TT	WT	15	75		17	81		9	45	
TA	H	4	20	0,33	4	19	0,63	10	50	0,39
AA	MUT	1	5		0	0		1	5	
rs7639618										
GG	WT	15	75		17	81		9	45	
GA	H	4	20	0,33	4	19	0,63	10	50	0,39
AA	MUT	1	5		0	0		1	5	
rs7651842										
AA	WT	14	70		15	71,4		15	75	
AG	H	6	30	0,43	5	23,8	0,51	4	20	0,33
GG	MUT	0			1	4,8		1	5	
rs7639807										
GG	WT	14	70		15	71,4		15	75	
GA	H	6	30	0,43	5	23,8	0,51	4	20	0,33
AA	MUT	0			1	4,8		1	5	
rs17040821										
CC	WT	14	70		15	71,4		15	75	
CT	H	6	30	0,43	5	23,8	0,51	4	20	0,33
TT	MUT	0			1	4,8		1	5	

HWE: Hardy Weinberg Equilibrium
n=number of patients

Table 11 Genetic analysis results according to KL grading

KL and genotype association analysis

To evaluate a potential association between genotypes, WT group or Mutated (Mut + H) one and the KL groups (A, B, C), Mann Whitney-U test, Chi-Square test and Fisher's exact test were performed (Table 4). Analysis showed a statistically significant association between genotype and KL grading scale for the rs11718863 and rs7639618 DVWA polymorphisms ($p = 0.03$). These results are in line with the study of Valdes AM et al (65), where these polymorphisms are associated with the risk of knee OA in the UK population, but to our knowledge, this is the first study that reports the simultaneous presence of these two genetic alterations associated with KL in a Sicilian group. Finally, it is possible to note in Table 4, that rs11718863 and rs7639618 DVWA SNPs (genotype H + Mut) are

more represented in group C (55%), compared to the other two groups A (25%) and B (19%), suggesting that they can be associated with a more severe OA radiographic grade.

For rs7651842, rs7639807 and rs17040821 DVWA SNPs, we did not observed significant statistical association with radiographic KL grade (Table 12).

	KL and Genotype						**KL and Alleles**					
DVWA Polymorphism		**WT**	**%**	**H/Mut**	**%**	***p-value****		**Allele T**	**%**	**Allele A**	**%**	***p-value****
	A	15	75	5	25		A	34	85	6	15	
rs11718863	B	17	81	4	19	0,03	B	38	90,5	4	9,5	0,04
	C	9	45	11	55		C	28	70	12	30	
								Allele G		**Allele A**		
	A	15	75	5	25		A	34	85	6	15	
rs7639618	B	17	81	4	19	0,03	B	38	90,5	4	9,5	0,04
	C	9	45	11	55		C	28	70	12	30	
								Allele A		**Allele G**		
	A	14	70	6	30		A	34	85	6	25	
rs7651842	B	15	71,4	6	28,6	0,936	B	35	83,3	7	16,7	0,9713
	C	15	75	5	25		C	34	85	6	25	
								Allele G		**Allele A**		
	A	14	70	6	30		A	34	85	6	25	
rs7639807	B	15	71,4	6	28,6	0,936	B	35	83,3	7	16,7	0,9713
	C	15	75	5	25		C	34	85	6	25	
								Allele C		**Allele T**		
	A	14	70	6	30		A	34	85	6	25	
rs17040821	B	15	71,4	6	28,6	0,936	B	35	83,3	7	16,7	0,9713
	C	15	75	5	25		C	34	85	6	25	

* Chi-Squared Test

Table 12 Genotype and alleles statistical association with KL grade

Synovial fluid Cytokines assay result

Table 13 shows the quantities of cytokines, chemokine and grow factor measured in the synovial fluid of each patients knee joint

Sample	Hu IL-6	Hu IL-7	Hu IL-8	Hu IL-10	Hu IL-12(p70)	Hu IL-13	Hu GM-CSF	Hu MCP-1	Hu MIP-1b	Hu TNF-a
	Conc in Range	Conc in Range	Conc in Range	Conc in Range	Conc in Range	Conc in Range	Conc in Range	Conc in Range	Conc in Range	Conc in Range
1	32,65	3,36	22,6	2,32	1,92	1,94	59,91	394,1	161,2	0,55
2	39,68	4,37	6,9	3,64	OOR <	4,8	13,5	96,0	53,86	OOR <
3	2655,08	2,38	22,84	7,16	11,17	1,17	127,28	138,4	173,1	18,2
4	33,7	3,09	22,08	3,88	6,99	2,07	73,87	398,31	162,8	0,68
5	40,53	4,44	6,39	3,64	0,9	5,74	15,5	92,33	55,08	OOR <
6	2529,01	3,15	22,74	9,39	8,15	1,53	131,73	150,53	172,3	18,69
7	1546	5,35	50,73	11,53	5,2	1,98	49,11	225,68	133,6	3,69
8	1596,19	5,5	51,1	11,12	6,43	1,25	50,16	229,17	138,6	3,66
9	83,61	7,67	25,92	OOR <	4,18	3,63	16,35	224,68	216,4	0,6
10	102,45	3,74	17,25	2,32	9,64	4,36	25,14	105,49	173,1	0,48
11	733,67	4,13	19,6	4,52	3,9	0,98	36,81	151,53	149,0	2,68
12	0,65	1,18	3,28	2,03	OOR <	3	8,56	22,07	8,5	OOR <
13	1,3	2,13	4,68	2,71	9,42	0,86	OOR <	63,97	53,96	OOR <
14	720,04	4,26	18,71	5,59	OOR <	1,04	82,78	141,03	60,36	2,31
15	440,52	4,02	18,36	14,56	84,24	1,3	OOR <	192,87	166,0	0,86
16	469,49	4,14	19,57	18,85	78,32	1,27	OOR <	205,08	172,4	1,17
17	166,81	2,85	29,16	2,78	5,87	1,32	46,28	331,94	188,5	0,55
18	2,64	8,89	12,21	3,23	OOR <	5,65	80,46	124,25	61,2	6,27
19	4,78	2,85	6,59	3,84	2,98	0,99	15,03	122,05	57,93	0,93
20	371,03	6,84	23,4	5,43	21,89	7,5	35,97	259,73	243,0	2,28
21	377,39	7,08	20,97	5,27	14,15	8,08	37,73	250,21	240,1	2,38
22	19,83	4,01	23,34	1,75	61,66	OOR <	6,48	146,23	126,6	0,77
23	35,97	3,94	23,86	3,75	1,15	1,78	37,31	65,13	102,3	OOR <
24	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR<	OOR <
25	46,54	4,08	9,22	OOR <	5,82	2,14	4,07	117,7	170,9	OOR <
26	124,69	2,56	12,06	2,49	1,86	2,53	OOR <	54,39	140,8	OOR <
27	84,17	13,61	30,39	2,87	1,92	4,34	31,99	124,58	89,53	1,31
28	1515,93	4,96	194,63	6,58	6,43	4,9	87,12	428,43	210,5	3,81
29	15,48	4,87	9,78	3,12	OOR <	3,92	51,44	115,91	66,91	0,51
30	180,42	OOR <	1316,43	22,94	139,76	3,23	21,3	410,7	217,5	3,37
31	2244,31	2,67	868,19	71,27	69,87	3,15	196,59	185,77	98,47	44,21
32	3069,94	2,1	3683,65	63,48	14,74	3,94	205,08	250,15	961,0	24,16
33	113,73	4,08	24,13	0,94	OOR <	4,21	OOR <	161,62	160,2	OOR <

34	107,97	3,69	90,27	3,03	2,28	6,34	37,98	281,01	191	1,03
35	107,86	3,81	89,09	3,43	2,92	5,5	32,93	284,66	184,9	0,89
36	33,89	5,01	5,2	4,25	OOR <	3,43	61,7	207,01	45,85	0,53
37	18	3,54	7,37	0,56	OOR <	0,46	32,59	141,56	142,7	OOR <
38	310,93	3,77	19,9	6,43	4,75	8,15	15,41	262,38	184,5	2
39	193,17	1,19	25,71	1,22	13,34	2,15	OOR <	143,05	125,1	1,07
40	159,33	4,45	10,11	2,57	OOR <	5,76	16,72	171,38	110,5	OOR <
41	515,84	4,49	49,64	2,33	10,19	4,94	44,98	301,77	133,7	1,59
42	190,93	2,83	49,12	1,57	12,9	OOR <	OOR <	170,51	313,5	0,39
43	314,55	3,19	32,83	1,18	OOR <	6,45	28,72	120,15	52,59	0,41
44	79,63	2,15	18,23	3,03	32,4	4,51	66,82	56,47	29,61	6,34
45	167,66	4,65	13,57	0,49	OOR <	1,64	38,81	167,45	108,2	0,77
46	1079,31	3,4	116,48	0,75	OOR <	0,88	19,49	87,03	95,52	2,9
47	530,5	3,46	39,85	OOR <	OOR <	2,35	45,15	184,35	94,91	0,93
48	1565,55	4,01	91,13	21,94	200,92	3,3	32,84	215,65	105,1	4,73
49	40,5	4,6	17,6	1,75	2,74	1,52	OOR <	69,62	180,6	OOR <
50	219,76	2,16	141,74	3,14	13,71	1,35	23,19	88,16	304,1	6,07
51	55,68	3,54	8,08	OOR <	0,65	1,04	11,07	29,82	168,7	OOR <
52	613,97	5,44	44	2,46	13,07	3,27	44,9	158,75	252,4	1
53	748,86	5,63	35,28	4,49	10,51	3,28	57,47	72,85	227,3	2,21
54	1340,41	4,86	1506,94	11,25	86,24	1,88	OOR <	75,75	380,2	4,71
55	28,5	4,71	25,02	2,42	3,49	6,07	37,06	130,79	160,9	OOR <
56	1624,04	4,5	20,12	5,41	1,92	3,23	22,92	556,04	158,4	3,69
57	968,99	6,97	82,45	3,14	12,04	4,9	32,76	338,95	217,0	2,88
58	1725,25	1,72	136,93	2,96	6,71	4,78	43,19	221,15	161,8	7,64
59	1602,13	5,31	20,53	4,63	5,43	4,3	33,61	518,23	165,0	4,55
60	197,04	4,95	15,02	0,64	17,63	3,27	30,02	118,02	103,3	0,89
Hu IL-1b Hu IL-2 Hu IL-4 Hu IL-5 Hu IL-17 Hu G-CSF Hu IFN-g are Out of Range (OOR)										

Table 13 Synovial fluid analysis. Concentration of Cytokines

Measurable levels of cytokines were not detected in all samples. Among the panel of 17 cytokines, Hu IL-1b, Hu IL-2, Hu IL-4, Hu IL-5, Hu IL-17, Hu G-CSF and Hu IFN-g are Out of Range (OOR) and for these reason excluded from the analysis.

Samples are divided into three groups, related to K&L classification,

-group A, from 1 to 20, early OA, grade 1-2

-Group B from 21 to 41, medium OA, grade 3

-Group C from 42 to 60, late OA, grade 4

In order to better analyse the relation between knee joint inflammation and immune modulation and K&L grade, the cytokines levels were compared among the three groups, using the Mann–Whitney tests. Table 14 reports the medians of cytokines levels for each K&L group, , and the Mann-Whitney test' results for the comparisons studied (A vs B; B vs C; A vs C) $P \leq 0.05$ was accepted as statistically significant.

Analytes (pg/ml)	Hu IL-6	Hu IL-7	Hu IL-8	Hu IL-10	Hu IL-12(p70)	Hu IL-13	Hu GM CSF	Hu MCP-1(MCAF)	Hu MIP-1b	Hu TNF-a
Group A	134,6	4,1	19,6	3,9	6,7	1,7	46,3	151,0	155,1	1,7
Group B	119,2	4,0	24,0	3,1	6,4	3,9	37,3	178,6	141,8	1,5
Group C	530,5	4,5	35,3	3,0	12,0	3,3	33,2	130,8	161,9	2,9
MANN WHITNEY (p-value)	**Hu IL-6**	**Hu IL-7**	**Hu IL-8**	**Hu IL-10**	**Hu IL-12(p70)**	**Hu IL-13**	**Hu GM CSF**	**Hu MCP-1(MCAF)**	**Hu MIP-1b**	**Hu TNF-a**
A vs B	0,92	0,94	0,07	0,27	0,98	**0,01**	0,76	0,38	0,47	0,85
B vs C	**0,04**	0,84	0,53	0,31	0,63	0,18	0,79	0,18	0,68	0,57
A vs C	0,13	0,70	**0,01**	**0,03**	0,36	0,17	0,34	0,36	0,29	0,49
A vs BC	0,35	0,79	**0,01**	0,06	0,58	**0,02**	0,47	0,19	0,30	0,59

Table 14 Medians of analytes measured divided in Group A - B - C

Moreover, in order to evaluate differences between early OA and late OA, the Mann-Withney test has been applied between the A group vs the BC group.

In our patients cohort, an unbalanced and enhanced knee joint inflammation can be observed to be related with the grading progression. Indeed, a trend for the increasing of inflammatory molecules such as IL-6, TNF-α, IL-12, IL-8 together to the decreasing of IL-10 levels can be described toward A vs C K&L grading.

Taking together these results justify and sustain the role of inflammation in the disease progression.

Particularly, the significant different comparisons were: the Hu IL-6 values in the B versus C group; the Hu IL-8, also known as chemokine CXXL8, in the A versus C group and A vs BC groups; , the Hu IL-10 in the A versus C group; the Hu IL-13 in the A versus B group and in A vs BC groups.

DISCUSSION

Pathological changes that occur with OA are loss of cartilage, osteophytes, subchondral sclerosis and cysts and deformation of the bone. Soft tissue, such as ligaments, can also be affected. The articulation object of our study is the knee, both because it's the most affected joints and for numerous diagnostic tests available. These features make the knee joint ideal for clinical, radiographic and proteogenomic studies.

Genetic and biochemical analyses have been developed to evaluate disease progress and severity, allowing a non radiographical alternative for an early detection of osteoarthritis or, linked by K&L grading, to better define the pathology.

The aim of our pilot study was to update the knee OA grading with further clinical and genetic associated data, since OA is nowadays considered a polygenic and multifactorial disease (69).

We studied the possible association between KL grade, clinical features (AKSS, age), susceptibility polymorphisms and cytokines expression to OA, in order to better define the grading of this disorder.

In our cohort of 66 patients, a statistical association was observed between the variables analysed: KL data versus: KS, FS, age. In particular, statistical association between KL grade versus KS and FS showed that KL group A can be associated with a medium clinical score, while KL group B and KL group C are related with low KS and FS. This suggests that a mild to severe OA radiographic grade is linked to severe clinical conditions and loss of articular function. In addition, association analysis between KL grade and age of patients confirms that severity of symptoms increases with age: the majority of our patients with KL grade A were 54 to 65 years old, and most of the patients with grade C were 65 to 86 years old.

Concerning the mutational analysis, we genotyped the patients for FRZB (OS1A and OS1B), MATN3 (OS2), PTHR2 (OS3), ASPN

D14 (OS4), GDF5 (OS5) and DVWA (OS6). Results revealed a significant statistical association between KL grade and GDF5 rs143383 (OS5) and DVWA rs11718863 (OS6) genetic alterations.

The OS5 rs143383 polymorphism, localized in the 5′-untranslated region, causes a decrease in the transcriptional activity of GDF5 and is still the most robustly replicated polymorphism associated with OA. This gene encodes growth differentiation factor 5, a bone morphogenic protein involved in the development, homeostasis and repairing of the bone, cartilage and other articular tissues (70). The Sicilian OA patients' odds ratio of 1.53 (confidence interval, 1.11 to 2.11) describes a positive association between rs143383 GDF5 and KL (KL $\leq$2 vs. KL > 2), a trend in line with other studies [48]. Moreover, even if our cohort isn't larger than other, the data are comparable for the variables analysed, supporting this variant as an OA progression marker.

The OS6 rs11718863 polymorphism is localized in an exonic region of the DWVA gene and causes a missense mutation (71). The DWVA protein, interacts with β-tubulin of microtubules and has an important role in the regulation of chondrocyte differentiation, protecting articulate joints from OA onset. In particular, the OS6 rs11718863 SNP induces a decreasing interaction between DVWA and β-tubulin (72-73).

In studied patients cohort, this genetic alteration was more represented in the KL group C (55%) compared with the other groups, KL group A (25%) and KL group B (19%), respectively. Therefore, it is possible to suggest that OS6 can be associated with a more severe OA radiographic grade, displaying its predictive role as OA marker progression.

Concerning the rs11718863 (OS6) and rs7639618 DVWA genetic polymorphisms, alleles frequencies analyzed were different in the Sicilian group with respect to those reported in dbSNP database for European individuals of various geographic areas.

In line with data obtained by other researchers (65), the minor allele frequency (MAF) for these SNPs were much lower in the European samples than in other ones. In addition, for the two above-mentioned genetic alterations, MAF values were greater in the Sicilian individuals than in the European ones: 18% rather than 14.6% and 13%, according to data reported by colleagues and in SNPs database respectively (52).

Moreover, a perfect genotypic correspondence in all individuals investigated (100% of the cases – 161 patients) has been displayed in between rs11718863 and rs7639618 DVWA SNPs. All individuals are double WT or double H or double MUT carriers. Linkage Disequilibrium Analysis was performed in order to verify the degree of allelic segregation among these SNPs. The *r2* test suggests that rs11718863 and rs7639618 DVWA SNPs segregate as haplotype, according to the observation of a coinheritance of SNP alleles.

(r2 = 1) (Figure 14). Considering that rs11718863 DVWA SNP is marked as susceptibility site, and that it is in LD with rs7639618 DVWA, we suggest to assay also this genetic alteration in OA patients in order to define the functional role of DVWA in OA grading and progression.

In addition, even for rs7651842, rs7639807 and rs17040821, sequencing data analysis have displayed a perfect genotypic correspondence (100% of the cases) in all of the 161 individuals investigated. In other words, all individuals were triple WT or triple H or triple MUT carriers. Interestingly, three of the 161 individuals investigated were triple MUT, also taking into consideration that in the literature data these genotypes occur separately at low percentages (rs7651842: 0.9%; rs7639807: 1.7%; rs17040821:1.7%). Therefore, we calculated r^2 LD coefficient, using a pairwise approach which resulted $r^2 = 1$ in every SNP pair analyzed, suggesting that the above-mentioned SNPs segregate as haplotype (Figure 1).

In order to extend the association with KL grade also for the rs7639618 DVWA SNP, we tested the significance of rs7639618 allele dose distribution among the KL OA groups showing a significant association between rs7639618 and KL in our OA patients cohort. Sequencing analysis of the electropherograms revealed the presence of other three less known DVWA SNPs: rs7651842, rs7639807 and rs17040821, for which no literature data were available. Also for these SNPs, MAF values were approximately three fold greater in the Sicilian individuals than in the European ones. Unlike rs11718863 and rs7639618, these polymorphisms are not marked as clinically relevant in dbSNP database probably due to absence of bibliographic data. These three genetic alterations are located in the same exonic region of DVWA gene and for this reason it is possible to hypothesize that they could cause protein functional changes, not still investigated.

Therefore, in this study a high percentage of the 161 Sicilian individuals are carriers of DVWA SNPs mutated alleles. In particular, 29.8% were H or homozygous MUT for rs11718863 (OS6) and rs7639618, whereas, 19.9% were H or homozygous MUT for rs7651842, 7639807 and rs17040821 SNPs.

Finally, as inflammation is increasingly being considered as an important component of OA's pathophysiology, cytokines are being assessed as possible candidates for biochemical markers. Biochemical analyses have been developed to evaluate disease progress and severity, allowing a non radiographical alternative for an early detection of osteoarthritis. (74-76). Grouping appropriate cytokine markers together and assessing them collectively with other markers as well as K&L provide a more statistically powerful tool in research and clinical applications, and additionally aid in distinguishing between early and late OA. Synovial fluid (SF) reflects the biological milieu of the joint and offers a direct measure of joint pathophysiology representing an important potential source

of biomarkers in osteoarthritis (63). Furthermore, the OA joint viscosity is greater than that of inflamed joints.

In our patient cohort, an unbalanced and enhanced knee joint inflammation can be observed to be related with the grading progression. Indeed, a trend for the increasing of inflammatory molecules such as IL-6, TNF-α, IL-12, IL-8 together to the decreasing of IL-10 levels can be described toward A vs C K&L grading.

IL-6 is a glycoprotein consisting of 184 amino acid residues (77) that strongly activates the immune system and enhances inflammatory response and it may be classified as pro-inflammatory cytokine. The increased concentration of IL-6 is present in synovial fluid, with an high level in Group C and is positively correlated with the intensity of lesions in X-ray imaging (78-80).

IL-8, also known as CXXL8, is a potent chemokine in the immune system. Few studies have examined this chemokine in synovial fluid and its relationship with OA. IL-8 is a key mediator associated with inflammation, classified as pro-inflammartory cytokine. IL-8 secretion is increased by oxidant stress, causing the recruitment of inflammatory cells and inducing a further increase in oxidant stress mediators. In this study, IL-8 can be suggested as a good progression marker to better define OA grading (78).

IL-13 has an anti-inflammatory and chondoprotective effects. It is well documented its capacity to transfer the intracellular signal both by the cascade JAK2/STAT3 and IL-13Rα1/TYK2/STAT1/STAT6.

Literature results indicate the potential utility of IL-13 in the treatment of OA, as a compound that inhibits the inflammatory processes, protects chondrocytes, reduces the secretion of inflammatory cytokines and metallo-proteinases, while stimulating the synthesis of IL-1Ra. In agreement with its protective role, in this study, the IL-13 is the only cytokine, having an anti-inflammatory role, which significantly increases passing from the A versus B and C groups of the K&L grading (81).

On the other hand, the major anti-inflammatory cytokine, Interleukin-10 (IL-10), which also have a chondoprotective effect and is also involved in stimulating the synthesis of type II collagen and aggrecan, in our study, it does not seem to increase adequately to the disease progression (82).

However, IL-10 contribute to the suppression of the inflammation of the synovial membrane (83-84). By reducing inflammation, these mediators can support cartilage production acting as anabolic effectors which can slow the progress of OA.

In our study, the IL-10 expression is progressively reduced going from A vs C group. Results of studied cohort show differential expression among A group vs C group, showing also in this cases a possible target to distinguish between early and late OA.

Summarized, nonetheless large scale studies are necessary to asses the effectiveness of these biomarkers, some inflammatory molecules could represent potential prognosis OA biochemical markers .

Conclusion

This work represents a multidisciplinary and translational medicine approach to study OA where clinical, radiographic, genetic and biochemical evaluation could contribute to better define the disease grading and progression for the development of new therapies.

The statistically significant association between clinical, radiographic and genetic signs observed suggests the extension of the actual grading of knee OA based mainly on X-ray features. Moreover, research using a similar multiplex ELISA approach or other proteomic techniques may enable researchers and clinicians to develop more accurate biochemical profiles of synovial fluid to help diagnose OA, identify subsets of OA or individual characteristics, guide clinical decisions, and identify patients at risk for OA after knee injury. (Figure 15)

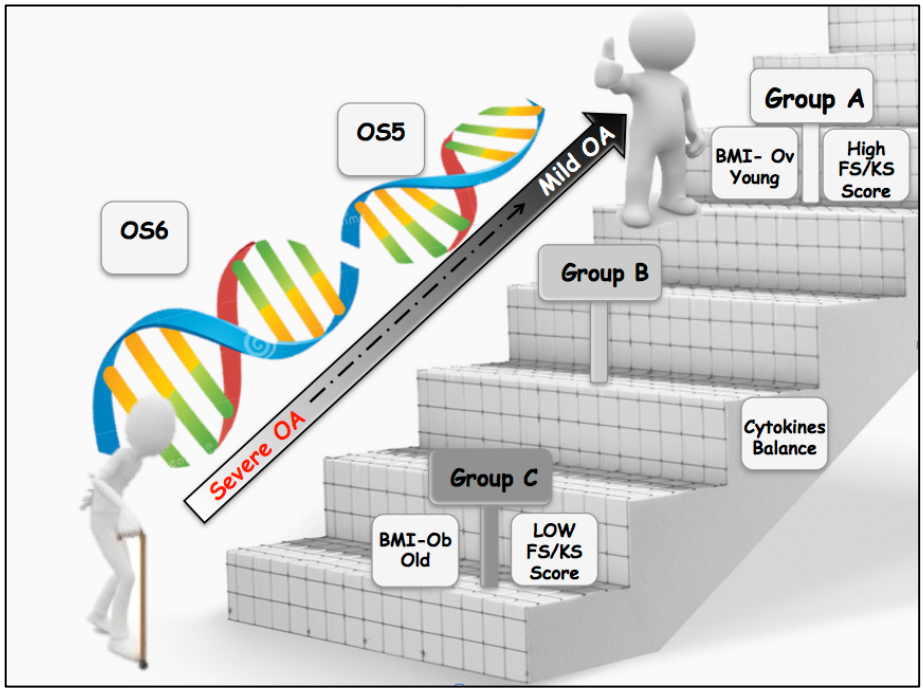

Figure 15 Model of relation between KL grading groups and selected features described in the study.

Bibliography

1. Goldring MB, Goldring SR. Osteoarthritis. J Cell Physiol. 2007;213:626–34.
2. G. Blumenkrantz, C. T. Lindsey, T. C. Dunn, H. Jin, M. D. Ries, et all. A pilot, two-year longitudinal study of the interrelationship between trabecular bone and articular cartilage in the osteoarthritic knee. Osteoarthritis and Cartilage, 2004; 12:997–1005.
3. Lane NE, Brandt K, Hawker G, Peeva E, Schreyer E, et all. OARSI-FDA initiative: defining the disease state of osteoarthritis. Osteoarthritis Cartilage 2011, 19:478–482.
4. Pereira D, Peleteiro B, Araújo J, Branco J, Santos RA, Ramos E: The effect of osteoarthritis definition on prevalence and incidence estimates: a systematic review. Osteoarthritis Cartilage 2011, 19:1270–1285.
5. Srikanth VK, Fryer JL, Zhai G, Winzenberg TM, Hosmer D, Jones G: A metaanalysis
   of sex differences prevalence, incidence and severity of osteoarthritis. Osteoarthritis Cartilage 2005, 13:769–781.
6. Oliveria SA, Felson DT, Reed JI, Cirillo PA, Walker AM: Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization. Arthritis Rheum 1995, 38:1134–1141.
7. Belo JN, Berger MY, Reijman M, Koes BW, Bierma-Zeinstra SM: Prognostic factors of progression of osteoarthritis of the knee: a systematic review of observational studies. Arthritis Rheum 2007, 57:13–26.
8. D. T. Felson, R. C. Lawrence, P. A. Dieppe, R. Hirsch, C. G. Helmick, et all. Osteoarthritis: New insights. part 1: the disease and its risk factors. Annals of Internal Medicine, 133(8):635 – 646,2000.
9. Lopez AD Murray CJL. The global burden of disease. In World Health Organization, 1997.
10. Heine J. Arthritis deformans. Virchow's Arch Path Anat 1926; 260:52 1-663.

11. Lawrence RC, Felson DT, Helmick CG, Arnold LM, Choi H, Deyo RA, et al. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. Arthritis Rheum. 2008;58:26–35
12. 4. Altman R, Asch E, Bloch D, Bole D, Borenstein K, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Arthritis Rheum. 1986;29:1039–49.
13. Anna Litwic, MD, Mark Edwards, MRCP, Elaine Dennison, PhD and Cyrus Cooper. Epidemiology and Burden of Osteoarthritis. Br Med Bull. 2013; 105: 185–199.
14. Altman R, Alarcon G, Appelrouth D, Bloch D, Borenstein K, Brandt K, et al. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hand. Arthritis Rheum. 1990;33:1601–10
15. Van Saase JL, van Romunde LK, Cats A et al. Epidemiology of osteoarthritis: Zoetermeer survey.Comparison of radiological osteoarthritis in a Dutch population with that in 10 other populations Annals of the Rheumatic Diseases 1989; 48(4): 271–280.
16. Felson DT, Naimark A, Anderson J, Kazis L, Castelli W, Meenan RF. The prevalence of knee osteoarthritis in the elderly. The Framingham Osteoarthritis Study. Arthritis Rheum. 1987; 30:914-8.
17. Hochberg M C. Osteoarthritis year 2012 in review: clinical. Osteoarthritis and Cartilage. 2012
18. Lawrence JS, Sebo M. The geography of osteoarthritis. In: Nuki G, ed. The Aetiopathogenesis of Osteoarthritis. Kent, UK: Pitman Medical; 1980:155-83
19. Poole A, Guilak F, Abramson S. Etiopathogenesis of Osteoarthritis in Osteoarthritis: Diagnosis and Medical/Surgical Management di Moskowitz R, Altman R, Hochberg M, Buckwalter J, Goldber V. Lippincott Williams & Wilkins 2007
20. Felson DT: Weight and osteoarthritis. J.Rheumatol. 1995;43:7-9.

21. Anderson J, Felson DT: Factors associated with osteoarthritis of the knee in the First National Health and Nutrition Examination (HANES I). Am.J.Epidemiol. 1988;128:179-189.
22. Felson DT, Anderson JJ, Naimark A, Walker AM, Meenan RF: Obesity and knee osteoarthritis: The Framingham study. Ann.Int.Med. 1988;109:18-24.
23. Felson DT, Chaisson CE: Understanding the relationship between body weight and osteoarthritis. Baillieres Clinical Rheumatology 1997;11:671-681.
24. Schouten JS, van den Ouweland FA, Valkenburg HA: A 12 year follow up study in the general population on prognostic factors of cartilage loss in osteoarthritis of the knee. Ann.Rheum.Dis. 1992;51:932-937.
25. Spactor TD, Cicuttini J, Baker J, Loughlin J, Hart D. Genetic influences on osteoarthritis in women: a twin study. British Medica Journal 1996. 13;312(7036):940-3.
26. Valdes AM, Spector TD. Genetic epidemiology of hip and knee osteoarthritis. Nat Rev Rheumatol. 2011; 7(1):23-32.
27. Buckwalter JA, Mankin HJ. Articular cartilage. Part I: Tissue design and chondrocyte matrix interactions. J Bone Joint Surg 1997; 79-A: 600–11.
28. Loughlin J. Osteoarthritis year 2010 in review: genetics. Osteoarthritis Cartilage. 2011; 19(4):342-5
29. Michael J, Schluter-Burst K, Eysel P. The epidemiology, Etiology, Diagnosis and Treatment of Ostearthritis of the knee. Deutsches Arztebl Int 2010; 107(9): 152-62.
30. Hooper M and Moskowitz R. Osteoarthritis: Clinical Presentations in Osteoarthritis: Diagnosis and Medical/Surgical Management di Moskowitz R, Altman R, Hochberg M, Buckwalter J, Goldber V. Lippincott Williams & Wilkins 2007
31. Buckland-Wright C. Which radiographic techniques should we use for research and clinical practice? Best Pract Res Clin Rheumatol. 2006;20:39-55.
32. Braun HJ, Gold GE: Diagnosis of osteoarthritis: imaging. Bone 2012,

51:278–288.

33. Bijlsma JW, Berenbaum F, Lafeber FP: Osteoarthritis: an update with relevance for clinical practice. Lancet 2011, 377:2115–2126.
34. Busija L, Bridgett L, Williams SR, Osborne RH, Buchbinder R, March L, Fransen M: Osteoarthritis. Best Pract Res Clin Rheumatol 2010, 24:757–768.
35. Kellgren JH, Lawrence JS: Radiological assessment of osteoathrosis. Ann Rheum Dis 1957, 16:494–502.
36. Reijman M, Hazes JM, Pols HA, Bernsen RM, Koes BW, Bierma-Zeinstra SM: Validity and reliability of three definitions of hip osteoarthritis: cross sectional and longitudinal approach. Ann Rheum Dis 2004, 63:1427–1433.
37. Ju Hee Ryu, Aeju Lee, Myung Sook Huh, Junuk Chu, Kwangmeyung Kim, et al. Measurement of MMP Activity in Synovial Fluid in Cases of Osteoarthritis and Acute Inflammatory Conditions of the Knee Joints Using a Fluorogenic Peptide Probe-Immobilized Diagnostic Kit. Theranostics 2012; 2(2):198-206.
38. Van Meurs JB, Uitterlinden AG: Osteoarthritis year 2012 in review: genetics and genomics. Osteoarthritis Cartilage 2012, 20:1470–1476.
39. Loughlin J: Genome studies and linkage in primary osteoarthritis. Rheum Dis Clin N Am 2002, 28:95–109.
40. Van der Kraan PM: Osteoarthritis year 2012 in review: biology. Osteoarthritis Cartilage 2012, 20:1447–1450.
41. Karlsson C, Dehne T, Lindahl A, Brittberg M, Pruss A, Sittinger M, Ringe J: Genome-wide expression profiling reveals new candidate genes associated with osteoarthritis. Osteoarthr Cartil 2010, 18:581–592.
42. Loughlin J: Genetics of osteoarthritis. Curr Opin Rheumatol 2011, 23:479–483.
43. Rodriguez-Fontenla C, López-Golán Y, Calaza M, Pombo-Suarez M, Gómez- Reino JJ, González A: Genetic risk load and age at symptom onset of knee osteoarthritis. J Orthop Res 2012, 30:905–909
44. Min JL, Meulenbelt I, Riyazi N, Kloppenburg M, Houwing-Duistermaat JJ, et al. Association of the frizzled-related protein gene

with symptomatic osteoarthritis at multiple sites. Arthritis Rheum 2005, 52:1077–1080.

45. Valdes AM, Loughlin J, Oene MV, Chapman K, Surdulescu GL, Doherty M, Spector TD: Sex and ethnic differences in the association of ASPN, CALM1, COL2A1, COMP, and FRZB with genetic susceptibility to osteoarthritis of the knee. Arthritis Rheum 2007, 56:137–146.
46. Stefánsson SE, Jónsson H, Ingvarsson T, Manolescu I, Jónsson HH, et al. Genomewide scan for hand osteoarthritis: a novel mutation in matrilin-3. Am J Hum Genet 2003, 72:1448–1459.
47. Kizawa H, Kou I, Iida A, Sudo A, Miyamoto Y, et al. An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis. Nat Genet 2005, 37:138–144.
48. Evangelou E, Chapman K, Meulenbelt I, Karassa FB, Loughlin J, et al. Large-scale analysis of association between GDF5 and FRZB variants and osteoarthritis of the hip, knee, and hand. Arthritis Rheum 2009, 60:1710–1721.
49. Min JL, Meulenbelt I, Kloppenburg M, van Duijn CM, Slagboom PE. Mutation analysis of candidate genes within the 2q33.3 linkage area for familial early-onset generalised osteoarthritis. Eur J Hum Genet 2007, 15:791–799.
50. Southam L, Rodriguez-Lopez J, Wilkins JM, Pombo-Suarez M, Sarah S, et al. An SNP in the 5′-UTR of GDF5 is associated with osteoarthritis susceptibility in Europeans and with in vivo differences in allelic expression in articular cartilage. Hum Mol Genet 2007, 16:2226–2232.
51. Chapman K, Takahashi A, Meulenbelt I, Watson C, Rodriguez-Lopez J, et al. A meta-analysis of European and Asian cohorts reveals a global role of a functional SNP in the 5′ UTR of GDF5 with osteoarthritis susceptibility. Hum Mol Genet 2008, 17:1497–1504.
52. Meulenbelt I, Chapman K, Dieguez-Gonzalez R, Shi D, Tsezou A, et al. Large replication study and meta-analyses of DVWA as an osteoarthritis susceptibility locus in European and Asian populations. Hum Mol Genet 2009, 18:1518–1523.

53. Michael JW1, Schlüter-Brust KU, Eysel P. The epidemiology, etiology, diagnosis, and treatment of osteoarthritis of the knee. Dtsch Arztebl Int. 2010 Mar;107(9):152-62.
54. MohingW: Die Arthrosis deformans des Kniegelenkes. Springer-Verlag Berlin 1966.
55. Pendelton AN, Arden N, Dougados M, et al.: EULAR recommendations for the management of knee osteoarthritis: report of a task force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCICIT). Ann Rheum Dis Dec 2000; 59: 936–44.
56. Forte GI, Piccione M, Scola L, Crivello A, Galfano C, et al. Apolipoprotein E genotypic frequencies among down syndrome patients imply early unsuccessful aging for ApoE4 carriers. Rejuvenation Res 2007, 10:293–299.
57. Wilcken B, Bamforth F, Li Z, Zhu H, Ritvanen A, et al. Geographical and ethnic variation of the 677C > T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas worldwide. J Med Genet 2003, 40:619–625.
58. Bravatà V, Stefano A, Cammarata FP, Minafra L, Russo G, Nicolosi S, Pulizzi S, Gelfi C, Gilardi MC, Messa C: Genotyping analysis and 18FDG uptake in breast cancer patients: a preliminary research. J Exp Clin Cancer Res 2013, 32:23.
59. Reijman M, Hazes JM, Pols HA, Bernsen RM, Koes BW, Bierma-Zeinstra SM: Validity and reliability of three definitions of hip osteoarthritis: cross sectional and longitudinal approach. Ann Rheum Dis 2004, 63:1427–1433.
60. Human Gene Mutation Database. [http://www.hgmd.cf.ac.uk]
61. dbSNP Short Genetic Variations database. [http://www.ncbi.nlm.nih.gov/snp]
62. Primer3. [http://fokker.wi.mit.edu/primer3]
63. Jayadev C1, Rout R, Price A, Hulley P, Mahoney D Hyaluronidase treatment of synovial fluid to improve assay precision for biomarker research using multiplex immunoassay platforms. C J Immunol

Methods. 2012 Dec 14;386(1-2):22-30.
64. GraphPad InStat software version 3.05. [http://www.graphpad.com]
65. Valdes AM, Spector TD, Doherty S, Wheeler M, Hart DJ, Doherty M. Association of the DVWA and GDF5 polymorphisms with osteoarthritis in UK populations. Ann Rheum Dis 2009, 68:1916–1920.
66. The International HapMap Consortium. The International HapMap Project. Nature. 2003;426:789–96.
67. The International HapMap Consortium. A haplotype map of the human genome. Nature. 2005;437:1299–320.
68. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005;21:263–5.
69. Reynard LN, Loughlin J: The genetics and functional analysis of primary osteoarthritis susceptibility. Expert Rev Mol Med 2013, 15:e2.
70. Miyamoto Y, Mabuchi A, Shi D, Kubo T, Takatori Y, et al. A functional polymorphismin the 5′-UTR of GDF5 isassociated with susceptibility to osteoarthritis. Nat Genet 2007, 39:529–533.
71. Miyamoto Y, Shi D, Nakajima M, Ozaki K, Sudo A, et al. Common variants in DVWA on chromosome 3p24.3 are associated with susceptibility to knee osteoarthritis. Nat Genet 2008, 40:994–998.
72. Nakajima M, Miyamoto Y, Ikegawa S: Cloning and characterization of the osteoarthritis-associated gene DVWA. J Bone Miner Metab 2011, 29:300–308.
73. Wagener R, Gara SK, Kobbe B, Paulsson M, Zaucke F: The knee osteoarthritis susceptibility locus DVWA on chromosome 3p24.3 is the 5′ part of the split COL6A4 gene. Matrix Biol 2009, 28:307–310.
74. Han MY, Dai JJ, Zhang Y, et al. Identification of osteoarthritis biomarkers by proteomic analysis of synovial fluid. J Int Med Res 2012; 40: 2243–2250. 5.
75. Honsawek S, Yuktanandana P, Tanavalee A, et al. Correlation between plasma and synovial fluid basic fibroblast growth factor with radiographic severity in primary knee osteoarthritis. Int Orthop 2012;

36: 981–985.

76. Ning L, Ishijima M, Kaneko H, et al. Correlations between both the expression levels of inflammatory mediators and growth factor in medial perimeniscal synovial tissue and the severity of medial knee osteoarthritis. Int Orthop 2011; 35: 831–838.
77. A. Hammacher, L. D. Ward, J. Weinstock, H. Treutlein, K. Yasukawa, and R. J. Simpson, "Structure-function analysis of human IL-6: identification of two distinct regions that are important for receptor binding," Protein Science, vol. 3, no. 12, pp. 2280–2293, 1994.
78. S. Kaneko, T. Satoh, J. Chiba, C. Ju, K. Inoue, and J. Kagawa, "Interleukin-6 and interleukin-8 levels in serum and synovial
79. O. Stannus,G. Jones, F. Cicuttini et al., "Circulating levels of IL-6 and TNF-α are associated with knee radiographic osteoarthritis and knee cartilage loss in older adults," Osteoarthritis and Cartilage, vol. 18, no. 11, pp. 1441–1447, 2010.
80. G. Livshits, G. Zhai, D. J. Hart et al., "Interleukin-6 is a significant predictor of radiographic knee osteoarthritis: the Chingford Study," Arthritis and Rheumatism, vol. 60, no. 7, pp. 2037–2045, 2009.
81. F. Iannone, C. De Bari, F. Dell' Accio et al., "Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes," Clinical and Experimental Rheumatology, vol. 19, no. 2, pp. 139–145, 2001.
82. D. Umulis, M. B. O'Connor, and S. S. Blair, "The extracellular regulation of bone morphogenetic protein signaling," Development, vol. 136, no. 22, pp. 3715–3728, 2009.
83. Ritchlin C, Haas-Smith SA. Expression of interleukin 10 mRNA and protein by synovial fibroblastoid cells. J Rheumatol 2001; 28: 698-705.
84. Miossec P, Briolay J, Dechanet J, Wijdenes J, Martinez-Valdez H, Banchereau J. Inhibition of the production of proinflammatory cytokines and immunoglobulins by interleukin-4 in an ex vivo model of rheumatoid synovitis. Arthritis Rheum 1992; 35: 874-883 [PMID: 1642654].