UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIA

THE ROLE OF VITAMIN K IN OSTEOARTHRITIS

Carla Margarida da Silva Pereira

Dissertação para obtenção do Grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob a orientação da Professora Doutora Dina Simes

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

_____

(Carla Margarida da Silva Pereira)

"O conhecimento torna a alma jovem e diminui a amargura da velhice. Colhe, pois, a sabedoria. Armazena suavidade para o amanhã."

(Leonardo da Vinci)

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Abbreviations

- ACR - American College of Rheumatology
- ADAMTS - A Disintegrin And Metalloproteinase with Thrombospondin motifs
- AGEs - Advanced Glycation End-products
- ANK - Ankylosis
- AP - Alkaline Phosphatase
- ASU - Avocado Soybean Unsaponifiables
- BCP - Basic Calcium Phosphate
- BMI - Body Mass Index
- BM - Bone Mass
- BMLs - Bone Marrow Lesions
- BMPs - Bone Morphogenetic Proteins
- BSP - Bone Sialoprotein
- Ca - Calcium
- CM - Chylomicrons
- COMP - Cartilage Oligomeric Matrix Protein
- COX - Cyclooxygenase
- CPPD - Calcium Pyrophosphate Dehydrated
- CR - Chylomicrons Remnants
- CT - Computed Tomography
- DMDs - Disease Modifying Drugs
- DMOAD - Disease Modifying Osteoarthritic Drug
- DRIs - Dietary Reference Intakes
- ECM - Extracellular Matrix
- EIA - Enzyme Immunoassay
- ELISAs - Enzyme Linked Immunosorbent Assays
- EMA - European Medicines Agency
- EULAR - European League Against the Rheumatism
- F IX - Factor IX
- F VII - Factor VII
- F X - Factor X
- FDA - Food and Drug Administration
- FGFs - Fibroblast Growth Factors
- GGCX - Gamma Glutamyl Carboxylase
- Gla - Gamma-carboxyglutamate

- Glu – Glutamate
- GRP - Gla-Rich Protein
- HDL - High Density Lipoprotein
- IGF-1 - Insulin-like Growth Factor 1
- IUPAC - International Union of Pure and Applied Chemistry
- HA - Hyaluronic Acid
- HIF - Hypoxia Inducible Factor
- HPLC - High Performance Liquid Chromatography
- Ihh - Indian hedgehog
- ILs – Interleukins
- JSN - Joint Space Narrowing
- JSW - Joint Space With
- KO - Vitamin K 2,3-epoxide
- LC-MS - Liquid Chromatography-Mass Spectrometry
- MAPK - Mitogen Activated Protein Kinases
- MGP - Matrix Gla Protein
- MMPs - Matrix Metalloproteinases
- MRI - Magnetic Resonance Imaging
- MVs - Matrix Vesicles
- NF-kB - Nuclear Factor Kappa light chain enhancer of activated B cells
- NIH - National Institutes of Health
- NLRP - Nucleotide-binding oligomerization domain Like Receptors
- NO - Nitric Oxide
- NPP1 - Nucleotide Pyrophosphatase Phosphodiesterase 1
- NSAID - Non Steroidal Anti-Inflammatory Drugs
- OA - Osteoarthritis
- OARSI - Osteoarthritis Research Society International
- OC - Osteocalcin
- ON - Osteonectin
- OP – Osteoporosis
- OPN – Osteopontin
- PC - Protein C
- PGE2 - Prostaglandin E2
- Pi - Inorganic Phosphate

- PPi - Inorganic Pyrophosphate
- PRGP1 - Proline-Rich Gla Proteins-1
- PRGP2 - Proline-Rich Gla Proteins-2
- PS - Protein S
- PTHrP - Parathyroid Hormone-related Peptide
- PZ - Protein Z
- RA - Rheumatoid Arthritis
- RANKL - Receptor Activator of Nuclear factor κB Ligand
- RIA - Radioimmunoassay
- ROS - Reactive Oxygen Species
- Runx2 - Runt-related transcription factor 2
- SBCs - Subchondral Bone Cysts
- SNRI - Serotonin and Norepinephrine Reuptake Inhibitor
- Sox9 - SRY-type high-mobility-group box transcription factor-9
- TG - Triglyceride
- TGF-β - Transforming Growth Factor Beta
- TIMPs - Tissue Inhibitors of Metalloproteinases
- TMG3 - Transmembrane Gla Proteins-3
- TMG4 - Transmembrane Gla Proteins-4
- TNAP - Tissue Nonspecific Alkaline Phosphatase
- TNF-α - Tumor Necrosis Factor Alpha
- tPA - tissue Plaminogen Activator
- TLRs - Toll-Like Receptors
- UNO - United Nations Organization
- US - Ultrasound
- uPA - urokinase Plaminogen Activator
- VEGF - Vascular Endothelium Growth Factor
- VKAs - Vitamin K Antagonists
- VKDP - Vitamin K Dependent Proteins
- VKOR - Vitamin K epoxide Reductase
- VKR - Vitamin K Reductase
- VSMCs - Vascular Smooth Muscle Cells
- Wnt - Wingless Type

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Abstract

Osteoarthritis (OA) is the most frequent chronic rheumatic disease, affecting approximately 15% of the population, with a higher prevalence among the elderly; occurring in synovial joints such as the hips, knees and the ankle.

This condition develops when the joint organ homeostasis is affected, causing abnormal remodeling of the articular tissues, leading to degradation of the cartilage, thickening of the subchondral bone, formation of osteophytes and variable degrees of inflammation.

The burden of OA clinically characterized by chronic pain and significant disability is high, and only few nonpharmacologic and pharmacologic treatment options are available, mostly focused on providing symptomatic relief and showing limited efficacy and several side effects. The research on this disease in need for novel therapeutic alternatives has increased and lately is becoming fully recognized that joint's calcification and the crosstalk with inflammation should be considered as an OA therapeutic target. In this context, vitamin K has been recognized as playing multifunctional roles that may modulate the pathogenesis of the disease.

Vitamin K acts as an essential coenzyme in the post-translational modification of specific glutamic acid residues (Glu) into γ-carboxyglutamic acid residues (Gla) in target proteins, known as vitamin K-dependent proteins (VKDPs), to make them biologically active.

Mineral-related Gla proteins, have been proposed as regulators of cell differentiation and inhibitors of mineralization in articular systems, so impairment in their γ-carboxylation status should have an impact in joint's health, showing a plausible rationale for the connection of vitamin K through the OA stages.

This old vitamin is now presented in a new perspective, with emerged value in human's health, crucial in the prevention of pathological calcification and an important protective tool against inflammation and oxidative stress; revealing a promising potential as a prophylactic and therapeutic agent in OA.

Keywords: Osteoarthritis; Vitamin K; Gla Proteins; Pathological Calcification; Inflammation.

Resumo

A osteoartrite (OA) é considerada a doença reumática crónica mais frequente, atingindo cerca de 15% da população e afetando pessoas de todas as idades, embora com uma maior predominância entre os idosos. A maioria dos indivíduos com idade superior a 65 anos apresenta evidências radiográficas e/ou clínicas desta patologia.

Esta doença representa um problema de saúde pública crescente, estimando-se que em 2020 seja a quarta principal causa de incapacidade motora. Apesar de não ser uma consequência inevitável do envelhecimento, mantém uma relação estreita e direta com a idade, em termos de incidência e prevalência. Assim, num futuro próximo, um aumento na esperança de vida conduzirá provavelmente a um crescimento do seu número de casos.

Estudos epidemiológicos mostram que esta é uma doença multifactorial muito complexa, dependente da associação de fatores genéticos e epigenéticos, sexo, etnia e idade, estando também associada com outras comorbidades, sedentarismo, lesões desportivas e fatores nutricionais.

Apresenta uma maior incidência de desenvolvimento em articulações que suportam o peso, tais como os joelhos, as ancas e os tornozelos, embora possa surgir em qualquer articulação sinovial, individualmente ou em simultâneo e com variáveis níveis de severidade.

A nível fisiológico, esta condição desenvolve-se quando a homeostasia da articulação é afetada, causando uma remodelação anormal dos tecidos articulares. As principais alterações patológicas comumente observadas em articulações atingidas por OA incluem a degradação irreversível da cartilagem articular, o espessamento do osso subcondral, formação de osteófitos, graus variáveis de inflamação, nomeadamente a nível da membrana sinovial e a presença de focos de mineralização ao nível da matriz extra celular.

O fardo da OA é elevado, sendo uma patologia caracterizada clinicamente por dor crónica, redução gradual do espaço interarticular e da mobilidade articular, podendo evoluir até causar uma incapacidade significativa. As opções de tratamento não farmacológico e farmacológico atualmente disponíveis são poucas e principalmente focadas em fornecer alívio sintomático, revelando uma eficácia limitada e apresentando vários efeitos colaterais.

A pesquisa sobre esta doença, com necessidade de novas terapias alternativas tem aumentado e, ultimamente, já se reconhece que a interrelação entre a calcificação patológica da articulação e a inflamação deve ser considerada um alvo terapêutico na OA. Por outro lado, o conhecimento sobre mecanismos moleculares da OA é ainda muito limitado e o seu

diagnóstico tardio. Neste contexto e dada a falta de medicamentos eficazes para o tratamento da doença, é essencial a descoberta de novos alvos moleculares e biomarcadores que beneficiem a patologia. Neste âmbito, a vitamina K tem sido reconhecida como um alvo muito interessante a explorar pelo seu papel multifuncional que pode ser modulador da patogénese da doença e contribuir para o seu tratamento e prevenção.

A vitamina K corresponde a uma família que inclui uma série de compostos essenciais e lipossolúveis. O termo vitamina K é usado de forma genérica para os compostos estruturalmente relacionados, que possuem um núcleo 2-metil-1,4-naftoquinona comum, mas diferem na composição de uma cadeia lateral na posição 3. As três diferentes formas de vitaminas K são classificadas de acordo com a estrutura química dessa cadeia lateral: K_1-Filoquinona, K_2-Menaquinonas e K_3- Menadiona.

A fonte dietética predominante de vitamina K é a filoquinona, obtida a partir de plantas, sendo as suas maiores concentrações encontradas em vegetais de folhas verdes, óleos vegetais, frutas e grãos. Relativamente às menaquinonas, para além da sua presença no fígado de animais, podem ser também encontradas em alimentos fermentados por bactérias, que nas dietas ocidentais são tipicamente representados por queijos e no Japão pelo natto, que é um alimento feito de soja fermentada.

O papel metabólico da vitamina K é atuar como cofator numa reação de carboxilação específica que transforma determinados resíduos de glutamato (Glu) em gamacarboxiglutamato (Gla), tendo um papel essencial para a função de proteínas dependentes da vitamina K (VKDP). Este processo de carboxilação ocorre no retículo endoplasmático e é catalisado pela enzima gamaglutamil carboxilase (GGCX), utilizando como cofator ativo, a forma reduzida de vitamina K (KH_2). Em simultâneo com a conversão Glu, KH_2 é oxidado a vitamina K 2,3-epóxido (KO), numa reação catalisada pela enzima redutase VKOR e convertido de volta para KH_2, num processo de reciclagem que é crucial para a função da vitamina K e para manutenção de níveis adequados de biodisponibilidade desta vitamina no organismo. Esta capacidade de reciclagem pode explicar a necessidade diária muito baixa de vitamina K, quando comparado com outras vitaminas e cofatores.

Este processo de gamacarboxilação resulta na formação de proteínas Gla, biologicamente ativas, capazes de ligar o cálcio e minerais de fosfato de cálcio. No entanto, na ausência de vitamina K, ou da presença de inibidores de VKOR (fármacos anticoagulantes, como por exemplo, 4-hidroxicumarinas: varfarina), a carboxilação das proteínas VKD é

incompleta e as proteínas são secretadas em várias formas menos funcionais, em todos os tecidos.

Durante as últimas décadas, a vitamina K inicialmente descoberta e associada a uma função hemostática, considerada como necessária para a correta função dos fatores de coagulação do sangue produzidos no fígado, para uma vitamina mais abrangente em termos funcionais, com a descoberta de outras VKDPs extra-hepáticas e associadas á calcificação como a osteocalcina (OC), a proteína Gla da matriz (MGP) e a proteína rica em Glas (GRP); Estas proteínas são caracterizadas por uma distribuição tecidual muito generalizada e um amplo impacto fisiológico com um papel em vários processos biológicos e fisiológicos, ao nível do osso, tecido vascular, pele e cartilagem.

Este grupo de VKDPs extra-hepáticas associadas á calcificação foi descrito como tendo um papel crucial na saúde, principalmente devido à sua função na regulação do cálcio e deposição e mineral nomeadamente patológicos de calcificação em tecidos moles.

Na última década, vários investigadores desenvolveram um conjunto de estudos genéticos e farmacológicos para adquirir mais informação sobre o papel das VKDPs extra-hepáticas, OC, MGP e GRP no processo de calcificação. Na verdade, OC e MGP são descritas como estando implicadas na regulação da calcificação endocondral. A MGP é considerada um poderoso inibidor da calcificação vascular. Mais recentemente, foi descrita uma associação direta das formas não funcionais da MGP e GRP á doença osteoartrítica.

A população em geral apresenta níveis de carboxilação baixos (10-40%) das proteínas VKDPs extra-hepáticas, pelo que, a atividade biológica destas proteínas em circulação pode ser considerada insuficiente. Esta baixa funcionalidade das VKDPs, não é essencial para a sobrevivência a curto prazo, o que sugere que a biodisponibilidade de vitamina K nos tecidos extra-hepáticos seja inferior ao necessário para assegurar a correta gamacarboxilação das proteínas. Em consequência da restrição da vitamina K, aumenta a vulnerabilidade a doenças associadas com o envelhecimento, com implicações importantes no osso e cartilagem, como é o caso da OA.

Uma vez que a calcificação e inflamação são processos comuns e bastante interligados em OA, a importância da vitamina K através da ação de OC, MGP e da GRP, abre novas perspetivas sobre o potencial da vitamina K como novo alvo terapêutico da OA.

Com o conhecimento emergente do seu valor na modulação da patogénese da OA esta vitamina é agora apresentada com uma nova perspetiva. A gama de ação da vitamina K provou ser crucial na prevenção da calcificação patológica, nomeadamente a nível vascular e

da pele, assim como ser essencial na proteção contra a inflamação e o stress oxidativo. Globalmente a informação disponível permite concluir que esta vitamina é uma promissora candidata a ser um potencial agente profilático e terapêutico na OA.

Palavras-chave: Osteoartrite; Vitamina K; Proteínas Gla; Calcificação Patológica; Inflamação.

1. Introduction

In our society, musculoskeletal conditions pose a huge burden on individuals, health systems and social care organisms; accounting for a significant economic impact. They represent the major cause of severe long-term pain and physical disability, affecting hundreds of millions of people in all continents, leading to more functional limitations in the adult population and reduction of the quality of life than any other series of disorders [1-3].

These conditions comprise a wide diversity in terms of pathophysiology, but share common anatomic features and are associated with pain and impaired physical function. They include a wide spectrum of situations, from acute onset and short duration, to lifelong disorders that include osteoarthritis (OA), rheumatoid arthritis (RA) and osteoporosis (OP) [1].

OA is the most frequent form of arthritis, affecting all the populations and ethnic groups investigated thus far [3-6]. It should not be thought of as a single disease, but rather as the pathologic endpoint of a variety of events that conspire to incite a cascade of events within the whole joint, that perpetuate its progressive degeneration and eventual failure [7, 8].

The OA disease occurs when the dynamic steady state between destructive forces and repair mechanisms destabilises the joint organ homeostasis. This imbalance is thought to be the driving force for this irreversible and debilitating condition, which slowly progresses in a cyclic interaction between systemic and local factors. Over the years, this interplay between biological, structural and mechanical changes, compromises the articular cartilage and causes disturbances in the underlying bone, soft tissues of the joint and surrounding muscles [4-6, 9-14].

Commonly, OA develops in weight bearing joints such as the hips, knees and the ankle, but it can occur in any synovial joint of the body, individually or simultaneously, with variable degree intensities [9]. The disease is clinically characterized by joint pain, tenderness, crepitus, stiffness and limitation of movement with occasional effusion and unpredictable levels of local inflammation [13, 14].

From prehistoric times to the present day, OA has proven to be a challenging disease, found in nearly every period and civilization. The disease history is rich and ancient, since it can be traced back in time, with historical depictions and paleopathological findings in Neanderthal and Cro-Magnon skeletal remains and Egyptian mummies. The pathological changes observed in a 100 million year old bone and in a contemporary bone are remarkably similar, suggesting that OA has not changed much throughout evolution [15].

Despite the nearly ubiquitous presence of the pathology, OA was not recognized until the late 18th century, possibly due to its few obvious clinical signs [15].

Further a nomenclature confusion delayed its identification, because OA and RA were for some time considered to be the same entity, known as arthritis deformans. Only in 1859, Sir Alfred Baring Garrod proposed a clinical distinction between these two diseases [15].

Historically the disease knowledge development and treatment innovation in OA has been considered slow, dependent upon the leisurely evolution of the understanding of the elaborate nature of joint tissue biology. OA is an extraordinarily intricate pathology with marked heterogeneity in onset, clinical presentation, rate of disease progression, pattern of joint involvement and synovial tissue structures affected [5].

Nowadays, OA is considered the most frequent chronic rheumatic disease. Several data indicate that affects people of all ages, with higher predominance among the elderly. A majority of individuals over the age of 65 have radiographic and/or clinical evidences of that condition [4, 5, 16, 17]. Although not an inevitable consequence of aging, OA maintains a close relationship with age, its incidence and prevalence grows with it, thus a longer life expectancy will lead to a possible increase in the number of cases in a near future [1, 4, 17-20].

In recent decades, there was a clear process of aging and the period between 1975 and 2025 was considered by the United Nations Organization (UNO) as "The Era of the Aging". In developing countries, this population aging is even more significant and accelerated. Due to the demographic changes OA is a growing public health problem across the globe, estimated to be the fourth leading cause of disability by the year 2020 [1-5, 20]. A rather worrying situation, aggravated by the fact that this is a condition with still no known disease modifying drugs (DMDs) available for treatment.

Some epidemiological studies tend to present different prevalence numbers for OA, according to geographical regions. Possible explanations for these differences range from genetic, socio-economic conditions, health-care access, environmental factors or different lifestyles. Indicating that predisposition and susceptibility, for this old disease that affects humankind, depends on the association of various risk factors [4, 22, 23].

Thanks to the increasing research and the use of molecular biology and proteomic tools, OA is being defined as a very complex, multifactorial disease. Although the disease can be dependent on genetic and epigenetic factors, sex, ethnicity and age, it is also associated with obesity, sedentary lifestyle, sport injuries and dietary factors [1, 2, 4, 19, 20, 24].

The requirements of this society and increased costs of living, force more and more people to acquire wrong routines, reducing the time that they used to dedicate to themselves; as a consequence, people devote less time to sports activity and choose fast food instead of healthy meals [4].

Our body is in an extremely controlled balance, where every little change may be responsible for several alterations. An equilibrated diet, including all required nutritional factors and moderate physical activity are necessary to maintain the body equilibrium and contribute to avoid pathological modifications in the joints [4].

Several studies have shown that insufficient intake of vitamin K, over long periods of time, is a risk factor for development of a wide range of diseases, including OA, OP, vascular calcification and cardiovascular disease, and even some types of cancer [18, 25-29]. Some clinical studies have demonstrated that the nutritional intake and concentration of vitamin K in circulation decreases with age [30, 31]. This suggests that the increase in vitamin K consumption can be made, either by using fortified foods or food supplements and might therefore represent a potential modifiable risk factor for several health disorders, including OA [25, 31].

Unfortunately, there are serious limitations for OA management, since it lacks specific and sensitive biomarkers for early diagnosis, prognosis and therapeutic monitoring [5, 8, 24]. Furthermore, there are few nonpharmacologic and pharmacologic treatment options, mostly focused on providing general symptomatic relief, although showing a quite limited efficacy and several side effects, prompting the need for novel therapeutic alternatives [18, 32-34].

Till date, there are no therapies approved by regulatory authorities to modify the onset or arrest the progression of OA structural damages. So, these limitations of pharmacological approaches, with no treatments available to prevent or halt the development of the illness, hasten the increased need of joint replacement surgeries. Consequently, there is an urgent need to identify novel prophylactic and therapeutic agents, which prove to be safe for clinical use and efficacy to prevent the initiation or slow the progression of OA [5, 34, 35].

In this context, vitamin K is a potential OA target and an interesting candidate for a disease modifying osteoarthritis drug (DMOAD), due to its multifunctional roles in health. Vitamin K has been described to have a determinant role in regulating bone and cartilage mineralization metabolisms, preventing soft tissue mineralization and further suggested as a protective agent against inflammation, suppressing the signaling of inflammatory mediators which have been reported to be involved in OA pathological pathways. More recently, a low plasma vitamin K has been suggested to be associated with higher prevalence and progression of knee OA features [18, 27, 29, 31, 36]. Altogether the information available strongly indicates that vitamin K should represent a promising tool that might help to alter the course of this pervasive disease.

2. Osteoarthritis

2.1. Definition

The Osteoarthritis Research Society International (OARSI) Disease State Working Group defined OA as "progressive disease representing the failed repair of joint damage that, in the preponderance of cases, has been triggered by abnormal intra-articular cell stress"[37, 38].

Presently, there is a consensus that OA represents a heterogeneous and complex group of conditions difficult to define. The process involves all the tissues of the movable joints affected, starting first as a molecular disturbance, followed by anatomic, and/or physiologic derangements that can culminate in illness. This degenerative syndrome, complicated by inflammatory reactions, leads to eventual failure of one or more joints of the body, causing chronic pain and significant disability [4, 9, 39-41].

2.2. Epidemiology

OA is the most common form of arthritis, affecting every population and ethnic group investigated thus far. This chronic rheumatic pathology represents a huge cause of morbidity, activity limitation, physical disability and excess health care utilization, especially in people aged 45 years and above [1, 3, 42-46].

Worldwide estimates show that this musculoskeletal condition affects approximately 15% of the population, with an incidence of about 100.000 new cases per year [1, 9, 12, 44]. In our country, general data presented by the Portuguese League Against Rheumatic Diseases revealed that around 6% of the Portuguese population has the disease [13].

Most of the OA burden is attributable to the involvement of the hip and the knee joints and women have higher rates than man, especially after the age of 50. The World Health Organization's Scientific Group on Rheumatic Diseases estimates that 18.0% of women and 9.6% of men aged 60 or older suffer from these disorders [1, 46-49].

Due to demographic changes, the ageing of population and rising obesity, it is anticipated that the impact of OA will grow and become a major problem, in the near future; with its prevalence projected to double by the year 2020 [1, 5, 43, 47].

Depending on the geographical regions, the epidemiological studies tend to report different numbers for OA. In general, this condition is more prevalent in Europe and the United States of America than in other parts of the world [46]. Possible explanations for these variances range from genetics, socio-economic conditions, environmental characteristics or different lifestyles; indicating that predisposition and susceptibility to the development of OA depends on the association of several factors [4, 38, 48].

A major goal of epidemiologic studies is to evaluate the disease risk factors. For OA, understanding whether and why certain individuals or groups are at high risk provides insights into disease biology and leads to novel opportunities for its prevention or treatment [41].

2.3. Risk Factors

OA is recognized as a multifactorial disorder that can be considered the product of a complex interplay between systemic and local factors [48-51].

2.3.1. Systemic Risk Factors

The systemic factors increase the individual predisposition to develop OA. This category of factors may act by raising the susceptibility of the joints to injury, by direct damage to joint tissues or by impairing the process of repair in damaged joint tissues [12, 48].

2.3.1.1. Non Modifiable Systemic Risk Factors

Some specific non modifiable systemic risk factors have been established.

OA in all its heterogeneous forms is, to a large extent, genetically determined. Evidences of the genetic influence come from a number of sources, including epidemiological studies of family history and family clustering, twin studies and exploration of rare genetic disorders related to OA. Taken together, these estimates suggest a heritability of 50% or more [51, 52]. The OA genetic architecture is similar to other complex polygenic diseases with contributions of several or even perhaps hundreds of genes, most of them affecting the occurrence in many joints, although there may be specific genes for specific sites, with some having small effects and a few having large effects [13, 51]. Overall findings indicate that half the variation in predisposition and susceptibility to OA in the population is explained by inherited components but, we need to keep in mind that, environmental and lifestyle factors are key modulators of gene expression [15, 48].

Many studies support the role of ethnicity in the development of OA based on variations among ethnic and racial groups [12, 15].The better characterized data of racial and ethnic differences in OA patterns of joint involvement come from large database studies, but the evidences are conflicting. The relative contributions of biological, lifestyle, and socioeconomic factors to ethnic differences in OA are still unclear [50, 51].

Even though aging is not sufficient for the development of OA, it is known as one of the strongest risk factor for the disease in all joints [15, 20]. Several studies and evidences confirm the high correlation between aging and OA, but the real mechanism behind this strongest predictor to the condition is still poorly understood [4, 43].

The increased incidence and prevalence of OA on the elderly, probably, is a consequence of cumulative exposure to various risk factors and biologic changes that occur with aging and can turn a joint less able to cope with adversity [12, 20]. The basic cellular mechanisms that maintain tissue homeostasis decline with age, leading to an inadequate response to stress or injury, resulting in joint tissue destruction [12, 48].

Several epidemiologic studies of OA suggest the relevant difference between gender in the onset and development of this disease in males and females. Before the age of 50, males are reported to have a higher prevalence of OA, but after this age it is higher in females and with an enlarged severity [4, 15]. In addition, the definite increase in OA in women following menopause has led investigators to hypothesize that hormonal factors may play a role in the onset of OA. However, the results on effect of estrogen, either endogenous or exogenous, on OA from observational studies have been inconsistent. Gender disparities may also be caused by differences in anatomy, mechanical alignment, bone strength and neuromuscular strength, pregnancy, etc. [53, 54].

2.3.1.2. Modifiable Systemic Risk Factors

Numerous studies have shown a strong positive association between body mass index (BMI) and OA. Situations of overweight or obesity could precede the development and increase the progression of OA in weight-bearing joints, such as the hip and knee, as well as in non-weight-bearing joints, like the hand [55, 56]. Prospective studies indicate that obesity increases the relative risk of developing knee OA by two to tenfold [57, 58]. The pathogenesis of obesity-associated OA is not completely understood, but recent studies indicate that adipose tissue, and in particular infrapatellar fat, is a local source of pro-inflammatory mediators that are augmented with obesity and have been shown to increase cartilage degradation in cell and tissue culture models [13, 56]. Adipose tissue, once considered a passive storage portal of energy, is now recognized as a highly metabolic endocrine organ, with its adipocytes implicated in the secretion of active agents, including adipocytokines that play pleiotropic functions in bone, cartilage and others tissues of the joint formation [8, 56-58].

Emerging studies have also suggested clustering between OA and comorbidities, vascular health and cardiovascular risk factors, such as hypertension, hypercholesterolemia and diabetes [48, 49, 59]. It has been reported that approximately 55% of knee OA patients over 65 years old have hypertension and 13% diabetes type 2 [60]. The association of OA with diabetes is based on the suggestion that high glucose concentration produces reactive oxygen

species (ROS) and advanced glycation end-products (AGEs), which are proteins or lipids that become glycated after exposure to sugars, accumulate in aged cartilage causing its degradation. In addition, long-term insulin therapy often needed to treat diabetes may overload tissues such as cartilage. Indeed, joint damage severity is higher in diabetic patients [4, 61].

The role of diet and nutrition in OA has been a recent area of research. There is evidence, from longitudinal studies, that low dietary intake and serum levels of vitamin D may be associated with the risk of knee and hip OA development. Without sufficient vitamin D, bones became thin, brittle or misshapen [15, 23].

Since antioxidants provide defense against tissue injury, high dietary intake of antioxidant vitamins could be postulated to protect against OA. For example, high vitamin C intake has been shown to reduce the progression of radiographic knee OA threefold as well as reducing the risk of developing knee pain [43, 51].

Several studies indicate that lack of adequate vitamin K may be a significant risk factor, for OA condition. Vitamin K has several potential effects that may modulate the development of OA. Some investigators show that a poor intake of vitamin K can result in abnormal bone and cartilage mineralization. Research demonstrated that high levels of vitamin K are associated with low OA prevalence ratios [18, 27, 29, 36]. Moreover, a recent study reported the beneficial effect of the assumption of extra virgin olive oil, rich in antioxidants such as vitamin A, E and K, in rats suffering from OA, underlining its possible application as preventive agent [62].

A few studies have identified high bone mass (BM) as a potential OA risk factor. Cross-sectional studies, in a variety of populations, have demonstrated associations between increased BM and radiographic OA in the large joints of the hip and knee. Longitudinal studies have also associated high BM with incident knee and hip OA [63, 64]. Like OA, OP is a common age-related skeletal disorder and the preponderant evidence suggests an inverse relationship between those two diseases [12, 51].

2.3.2. Local Risk Factors

Local factors are most commonly biomechanical affecting adversely the forces applied to the joint. Abnormal joint biomechanics, whether from trauma or other cause, may be the initiator of changes in the local joint environment that can, if ignored, result in OA development [12, 48]. These factors have the potential for intervention and risk modification [50].

Numerous cases prove that acute injury to the structures of the joint increase the risk for OA development and musculoskeletal symptomatology; especially in the knees, but nearly all joints can be affected. It is reported that knee injury is the leading modifiable risk factor in men and the second in women [48, 49]. Individuals with a history of joint injury are three to six times more likely to develop OA and are diagnosed approximately ten years earlier than individuals without any joint trauma. It is concerning that certain types of injuries may be associated with a rapid cascade towards joint failure in less than one year [44, 65].

The professional occupation is another appointed risk factor for OA onset. It appears that people who are exposed to certain physically demanding activities in their jobs, which involve repetitive use of joints during their tasks, are at increased risk of developing localized OA [47, 51]. Workers whose occupations demand squatting or kneeling have twice the risk for knee OA, prolonged standing and lifting might cause hip OA and work that require increased manual dexterity have been associated with features of hand OA [20, 44].

Participation in certain competitive sports, such that done by elite level athletes, that demand high intensity, torsion, direct impact as a result of contact with others participants, playing surfaces or equipment, increases OA risk too [50, 51].

The relationship between muscle strength and OA is complex and may vary, depending on specific muscles and joints examined. Recent reviews conclude that muscle weakness and atrophy may predispose for knee OA onset and progression. Sarcopenia is common in patients with knee OA and can occur as a consequence of OA related to disuse due to pain avoidance [23, 48]. Greater muscle strength is not, however, always protective as it corresponds to higher forces and thus increased joint loading during activity [12, 48].

A concerning factor is joint alignment, because any shift from a neutral or collinear alignment of the hip, knee and ankle will alter load distribution across the articular surfaces of the knees. A systematic review confirmed that knee malalignment is one of the strongest predictors of knee OA progression. Abnormal increases in compartmental loading are thought to increase stress on the articular cartilage, and other joint structures, subsequently leading to degenerative change. Medial progression of knee OA was four times more likely in individuals with varus deformity, whilst lateral progression was five times more likely in individuals with valgus alignment [12, 44].

Relative importance of the risk factors may vary in the different joints and according to the stages of the disease. The multifactorial etiology, with such a different set of intrinsic and extrinsic factors, turns it difficult to make a distinction between single and clustered factors. Each aspect is thought to be a risk not just because of one of the abovementioned reasons, but as a combination of them, presenting a synergistic or cumulative effect [13].

2.4. Physiopathology

2.4.1. Physiology of the Synovial Joints

Humans have the capacity to maintain shape and produce a great variety of postures and movements that require that the musculoskeletal system of the body both generate and respond to forces that produce and control movement at the body's joints. These joints, or articulations, are the points where two or more adjacent bones of the skeleton come together to form a connection and articulate with each other [66, 67].

The three major kinds of joints can be classified structurally as: fibrous, cartilaginous, and synovial. In this classification scheme, joints are categorized according to the major connective tissue type that covers the surfaces of the adjacent bones and whether they are strongly anchored or not to each other [66, 67].

In the human body, synovial joints are the most common. They are formed by articular cartilage of dense irregular connective tissue (2-5 mm thick) and present a joint cavity, surrounded by walls of articular capsule, filled with a lubricating fluid. These joints fundamental structural characteristics (figure 2.1) allow the bones to move smoothly, providing great mobility. However, since the bones are free to move in relation to each other, being only held together by ligaments, the synovial joints are less stable and more vulnerable.

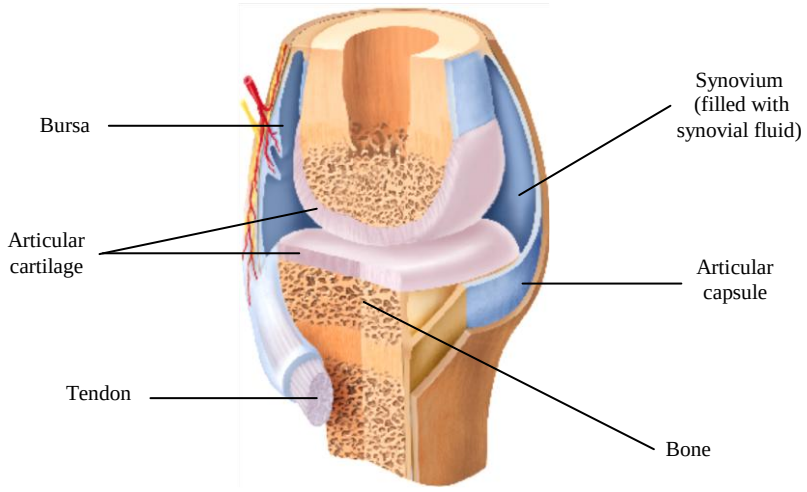


Figure 2.1- Structure of synovial joint (Adapted from reference 67).

These diarthrodial joints are quite important for normal human body functions and autonomy. Fortunately, they are self maintaining, but damage, disorder or disease of a synovial joint, like OA, can lead to huge difficulty in movement and chronic pain, hampering the quality of life.

2.4.2. Pathological Changes in the Synovial Joints Structures

Findings of detrimental pathologic changes in all of the synovial joint tissues lead to consider OA as a disease of the whole joint as an organ, being difficult to understand when and where the changes begin [6, 68-72].

OA is not a uniform disease, but a complex network of processes that occur when the dynamic steady state between destructive and repair mechanisms destabilises the joint homeostasis, adversely affecting the multiple articular components, including cartilage, subchondral bone, synovium, ligaments and neuromuscular support structures [68-71, 73, 74].

In the long-term, is reasonable to conceive that the pathologic bioreactivity of the individual tissues and the crosstalk between them likely contribute to the disease perpetuation, underlining that the pathological pathways are, in many respects, intertwined [70, 74-76].

2.4.2.1. Articular Cartilage

Adult articular cartilage is a hyaline cartilage, avascular, aliphatic and aneural structure subjected to a harsh biomechanical environment. This highly specialized tissue is designed for efficient gliding motion, with a limited capacity for intrinsic repair so, in this regard, its preservation is vital for the synovial joints health [77, 78].

The resilience, integrity and function of articular cartilage are highly dependent on the specialized mesenchymal cells, the chondrocytes, the sole cellular component found in this tissue, responsible for the production and maintenance of its surrounding extracellular matrix (ECM) infrastructure [79]. This abundant ECM, that comprises 95% or more of the cartilage volume, is composed of a big amount of water with dissolved inorganic ions (calcium, sodium, potassium) and organic macromolecules, mainly collagens, proteoglycans and small amounts of other noncollagenous proteins, glycoproteins and lipids [77-80].

Normal structure of the articular cartilage ECM is made of highly crosslinked fibrils, mostly of type II collagen molecules and, in a minor proportion, of type I, III, VI, IX, X, XI, XII and XIV; intertwined with two major classes of proteoglycans (figure 2.2), large monomers or aggrecans and small proteoglycans including decorin, biglycan, fibromodulin and lumican [74, 77-81].

The aggrecan is composed of a core protein with covalently attached glycosaminoglycans side chains of chondroitin and keratin sulfates that interact with hyaluronic acid (HA) (figure 2.2), which creates an hydrophilic environment, providing cartilage osmotic properties that are crucial to give tensile strength and withstand compressive forces [77-79].

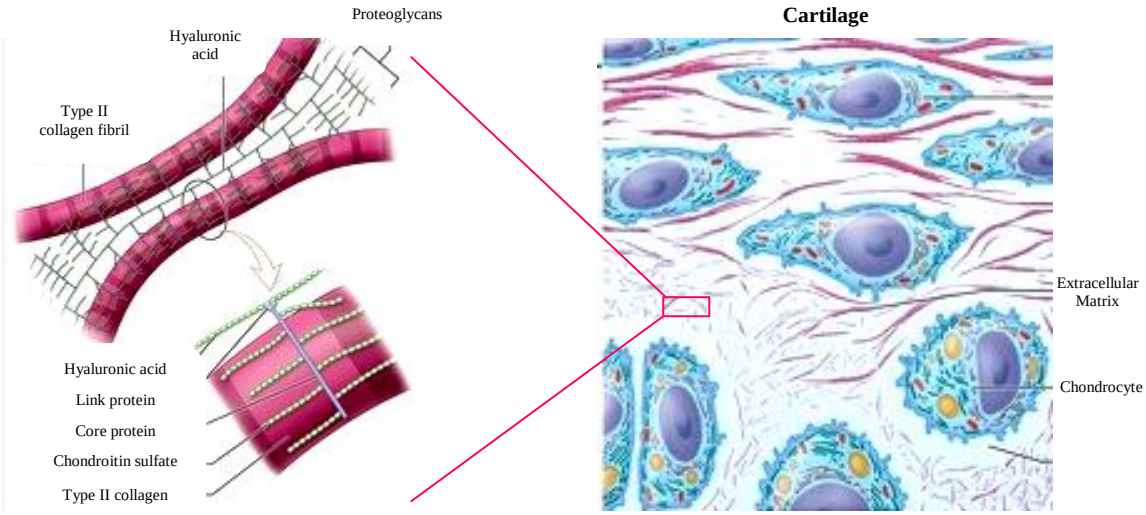


Figure 2.2- Principal molecular components of the articular cartilage (Adapted from reference 77).

This specific organization of articular cartilage results from the joints embryogenesis, in a process called endochondral ossification, comprising four steps: chondrogenesis from mesenchymal condensations, chondrocyte differentiation and hypertrophy, mineralization of the matrix with invasion of bone cells and the definitive formation of bone [74].

Histologically, cartilage tissues can be divided into a thick nonmineralized layer composed by superficial, transitional and radial zones, separated by a tidemark to a thin calcified deeper area. All of them characterized by a different phenotypic and gene expression patterns of the resident chondrocytes, as well as distinct ECM composition and organization [80].

In healthy adult cartilage in a non-stressed steady state, the chondrocytes are quiescent cells and the articular cartilage equilibrium is regulated by a complex interplay between the anabolic growth factors, including insulin-like growth factor 1 (IGF-1), transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) and the catabolic proinflamatory factors, particularly some interleukins (ILs), tumor necrosis factor alpha (TNF-α), prostaglandin E2 (PGE2) and nitric oxide (NO) [74].

Much work has been devoted to the knowledge of how the homeostatic balance of the cartilage is perturbed at molecular level and how this leads to disease. Changes in chondrocytes activity are pivotal for OA development, through mechanisms that are not completely understood. It is highly feasible that normal cartilage, in spite of the low cellular activity and matrix turnover, possesses a strong metabolic potential. Activation of chondrocytes is driven by a cascade of signals, particularly by nuclear factor kappa light chain enhancer of activated B cells (NF-kB) and mitogen activated protein kinases (MAPK) pathways, which are critical in cell survival, differentiation and chondrogenesis [6, 72, 81, 82].

Disruption of the chondrocytes resting state may be viewed as an active response, with the recapitulation of developmental programs of the matrix substitution. A disturbed cell-matrix relationship lies at the centre of OA pathogenesis, promoted by chondrocytes phenotypic shift, rising cell proliferation and production of diverse cytokines, chemokines and matrix proteinases, either as initiating or as feedback amplification events [6, 72, 81, 82].

Notably, there appear to be significant differences between the individual zones of OA cartilage. In the upper zones, expression of collagen type II is significantly suppressed while collagen type III, fibronectin and cartilage oligomeric matrix protein (COMP) are upregulated during the progression of matrix destruction. Thus, chondrocytes produce a different kind of matrix structure, which is more susceptible to erosion. In contrast, the middle and deeper zones exhibit normal metabolism producing predominantly cartilaginous molecules [10, 74].

The involvement of several proteolytic enzymes is well established in the degradation of cartilage proteoglycans and collagens (table 2.1). Members of both matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families are important mediators of proteoglycan degradation, while collagen catabolism is majorly attributed to the action of MMPs [14, 83, 84]. Initial OA changes may be due to MMP-3 and ADAMTS-5 that degrade aggrecan, followed by MMP-13, which is highly efficient in degradation of type II collagen. At some point, the biosynthetic anabolism is unable to keep pace with the catabolic activity and, once the collagen network is broken, the process is irreversible [6, 72, 83-85].

Table 2.1- Principal proteolytic enzymes involved in cartilage tissues degradation [14, 79, 83, 84].

Metalloproteinases	Aggrecanases	Other Proteinases
Collagenases: MMP-1, MMP-8, MMP-13	ADAMTS-4	Cathepsins K,B,D,G,L
Stromelysins: MMP-3, MMP-10, MMP-11	ADAMTS-5	Tissue Plaminogen activator (tPA)
Gelatinases: MMP-2, MMP-9		Urokinase Plaminogen activator (uPA)

In OA advanced stage, the cartilage becomes hypocellular, often accompanied by lacunar emptying, which has been considered as evidence that chondrocyte death is a feature in OA progression. Most eukaryotic cells attach to neighboring cells or to the surrounding matrix for survival, a phenomenon called anchorage dependence. Indeed, chondrocyte survival is mediated by integrins that are responsible for the connection of ECM components to various intracellular cytoskeletal proteins. So, it is plausible that chondrocyte anchorage to the ECM is disturbed to a significant degree that leads to cellular death [4, 86].

Additionally, OA cartilage produces endogenously NO and ROS, causing oxidative stress and mitochondrial dysfunction [10, 87]. The excess production of NO in OA tissues has been linked with chondrocyte apoptosis both *in vitro* and *in vivo*. The activation of the caspase cascade seems to play an essential role, along with another possible mechanism that has recently been identified. In the superficial layer where most of the apoptotic cells are located, a subpopulation of OA chondrocytes expresses the Fas antigen, which upon ligand binding could induce cell apoptosis [86, 88].

However, the relative contribution of chondrocyte apoptosis in the OA pathogenesis is difficult to ascertain. It has been suggested that matrix loss and chondrocyte death may form a vicious cycle with chondrocyte apoptosis being an inducer of cartilage degeneration and at the same time, a byproduct of cartilage destruction. The release of intracellular contents from apoptotic cells, as well as local production of inflammatory mediators might also play a further role in the disease progression [4, 86, 89].

2.4.2.2. Synovium

Synovium structure consists of a soft tissue covering the spaces of diarthrodial joints, tendon sheaths and bursae. Under normal physiological conditions it includes a thin surface layer, named intima, of macrophages and fibroblasts surrounded by an amorphous fine fibrillar ultrastructure of collagens type I, III, IV, V and VI, with laminin, fibronectin and chondroitin-6-sulfate-rich proteoglycan and an underlying subintima, containing scattered blood and lymphatic vessels, fibroblasts and fat cells in a collagenous matrix [70, 90, 91].

The synovium is an important source of synovial fluid components, which provide the major route of nutrition and help to modulate chondrocyte activity, while contribute to maintain the integrity and functional properties of the articular cartilage. Two of those essential synovium secreted molecules are HA and lubricin, which together reduce friction, providing boundary lubrication at the articular joint and lubricin reducing the pathologic deposition of proteins at the cartilage surface [70, 90-92].

As adult articular cartilage has no intrinsic vasculature it relies on the adjacent tissues, including the synovium, for removal of products of the chondrocytic metabolism and the matrix turnover. The synovium acts as a semipermeable membrane, controlling molecular traffic in the joint cavity, maintaining the composition of the synovial fluid and preserving the physiologic state. Therefore, alterations in the synovial membrane can result in decreased concentrations of cartilage protecting factors and increased production of degradation factors [91].

The synovium produces some of the chemokines and metalloproteinases that degrade cartilage (figure 2.3), even though the cartilage itself produces most of these destructive molecules in a vicious autocrine and paracrine manner. In turn, cartilage breakdown products can provoke the release of collagenases and other hydrolytic enzymes from synovial cells and lead to synovial hypertrophy and vascular hyperplasia, with an increased number of lining cells, often accompanied by infiltration of the sublining tissue with scattered foci of lymphocytes [8, 69, 75, 89].

In the past, OA was categorized as a non-inflammatory condition because of the neutrophils absence in the synovial fluid and the lack of systemic manifestations of inflammation. Recent studies have been proving that, even in early OA, some degrees of synovitis may be observed. The synovium tissue is the most inflammatory on molecular level (figure 2.3) and provides the best evidence of a coordinated biochemical process along with cartilage and subchondral bone. However, in contrast to RA, synovial inflammation in OA is mostly confined to adjacent areas of pathologically damaged cartilage and bone [8, 75, 76].

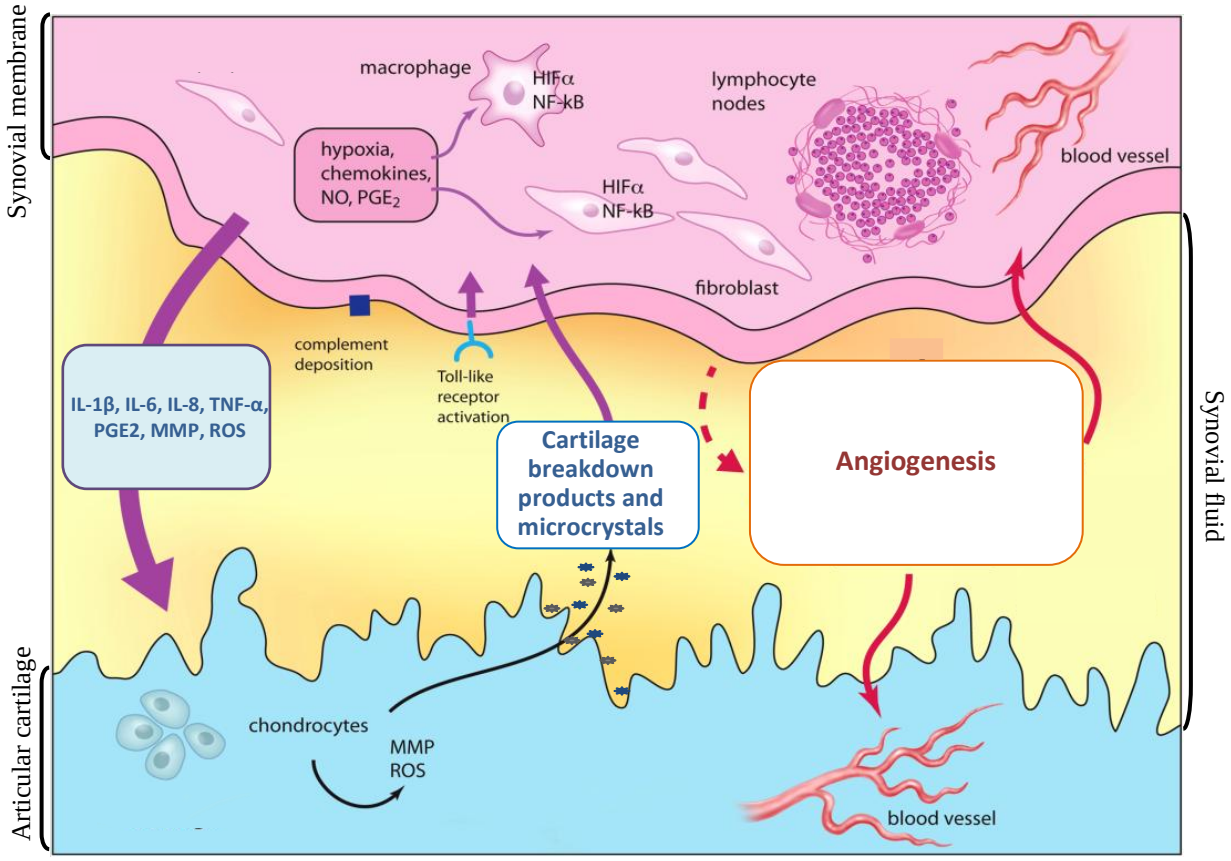


Figure 2.3- Interaction between inflammation, angiogenesis and cartilage degradation in OA (Adapted from reference 92). HIF- hypoxia-induced factor; IL- interleukin; MMP- matrix metalloprotease; NF-κB- nuclear factor-κB; NO- nitric oxide; PGE2- prostaglandin E2; ROS- reactive oxygen species; TNF-α- tumor necrosis factor alpha.

Innate immunity is the first level of immune system activation in response to inflammatory challenges. The available information suggest that OA synovitis may be established through at least two mechanisms, via stimulation of cell receptors known as toll-like receptors (TLRs) and the activation of the complement cascade (figure 2.3) [72, 81, 91, 93].

A consequence of the low-grade inflammatory processes is the induction of synovial IL-1β and TNF-α, which are active contributors to the degradative cascade. There are also reports of increased numbers of immune cells in synovial tissue, such as activated B cells and T lymphocytes, including evidence for a clonally expanded, antigen-driven B-cell response that may contribute to the development of the disease [8, 89, 91].

Local hypoxia is a major feature of the inflammatory tissue that also triggers angiogenesis. Hypoxia stimulates the expression of hypoxia inducible factor (HIF) which acts predominantly via upregulation of the vascular endothelium growth factor (VEGF). Angiogenic factors produced by various cell types in the synovium activate local endothelial cells which, in turn, release proteolytic enzymes that degrade the endothelial basement membrane and the perivascular ECM. Endothelial cells then proliferate and migrate into the interstitial tissue forming a 'primary sprout'. The lumen formation within these sprouts leads to the formation of 'capillary loops' followed by synthesis of a novel basement membrane and ultimately capillary formation (figure 2.3) [92]. Then, the blood vessel permeability and upregulation of adhesion molecules that are seen as part of angiogenesis potentiates the inflammatory response. A new road map is created to perpetuate the transport of these inflammatory cells and nutrients to the sites of inflammation, in a vicious way [71, 76].

Pathologic studies describe that patterns of synovial change in OA are diverse, varying with the stage of the disease. Nonetheless there is a common agreement that persistence of synovitis is correlated with symptom severity, faster rates of cartilage erosion and osteophytosis [6, 16, 90-93].

2.4.2.3. Subchondral Bone

Despite the focus on the contribution of subchondral bone to the pathogenesis of OA, there remains a controversy whether the alterations within subchondral bone are a driving force or a consequence of cartilage breakdown, being nowadays still a matter of debate if bone changes are preceding, concurring with or following cartilage degradation [71, 94, 95].

Subchondral bone refers to the zone of epiphyseal bone just underneath the articular cartilage and includes two distinct anatomic entities, the subchondral bone plate and the

underlying trabecular bone. The subchondral bone plate is a thin cortical lamella, lying beneath the calcified cartilage and separated from the deeper calcified cartilage by a sharp borderline, called the cement line. This cortical endplate is a slightly porous structure with channels that provide a direct connection between cartilage and the subchondral trabecular bone that is more porous and metabolically active, containing blood vessels, sensory nerves, and bone marrow [70].

Evidently, a close contact exists between the deeper layer of non-calcified cartilage, the tidemark, calcified cartilage, cement line and the subchondral bone forming a strictly functional unit called the osteochondral junction. There is intensive biomechanical and biochemical cross-talk across this region that plays a role in maintenance of the joint [96, 97].

Subchondral bone is a dynamic specialized connective tissue made up of several components including the specific cells osteoblasts, osteocytes and osteoclasts, inorganic non-collagenic substances such as proteoglycans and a collagenic component, majorly of collagen type I [69, 71]. Its integrity is maintained by a distinct equilibrium through processes of bone remodeling and modeling, which are regulated by the osteocytes that are the major source of the osteoclast differentiating cytokine, receptor activator of nuclear factor κB ligand (RANKL). Control of skeletal patterning, tissue remodelling and cell development involves a complex network interaction of signaling molecules including hormones and local growth factors, like IGF-1, TGF-β, BMPs, MAPK and wingless type (Wnt) proteins [97, 98].

The detection of bone changes in OA prior to the appearance of detectable changes in the cartilage can be attributed, in part, to the marked differential capacity of adaptation to altered mechanical loads and damage. Bone can rapidly alter its architecture and structure via the cell-mediated processes of modeling and remodeling. In contrast, the capacity of chondrocytes to repair and modify their surrounding matrix is relatively limited [69, 71]. Under physiological conditions, bone and cartilage turnover are coupled. Along the progression of OA, subchondral bone turnover appears to be 20 fold increased compared to normal turnover [94, 99].

In OA, the fissuring and flanking along with the augmented vascularization across the osteochondral junction seem to operate as instigators of the bone remodeling process and provide a mean of direct passage of the catabolic mediators secreted from cartilage to bone and *vice versa* (figure 2.4), affecting the homeostasis of all neighboring tissues. The osteochondral junction zone undergoes uncontrolled catabolic and anabolic remodeling processes to adapt to local biochemical and biological signals. The changes in cartilage and bone are not merely secondary manifestations of OA, they are active components of the disease, contributing to its severity [70, 96, 97].

Regardless all research efforts around the numerous OA pathophysiological alterations detected in subchondral bone, the molecular mechanisms underpinning these phenomena and how the different aspects are interrelated with each other, are still not clear [94]. Based on the current state of knowledge, the changes in subchondral bone structure and remodeling are due, ultimately, to abnormal cellular metabolism, driven in turn by altered expression of key regulatory genes. Previous *in vitro* and *in vivo* studies have been supporting a disturbed subchondral bone cell behavior in OA, with fundamental findings that some osteoblasts are phenotypically different and may produce increased levels of alkaline phosphatase (AP), osteocalcin (OC), TGF-β1, IGF-1 and uPA, while levels of IGF binding proteins 3, 4, 5 are lower and PA inhibitor 1 and IL-1 levels remain unchanged, demonstrating the atypical development and function of OA osteoblasts [100-103].

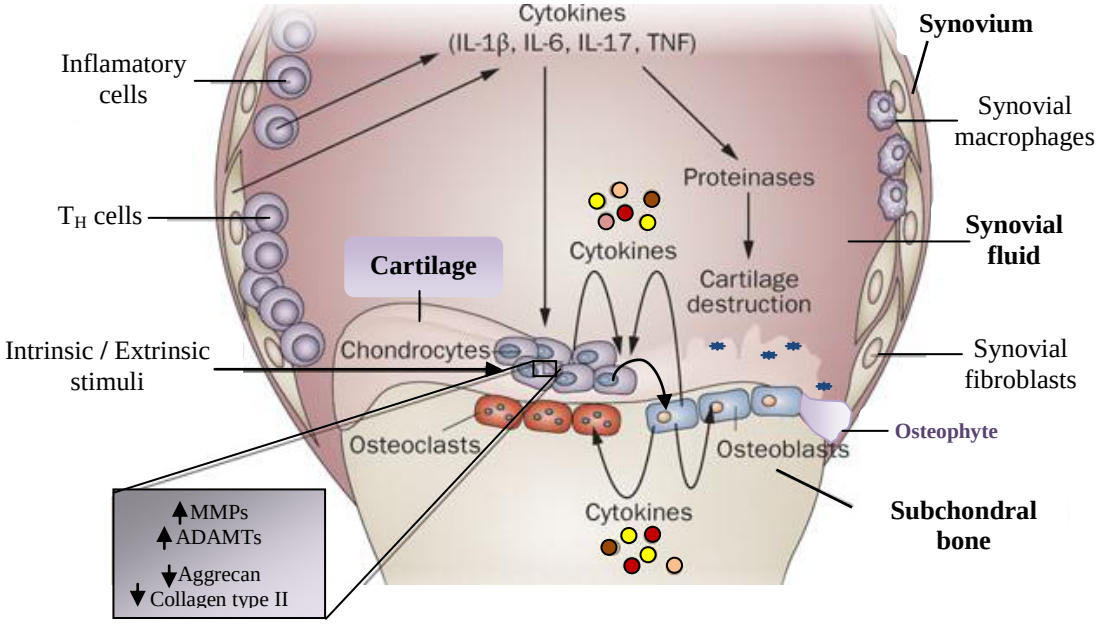


Figure 2.4- Joint intertwined pathways in the pathophysiology of OA (Adapted from reference 104).

Recently, chemokines, cytokines and proteases secreted from chondrocytes have been also implicated to alter biochemical and functional abilities of subchondral bone osteoblasts (figure 2.4), For example, IL-6 in combination with other cytokines like IL-1β can switch osteoblasts from a normal phenotype to a sclerotic phenotype [105]. Chondrocytes undergoing apoptosis are also known to secrete increased amounts of osteoclastogenesis inducing factor RANKL associated with increase turnover of subchondral bone in the early stages of OA [106].

Apart from the stimulatory role of chondrocytes on subchondral bone, increasing evidence for a number of subchondral bone factors that are involved in both tissue remodeling and the modulation of cartilage catabolism has been demonstrated. Subchondral bone osteoblasts cultured from OA patients have shown aggrecan and collagen type II increased degradation in cartilage compared to healthy controls. Osteoblasts stimulated production of MMP-2 may also mediate proteoglycan degradation in cartilage [96, 107].

The microarchitectural alterations of subchondral bone occur during different stages of the OA disease process. At the beginning, OA is characterized by a thinning subchondral plate with increased porosity. During progression of OA the subchondral bone gradually becomes sclerotic, although this occurs without an increase in bone mineralization. An imbalance between the production of collagenous and noncollagenous proteins can lead to an augment in bone volume without a concomitant increase in bone mineralization pattern; occurs trabecular separation and deterioration with decreased bone density and trabecular thickness [69, 70, 94]. The matrix in sclerotic areas is characterized by an accumulation of osteoid substance, composed primarily of elevated levels of abnormal homotrimeric collagen type I and noncollagenous bone matrix proteins, mainly OC, osteopontin (OPN) and bone sialoprotein (BSP), potent mineralization inhibitors [99-102]. Although the subchondral bone tissue is hypomineralized, the increase in trabecular number and volume compensates for this situation, thus providing an apparent stiffer structure [69, 71].

In a late OA stage, the sclerotic bone is less able to absorb and dissipate energy, thereby increasing forces transmitted through the joint and predisposing the articular surface to deformation. Subchondral bone attrition may be caused by altered mechanical loading resulting in subchondral remodeling and is associated with development of bone marrow lesions (BMLs). These histopathological alterations generally appear in the proximity of focal cartilage damage, associated with high bone turnover and mineralization. BMLs are degenerative lesions that may originally correspond to an acute inflammatory response, edema, contusion and/or necrosis, which over time are replaced by more permanent bone marrow remodelling such as fibrosis and myxomatous connective tissue [94, 97].

Severe remodeling processes take place in particular areas of total cartilage destruction (the eburnated bone plate), synovial fluid gets access to the bone marrow and presumably leads to cavitary lesions, normally referred to as subchondral bone cysts (SBCs), frequently seen within or adjacent to BMLs, associated with stronger disease symptoms and severity [71, 94].

There is a progressive increase in the subchondral bone plate thickness, a modification in the architecture of the trabeculae, from rod-like into plate-like and an additional mechanism for skeletal adaptation occurs at the joint margins and entheseal sites, where new bone is added by endochondral ossification; recapitulating the cellular mechanism of skeletal growth, in a process involving local production of growth factors TGF-β and BMP-2, giving rise to the formation of prominent osteochondral nodules known as osteophytes (figure 2.4) [71, 85, 99].

2.4.3. Pathological Calcification

During growth and in healthy adult life, mineral formation is restricted to specialized tissues of the vertebral skeleton (dental enamel, dentin, bone, cementum and calcified cartilage), where it provides mechanical strength. Within these tissues, the regulation of physiological mineralization is tightly controlled, mediated at molecular and cellular levels, involving the coordination between stimulatory and inhibitory factors. Nevertheless, uncontrolled or pathological mineralization, due to an unbalance between pro and anti-mineralization factors, can take place as an unwanted event, like the excessive mineral deposition that happens in articular cartilage through OA, contributing to the wear of joints. This inappropriate biomineralization occurring in soft tissues is called ectopic calcification, a common problem associated with several pathological situations such as vascular calcification associated with cardiovascular and chronic renal diseases, certain cancers, skin calcinosis, organ injury, bioprosthetic implants and OA [108-112].

The exact triggers for the hyaline cartilage calcification, by the inappropriate deposition of calcium (Ca^{2+}) and phosphate (PO_4^{3-}) minerals in OA are currently unknown, but several histochemical studies demonstrate that, in the course of the disease, the cartilage undergoes marked alterations in cellular composition and structure. Cartilage zonal organization is disturbed, the superficial zone degradation produces rough fibrillated surface and microfissures extending to the deep area of the calcified cartilage. Despite the previous belief that the tidemark between articular cartilage and subchondral bone was impermeable, OA calcified cartilage is occupied by neovascular elements, extending from the subchondral bone and adjacent marrow space, recapitulating the vascular invasion of the growth plate that occurs during development. This process results in osteochondral junction obliteration, duplication of the tidemark and advancement of the calcified cartilage into the deep zones of the articular cartilage, with its concomitant thickening (figure 2.5) [94-96, 113].

Multiple theories have been proposed regarding the mechanisms to explain this observed aberrant advancement of the calcification front into the uncalcified cartilage. Current understanding of cartilage physiological and pathological mineralization phenomenons derives largely from the knowledge of endochondral ossification during embryonic limb development, fracture healing and from studies of mineralization associated with crystal deposition arthropathies. Surprisingly, increasing evidence supports the contention that the mechanisms of soft tissue calcification are comparable to those seen in normal skeletal growth [74, 108, 114].

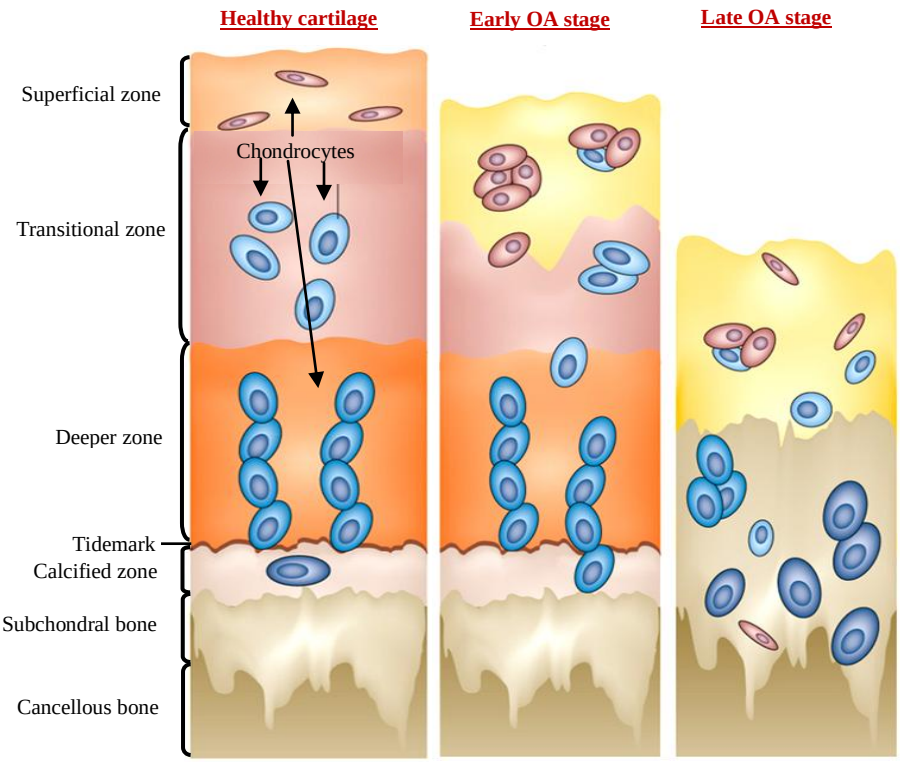


Figure 2.5- Changes across cartilage zones in OA pathological calcification (Adapted from reference 115).

During development, the rate at which the growth plate cartilage mineralizes is highly regulated. It has been shown to be controlled via the parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (Ihh) negative feedback. Articular cartilage is supposed to be resistant to ECM mineralization; this resistance is achieved by the synthesis of natural calcification inhibitors and the maintenance of articular chondrocytes in a mature state, without undergoing differentiation. The mineralization potential is regulated by the communication between zonal sub-populations of articular chondrocytes. Cellular interactions, especially those between chondrocytes derived from the surface and deep zones, are critical for the inhibition of deep zone calcification. Moreover, the regulation of chondrocyte mineralization is mediated by local paracrine factors, most likely through the PTHrP/Ihh negative feedback loop reminiscent of endochondral ossification [111, 113, 114].

In articular tissues that are not meant to calcify, pathologic calcification can be both a response to up-regulated action of promoters of calcification and the consequence of deficiencies of calcification inhibitors. The balance between the pro and anti-calcification mechanisms dictates the formation of ectopic calcification at a given site. Calcification can proceed when the equilibrium is disrupted by factors of multiple origins such as genetics, aging, ECM changes, imbalance between inhibitors and promineralizing factors, alterations in

extracellular Ca^{2+} levels, dysregulation of inorganic pyrophosphate (PPi) and inorganic phosphate (Pi) metabolism, chondrocyte phenotype modifications and altered responses to growth factors, inflammatory cytokines and other mediators of inflammation [17, 110, 111, 116].

Whereas in healthy articular cartilage most chondrocytes maintain a stable resting phenotype and resist proliferation and differentiation, articular chondrocytes in OA joints form clusters by proliferating more strongly and eventually develop hypertrophy; typically close to areas of mineralized cartilage matrix and near sites of surface lesions. Changes in chondrocyte behavior and viability mediate chondrocalcinosis, including the development of chondrocyte heightened hypertrophy associated with expression of stereotypic bone matrix proteins and of chondrocytes apoptosis. Ectopic calcification of hyaline cartilage is a regular event in human OA. The mineralization of cartilage is re-initiated in the deepest zones of cartilage, as part of the pathogenic modifications associated with the terminal hypertrophic differentiation of diseased chondrocytes [116-119].

A current paradigm involves chondrocyte hypertrophy traits of OA cartilage with certain similarities to those of the hypertrophic chondrocytes in the mineralizing growth plate, but other phenotypic changes are also reported [74, 117, 118]. Expression of the pivotal chondrogenic SRY-type high-mobility-group box transcription factor-9 (Sox9) is down regulated in human OA cartilage in comparing with terminal differentiating growth plate chondrocytes. The master factor of hypertrophic maturation runt-related transcription factor 2 (Runx2) is highly expressed in OA cartilage as well as VEGF, PTHrP, Ihh, MMP-13, BMP-2, OC, OPN, osteonectin (ON), AP, amongst others [113, 118]. The most relevant shared features of hypertrophic chondrocytes in the growth plate and in OA hypertrophic chondrocytes include the synthesis of type X collagen and the release of matrix vesicles responsible for the initial formation of mineralization. Interestingly, a significant correlation exists between the expression of type X collagen, a marker of chondrocyte hypertrophy, and mineral growth. Chondrocyte hypertrophy is a key factor in articular cartilage mineralization that is indissociably linked to OA disease process [74, 117].

Much still remains to be dissected about the pathways that link chondrocyte differentiation to the calcification of the surrounding matrix. In addition to synthesizing and secreting the organic matrix, cells maintain the ion composition of the extracellular space through the action of Ca^{2+} and PO_4^{3-} ion pumps. The activity of these ions in the extracellular space is modulated by non-collagenous proteins (table 2.2) that enhance mineral formation, inhibit mineral formation, or do both and by the presence of PPi [108].

Table 2.2- Principal non-collagenous proteins involved in cartilage mineralization regulation [17, 109, 112, 116].

Proteoglycans	Glycoproteins	γ-Carboxyglutamic Acid (Gla) containing proteins	Serum Protein
Aggrecan	Osteopontin (OPN)	Matrix Gla protein (MGP)	Fetuin
Biglycan	Bone Sialoprotein (BSP)	Osteocalcin (OC)	
Decorin	Fibronectin	Gla rich protein (GRP)	
Versican	Thrombospondin		

The mechanisms involved in ectopic calcification during OA are only elusive and need to be scrutinized. One surprising notation is that pathological calcification in normal soft tissues may utilize means similar to those found in hard tissues. A key constituent found in both normal and ectopic calcifications are matrix vesicles (MVs) and MV-like particles [74, 108, 119]. It is currently well accepted that cartilage calcifications are formed, at least partly, in chondrocyte-derived apoptotic bodies and MVs (figure 2.6), which are small (20-200 nm in diameter) membrane-enclosed particles released from the plasma membrane of mineralization competent cells [110, 119-121].

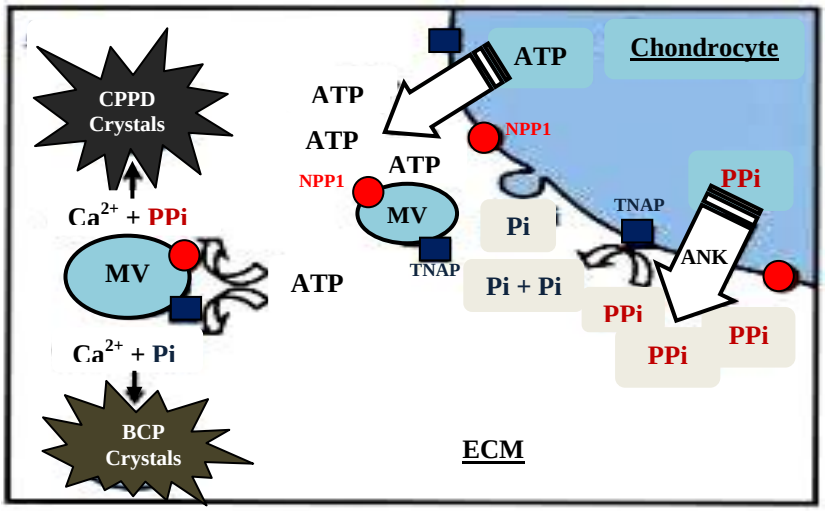


Figure 2.6- Schematic representation of crystal formation in articular cartilage (Adapted from reference 121).

ANK - Ankylosis protein; ATP - Adenosine Triphosphate; BCP - Basic Calcium Phosphate; CPPD - Calcium Pyrophosphate Dehydrated; ECM – Extracellular Matrix; MV- Matrix Vesicle; NPP1 - Nucleotide Pyrophosphatase Phosphodiesterase 1; Pi - inorganic Phosphate; PPi - inorganic Pyrophosphate; TNAP - Tissue Nonspecific Alkaline Phosphatase.

Hypertrophic chondrocytes elaborate membrane-bound MVs that play a key role in the maintenance of the PO_4^{3-}/PPi ratio in the matrix. These extruded vesicles contain a specific

combination of proteins, annexins (II, V and VI), phosphate transporters, and phosphatases which provide the nucleation site for mineralization. They have the unique ability to elaborate basic calcium phosphate (BCP) and calcium pyrophosphate dehydrated (CPPD) crystals from extracellular Pi and PPi, respectively (figure 2.6). The balance between extracellular Pi and PPi must be tightly regulated, since PPi potently suppresses the deposition and propagation of BCP, and the maintenance of relatively high extracellular PPi concentrations by chondrocytes is a vital physiologic mechanism to prevent articular cartilages from calcifying [17, 110, 121].

Three molecules have been identified as central regulators of PPi metabolism: the multiple-pass transmembrane ankylosis (ANK) protein, which mediates intracellular to extracellular channeling of PPi, the tissue nonspecific alkaline phosphatase (TNAP) that hydrolyzes PPi and the nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) that generates PPi from nucleoside triphosphates (figure 2.6) [108, 121]. The chondrocytes in OA articular cartilage overexpress ANK and NPP1, contributing to excessive accumulation of PPi in cartilage matrix and leading to deposits of pathological CPPD crystals [110]. Based on the observation that extracellular PPi is increased in articular cartilage in direct association with OA, it has been suggested that supersaturation of the ECM with PPi along with alterations of the structure and composition of the damaged OA matrix alter the solubility product of PPi and Ca^{2+}, ultimately causing cartilage abnormal calcification [74].

The breakdown of MV membranes releases the crystals to the ECM, where they serve as templates for the formation of crystalline arrays and continue to propagate along the extracellular hole zones of the collagen fibrils. In the articular tissues the two most common forms of pathologic minerals found are the BCP crystals that encompass a heterogeneous group of apatite crystals including carbonated apatite, hydroxyapatite, octacalcium phosphate, immature amorphous apatite, tricalcium phosphate and magnesium-substituted apatite (whitlockite) and the CPPD crystals. Their abnormal deposition may occur in hyaline cartilage, fibrocartilage and in the synovium. It is presumed that crystals are released from cartilage into the synovial space by injury or mechanical wear [109, 121-123].

Fuerst and colleagues recently demonstrated, in their researches, that human knee and hip cartilage specimens, harvested at the time of joint replacement for clinical OA, contained intraarticular BCP crystals in 100% and CPPD crystals in 20% of the cases [117, 124]. Synovial fluid studies demonstrate calcium crystals in 40-60% of the OA patients; some of the variation in that range may be due to different techniques used [117, 125, 126].

Only lately is becoming fully appreciated that joint soft tissues calcification could be considered as a potential therapeutic target in OA, so investigation in this area is increasing. Calcium containing crystals may precede or follow destructive joint changes, whether primary or secondary to tissue degeneration, may accelerate the OA deleterious process. Numerous clinical and experimental data provide strong evidences that the abnormal mineralization occurs as an active process and that calcification clearly plays a pathogenic role in OA, a phenomenon referred to as "microcrystal-induced stress" (figure 2.7) [17, 126-128].

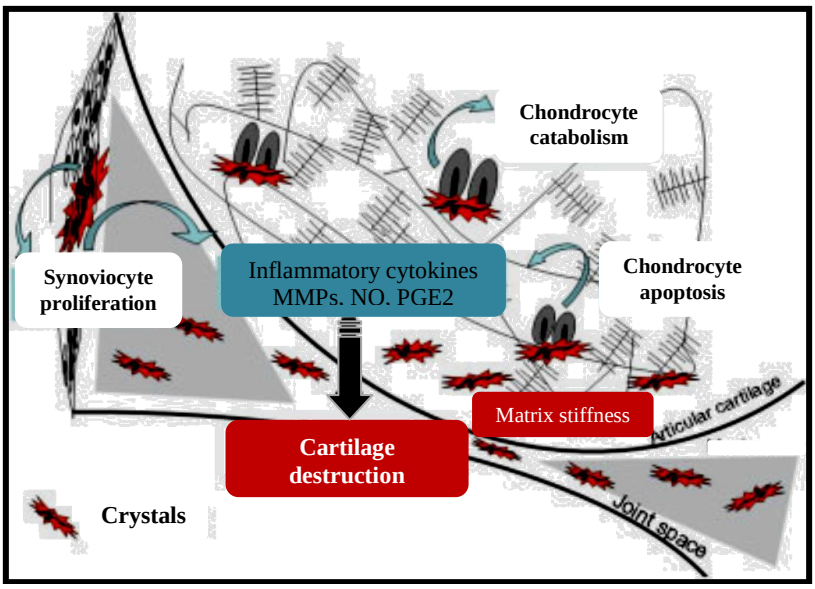


Figure 2.7- Model of microcrystal stress in OA joint (Adapted from reference 17).

BCP and CPPD crystals represent a real microcrystalline stress for the resident articular cells, clearly inducing alterations in the chondrocytes and synoviocytes (figure 2.7). Articular chondrocytes phenotypic changes can be summarized into: mitogenic chondrocytes, proinflammatory chondrocytes that produce cytokines (IL-1β), catabolic chondrocytes able to produce prodegradative soluble factors (NO, MMP-13) and apoptotic chondrocytes, enhanced by annexin V coating, that release promineralizing apoptotic bodies increasing stress [122, 129].

The BCP crystals present phlogistic properties, they are able to induce fibroblast proliferation and inflammation related events such as the production and/or activation of cytokines (IL-1β and TNF-α), production of NO, MMPs and the induction of cyclooxygenases (COX)-1 and 2, increasing PGE2 in OA synovial fibroblasts [123, 130-132]. Recently, the inflammatory effects of BCP and CPPD crystals have been implicated in both TLR-2 and TLR-4 [133, 134] and nod-like receptors (NLRP) [127, 132, 135]. These findings further indicate that Ca^{2+} containing crystals elicit and/or amplify synovitis and contribute to cartilage degradation in OA [7, 17, 125-128].

2.4.4. Concluding Remarks in the Description of the Disease

The overall characterization of OA disease is rather complex, because all patients present, with at least to some extent, different histories and the evolution of the disease happens at an unpredicted rate or pattern. Research into the development of the disease is fraught with "chicken and egg" syndrome, because it is difficult to ascertain whether a given finding is causal, a pathological effect, or secondary to other unrelated conditions.

A common edict in all OA patients is the early aging of the synovial joint(s) due to a premature senescence of the cells that maintain the articular integrity. By analogy to neurodegenerative disorders, OA could be designated as the "Morbus Alzheimer" of the joint [75, 136]. OA usually represents a long time-course degenerative disease that develops through progressive stages (figure 2.8), whose cumulative and irreversible result is the abnormal remodeling of the joint tissues, causing a decreased range of articular motion [6, 69, 136].

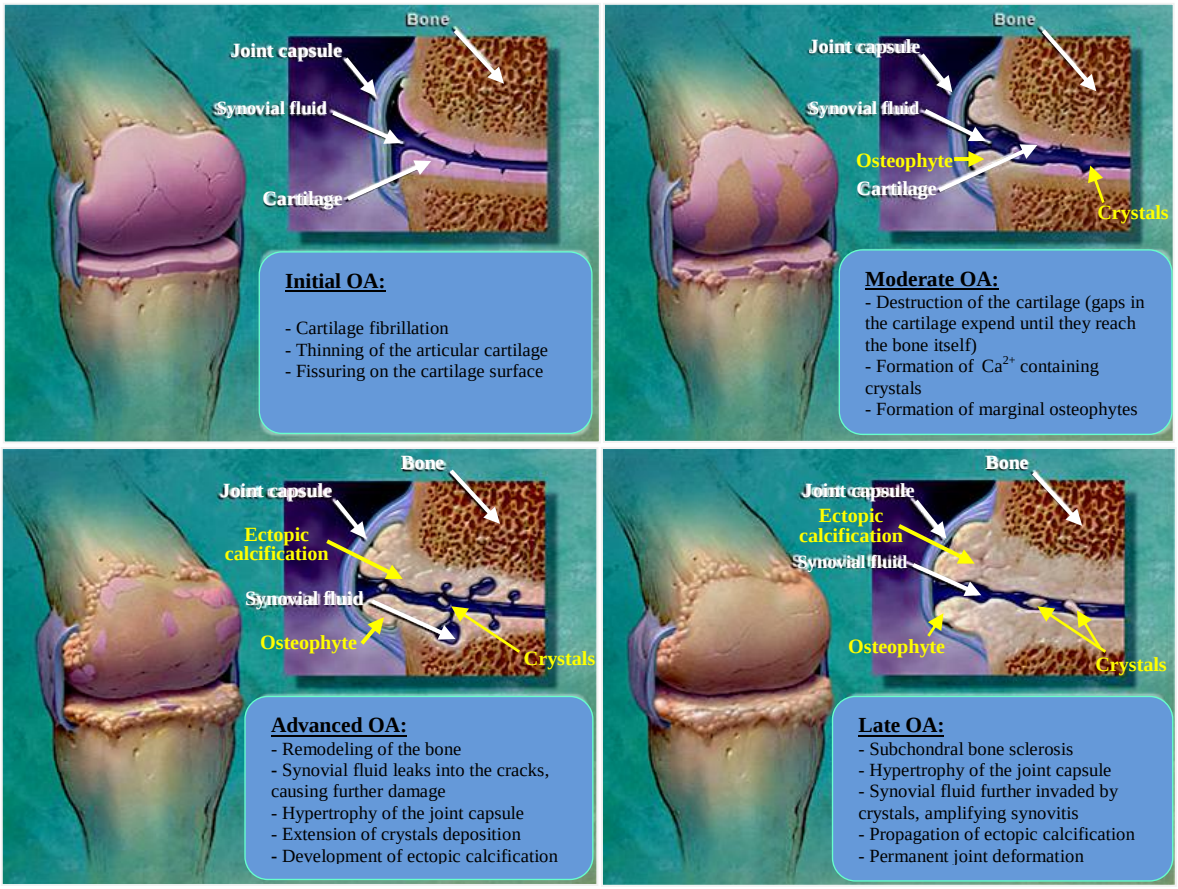


Figure 2.8- Progressive features along the OA illness (Adapted from reference 137).

Clinically, patients with OA may experience a spectrum of signs and intermittent symptoms that can include pain in and around the affected joints, tenderness, crepitus, stiffness, deformity, occasional effusion and variable degrees of local inflammation, with gradual loss of joint function and concomitant disability [1, 13, 70, 136].

2.5. Diagnosis

An accurate diagnosis of OA is the first important step in ensuring appropriate management of the disease. In a clinical setting, diagnosis is made in the course of a thorough medical examination and radiographic findings, normally using established methods and clinical guidelines that are used as a diagnostic reference; keeping always in mind that there is significant patient-to-patient variability in the time course of the disease [13, 138-140].

Evidence-based recommendations were defined by the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR). Regardless the pathophysiological features of OA being well described, differences between joint sites should be considered to improve case ascertainment, since inconsistencies are expected, as articular changes generate signs and symptoms within different joints (table 2.3) [15, 138-140].

Table 2.3- The ACR criteria for OA diagnosis of the hand, hip and knee [13, 15, 140].

Joints	Criteria	Items required for OA diagnosis
Hand	**Clinical**	1, 2, 3, 4 or 1, 2, 3, 5
	1. Hand pain, aching or stiffness for most days of prior month	
	2. Hard tissue enlargement of ≥ 2 of 10 selected hand joints	
	3. Metacarpophalangeal swelling ≤ 2 joints	
	4. Hard tissue enlargement of ≥ 2 distal interphalangeal joints	
	5. Deformity of ≥ 1 of 10 selected hand joints	
Hip	**Clinical and Radiographic**	1, 2, 3 or 1, 2, 4 or 1, 3, 4
	1. Hip pain for most days of the prior month	
	2. Erythrocyte sedimentation rate ≤ 20 mm/h	
	3. Radiographic femoral and/or acetabular osteophytes	
	4. Radiographic hip joint space narrowing	
Knee	**Clinical**	1, 2, 3, 4 or 1, 2, 5, or 1, 4, 5
	1. Knee pain for most days of prior month	
	2. Crepitus on active joint motion	
	3. Morning stiffness ≤ 30 min	
	4. Age ≥ 38 years old	
	5. Bony enlargement of the knee on examination	
	Clinical and Radiographic	1, 2 or 1, 3, 5, 6 or 1, 4, 5, 6
	1. Knee pain for most days of prior month	
	2. Osteophytes at joint margins	
	3. Synovial fluid typical of OA	
	4. Age ≥ 40 years old	
	5. Morning stiffness ≤ 30 min	
	6. Crepitus on active joint motion	

The EULAR evidence-based recommendations for the diagnosis of OA refer that, even though specific aspects are defined for each joint, a confident diagnosis should be done according to symptoms and signs on individual examination. The imaging diagnosis provides the morphological assessment of osteo-articular changes and occasionally other investigations should be considered for the analysis of atypical situations or to exclude other possible conditions [13, 138].

2.5.1. Imaging Analysis

2.5.1.1. Imaging Biomarkers

The National Institutes of Health (NIH) Biomarkers Definitions Working Group has defined a biomarker as ‘‘a characteristic, i.e. objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention’’. In essence biomarkers help to diagnose illness, measure its progression and verify how well preexisting or novel therapies work [141].

In OA, availability of biomarkers could provide useful diagnostic information by reflecting disease relevant biological activity, detecting joint degradation and predicting the course of pathological progression. But, although some biological markers of joint metabolism might be significantly increased in a group of patients with OA, these markers cannot be used as diagnostic tests in individual patients. The main biomarkers in current development for OA are imaging and biochemical markers; several studies are underway, but currently there are no reliable, quantifiable and easily measured biomarkers that provide an earlier diagnosis, information on the prognostic of disease and which can monitor responses to therapeutic modalities. There is still a huge and unmet need to identify, test, validate and qualify novel and well known biomarkers [5, 13, 141].

2.5.1.2. Radiography

Conventional radiography was the first broadly available radiological technique and it is the simplest, the least expensive and most widely used imaging technique for evaluation of a patient with a known or suspected diagnosis of OA. The X-ray has played a major role in the analysis and grading of OA, and is still considered to be the “gold standard” in cross-sectional and longitudinal studies. In research and clinical trials, radiography is the only European Medicines Agency (EMA) and Food and Drug Administration (FDA) recommended imaging modality for defining the inclusion criteria and efficacy end points of a clinical trial [142, 143].

Radiographic methods can detect OA-associated bony classical features including marginal osteophytes, subchondral sclerosis, and subchondral cysts and can also determine joint space with (JSW), an indirect surrogate of cartilage thickness and meniscal integrity (figure 2.9). These markers allow for the diagnosis and are used to assess disease development over time. Progression of joint space narrowing (JSN) is the most commonly used criterion for the estimation of OA evolution and the complete loss of JSW, characterized by bone-on-bone contact (figure 2.9), is one of the indicators for joint replacement surgery [13, 142, 143].

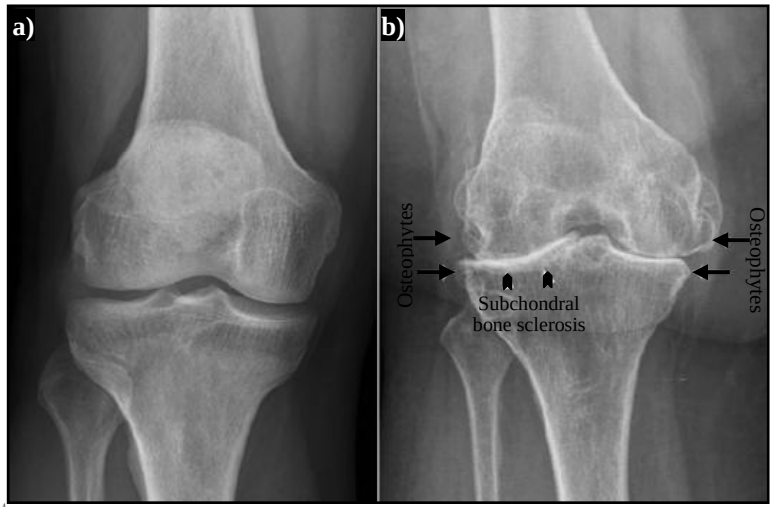


Figure 2.9- X-ray comparison of: **a)** normal knee;
b) end-stage OA knee, showing complete obliteration of the joint space (Adapted from reference 143).

The severity of OA can be estimated by semiquantitative scoring systems using published atlases that provide images that represent specific grades. All these criteria are recognized to have advantages and limitations, so there is currently debate as to what are the best radiographic definitions of OA. The most commonly accepted and widely used system was proposed by Kellgren and Lawrence (table 2.4) in their atlas of standard radiographs, more than five decades ago [15, 142-145].

Table 2.4- Kellgren-Lawrence radiographic grading system for OA diagnosis [15, 145].

Grade	X-ray findings	Diagnostic classification
0	None	Normal
1	Doubtful joint space narrowing (JSN) and possible osteophytic lipping	Doubtful
2	Definite osteophytes and possible JSN	Minimal
3	Moderate osteophytes, definite JSN, some sclerosis and possible deformity of bone	Moderate
4	Large osteophytes, marked JSN, severe sclerosis and definite deformity of bone	Severe

Radiography is a short time exam constituting a first line diagnostic imaging tool and an affordable technique. However, for OA diagnosis is a method with inherent limitations, namely, the lack of sensitivity and specificity for the detection of some pathologic features and the low reproducibility to follow-up in clinical studies. Although structural changes observed in X-ray images serve well as biomarkers for diagnosis in moderate to severe OA, they are not suitable for the identification of an early disease onset. The X-ray can only reliably evaluate bone and the other tissues of the joint and synovial effusion are either not evidenced or the visibility is of poor quality. The processes inherent to radiography project a three dimensional structure as a single two dimensional representation and, in doing so, lose important spatial and morphological information [13, 142, 143].

2.5.1.3. Magnetic Resonance Imaging (MRI)

The ability of MRI to image the joint as a whole organ and to directly and three-dimensionally assess cartilage morphology and composition plays a crucial role in understanding early disease onset and the natural history of the OA, representing one of the most promising fields for the assessment of pathological changes along the illness. MRI acquisitions are non-invasive and non-radiant, uniquely able to depict all the components of the diarthrodial joint and their pathologies, including hard and soft tissue structures [13, 142, 143].

This technique can detect the pathology of preradiographic OA and possible complications of the disease at a much earlier stage than radiography. MRI has a tomographic viewing perspective and thus provides cross-sectional images of the anatomy free of the projectional limitations of radiography [142].

In OA, MRI is nowadays used on a regular basis in longitudinal studies evaluation. Of special interest are the quantitative determination of cartilage (volume or thickness) and synovial fluid, semiquantitative assessment of subchondral BMLs, subchondral cysts, intra and periarticular cystic lesions and alterations in the synovial membrane [142, 143].

The main motives that are still limiting its use are the prolonged time to execute and evaluate the exam, its high cost and limited access [13, 15].

2.5.1.4. Ultrasound (US)

This method enables multiplanar and real-time imaging at a moderately low cost, without radiation exposure. High resolution US can demonstrate changes in cartilage, menisci, bone surface, synovial membrane, tendons, ligaments, joint capsule and bursae in initial to late stage OA. It has the ability to image soft tissue and to detect synovial pathology without the need for contrast administration. US have been demonstrated to be more sensitive than clinical examination in detecting synovial hypertrophy and joint effusion [142, 143].

The US major advantages lie in the safety and non-invasiveness of the technique, its increasing availability in rheumatology clinics, and the possibility of assessing multiple joints in the same session. Limitations of US are that it is an operator-dependent technique and that the physical properties of sound, the limited number and width of acoustic windows, hinder its application to deeper articular structures and the subchondral bone. The sound waves used for soft tissue assessment cannot penetrate bone which, in consequence, obstructs the view of many internal joint structures partially or completely. In addition, intrinsic bone alterations seen in MRI are not accessible with US. The sensitivity of detection of structural changes in early OA is not yet completely satisfactory but likely to improve with the help of enhanced techniques such as three-dimensional US. Regardless of these limitations, US have proven to be an interesting resource to guide therapeutic interventions [142, 143].

2.5.1.5. Computed Tomography (CT)

CT is ideally suited to the application of novel computational analysis techniques, it provides excellent imaging information on bone, being a valuable tool for the characterization of OA, particularly when imaging of osseous changes or detailed presurgical planning is required. It can provide superior information about bone structure, including joint morphology and standard morphometric parameters such as bone volume fraction and trabecular thickness [142, 146].

The advantages of CT are the short acquisition time and excellent depiction of bone and calcified periarticular structures. This technique could be used in OA for specific indications such as the assessment of axial joints which are difficult to assess by conventional X-ray, detection of calcified intra-articular loose bodies or joint infiltration [143]. Cortical bone and soft tissue calcifications are better portrayed than on MRI [142].

This technique is not suitable for detection of cartilage deterioration, synovial membrane, menisci or ligaments, making it a modality of restricted interest for use in OA. The major disadvantage of using CT lies in the exposure to radiation, especially in longitudinal studies requiring repetitive assessments [143].

CT arthrography is an alternative method for indirect visualization of cartilage and other intrinsic joint structures, especially in the knee joint. CT arthrography may be relevant especially where access to MRI facilities is limited or when MRI is contraindicated. Limitations of this modality are its insensitivity to changes of the deep layers of cartilage without surface alterations and its invasive nature [142, 146].

2.5.1.6. Nuclear medicine

This imaging method involves the administration into the patient of radioactive agents consisting of substances with affinity for certain body tissues labeled with radioactive tracer.

Scintigraphy uses radiopharmaceuticals to visualize skeletal metabolism, to contribute to the localization of disease, and to assess the severity of pathologic changes in subchondral bone in OA. As what happens with CT, the principal disadvantage of this technique lies in the exposure to radiation [142, 143].

Positron emission tomography uses radioactively labeled glucose (2-[18F]-fluoro-2-deoxy-D-glucose) to demonstrate metabolic changes in target tissues and can detect foci of inflammation in OA. Although this technique alone is highly nonspecific, its combination with CT is also available and combines the advantages of both techniques, the demonstration of elevated tissue metabolism and high image resolution of calcified tissue. Its low specificity, high radiation exposure, the high costs and low availability of this method, which is limited to a few specialized centers, make it not truly suitable to assess biomarkers in daily practice [142, 143].

2.5.2. Laboratory Analysis

The diagnosis of OA is generally based on clinical and radiographic changes, which occur very late during the disease pathogenesis pathway and have poor sensitivity for monitoring the dynamic changes in the joint and disease progression. Therefore, the OA research community has focused on measurement of biomarkers of joint tissue turnover in body fluids. The maintenance of the joint is compromised in OA and the components are degraded by catabolic enzymes in response to inflammatory mediators, producing fragments that are released into synovial fluid and the general circulation. The identification of such fragments in synovial fluid, serum and urine does not consistently correlate with radiographic changes and symptoms, such as pain and loss of mobility [15, 141].

Laboratory tests are not frequently required for OA diagnosis but could be used in patients with suggestive symptoms or signs, in conjunction with biomedical imaging, to complement the diagnosis, confirming or excluding coexistent inflammatory disease (e.g. RA, gout, pseudogout), helping to establish the stage of disease and predict its progression [8, 13].

2.5.2.1. Biochemical Biomarkers

Approximately 10 years ago it became apparent that the OA researchers needed a definition of biomarkers for their own community. The biomarkers group of the OARSI-FDA initiative stated that there are no FDA qualified OA-related biochemical biomarkers, although there are many that have shown associations with some aspect of OA and that fulfill one or more aspects of the BIPED classification scheme; this scheme classifies the major types of biomarkers into categories corresponding to Burden of disease, Investigational, Prognostic, Efficacy of intervention and Diagnostic biomarkers (table 2.5) [5, 8, 39, 141].

Table 2.5- Principal biochemical biomarkers of OA [8, 143].

Tissue	Biochemical Marker	Body Fluid	Putative Process	BIPED
Bone	NTX-I	Serum and Urine	Type I collagen degradation	PED
	CTX-I	Serum and Urine	Type I collagen degradation	BPD
	Osteocalcin	Serum	Anabolic bone turnover	BPED
Cartilage	C2C	Serum and Urine	Type II collagen degradation	PED
	CTX-II	Urine	Type II collagen degradation	BPED
	Coll 2-1 and Coll 2-1 NO_2	Serum and Urine	Type II collagen degradation	BPD
	Chondroitin Sulfate epitope 846	Serum	Cartilage aggrecan synthesis/turnover	PE
	Keratan Sulfate	Serum	Aggrecan degradation	BPED
	PII ANP	Serum	Type II collagen synthesis	BPD
	PII CP	Serum	Type II collagen synthesis	PD
	PII NP	Urine	Type II collagen neoepitope	BP
Multiple tissues	C1,2C	Serum and Urine	Types I and II collagen degradation	D
	Cartilage Oligomeric Protein	Serum	Cartilage degeneration	BPD
	Hyaluronic Acid	Serum	Increased HA turnover	BPED
Proteinases	Matrix Metalloproteinases-1,3,13	Serum	Joint tissue degradation	BPE
Synovium	Glucosyl-Galactosyl-Pyridinoline	Urine	Collagen fibril degradation in synovium	BED

C1,2C: assay that detects COL2-¾C (short) epitope; C2C: assay that detects COL2-¾C (long) epitope; Coll 2-1: 9-amino-acid peptide of type II collagen; Coll 2-1 NO2: nitrated form of Coll 2-1; CTX-I: carboxyl-terminal cross-linked telopeptide of type I collagen; CTX-II: C-terminal cross-linked telopeptide of type II collagen; NTX-I: N-terminal cross-linked telopeptide of type I collagen; PII ANP: N-propeptide IIA of collagen type II; PII CP: C-propeptide of collagen type II; PII NP: N-propeptide II of collagen type II.

The previous classification, although reflecting the current findings of the literature, is subject to ongoing change as new markers are developed on a continuous basis and knowledge of the known markers increases constantly. There is a need for reliable biomarker tools that can detect the disease in its molecular and pre-radiographic stage, long before structural and functional alterations in tissue integrity have occurred, to facilitate earlier OA diagnosis and understand its progression. However, finding biomarkers with the sensitivity and specificity to achieve this remains a challenging problem [13, 141, 143].

2.5.2.2. Assessment Methods

Measurement of the biochemical markers is, most commonly, done by enzyme linked immunosorbent assays (ELISAs). The commercially available assays are competitive inhibition and sandwich ELISAs. Other less frequently used techniques are enzyme immunoassays (EIA), radioimmunoassay (RIA) and liquid chromatography-mass spectrometry (LC-MS) assays [143].

2.5.2.3. Crystals Examination

The clinical and pathological relevance of cartilage mineralization in patients with OA is not completely understood. A clear understanding of the relationship between calcium crystal deposition and OA is limited by the lack of a widely available and simple technique for the examination of these crystals. Detection of calcium phosphate crystals in samples aspired from the intra-articular fluid from the OA affected joints is often pivotal for early diagnosis and appropriate management of the disease [124, 147, 148].

BCP crystals have been associated with OA and therefore their presence, can aid diagnosis of the disease. The detection of these crystals in the synovial fluid of patients with OA is fraught with challenges. Synovial fluid is a very viscous and an intricate biological matrix to work with analytically. It contains many different constituents that can interfere with the detection of the BCP crystals or even other crystal-associated arthropathies, as for example calcium pyrophosphate dihydrate (CPPD), monosodium urate (MSU) and calcium oxalate (CO). Hence, removal of the biological material before analysis of the crystals is often necessary. BCP crystals can be isolated from synovial fluid by means of chemical and/or enzymatic approaches. The utilization of hydrazine is a commonly used method for deproteination of the crystals and it induces only minor chemical transformations and no structural changes of the mineral phase [128, 147, 148].

Another reason for the difficulty in the identification of BCP is their sub-micron size dimension. BCP crystals are a group of ultramicroscopic crystalline substances primarily composed of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, and its precursors octacalcium phosphate $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$ and tricalcium phosphate $Ca_3(PO_4)_2$. The stability of these crystal structures within the synovial fluid environment is of great importance since it is believed that they are far from being inert. Crystals are exposed to many physical forces during laboratory procedures, thus, precautions have to be taken in the handling of specimens from joint fluid and of those made in the laboratory to avoid false negative/positive results during the analysis [128, 147, 148].

Some progress has been made in the past few years in the detection of intra-articular pathological microcrystals. Histological stains such as Alizarin Red and Von Kossa are sometimes used to analyze the presence of calcium deposits in the synovial fluid. There are a number of analytical techniques that have been applied to the detection of BCP crystals, either directly or indirectly (table 2.6) [124, 128, 147, 148].

Table 2.6- Analytical methods applied in BCP detection [148].

Analytical Methods	Principal Advantages	Principal Disadvantages
- Imaging:		
Light and Polarized Microscopy	Inexpensive, widely available and useful for the detection of larger crystals (>1μm) that can occur	Inaccurate, non-specific and cannot detect BCP crystal; polarized microscopy can aid detection of CPPD crystals
Microscopy with Staining	Inexpensive, widely available and can identify BCP crystal clumps	Sensitive but non-specific, with frequent false positive results
Multi-dimensional Microscopy	Coupling of multi-channel, spectrophotometer and 3D relief imaging system to a microscope laser light scattering spectroscope	Not usually available and do not evidence a clear identification of synovial fluid crystals
Transmission Electron Microscopy	Uses small sample size and can be used in conjunction with electron diffraction	Expensive, complex, not usually available and operator-dependent
Scanning Electron Microscopy	Small sample size and can be coupled to X-ray elemental analysis	Expensive, complex and relies on morphology
Atomic Force Microscopy	Uses small sample size with minimal preparation; can exploit hardness and lattice features in sample for detection and identification, with specificity	Operator-dependent, intricate to use on liquid samples and relies on morphology
-Spectroscopic:		
Fourier-Transform Infrared Spectroscopy	Accurate method, used for automated pattern recognition modalities	Can be misinterpreted, with water interferes in certain parts of the spectrum
Raman Spectroscopy	Accurate method, that water does not interfere, with unique spectral signatures for each crystal type	Expensive, fewer library spectra available; requires sample purification to distinguish between various crystal types
Fluorescence	With correct dyes, can be very sensitive and selective	Requires special equipment; dyes can be expensive and it is not suitable for *in vivo* use
Nuclear Magnetic Resonance / Magnetic Resonance Imaging	Able to visualize most types of pathologies, including crystal depositions	Very expensive, cannot identify nature of crystal deposition and is operator-dependent
- Other Techniques:		
Calcium and Phosphorus Analysis	Well-understood assays such as Atomic Spectrometry, UV-Vis Spectrophotometry, etc.; can be selective and sensitive	Practical only in analysis of dissociated crystals, other matter present in the fluid can interfere
X-Ray Diffraction	Accurate technique for unambiguous identification	Requires sample purification, dried and quantity
Capillary Electrophoresis	Can be applied for pattern recognition analysis of synovial fluids containing crystals	Not applicable for direct separation of crystals
Radioassay	Allows semi-quantitative determination of BCP	Involves radioactive reagents
Ferrography	Provides separation of particles by size/magnetic properties	Requires special sample preparation, only reflects general content in the synovial fluid

2.6. Pharmacological Therapies

Following the expanded understanding of OA disease and of its clinic manifestations, the prescribing approaches have changed over time. The intricacy and variability of OA condition infers that illness management should be tailored specifically to each individual case and the identification of the affected joints should be taken in consideration.

The recommendations made by the foremost institutional organizations in the field (OARSI, ACR, EULAR) were recently reviewed and updated. They assert that no single therapy is adequately effective to control OA affliction and suggest that a proper treatment should involve a combination of nonpharmacological and pharmacological modalities [149-153].

Because OA is a chronic disease, most common in elderly and polymedicated people, safety remains critical; the pharmacological treatments should provide improvement of health related quality of life, avoiding drug interactions and toxicity. When choosing an appropriate treatment, from among the available agents, the balance between improvements and adverse events should always be considered, trying to achieve a positive benefit-risk profile.

2.6.1. Symptomatic Treatments

Despite the increasing number of patients, currently, there is no known cure for OA and there are no therapies which slow or arrest the disease progression. The major goals of OA pharmacological approaches remain symptomatic (table 2.7), by treatment interventions focused on pain relieve and maintenance of joint mobility and function [154].

Table 2.7- Pharmacological symptomatic treatment options for OA management [149-154].

Treatment Type			Adverse Effects
Symptomatic Treatment	**Oral**	Paracetamol (Acetaminophen)	Gastrointestinal discomfort and possible bleeding; renal failure; hypertension; hepatotoxicity.
		Non selective Non Steroidal Anti-Inflammatory Drugs (NSAID)	Gastrointestinal ulcer and bleeding; cardiovascular events; renal events.
		Selective NSAID	
		Opioids	Tolerance; dependence; constipation; urinary retention; vomiting; nausea; dizziness; somnolence; morbidity and mortality in the elderly.
		Duloxetine	Constipation; nausea; hyperhidrosis.
	Topical	NSAID	Skin reactions; mild gastrointestinal events.
		Capsaicin	Skin burning sensation and skin desensitization.
	Injectable	Intra-articular Corticosteroids	Local infection; systemic effects.
		Intra-articular Hyaluronic Acid	Local reactions at the site of injection, swelling, flares of pain.
Slow-acting Symptomatic Treatment	**Oral**	Glucosamine and Chondroitin Sulfate	Epigastric pain or tenderness, heartburn, diarrhea, and nausea.
		Diacerein	Lower gastrointestinal effects, diarrhea, and discoloration of urine.
		Avocado Soybean Unsaponifiables (ASU)	Skin hypersensitivity; mild gastrointestinal discomfort; possible interference with blood coagulation.

2.6.1.1. Oral Drugs

Pharmacological guidelines for the management of OA recommend paracetamol as the first-line therapeutic agent for mild-to-moderate pain. If found to be successful at alleviating pain, it is suggested that paracetamol be the preferred long-term oral analgesic, because of its low cost, efficacy and safety profile, for doses not exceeding 4 g per day [149-153].

Obviously, the use of analgesics should take in to consideration the clinical context; even at therapeutic doses, paracetamol can provoke asymptomatic elevation of liver enzymes in healthy people, so it is recommended that acetaminophen be avoided in patients with chronic alcohol abuse and should not be used in people with existing liver dysfunction. Furthermore, with long-term consumption and doses of up to 3 g per day, some adverse effects have been reported, including gastric ulcerations, increased risk of mild loss of renal function and hypertension (table 2.7) [149].

For patients who are unresponsive to appropriate dosages of paracetamol or with signs of clinical inflammation, a switch to nonsteroidal anti-inflammatory drugs (NSAIDs) is generally recommended, prescribed at the lowest effective dose and for the shortest possible time duration. NSAIDs are known to be more effective for pain relief, although, their use is limited by a number of adverse situations, including gastrointestinal, renal and cardiovascular adverse effects that usually increase with age. In individuals with higher gastrointestinal risk, guidelines recommend the use of the nonselective NSAID combined with a proton-pump inhibitor or misoprostol or even a selective COX-2 inhibitor. The COX-2 inhibitory drugs celecoxib and rofecoxib are usually as effective as conventional NSAIDs for pain relief; with fewer gastrointestinal complications, but might carry an augmented cardiovascular risk [149-154].

In the latest years, opioids have become a widely prescribed class of medicines for patients with symptomatic knee and hip OA, who have not had an adequate response to the other pharmacological agents and are either unwilling to undergo or are not candidates for joint arthroplasty. It is common to start the treatment with a weak opioid, such as codeine or tramadol, often in combination with paracetamol and, if ineffective or not tolerated, to use a stronger opioid like hydrocodone, oxycodone, morphine, or transdermal fentanyl. However, adverse conditions are frequent and significant, including constipation, urinary retention, nausea and vomiting, sedation and respiratory depression (table 2.7). The use of opioids is still controversial, being recommended that the treatment starts at a low dose and gradually adjusted upwards; avoiding long-term use, or at least regularly reevaluated. The benefits of using opioids should be evaluated as judiciously as possible. In elderly people, opioids may induce severe injuries from falls and sometimes are even responsible to cause death [149-153].

The chronic pain often observed in OA has been shown to involve centrally-mediated pain pathway dysfunction, promoting the study of pharmacologic agents that have a centrally-mediated action. Duloxetine is a selective serotonin and norepinephrine reuptake inhibitor (SNRI), with central nervous system activity; its analgesic efficacy in central pain is putatively related to its influence on descending inhibitory pain pathways, improving pain and function in knee OA [150, 151, 155].

2.6.1.2. Topical Drugs

In the case of persons with hand or knee OA, who have mild to moderate pain, it is appropriate the use of topical analgesics, as adjunctive treatment or as an alternative monotherapy. Topical therapies should be applied to the symptomatic joint tree, four times a day and represent an interesting treatment modality that can be used to decrease the consumption of analgesics.

The analgesic and anti-inflammatory action of topical NSAIDs, such as diclofenac, etofenamate, ketoprofen, indometacine and some others, is related with the local suppression of prostaglandin synthesis. They seem to be as effective as oral NSAIDs, with the advantage of lower risk of systemic exposure and gastrointestinal complications; their principal reported adverse effect is local skin reactions (table 2.7). For patients older than 75 years of age, the main guidelines recommend their use in the initial management of OA and prefer them to oral NSAIDs [149-153].

Another topical agent frequently recommended is capsaicin that can cause depletion of substance P from sensory nerve endings, reducing or abolishing the transmission of painful stimuli. A local burning sensation is the most common adverse effect, particularly during the first week of application, but rarely leads to the discontinuation of the treatment. However, its effectiveness and safety remains controversial; is still unclear if long-term capsaicin treatment can cause persistent skin desensitization that may not be totally reversible [149-152].

2.6.1.3. Injectable Drugs

When OA patients do not respond to oral analgesics neither to topical options, then usually the application of intra-articular injections is considered.

The ACR guidelines recommend that knee and hip OA patients receive intra-articular anti-inflammatory corticosteroid injections. Short-term pain reduction in knee OA occurs after 2 to 3 weeks, but without significant effect on function. For pain relief from OA flares, especially if accompanied by effusion, is recommended intra-articular injection of a long-acting corticosteroid; but they present the disadvantage of the systemic steroidal effects [150, 151].

Intra-articular hyaluronate (HA) injections (viscosupplementation) is recommended for knee OA and could present beneficial symptomatic effect, despite some reported local acute reactions such as transient pain and swelling at the injection site (table 2.7). Compared with intra-articular corticosteroids, intra-articular hyaluronic acid injections show delayed but prolonged efficacy [149, 151].

2.6.1.4 Slow-acting Symptomatic Drugs

There is a wide variability throughout the world in the use of these agents and how they are classified. In some countries, they are considered health food supplements, rather than prescribable drugs, only available over-the-counter and usually self-administered.

During the past 10 years, glucosamine and chondroitin sulfate have been prescribed and used for symptom relief by OA patients. These compounds appear to be gaining popularity among consumers. Glucosamine is a structural component of glycosaminoglycans (GAGS) and chondroitin a sulfated GAG and a component of the normal articular cartilage. Concern in these substances has been tempered by lack of a plausible mechanism to enlighten how they might achieve a therapeutic effect.

The symptomatic effect of glucosamine varies and is still considered controversial. In fact the results of the clinical trials greatly depended on the different products used, the study population and study design quality. Glucosamine and chondroitin sulfate have been approved in Europe [151-153], but not in North America, where they are regulated not as medicines but as nutraceuticals; consequently, a substantial variation in their content is possible. This explains why, in the latest ACR guidelines, these agents were not recommended as options for OA treatment [150]. Nevertheless, OARSI guidelines recommend the use of glucosamine sulfate and chondroitin sulfate, as they demonstrate OA pain relief with a moderate to large effect size [149].

These compounds are associated to few side situations, the most common adverse effects are epigastric pain or tenderness, heartburn, nausea and diarrhea (table 2.7). Glucosamine, as a product derived from lobster, crab or shrimp shells, may cause allergic reactions in patients with seafood allergies and may also interact with some pharmaceuticals, such as warfarin and diabetic medications, dangerously modifying their therapeutic efficacy.

Diacerein is an anthraquinone derivative that provides sustained pain relief during several weeks after discontinuation and an analgesic-sparing effect in patients with knee and hip OA. Moreover, their effects have been found to be additive to that of NSAIDs. Diacerein presents a reasonable tolerability and a good overall safety profile; the most frequent adverse events are loose stools and diarrhea (table 2.7) [149, 151, 152, 156].

Avocado soybean unsaponifiables (ASU) are fractions of natural vegetable extracts, made of one third avocado and two thirds soybean oils. ASU is a complex mixture of several compounds including fat-soluble vitamins, sterols, triterpene alcohols and possibly furan fatty acids. The symptomatic efficacy of ASU has been assessed and the preclinical studies demonstrated that they present some beneficial effects in patients with knee and hip OA, with a carry-over effect that persists after treatment discontinuation [157-159].

Although significant progresses have been made in amelioration of the secondary effects of the disease, the available medicines fail to address the evolving and complex nature of OA. These aforementioned therapeutic options are scarce, neither exclusive nor sufficient; with more than 75% of the OA patients reporting need for additional treatment. The major unmet necessity is for pharmacological entities that are able to stop, delay, or reverse the progression of the joint structural damages [160-162].

2.6.2. Structure Modifying Treatments

Application of new sources of knowledge about the disease process holds promise for moving toward the discovery of substances with the potential to become disease modifying pharmaceuticals that can be taken in association with the conventional therapeutic strategies to provide more effective treatment for OA.

Along the latest years, various attempts are being developed to find new pharmacological agents to halt or slow the progression of OA, and they are collectively known as disease modifying osteoarthritis drugs (DMOADs) [160-162]. There are draft guidelines from the EMA and FDA both requiring that a DMOAD should not only inhibit radiographic structural disease progression, but also ideally achieve patient-reported long-term clinical benefit, improving symptoms manifestations and function [163, 164].

The major challenges in the DMOADs search include the establishment of suitable preclinical animal models that reflect human OA, restrictions of the imaging tools for structural evaluation, the few biochemical biomarkers and the limitations in the clinical trials that are hindered by slow and unpredictable disease progression. Moreover, an ideal DMOAD should be used in the early disease stage, before irreversible molecular and biomechanical pathology is established, and should demonstrate a good safety profile with respect to both patient tolerance and drug interactions. So far, there are no licensed DMOADs but, alongside the contemporary innovations in the pharmaceutical drug discovery environment, some prospective agents are under investigation (table 2.8) [35, 160-162].

Table 2.8- Prospective Disease Modifying Osteoarthritis Drugs (DMODs) under investigation [160-162].

Treatment Target	Potential DMOADs	Mechanism of Action
Cartilage	Matrix Metalloproteinase (MMP-13) Inhibitors	Anticatabolic
	Tissue Inhibitors of Metalloproteinases (TIMP-3)	Anticatabolic
	Fibroblast Growth Factor 18 (FGF-18)	Anabolic
	Bone Morphogenetic Protein-7 (BMP-7)	Anticatabolic and Anabolic
	Inducible Nitric Oxide Synthase (iNOS) Inhibitors	Anticatabolic
	Cathepsin K Inhibitors	Anticatabolic
	Diacerin	Anticatabolic
	Doxycycline	Anticatabolic
	Glucosamine and Chondroitin Sulfate	Anticatabolic and Anabolic
	Avocado Soybean Unsaponifiables (ASU)	Anticatabolic and Anabolic
	Vitamin E	Anticatabolic
Subchondral Bone	Calcitonin	Anticatabolic
	Bisphosphonates	Anticatabolic
	Strontium Ranelate	Anticatabolic and Anabolic

2.6.2.1. Disease Modifying Osteoarthritis Drugs Targeting Cartilage

Synthetic metalloproteinases (MMP) inhibitors have failed the clinical trials due to the frequent development of a painful musculoskeletal syndrome that has previously held back research in this area [161, 162]. More recently, more selective MMP-13 inhibitors were developed, with chondroprotective properties and the potential to modulate joint pain [165]. Until now there is no report of MMP inhibitors entering the phase III of trials for their clinical use as DMOADs [162].

Tissue inhibitors of metalloproteinases (TIMPs) are regulatory proteins consider as endogenous inhibitors of the metalloproteinases and consequently important regulators of ECM turnover, tissue remodelling and cellular behaviour. Deficiency of TIMP-3 was associated with mild cartilage degradation in knockout mice similar to changes seen in patients with OA. Hence, TIMP represent a potential DMOAD target and selective MMP inhibitors have been engineered based on these proteins [166].

Fibroblast growth factor-18 (FGF-18) was reported to have significant anabolic effects on cartilage. In animal models, it has been shown to promote chondrogenesis, cartilage repair, and subchondral bone remodelling [167]. This potential DMOAD is currently undergoing phase II clinical trials, to evaluate changes in cartilage volume [161, 162].

Human BMP-7, also known as osteogenic protein-1 (OP-1), is member of the bone morphogenetic protein (BMP) family, involved in cartilage homeostasis that, unlike other members of its family, has not only pro anabolic activities but also very strong anti-catabolic properties. OP-1 levels in joints can show a significant decrease with aging, if this decline is pathologic, the use OP-1 to slow the progression of OA associated to cartilage loss could be considered [168]. The efficacy of this agent in regeneration of articular cartilage lesions was examined in animal studies, demonstrating that a recombinant BMP-7 stimulates ingrowth of mesenchymal cells into the chondral defects, which then transforms into newly formed articular cartilage-like tissue [169]. Human chondrocytes also promote cartilage formation in response to treatment with low doses of BMP-7, which leads to a production of ECM components [170]. Clinical trials have already started to evaluate the efficacy of BMP-7 in the modification of structural damage of human knee OA [162].

Several compounds that inhibit iNOS are under investigation for their use as potential DMOADs. This enzyme is upregulated in OA and produces NO that, along with its primary metabolites, is toxic to cells and contributes to the damage and degradation of cartilage matrix and synovial tissue. In vivo studies using a canine model of OA, the selective inhibition of iNOS significantly reduced articular cartilage degradation and the number and size of osteophytes [171-173]. More studies need to be performed to clear de role of iNOS inhibition, since the results of a recent two year randomized, double-blind, placebo-controlled trial testing an oral and selective iNOS inhibitor, showed only a transient slowing of JSN, in Kellgren-Lawrence grade 2 Knee OA. Moreover the results were not sustained after two years and no significant evidence of inhibition of structural progression was seen in OA of higher severity [174].

Cathepsin K is a cysteine proteinase involved in bone resorption and appears to contribute to cartilage damage, being correlated with OA severity. In preclinical studies, cathepsin K inhibition resulted in a reduced evidence of cartilage degeneration in the anterior cruciate ligament transection rabbit and murine models of OA [175]. Contradictory results have been found in a 6 months trial with knee OA patients treated with cathepsin-k inhibitor, which during the time of the treatment showed no significant differences in cartilage volume measured by MRI [176].

Diacerein, a purified compound with anthraquinonic structure, has been shown to inhibit the production and activity of IL-1 and the secretion of metalloproteases, which may lead to a positive influence on cartilage homeostasis and subchondral bone remodelling to

prevent joint destruction. A study has confirmed the previous clinical findings, indicating that the demonstration of a structure-modifying effect in hip OA is feasible; showing that a 3 years treatment with diacerein resulted in a reduction in the macroscopic and microscopic lesions of the articular tissues [177]. In this case a secondary side-effect of diarrhea may have limited the validity of the double-blind design of the trial. Another study evaluating the efficacy and safety of diacerein in knee OA patients, showed radiographic structural improvement, including reduction in osteophytes and widening of the joint spaces [178].

Doxycycline is a tetracycline antibiotic and, although there is no evidence to support an infectious etiology in OA, tetracyclines inhibit collagenase levels and iNOS activity, thereby decreasing chondrocyte MMP activity and increasing proteoglycan synthesis [179]. A randomized, placebo-controlled, double-blind trial was conducted including 431 obese women with unilateral radiographic knee OA and doxycycline was shown to reduce the mean loss of joint space width (JSW) by approximately 30% at 30 months; however, the mean progression of JSN in both groups was limited. It is unknown whether a statistically significant slowing of radiographic progression is clinically significant and can predict an enhanced clinical outcome. Moreover, doxycycline did not significantly prevent the onset of progressive JSN in the contralateral knee, and did not improve measures of pain or function in this study [180].

The availability of glucosamine and chondroitin sulfate substrates may limit the formation of cartilage; therefore oral supplementation with these components could be considered a sound strategy for positively influence OA development. Clinical studies of these two agents, given either in combination or separately, have yielded variable results with regard to reducing both symptoms and progression of OA. This may be, in part, due to mostly small numbers of patients enrolled and methodological differences, as well as different bioavailability of the preparations. A recent meta-analysis concluded that there is no structural modifying effect using these substances, based upon trials using JSN as clinical end point [181]. Inferences cannot be drawn about the potential of glucosamine and chondroitin sulfate in changing the OA pathologic progression; the structural effects of these substances remain under debate, so these treatments are not registered as structure-modifying agents [149, 151-153].

A growing body of evidence has shown that the dietary supplement avocado/soybean unsaponifiables (ASU) presents beneficial effects on cartilage, due to its chondroprotective, anabolic and anticatabolic roles, as well as anti-inflammatory properties [157, 182, 183]. An experience developed with a mice OA model, treated with ASU, pointed to a significant

reduction in cartilage degeneration [184]. One recent *in vitro* study proposed a mechanism of action for ASU, as preventing the OA osteoblasts-induced inhibition of matrix molecules production, suggesting that this supplement may promote OA cartilage repair by acting on subchondral bone osteoblasts [185]. Another more recent trial reinforced the promising potential structure-modifying effect of ASU, reporting that a three year treatment with this supplement reduced the percentage of JSW progression in hip OA [186]. Further research is required to determine the specific bioactive compounds, target molecules and mechanisms of actions associated with ASU that might be beneficial as novel nutraceuticals for treatments of OA patients.

One of the etiological factors for OA is free radical injury, therefore it is conceivable that exogenous supplementation with vitamin E, a phenolic antioxidant with additional anti-inflammatory action, should be useful for the prevention and the control of disease progression [187, 188]. Vitamin E was identified as a potential DMOAD following an *in vitro* study using rabbit articular chondrocytes, evidenced a decreased in cartilage collagen oxidation and degradation in the presence of the antioxidant [189]. Unfortunately, clinical trials testing the efficacy of vitamin E in the treatment of OA have been methodologically weak and have produced contradictory findings [190, 191].

2.6.2.2. Disease Modifying Osteoarthritis Drugs Targeting Subchondral Bone

Calcitonin is one of the natural regulators of calcium and phosphate homeostasis in the body. It has the ability to counteract the blood calcium increasing effects of PTH and therefore inhibits osteoclast bone reabsorption [192]. Its inhibition of subchondral bone turnover could be chondroprotective, since it may restrain the structural disease progression of OA [193]. A recent human trial, with 14 days of administration of oral salmon calcitonin resulted in a reduction of the biomarkers of bone resorption and cartilage degradation [194]. In a 2-year phase III trial of knee OA, oral calcitonin modified symptoms and increased cartilage volume, although improvement on the primary endpoint of JSW was not reached [195].

Bisphosphonates are interesting candidates for OA treatment based on their known pharmacology of altering bone remodelling through a direct inhibitory effect on the osteoclasts and because they may also have activity as immunomodulators, via inhibition of pro-inflammatory cytokines [195]. Some animal models identified the beneficial effects of bisphosphonates in OA, through their impact on subchondral bone, which includes inhibition of remodeling and osteophyte formation along with decreased vascular invasion of calcified

cartilage [197, 198]. Several human trials are being developed with different antiresorptive agents, even though, at present, evidences of clinical benefit for OA patients treated with bisphosphonates are still lacking [161].

Strontium ranelate influences bone remodeling through calcium-sensing receptors on osteoclasts and osteoblasts in subchondral bone and by an antiresorptive action via inhibition of osteoclastogenesis. *In vitro* studies suggest that this agent has anabolic effects on cartilage by directly promoting formation of human cartilage matrix [199]. In a study of human spinal OA, strontium ranelate reduced cartilage degradation markers and inhibit clinical symptoms and radiographic features [200]. A double-blind, placebo-controlled, randomized, international 3-year study of knee OA demonstrated a chondroprotective effect and symptomatic improvement, suggesting strontium ranelate as a potential DMOAD [201].

In the current OA pharmacological treatment, agents that relieve pain rarely have structure modification benefits and might, in some cases, be detrimental to the joint, whereas potentially successful structure modifying agents often fail to provide pain relief. Evidences to support the DMOADs do not yet provide adequate proofs for their use in clinical practice, but clearly suggest that the concept is feasible, with the recent discovery of several effective substances and the identification of the subgroups of OA patients in which they could be helpful.

3. Vitamin K

3.1. Discovery

Vitamin K was discovered during the years 1928-1930, when Carl Peter Henrik Dam, was studying the metabolism of cholesterol in chicks and noted that when their food was extracted to remove the sterols, the animals suffered from hemorrhages and their blood clotted slowly. He also found that supplementing the diet with cholesterol did not solve the problem and he hypothesized that this might be due to another lipid soluble compound that had been extracted from the animal food. This compound was designated as vitamin K according with the Scandinavian word 'koagulation' [202, 203].

McFarlane and his collaborators, also working with chicks, at the Ontario Agricultural College in Guelph, Canada, also described a clotting defect on the birds, in experiments using different protein diets sources [204].

For the isolation of the vitamin, Dam in collaboration with Paul Karrer in 1939, succeeded to separate the compound as a yellow oil from alfalfa [205]. Another research group led by Edward Adelbert Doisy, also working on vitamin extracts of alfalfa and putrefied fish products; identified the active substances of the lipid extract as quinones [206].

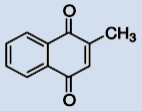
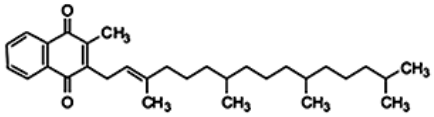
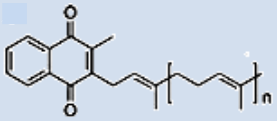
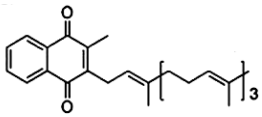
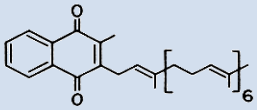
In 1943, both researchers Dam and Doisy received the Nobel Prize for Physiology or Medicine for their findings in vitamin K studies.

3.2. Characterization

Vitamin K includes a series of essential fat soluble family of compounds synthesized by plants and bacteria. The term vitamin K is used as a generic descriptor for the structurally-related compounds that have a common 2-methyl-1,4-naphthoquinone nucleus but differ in the constitution of a side chain at the 3-position and the three different forms of K vitamins are classified accordingly to the chemical structure of this side chain (table 3.1) [207, 208].

Since its discovery, the nomenclature of compounds possessing vitamin K activity has been modified and the most recently adopted is the one of the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry Subcommittee on Nomenclature of Quinones (table 3.1).

Table 3.1- Classification of the principal forms of vitamin K [207-209].

Chemical Compound	Chemical Structure	Original Nomenclature	IUPAC Nomenclature
2-methyl-1,4-naphthoquinone		K_3	Menadione
2-methyl-3-phytyl-1,4-naphthoquinone		K_1	Phylloquinone (K)
2-methyl-3-multiprenyl-1,4-naphthoquinone (class)		$K_{2(n)}$	Menaquinone-n (MK-n)
2-methyl-3-geranyl-geranyl1,4-naphthoquinone		$K_{2(20)}$	Menaquinone-4 (MK-4)
2-methyl-3-farnesylgeranylgeranyl-1,4-naphthoquinone		$K_{2(35)}$	Menaquinone-7 (MK-7)

In nature, the 3-position substituent has an isoprenoid structure with varying lengths and degrees of saturation depending on the organism by which it is synthesized. Plants and cyanobacteria, almost invariably, synthesize the form phylloquinone, which has a phytyl side chain (table 3.1). All the other bacteria that possess the machinery for vitamin K synthesis produce a plethora of isoprenologues named menaquinones, which comprise repeating prenyl units. This family of menaquinones can be subclassified, depending on the length of their side chain. The various forms are denominated as menaquinone-n, where the suffix n denotes the number of isoprenyls (table 3.1) that could vary between 4-13 units.

The menadione form (table 3.1) is a synthetic analog of vitamin K, which can be regarded as a provitamin, since vertebrates can endogenously convert it into menaquinone, with the addition of a prenyl side chain.

3.3. Sources

Until the mid1980s, the lack of adequate analytical methodology and the relatively unusual occurrence of insufficient intake of vitamin K, hindered the development of databases describing the amount of this vitamin in food or biological tissues. Furthermore, vitamin K quantification is hampered by the small amount of vitamin K in the initial extracts.

Modern analyze by high performance liquid chromatography (HPLC) with fluorescent detection is the standard tool to determine vitamin K concentration in various sources. To separate vitamin K from interfering lipids and to separate the various homologs of the vitamin, both adsorptive and reversed phase partition HPLC have been developed and utilized in various methods [207, 208].

3.3.1. Dietary

Following the development of standardized assays for vitamin K quantification in food sources numerous reports of the vitamin content in food became available [210]. Nevertheless, there is still some variability between different laboratories on vitamin K content data reported in food [207].

The predominant dietary source of vitamin K is phylloquinone obtained from plants. The highest concentrations of phylloquinone can be found in green leafy vegetables, vegetable oils, fruits and grains (table 3.2) [210-212].

Table 3.2- Phylloquinone content of selected common food [210].

Food Item	[Phylloquinone] (µg/100g)	Food Item	[Phylloquinone] (µg/100g)
Collards	440	Dry soybeans	47
Spinach	380	Margarine	42
Broccoli raab	242	Kiwi	41
Soybean oil	193	Okra	40
Broccoli	180	Cashew	35
Brussels sprouts	177	Green beans	33
Cabbage	145	Green peas	24
Canola oil	127	Dry lentils	22
Green leaf lettuce	127	Avocado	21
Red leaf lettuce	123	Cucumbers	20
Bib lettuce	122	Cauliflower	20
Romaine lettuce	103	Blackberries	19
Asparagus	60	Carrots	10
Cottonseed oil	60	Butter	7
Olive oil	55	Tomatoes	6
Nuts	54	Corn oil	3

Although phylloquinone is the main form of vitamin K available in the diet, menaquinones can substantially contribute to the total intake. This late form has a more restricted distribution in the diet, and can be found in some animal products and bacterial sources that contain a mixture of isoprenalogs of the menaquinone series (table 3.3).

Dietary amounts of menaquinones have been less well studied, but it is known that apart from its presence in animal liver, the richest dietary source of long chain menaquinones are bacterial fermented foods, typically represented by cheeses in Western diets and natto in Japan [207, 208].

Table 3.3- Menaquinone content of selected common food [207].

Food Item	[MK-4] (µg/100g)	[MK-7] (µg/100g)	[Other MK] (µg/100g)
Natto (fermented soybeans)	<1	998	105
Egg yolk	37	<1	1
Chicken meat	30	<1	<1
Hard cheeses	5	1	70
Soft cheeses	4	1	52
Butter	15	<1	<1
Beef liver	1	3	5
Beef roast	3	<1	<1

Moreover it seems that vitamin K is not destroyed by boiling or microwaving processes, but it is not stable when exposed to ultraviolet light [207].

The dietary reference intakes (DRIs) of vitamin K currently considered were published in 2001 and represent the adequate daily intake for different age groups (table 3.4). These values are defined as "the recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group or groups of apparently healthy people that are assumed to be adequate" [210].

Table 3.4- Adequate intakes of vitamin K for the different age groups [210].

Age Group		Vitamin K (µg/day)
Infants	0-6 months	2.0
	7-12 months	2.5
Children	1-3 years	30
	4-8 years	35
Adolescents	9-13 years	60
	14-18 years	75
Adult Women	19- >70 years	90
Adult Men	19- >70 years	120

3.3.2. Non Dietary

The human intestinal microflora produces large amounts of menaquinones, which are potentially available as a source of vitamin K. Most of them are synthesized in the distal colon by *Bacteroides* (MK-10 and MK-11), *Enterobacter* (MK-8), *Veillonella* (MK-7) and by *Eubacterium lentum* (MK-6); though their contribution to the vitamin K requirements and status maintenance has been difficult to assess [207, 208].

3.4. Metabolic Processes

The physiology of the absorption, transport, cellular uptake, metabolism and excretion of vitamin K is reasonably understood and in general seems to be similar to the pathway established for other lipophilic metabolites.

3.4.1. Absorption and Distribution

After ingestion, in the intestinal lumen, these vitamins are incorporated chemically unchanged into mixed micelles comprising bile salts, the products of pancreatic lipolysis, and other dietary lipids. In healthy adults, the efficiency of absorption is estimated at 40-80%, depending on the vehicle in which the vitamin is administered and the enterohepatic circulation [207].

The Mixed micelles are taken up by intestinal enterocytes of the small intestine and are incorporated into nascent chylomicrons (CM) that are secreted from within the intestinal villi into the lacteals, which join larger lymphatic vessels and empty into the blood circulation via the thoracic duct. In the bloodstream, it is rapidly cleared at a rate consistent with its continuing association with CM and the chylomicron remnants (CR), which are produced by lipoprotein lipase hydrolysis at the surface of capillary endothelial cells. After an overnight fast, more than half of the circulating vitamin is still associated with triglyceride-rich lipoproteins, with the remainder being equally distributed between low density lipoproteins (LDL) and high density lipoproteins (HDL) [207, 208].

In many species, including human, vitamin K_1 is incorporated into triacylglycerol rich lipoproteins and delivered to the liver, whereas vitamin K_2 is transported via LDL and HDL particles to other organs; further significant uptake organs are the bone and could be the adipose tissue, skin, heart, pancreas, kidney, brain and lungs [207, 210].

The relationship between hepatic and extrahepatic total body stores of vitamin K is not known. Whilst, there is evidence of a high turnover of the hepatic reserves of phylloquinone; liver stores are very labile, under conditions of severe dietary depletion, liver concentrations after only 3 days are usually reduced to about 25 % of the initial phylloquinone levels [207, 208, 210].

3.4.2. Metabolism and Excretion

A very important advance in understanding the overall process of vitamin K metabolism was the finding that a significant portion of phylloquinone is subjected to side-

chain cleavage to generate menadione (figure 3.1). Although the majority of menaquinones found in animal tissues are the product of bacterial menaquinone biosynthesis in the gut, very little MK-4 is produced by these organisms, but substantial amounts are present in some tissues and organs. Since the 1950s, It is known that animals have an intrinsic capacity to transform menadione to MK-4, the question arises as to whether the tissues that accumulate MK-4 do so through local biosynthesis or whether MK-4 is delivered to the target organ via the blood, from a separate synthesizing organ such as the liver [207, 208].

The essential metabolic role of vitamin K is to act as a coenzyme for a specific carboxylation reaction that transforms selective glutamate (Glu) residues to gamma-carboxyglutamate (Gla), in a number of vitamin K dependent proteins (VKDP). This process of carboxylation occurs in the endoplasmic reticulum, requiring the reduced form of vitamin K (KH_2), as one of the cofactors, and alongside with each Glu modification, KH_2 is oxidized to vitamin K 2,3-epoxide (KO) and converted back to KH_2, in a recycling salvage pathway that is crucial for both the function of vitamin k and to the conservation of its microsomal limited stores (figure 3.1) [207, 213, 214].

In humans, the liver is the site of vitamin K catabolic pathway, common to phylloquinone and menaquinones, whereby their respective polyisoprenoid side chains undergo ω-oxidation step carried out by cytochrome P450, followed by β-oxidation, leading to two major aglycone metabolites with side chain lengths of five and seven carbon atoms respectively; the metabolites are then conjugated, mainly with glucuronic acid, and excreted in the bile and urine. One notable feature of vitamin K metabolism, compared to other fat-soluble vitamins, is that the most abundant dietary form, phylloquinone is poorly retained in the body, with about 40% of a daily physiological dose being excreted via the bile and 20% via the urine (figure 3.1) [207, 208].

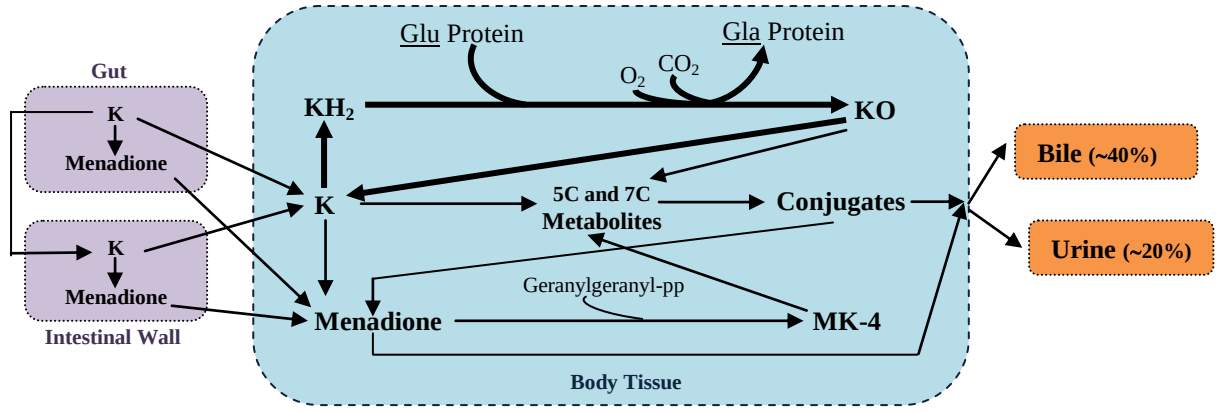


Figure 3.1-Schematic representation of metabolism and excretion of vitamin K (Adapted from reference 207).

4. Vitamin K Dependent Proteins

Several vitamin K dependent proteins (VKDPs) are known and characterized (table 4.1) and their biological function and tissue distribution can be rather diverse. These proteins require the post-translational modification of Glu residues into Gla, to become biologically active. This modification is widely described as crucial for its physiologic activities, including hemostasis, calcium regulation, bone mineralization, apoptosis, phagocytosis, growth control and signal transduction [207, 214-218].

Table 4.1- Identified vitamin K dependent proteins [207, 215-218].

Hemostatic Vitamin K Dependent Proteins	Prothrombin (F II)
	Factor VII (F VII)
	Factor IX (F IX)
	Factor X (F X)
	Protein C (PC)
	Protein S (PS)
	Protein Z (PZ)
Vitamin K Dependent Proteins associated with calcification	Bone Gla Protein or Osteocalcin (OC)
	Matrix Gla Protein (MGP)
	Gla-Rich Protein (GRP)

Carboxylation of VKDPs is a concerted mechanism (figure 4.1), accomplished by an enzymatic machinery that was first demonstrated to occur in hepatocytes, in 1975, shortly after the discovery of Gla residues. The key enzymes involved in the vitamin K-dependent carboxylation include the integral membrane proteins gamma-glutamyl carboxylase (GGCX), vitamin K epoxide reductase (VKOR) and an as-yet-unidentified vitamin K reductase (VKR) [214, 219].

All VKDPs contain a carboxylase recognition sequence that usually lies within the propeptide, adjacent to the Gla domain, which tethers the substrates to bind an exocyte on GGCX, initiating a structural reorientation, by which the Gla domain of the substrate is positioned at the catalytic site of GGCX. This dual-function enzyme also requires the reduced form of vitamin K (KH_2), O_2 and CO_2 as co-factors The Gla domains of VKDPs can include between 3 and 13 Gla residues, and the multiple modification of Glu to Gla is accomplished by a processive mechanism, occurring as a result of a single binding event (figure 4.1) [213-216, 220].

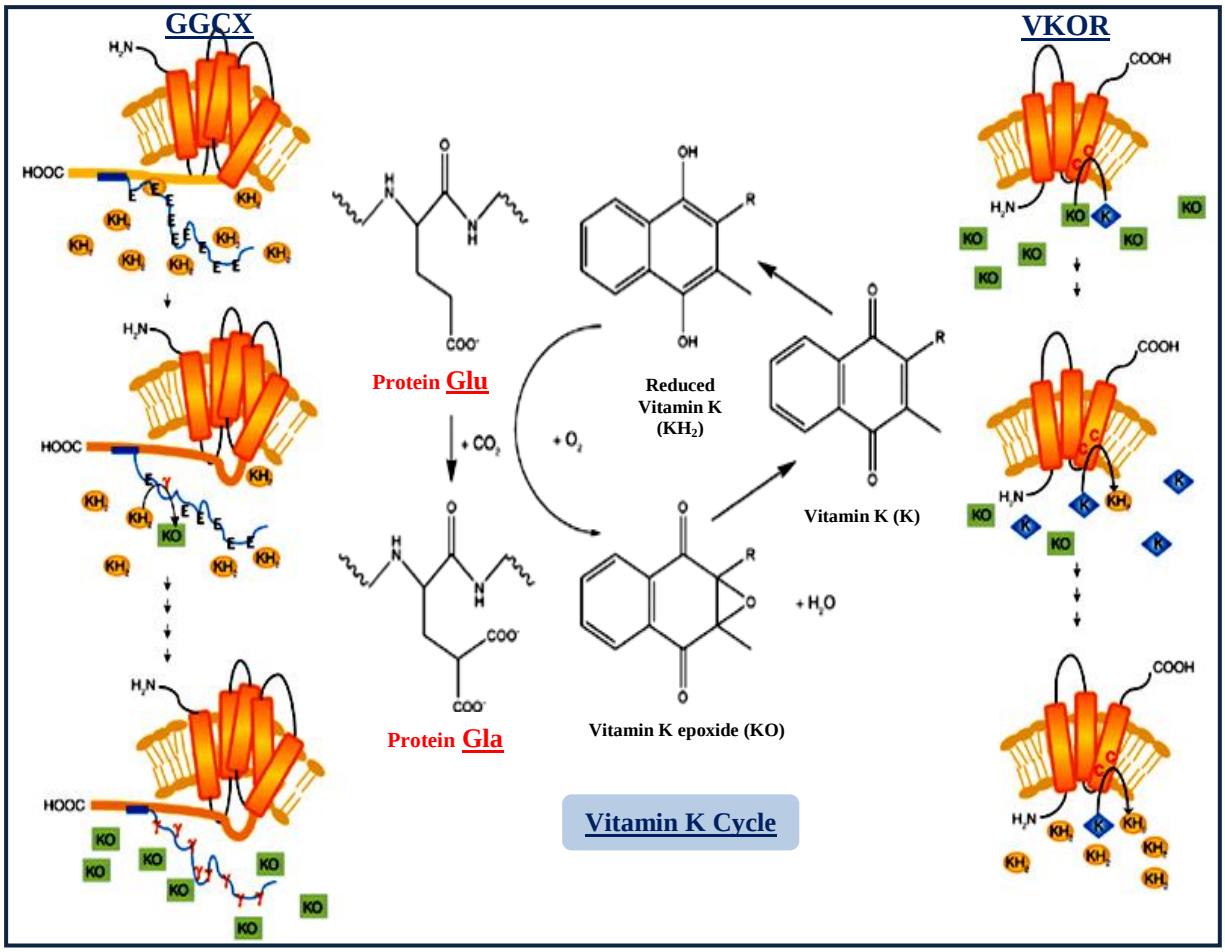


Figure 4.1-Post-translational enzymatic modification of Vitamin K Dependent Proteins (Adapted from reference 221). E - Glutamate; γ - Carboxyglutamate; GGCX - Gamma-glutamyl carboxylase; VKOR - Vitamin K epoxide reductase.

The addition of a carboxyl group to the gamma carbon of targeted glutamic acid (Glu) catalyzed by GGCX requires the reduced form of vitamin K (KH_2). In the course of the catalytic sequence, KH_2 is oxidized to the vitamin K 2,3 epoxide (KO) and the reaction provides the necessary energy for the carboxylation of Glu residues by removing a proton from the gamma carbon of the Glu residue, leading to the formation of a carbanion. Subsequent addition of CO_2 to the carbanion intermediate produces the Gla residue. In vivo carboxylation of VKD proteins requires continuous recycling of the KO, in a reaction catalyzed by the reductase enzyme VKOR (figure 4.1) [214, 218]. This recycle ability can explain the very low daily requirement of Vitamin K when compared with other vitamins and cofactors.

This process of γ-carboxylation results in the formation of Gla proteins that are described to be able to bind calcium and calcium mineral. Nevertheless, in the absence of vitamin K or the presence of VKOR inhibitors (e.g. 4-hydroxycoumarin anticoagulant drugs as warfarin), carboxylation of precursor proteins is incomplete and proteins are secreted in various undercarboxylated forms in all tissues and fail to bind calcium [218, 220].

4.1. Hemostatic Vitamin K Dependent Proteins

The orchestration of events that sustain hemostasis is performed by an interactive and highly regulated network of proteins that preserve the blood flow and the integrity of the vasculature, preventing a hemorrhagic or thrombotic event [207, 216].

Vitamin K-dependent plasma proteins are a group of structurally related secretory glycoproteins, synthesized in the liver, with molecular weights ranging from 50,000 to 72,000 Da, which correspond to the clotting factors involved in the coagulation cascade as prothrombin, factors VII, IX, X and the circulating anticoagulant proteins C, S, Z. The majority of these proteins, except protein S, are zymogen forms of a serine protease. Their distinguishing feature is the presence of from 9 to 13 γ-carboxyglutamic acid residues in the Gla domain, located within the first 40 residues of the N-terminus of the mature proteins [216].

The Gla domain in association with the adjacent aromatic amino acid stack domain functions as a membrane binding component of these proteins. These Gla residues confer unique properties for calcium ion-mediated binding to negatively charged phospholipid surfaces provided by blood platelets and endothelial cells at the site of injury. Because of that, Gla residues are crucial for the functional activity of these proteins and the extent of coagulation is proportional to the level of its carboxylation [207, 216].

The remaining vitamin K-dependent plasma proteins play a regulatory role in the inhibition of coagulation, limiting the extent of thrombosis that will occur when clotting is initiated. The main function of protein C is to degrade phospholipid-bound activated factors V and VIII in the presence of calcium; protein S acts as a synergistic cofactor to protein C by enhancing the binding of activated protein C to negatively charged phospholipids; while Protein Z interacts with a protease inhibitor, regulating the equilibrium of coagulation [207].

4.2. Vitamin K Dependent Proteins Associated With Calcification

In 1976 the first VKDP not related to blood coagulation was discovered and designated as bone Gla protein or osteocalcin (OC). This protein is present in relatively high amounts in most vertebral species, where it represents 15% to 20% of the noncollagenous bone protein. In human skeletal tissues, is the second most abundant protein in bone and is also found in dentine and in areas of ectopic calcification [207].

OC is expressed by both osteoblasts and vascular smooth muscle cells (VSMCs), being a small γ-carboxylated protein, with a molecular weight of 5.8 kDa that contains three Gla residues, typically located at positions 17, 21, 24 (figure 4.2). It binds calcium mineral in the form of hydroxyapatite, indicating a role in the physiological process of bone mineralization[222, 223].

Figure 4.2- Amino acid structure of mature human osteocalcin (Adapted from reference 224).

In 1983 another VKDP purified from the bovine bone matrix was discovered by Price and colleagues, and named matrix Gla protein (MGP). This protein is a 14 kDa secreted protein, expressed in a variety of tissues, including cartilage, heart, lung, kidney, skin and arterial vessel walls; and synthesized by chondrocytes, VSMCs, fibroblasts and endothelial cells. This mature protein consists of 84 amino acids, containing Gla residues at positions 2, 37, 41, 48 and 52 (figure 4.3). Of its nine glutamate residues only five can be γ-carboxylated and three of its five serine residues can be phosphorylated into phosphoserine, located in position 3, 6 and 9 (figure 4.3) [207, 225, 226].

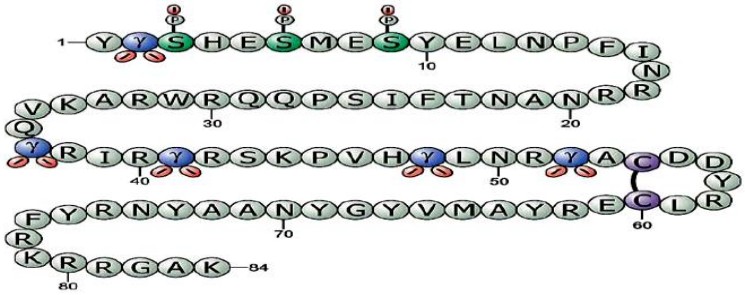


Figure 4.3- Amino acid structure of mature human matrix Gla protein (Adapted from reference 225).

The function of MGP was clarified when mice lacking the gene for MGP was produced and shown to develop spontaneous calcifications in both arteries and cartilage tissues. The studies performed on this knockout mice lead to the proposal of MGP as a strong inhibitor of vascular calcification. The importance of MGP in regulation of mineralization was also strengthened by the finding that Keutel syndrome, a rare human autosomal recessive condition, associated with mutations in the MGP gene, is characterized by abnormal cartilage calcification peripheral pulmonary stenosis and midfacial hypoplasia [227].

More recently in 2008, a novel Gla protein was isolated from the calcified cartilage of Adriatic sturgeon and described as a 10.2 kDa secreted protein exhibiting in its mature form, 16 Gla residues in its 74 residue sequence; the highest Gla percent of any known protein, so therefore it was termed Gla-rich protein (GRP). Its unprecedented high density of Gla residues, conferring an outstanding capacity for calcium-binding, together with its extensive pattern of tissue distribution and accumulation in amphibians, reptiles and mammals, and an high evolutionary conservation in vertebrates, suggests an important function as a global calcium modulator with a key role in the calcification process[228].

This group mineral-associated extrahepatic VKDPs has been described to have a crucial role in health and disease, mainly due to its function in calcium and mineral regulation involved in both physiological and pathological calcification processes. In the last decade investigators developed several genetic and pharmacological studies to gain further insights into the role of the extrahepatic VKDPs OC, MGP in the calcification process. In fact, OC and MGP are described to be implicated in the regulation of endochondral calcification, controlling HA deposition and deficiencies in these VKDPs interfere with endochondral bone formation, leading to pathological calcification [223, 225, 226]. With the discovery of GRP, its potential involvement with physiological and pathological calcification was hypothesized, and studies have been conducted in the past years to further elucidate this question [228].

The calcification inhibitory role of MGP has been directly linked to the presence of Gla residues that confer the ability to bind calcium crystals Accordingly, Keutel disease, caused by mutations in the human MGP gene and resulting in non-functional protein, originates, among other features, a severe cartilage calcification phenotype similar to those observed in the MGP-deficient mice Transgenic mice with a MGP mutant that could not be carboxylated, also acquired a similar phenotype [225, 226].

Although the function of GRP in humans has not yet been completely established, GRP has been associated to sites of pathological calcification in human and proposed as a modulator of calcium availability and a calcification inhibitor in the cardiovascular system, depending on its γ-carboxylation status [229, 230]. These findings highlight the possibility of GRP association to other ectopic calcification-related pathologies, such as certain types of cancer [231].

Even though their exact molecular mechanisms are not fully understood, based on the studies accumulated data, the overall conclusion is that these VKDPs can play a role in the initiation phase of mineralization and the amount of mineral deposited. The Gla residues of those proteins promote binding properties with calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions, so in mineralization competent cells they can inhibit or compete with Ca^{2+} influx into the vesicle's lumen and restrain the matrix vesicle's capacity to initiate calcification nidus. Likewise, it is quite possible that the Gla and other acidic residues could allow those proteins to adsorb to nascent crystals and thereby prevent or limit lattice ion addition for crystal growth. Another plausible mechanism by which pathological calcification of tissues could be prevented is by regulation of cell differentiation. In fact MGP is also described to affect osteogenesis, chondrocyte maturation and ECM calcification inhibition, by interacting with BMP2, a potent inducer of bone formation in both skeletal and soft tissues. Matrix Gla protein regulates BMP2 activity via binding through its Gla residues, emphasizing the need of MGP γ-carboxylation in bone and cartilage formation [225, 226, 232, 233].

5. The Role of Vitamin K and VKDPs in Osteoarthritis

During the past few decades, vitamin K has emerged from a single haemostatic function, considered as required only for the synthesis of blood clotting factors in the liver, to a multifunctional vitamin, with the continuous discovery of the last extra-hepatic VKDPs, characterized by widespread tissue distribution and broad physiological impact. This clearly showed that vitamin K functions lies beyond its function in blood coagulation, and that might have a role in multiple biological processes such as in bone and in cartilage.

The vitamin K recycling system is able to maintain an adequate level of vitamin K supply in the organism, and support a proper functioning of the blood coagulation system. Nevertheless, the extrahepatic VKDPs mentioned are found only partly carboxylated in the healthy adult population, suggesting a common vitamin K insufficiency [207, 226].

Insufficient γ-carboxylation either by dietary vitamin K deficiency or impairment of vitamin K recycling, has a direct effect on VKDPs such as OC, MGP and GRP, known to be of vital importance in bone and/or cartilage health and disease, leading to the production of inactive undercarboxylated VKDP forms [26, 207]. OC is presently considered a marker for bone formation and is undercarboxylated form has been proposed as a biomarker for vitamin K status [207, 226].

The vitamin K status of the elderly is a particular concern, it is estimated that at any given time as high as 7% of them are being prescribed with VKAs. Oral anticoagulant treatment (e.g. warfarin) is used in the prevention and treatment of deep vein thrombosis, pulmonary embolism, atrial fibrillation, myocardial infarction and hip and knee orthoplasty and surgery, therefore commonly administered [207]. A special care should be given to the widely used VKAs agents whose side effects are certainly still not completely unraveled [234].

The general population exhibit undercarboxylation (10-40%) of circulating OC, MGP, or GRP and thus the biological activity of these proteins could be considered sub-optimal. This low functionality of those VKDPs, not essential for short-term survival, suggests that they are likely to require higher intakes of vitamin K to achieve maximum activity [25, 28, 207].

Insidious changes may accumulate as a consequence of the vitamin K restriction, raising the vulnerability for diseases associated with aging, with important implications in bone and cartilage and deleterious health consequences, as in the case of osteoarthritis [14, 15, 17, 19].

Since calcification and inflammation are common and interconnected events in OA, the importance of vitamin K through the action of OC, MGP, and the new discovered GRP opens new perspectives on the potential range of action of vitamin K [32, 225, 226, 235].

Currently it is recognized that vitamin K plays several and crucial roles in joint's health. Subclinical vitamin K levels have been related with an increased risk of OA development [18, 27, 29, 236], while the undercarboxylated forms of OC, MGP and GRP have been associated with the disease [32, 36, 235, 237, 238].

Those VKDPs have been implicated in the crosstalk of key molecular processes responsible for OA development, progression and severity: mineralization and inflammation of joint tissues, the main pathophysiological features that feed the articular degradation cycle. Accumulated data have been proposing those Gla proteins as regulators of cell differentiation, modulators of calcium availability in the ECM and inhibitors of calcification in the articular systems [228, 232, 235].

OA disease is directly associated with high calcium availability in the joint environment and with deposition of BCP crystals in the articular cartilage, synovial fluid, or synovial membrane. Moreover, BCPs have been suggested to have a direct pathogenic role in OA, driving synovial inflammation and cartilage degradation [17, 117, 147, 123].

As previously mentioned, the calcium binding properties of Gla residues within the VKDP family have been associated with the affinity of functional MGP and GRP to bind BCP crystals. The consequence of this binding effect has been propose to change the dynamic of crystal growth and to interfere with crystal-cell membrane interaction, thus modulating the production of proinflammatory mediators and protecting cells from the increased expression of MMP13 and PGE2 production [235].

In an OA context, calcification can stimulate proinflammatory signaling that parallels phenotype alterations such as hypertrophic differentiation, apoptosis, accompanied by upregulation of OC, MGP and GRP expression, as well as altered responses to inflammatory cytokines and mediators of inflammation [32, 223, 225, 226, 235].

Comparative data clearly shows the colocalization of undercarboxylated MGP and GRP at sites of ectopic calcification on both cartilage and synovial membrane in OA, reinforcing the importance of γ-carboxylation status along calcium mineral formation inhibition (figure 5.1) [32]. These studies have clearly shown a predominance of undercarboxylated forms of GRP and MGP in biological OA samples. Remarkably, the increase of GRP and MGP expression in OA-derived cells was also shown to be associated with a down-regulation of GGCX and VKOR genes. These results suggested a reduced capacity of OA cells, and consequently a decrease in the γ-carboxylation of target proteins, with concomitant increased matrix mineralization, further supporting the link with disease worsening [32, 235, 237].

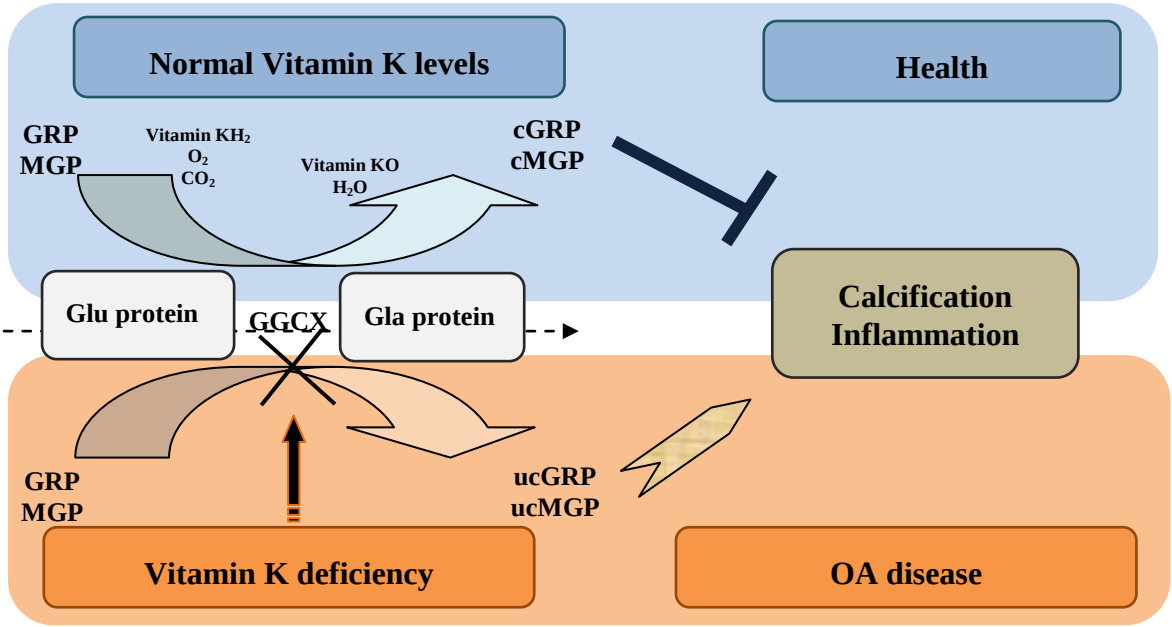


Figure 5.1- Schematic Representation of the Role of vitamin K and extra-hepatic VKDPs in OA (Adapted from 232) GGCX- Gamma Glutamyl Carboxylase; GRP- Gla rich protein; MGP- Matrix Gla protein; cGRP- carboxylated Gla rich protein; cMGP- carboxylated Matrix Gla protein; ucGRP- undercarboxylated Gla rich protein; ucMGP- undercarboxylated Matrix Gla protein; OA- Osteoarthritis.

Moreover, besides their role as a coenzyme of GGCX, other biological functions have been proposed for vitamin K, namely the anti-inflammatory effect of vitamin K_2 through suppression of the NF-kB pathway with a dual pro-anabolic and anti-catabolic activity in bone [239]. In fact, improper regulation of anti-catabolic activity in bone has been revealed as one of the main causes of setting an inflammatory state in OA and impelling articular degradation [240].

Furthermore, vitamin K by itself has been suggested to have a protective effect against oxidative stress damage through blocking of ROS generation [208].

All of these findings unveil the multifunctional roles of vitamin K in human joint's health. With the knowledge of its emerged value in the modulation of OA pathogenesis, this old vitamin is now presented in a new perspective. The vitamin K range of action proved to be crucial in the prevention of connective tissues pathological calcification and to be an essential protective factor against inflammation and oxidative stress. A promising tool, suggested as a potential prophylactic and therapeutic agent in OA.

6. Conclusion

Compelling evidence demonstrate the protective role of vitamin K against pathological mineralization, by improving the function of mineral-related extra-hepatic VKDPs, as OC, MGP and GRP, acting as calcification regulators and associated with ectopic calcification-related diseases. In addition to the association of pathological calcification with OA, the link of inflammatory processes to the etiology of this diseases and the increased attention to the novel biological functions of vitamin K, namely its anti-inflammatory and antioxidant role, lead to the suggestion that this vitamin might be a potential candidate for OA prevention and therapeutics.

Nevertheless, the unequivocal beneficial effect of maintaining an optimum vitamin K status to protect against OA is still under debate although no toxicity is known for high vitamin K dosages [234].

Little is known on how K vitamins tissue distribution and metabolism may change during the progression towards vitamin K deficiency and whether or not certain tissue stores can be mobilized to protect essential functions. Among the factors that need to be taken into consideration are differences in bioavailability, transport, tissue distribution, metabolic handling and turnover of the different vitamin K isoprenologues, to access and correctly compare their efficacy [208].

Accumulated data suggest that a high vitamin K_2 intake could be an effective interventional strategy to decrease the calcification risk in the general population [225]. Some studies suggest that MK-4 is more active than vitamin K_1 in extrahepatic tissues, with attributable health-promoting effects [241]. Another well studied example, the MK-7, obtained from natto, showed physic-chemical advantages compared with vitamin K_1; because due to its molecular structure, it is more lipophilic and presents a much longer half-life (3 days) than vitamin K_1. Although, as a result of the considerably higher bioavailability, the risk of a pharmacodynamic interaction of MK-7 with vitamin K antagonists (VKAs) is also shown to be markedly superior than with vitamin K_1 [218].

Additional and representative studies with OA patients are needed to compare their different K_1 and K_2 levels and evaluate the additional benefits in OA. More investigation is required to determine a precise necessary intake of one or other vitamins. It is also essential to establish whether this should be done by dietary intake increase, nutritional supplements or specific medication [32].

Further and long term research tests and clinic trials are warranted to further elucidate and support the mechanisms underlying the vitamin Ks role in OA, to definitively prove its efficacy as a prophylactic and therapeutic agent in OA; which might help to alter the burden of this insidious disease.

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