Università degli Studi di Ferrara

DOCTORAL COURSE IN
"BIOMEDICAL SCIENCES AND BIOTECHNOLOGY"

CYCLE XXXV

COORDINATOR Prof. Paolo Pinton

SEX DETERMINANT IN AGING AND OSTEOARTHRITIS: *IN VITRO* MODELS OF HUMAN CHONDROCYTES AND SYNOVIOCYTES AND *IN VIVO* EVALUATIONS

Scientific/Disciplinary Sector (SDS) BIO/17

Candidate
Dr. Contartese Deyanira

Supervisor
Prof. De Mattei Monica

Year 2019/2022

INDEX

1. INTRODUCTION

1.1 Joint biology

Joints are complex anatomical structures that bring two or more bones into contact with each other. The joints of the human body are numerous, and structurally very different one from each other. The task of the joints is to keep bone segments together with muscles, ligaments and tendons, so that the skeleton can carry out its function of support, mobility and protection. Most of the joints fall into the category of diarthrosis, that are mobile joints characterized by a particular anatomical structure. They are in fact, made up of different elements: the epiphysis of two bones, the cartilaginous tissue layer, the joint capsule, the joint cavity, the synovial membrane, the synovial fluid and the ligaments [1,2].

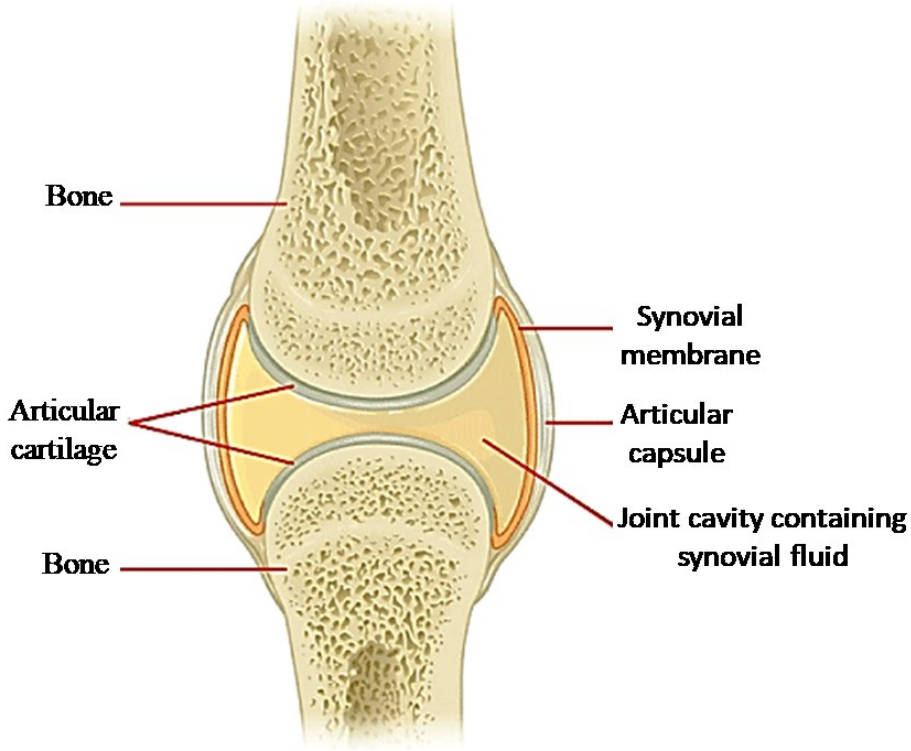


Figure 1. Joint anatomy.

1.1.1 Articular cartilage

Articular or hyaline cartilage is a particular highly specialized support connective tissue that covers the bone epiphysis, promoting the movement of the articular heads and performs a function of absorption and distribution of the mechanical stresses to which the joint is subjected. The structure and vascularisation of cartilage are such that its regenerative capacities are limited and insufficient to repair the damage caused by trauma or by the

catabolic effects of the consequent inflammatory reactions. The cartilage has a variable structure according to the distance from the contiguous surface to the joint cavity. In particular, four regions can be distinguished which differ one from each other in terms of orientation of the collagen fibers, concentration of proteoglycans and water content. Moving from the articular surface towards the subchondral bonethere are a surface area, an intermediate area, a deep area and finally a calcified area.

- The surface layer is the area in direct contact with the joint cavity in which the synovial fluid is contained. In this zone the collagen fibers run parallel to the surface, the proteoglycan concentration is low while the water content is the highest of the four zones.
- The intermediate layer consists of a high concentration of collagen. Here, the collagen fibers, contrary to the previous layer, do not develop along preferential directions; the percentage of water present is lower than the overlying area, while that of proteoglycans increases.
- In the deep layer, the concentration of proteoglycans is high, while the aqueous content is lower than in the previous layer. Contrary to the first layer, the collagen fibers, in lower concentration than in the previous areas, are oriented perpendicular to the bone surface.
- Finally, the calcified layer is the thinnest area and connects the cartilage to the subchondral bone.

Like all connective tissues, cartilaginous tissue is made up of two components: a cellular population and an extracellular matrix, which, in turn, consists of an amorphous component or ground substance and a fibrous component [1,2].

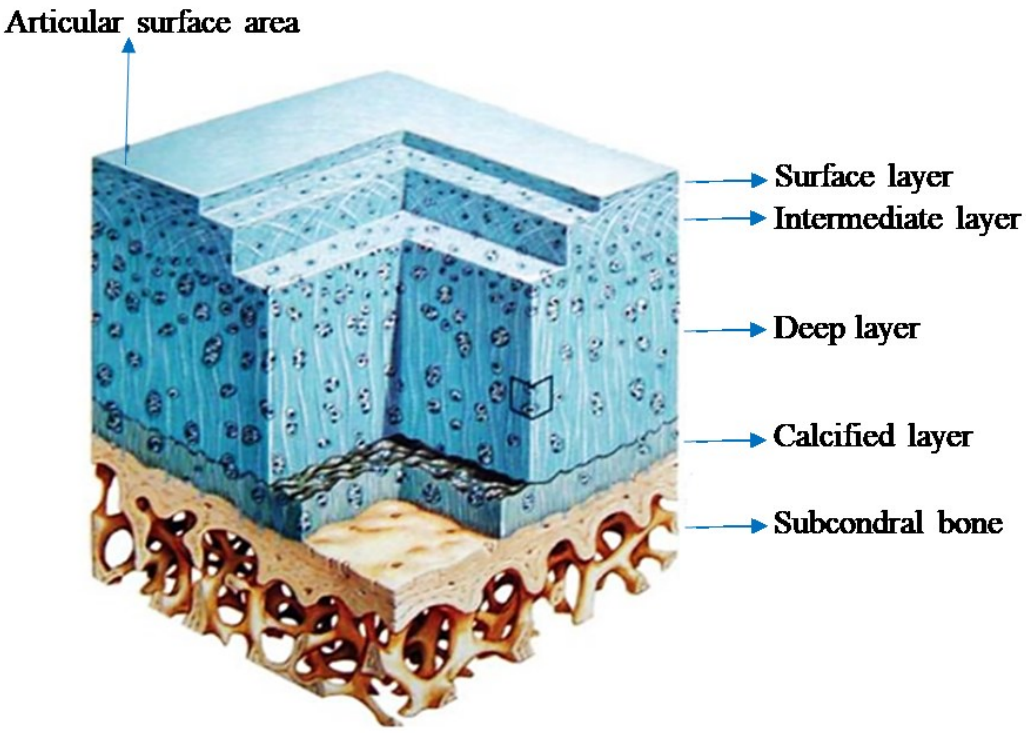


Figure 2. Schematic representation of articular surface and subchondral bone.

1.1.2 Extracellular matrix

The extracellular matrix (ECM) is an organized network of extracellular materials, which plays a key role in determining the shape and activities of cells. Its components are continually renewed, with a turnover that slows down with age. The ECM consists of water molecules and collagen fibers immersed in a highly hydrated amorphous substance rich in glycosaminoglycans, proteoglycans and glycoproteins. Collagen fibers are mainly made up of type II collagen, which has the characteristic of having three structurally identical polypeptide chains and forming small-sized fibrils organized in a delicate reticle. The fibrous component gives cartilage its compressive strength. In the amorphous substance there are glycoproteins, which bind to hyaluronic acid molecules to form aggrecans, but also other proteins such as chondronectin, fibronectin, laminin, decorin, thrombospondin etc., important for the proper functioning of the articular cartilage. All these structures are responsible for the high degree of hydration of the cartilage, which on the one hand ensures the nutrition of the tissue, and on the other gives it strength, lubrification and elasticity [1,2].

1.1.3 Chondrocytes

Chondrocytes are the functional cellular units of the cartilage tissue. They are contained in spaces carved into the extracellular matrix called lacunae. The lacunae may contain one or more chondrocytes. Their morphology and distribution areon function of the level of depth within the cartilage. In the central part of the cartilage the cells are distributed in groups, called isogenic groups, and have a spherical shape. Moving towards the peripheral part, they take on a more flattened, ovoid shape and lose their distribution in groups. Chondrocytes contain a voluminous nucleus, numerous mitochondria, elements of rough endoplasmic reticulum, ribosomes responsible for the synthesis of collagen fibrils, and an extensive Golgi complex, in which carbohydrates are synthesized and glycosaminoglycans are sulphured. After being surrounded by the matrix synthesized by themselves, chondrocytes continue throughout the growth period of the cartilage to elaborate cartilaginous substance, synthesizing and pouring into the matrix the constituents of the ECM (proteoglycans, glycoproteins and collagen) and to divide. These cells respond specifically to local needs of articular cartilage by performing both catabolic and anabolic activities, synthesize ECM proteins and enzymes that degrade the matrix itself leading to a modulation of inflammatory and catabolic responses. Therefore, they are important in guiding cartilage remodeling and regeneration. Chondrocytes occupy approximately 1-5% of the total volume of normal adult articular cartilage, and due to the environment in which they live, with a relatively low oxygen concentration, have an essentially anaerobic metabolism. Due to the limited number of cells and their pyknotic nature, damage caused by various stressful conditions, such as abnormal mechanical loading, trauma and inflammation, can lead to changes in cartilage structure and function. Their metabolism is influenced by chemical and mechanical factors, such as soluble mediators (growth factors and interleukins), matrix composition, mechanical loads, hydrostatic pressures and electric fields. Furthermore, mature chondrocytes have a limited ability to increase the synthesis of surrounding matrix components to repair tissue defects. There is in fact programmed cellular senescence, such that the ability to synthesize some types of proteoglycans and to increase cell division in response to stimuli, decreases with age [1,2].

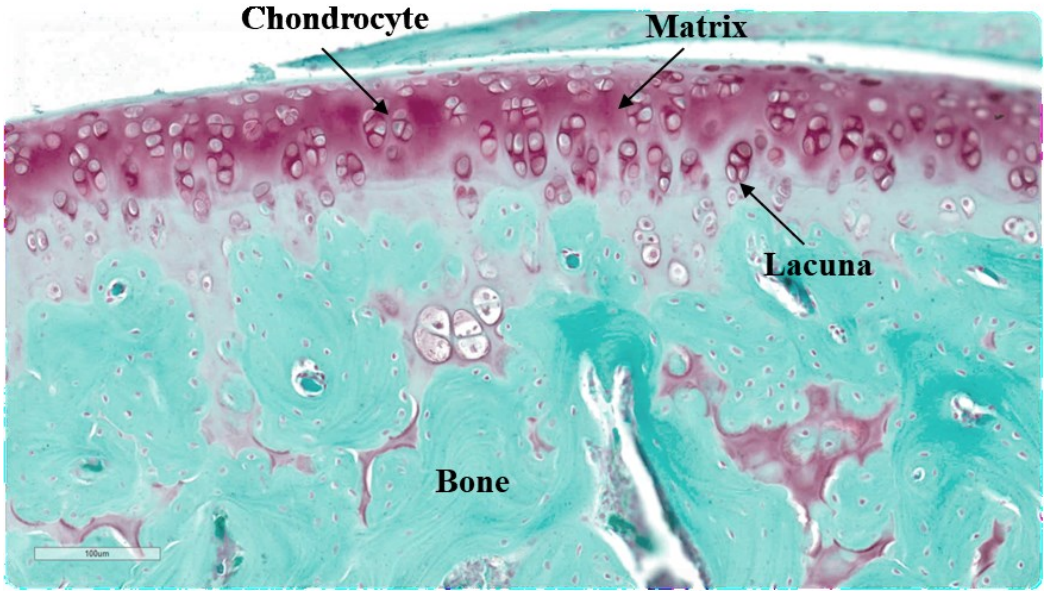


Figure 3. Histological representation of articular cartilage. Safranin O/Fast Green staining. Magnification: 20x.

1.1.4 Synovial membrane

Another fundamental component in the functioning of the joint is the synovial membrane or synovium. Synovium is a soft tissue found in the diarthrodial joints, tendon sheaths and bursae. They have consecutive layers of cells, where inner layer is called the intima and the outer layer is called the subintima. The intimal layer houses the macrophages and fibroblasts while the subintima has blood and lymphatic vessels, fibroblasts and infiltration of cells in a collagenous extracellular matrix. The intimal layer is 20-40mm thick in cross-section and the subintima can be up to 5mm in thickness. The synovium is responsible for maintaining the functional activity of articular cartilage and the well-being of chondrocytes by producing lubricin and hyaluronic acid (i.e. synovial fluid). Numerous factors contribute to synovial joint homeostasis, including regular expression of protective lubricin, fibroblast-like synoviocytes (FLSs) secretion of matrix metalloproteinase (MMPs), immune centralized role is played by resident macrophages and FLSs, regulated entry and exit of leukocytes involved in immune surveillance, and local regulation by cytokines and growth factors [3-5].

1.1.5 Synoviocytes

Synoviocytes are cells of the membrane and synovial fluid. These are metabolically very active cells, essential for nourishing the chondrocytes and maintaining the homeostasis of the ECM of the joint, both through the degradation of waste products and the production of mediators (cytokines and growth factors), enzymes (MMPs), hyaluronic acid and lubricin. The synovial membrane is composed mainly of two types of synovial cells. Synovial macrophage-like cells (type A), which are relatively small and are found on the inner surface of the joint cavity, have mainly a phagocytic function, are responsible for the removal of debris from the synovial fluid and produce proinflammatory cytokines. They represent 25 % of the resident cells in the synovial membrane. In contrast, synovial fibroblast-like cells (type B) are responsible for the synthesis and secretion of major ECM proteins into the synovial fluid. They produce hyaluronic acid, which traps water within the synovium, and lubricin, which lubricates the joint surfaces, thereby providing structure, nourishment, and lubrication. They represent about 75 % of the cells of the synovium. Furthermore, synovial fibroblast-like cells can migrate to the site of tissue remodeling and interact with ECM molecules via specific surface receptors. They can sense and respond to changes in the surrounding environment in the composition and structure of synovial tissue by regulating their interaction with the production of ECM components [1,2].

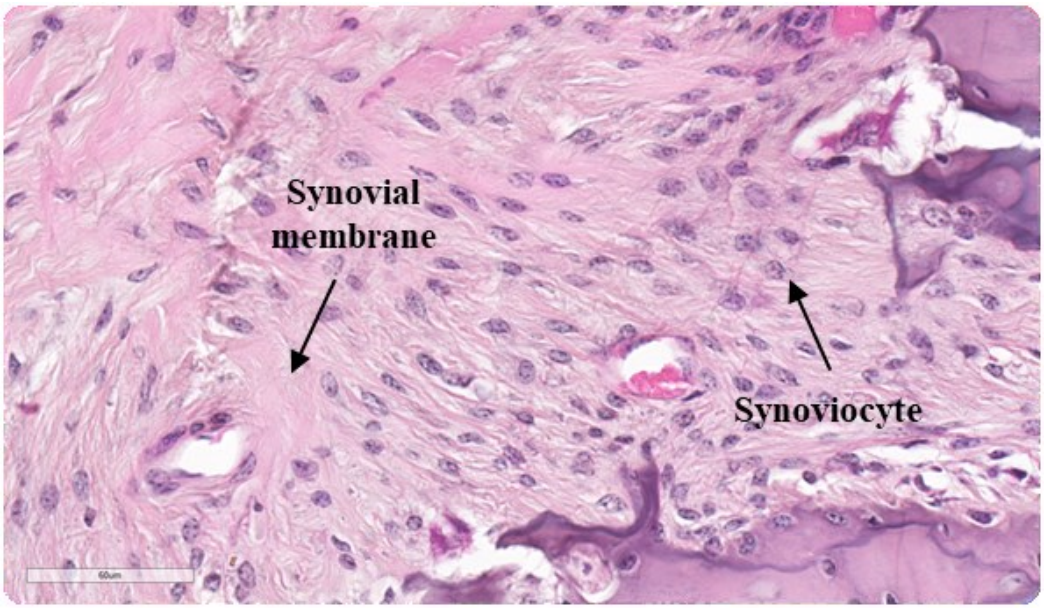


Figure 4. Histological representation of synovial membrane. Hematoxylin/Eosin staining. Magnification: 40x.

1.2 Osteoarthritis

Osteoarthritis (OA) is considered one of the most common degenerative musculoskeletal disease and one of the most frequent causes of the disability involving millions of people worldwide [6,7]. OA mainly affects the joints of the knees, hips, hands or spine [8]. Itis defined as a multifactorial condition of joint failure characterized primarily by loss of articular cartilage, subchondral bone sclerosis, hyperplasia, and inflammation [9]. Cartilage and subchondral bone are subject to irregular external mechanical stresses and internal biochemical or morphological changes, which result in a loss of function in the absorption of biomechanical forces [10]. Similarly, muscles, ligaments, and menisci can be altered due to injury or weakness, leading to disruption of function and amplifying physical stresses. During OA, the synovium also may exhibit inflammatory responses leading to further damage to surrounding tissues due to the release of pro-inflammatory mediators into the synovial fluid [11]. This complex nature of OA is evidenced by the multiplicity of pathological features and symptoms present during this condition.

Clinical signals often begin as stiffness and intermittent pain, with the symptoms becoming more constant, severe, and debilitating [12]. Diagnosis may be complicated by heterogenous sources of pain as the initial activity elicits peripheral pain, sometimes leading to central sensitization. Thus, the threshold of early OA symptoms evolving into “illness,” i.e., impacting the quality of life, still needs to be defined. In fact, currently, OA has a huge impact on the aged population. It is estimated that it affects over 300 million people in the global population, 10 % of men and 18 % of women aged 60 and over [13,14]. Furthermore, with the worldwide increases in aging, OA prevalence is anticipated to continuously increaseand become a major public health concern [15]. Despite the important and huge impact of the OA, current guidelines highlight a lack effective of treatment options that, at the moment, are sufficient for symptom modification, and there are currently no disease-modifying OA drugs approved by the Food Drugs Administration (FDA) [16]. Thus, numerous researches arecurrently aimed at identifying new, advanced and personalized strategies potentially able to engrave on the evolution of this pathology [6].

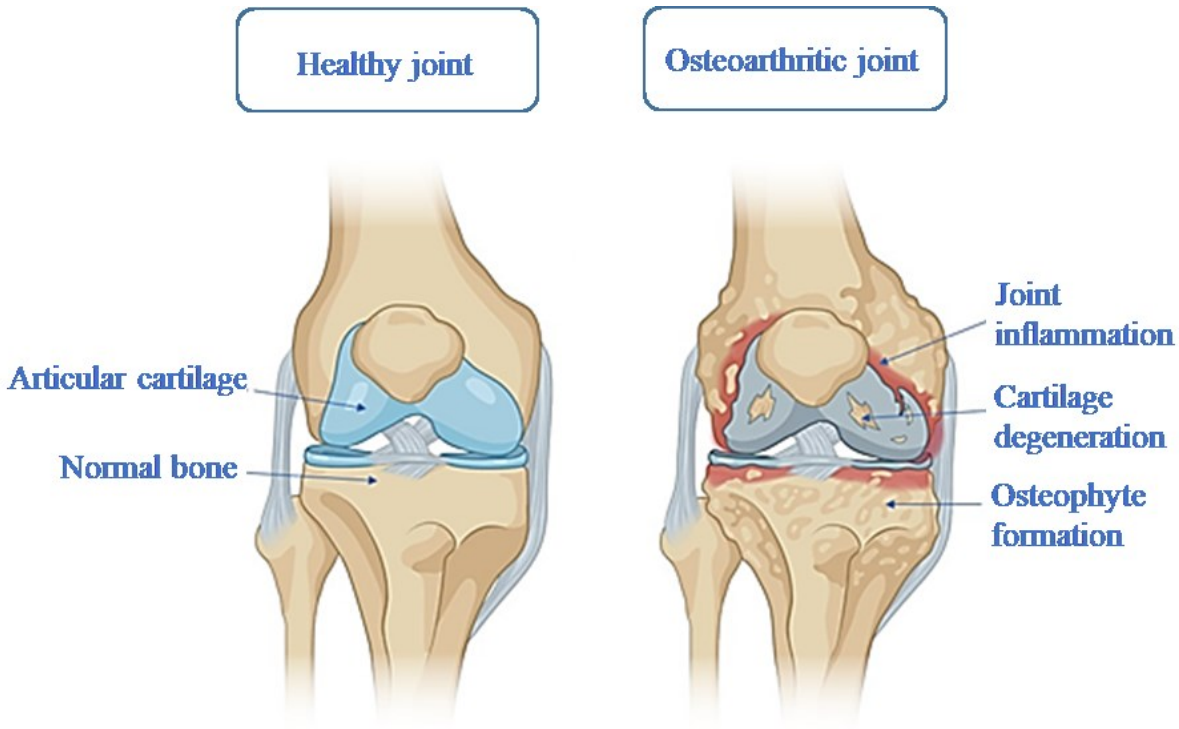


Figure 5. Healthy and osteoarthritic joint.

1.2.1 Pathogenesis

Alterations in the function of structures as meniscus, tendons, ligaments, joint capsule, cartilage, bone, synovial membrane, and synovial fluid are present in OA. During the early stages of OA, the cartilage surface remains intact due to the enhancement of compensatory mechanisms [17] while, during its progression, the ECM molecular composition and organization change [18]. Additionally, further mechanical abrasions lead to degenerative changes of the meniscus, with loss of type I and type II collagen simultaneously to distruption of cartilage matrix homeostasis by proinflammatory cytokines [19,20]. In this context, chondrocytes have poor regenerative capacity and low metabolic activity. They exhibit a transient proliferative response and hypertrophic differentiation with increased matrix synthesis, which attempts to initiate repairs in response to disease processes. The altered composition and structure of the cartilage causes the stimulation of chondrocytes to produce greater quantities of mediators involved in the degradation of the cartilage [21]. Then, the articular chondrocytes undergo apoptosis, the articular cartilage is destroyed [21]. Because of increased protein catabolism, there is an imbalance in the synthesis of collagen and proteoglycans. This causes the loss of the net woven fibers in the cartilage, weakening its strength. As a result, gaps are formed on its surface. At the same time, inflammatory mediators affect the surrounding tissues, leading to changes in the subchondral bone and

synovium. Cartilage undergoes fibrosis in regions affected by mechanical stress, with bone sclerosis and thickening of the synovium and joint capsule. The cartilaginous fragments of the degenerated joint surfaces induce the inflammatory process of the synovium, interrupting the synthesis of synovial components, such as hyaluronic acid, making the synovial fluid less viscous and elastic and eliminating its ability to hydrate the cartilage. In response to tissue damage, proinflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor (TNF-α) are released and stimulated the production of joint proteases like MMPs anda disintegrin and metalloproteinases with thrombospondin motifs (ADAMTSs).

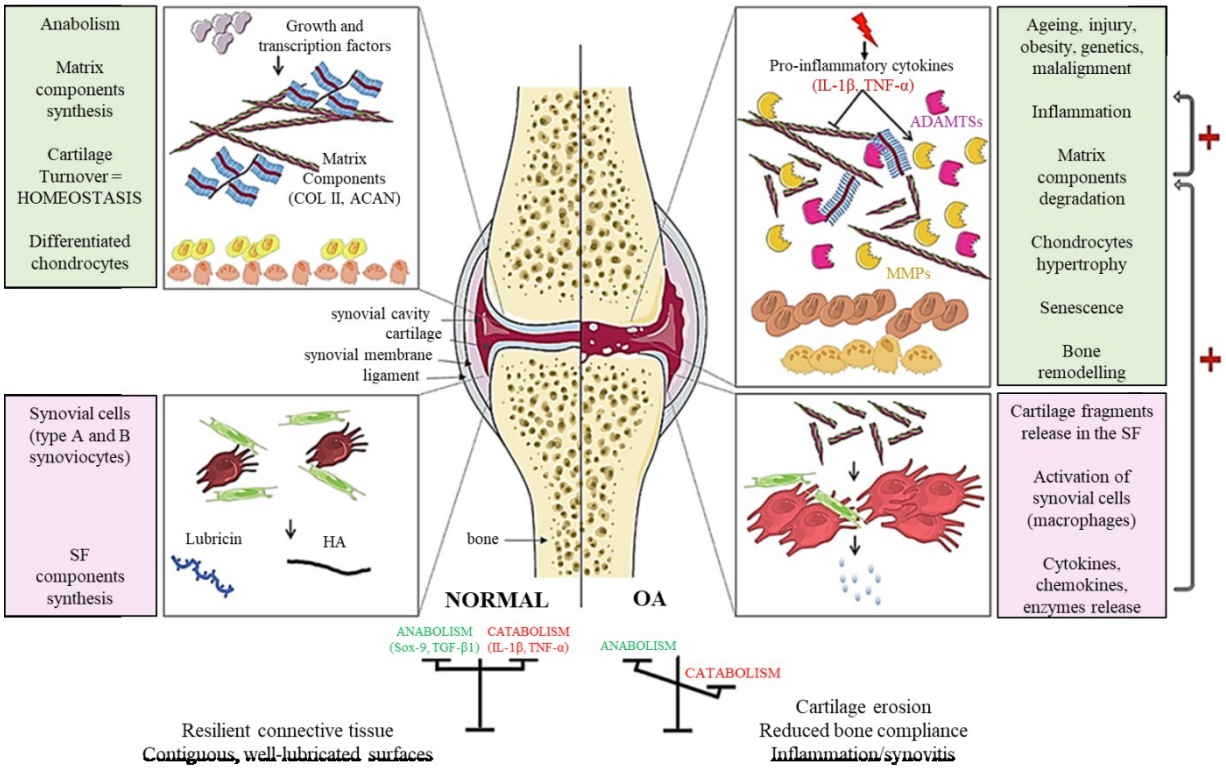


Figure 6. Schematic overview of structure and mechanism changes involved in an osteoarthritis joint and comparison with a normal joint [22].

1.2.2 Symptoms, diagnosis, and treatments

As previously said, symptoms of OA most commonly include pain, swelling, and stiffness in the affected joint, resulting in a degradation of articular cartilage, sclerosis, bone deformity, osteophyte formation, and inflammation of the synovial membrane [23,24]. Symptoms often develop slowly and get worse over time with aninsidious onset. In the primary/early stage of the pathology, occasional pain, joint stiffness, and discomfort characterize the initial course of the disease. In the later/symptomatic stages of OA a chronic inflammatory response in the synovium and bone, with a high level of discomfort during locomotion, is present. Finally, in the more severe stages of OA, all joint structures are affected, lubricating fluid is lost, leading to disability and total loss of movement. Once the degenerative evolution of OA reached this critical level, the solution involves a total joint replacement [25]. This highly invasive procedure requires extensive post-operative rehabilitation, has a high cost to healthcare systems, and does not rule out subsequent complications such as pain, dislocations, infections, etc. Depending on structural changes of the joint identifiable by radiographic evaluation, it is possible to define a degree of OA severity; one of the earliest and most used grading systems is that provided by Kellgren and Lawrence [26]. This system allows to evaluate the pathological status based on osteophytes formation, joint space narrowing, sclerosis and bone deformity, with a classification ranging from grade 0 to grade IV: Grade 0, no radiographic features typical of OA; Grade I, initial osteophytes formation and possible joint space narrowing; Grade II, well defined osteophytes; Grade III, joint space narrowing, sclerosis, possible bone deformity, and multiple osteophytes; Grade IV, large osteophytes, marked joint space narrowing, pronounced bone deformity, and severe sclerosis.

X-ray, computed tomography (CT) scan, magnetic resonance imaging (MRI) and clinical scores are the main diagnostic methods employed for OA, sometimes also supported by quantification of serum or plasma biomarkers, mainly used for early stage diagnosis, progression assessment and therapeutic monitoring [27,28]. Despite a wide range of treatments available, to date there is still a large lack of effective therapies capable of restoring the functionality of the cartilage tissue affected by OA [29]. Although the primary treatment strategies for OA are nonpharmacological interventions, such as education, weight loss, and exercise, pharmacological treatment with acetaminophen/paracetamol and nonsteroidal anti-inflammatory drugs (NSAIDs) are key modalities for patients with symptomatic OA. Alternative strategies also include infiltrative therapies. Specifically, intra-articular injections of therapeutic agents, such as hyaluronic acid viscosupplements,

allow the treatment of joint pain as an alternative to therapies with NSAIDs drugs [30]. Another existing approach is based on the injection of autologous platelet-rich plasma (PRP), used to induce a healing response based on the patient's pro-healing factors [31]. Although this technique has shown good clinical results, the treatment is affected by enormous inter-patient variability, a lack of total pain relief, and an unclear mechanism of action. Another alternative is represented by cell therapy; introduced in the 1980s, it has rapidly developed andcurrently offers a long-term solution to repair cartilage, alleviate symptoms, and delay OA progression. Cell therapy applies to both mature cells, such as chondrocytes, and stem cells [32]. However, it is still limited interventions, which seems to be decisive only in younger patients, on some joints and in early grade OA without affecting the underlying bone. Finally, another option can be represented by tissue engineering that uses cells, scaffolds, and bioactive factors to enhance tissue mechanical properties and promote cell migration, attachment, proliferation, and differentiation. To date, tissue engineering has shown promising outcomes in treating cartilage defects, including OA [32], but few clinical data are still available. Although current pharmacologic and regenerative therapies show promising results, several limits are still present. Thus, the identification and availability of new effective treatments are mandatory and could significantly lengthen the time of OA diagnosis, increasing the healthy and active lifespan of middle-aged and older people.

1.2.3 Risk factors

As for other pathologies, also for OA there is a combination among comorbidities, anatomy, genetic predisposition of the individuals and the influence of environmental factors in the onset and aggravation of the pathology. Risk factors associated with OA are multiple and can be divided into genetic, metabolic, biochemical and biomechanical factors, and patient-related factors, including age, sex/gender, obesity, and hormone levels.

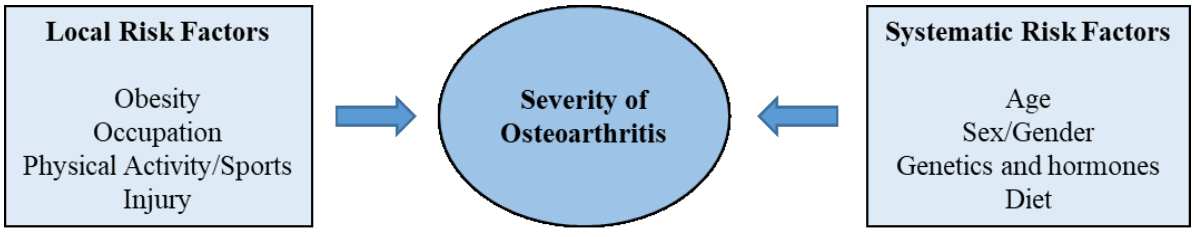


Figure 7. Risk factors of osteoarthritis.

These factors can act alone or in synergy to initiate a cascade of intra-articular pathophysiological reactions, which lead to: (1) alteration of joint homeostasis, (2) activation of the non-antigen-specific innate immune response with consequent (3) release of pro-inflammatory cytokines, accumulation of cells debris and disruption of the collagen fibrillary network within the joint. Between patient-related factors the sex and age certainly have a strong impact, but are usually considered as adjusting factors in regression models. A growing body of evidence suggested that sex differences exist in clinical and preclinical manifestations, symptoms, and OA progression. Women resulted a higher risk of OA, mostly after 50 years of age (increasing dramatically around the time of menopause) and experienced more debilitating pain than men. Concerning aging, it is characterized by the accumulation of cellular senescence and of degenerative stimuli inside and outside the cell; in OA a progressive degeneration of the articular cartilage and surrounding joint tissues together with an inflamed microenvironment is observed. In the following paragraphs will be explored the molecular and biological differences of sex and aging during OA.

1.2.3.1 Sex

Despite being understudied, sex is an important risk factor in the development of OA [33]. Sex differences were evidenced in the composition of synovial fluid and in cells (chondrocytes, chondroblasts, osteoblasts and FLSs) isolated from different tissues of OA patients, or from animal tissues, mainly at the molecular level and in gene expression of inflammatory cytokines and hormone receptors [34-36]. Furthermore, sex differences in the development and severity of OA were found in histopathological aspects, in terms of bone architecture, osteophyte formation, synovial inflammation, cartilage degeneration, loss of density bone loss, subchondral bone deterioration, and pain development [36,37].

At clinical level, sex differences were noted also in OA incidence and have been detected in symptoms severity and in pain perception [38]. To date, the results reported in the literature confirm that women compared to men show a higher prevalence of OA and more debilitating pain, especially after the age of 50, with a clear increase during the menopause period. This appears to be related to the role of hormones, especially estrogens, as possible explanatory factors [39]. A postmenopausal decrease in estrogen levels results in an increasing risk of OA [40]. Some studies have found evidence that hormone replacement therapy was significantly protective [41]. Treatment of postmenopausal women with a selective estrogen receptor modulator has show to decrease cartilage-specific type II collagen degradation products [41]. Conversely, an increased prevalence of OA has been found in patients on

hormone replacement therapy [42]. However, to date, results are conflicting and are based on a dual view in which estrogens appear to be protective for OA or linked to OA onset, pointing to a multitude of complex effects that may influence disease onset and evolution [43].

A recent study comparing female healthy cartilage samples with those of males also revealed 36 differential expression genes (DEGs), indicating the fundamental sex-related differences at the molecular level. The different signaling response to OA in the male and female cartilage was further enforced by recognizing 50 genes with significantly different OA responsive expression fold changes in males and females. Particularly, 14 Reactome pathways, such as "Extracellular matrix organization", "Collagen biosynthesis and modifying enzymes", "Dissolution of fibrin clot", and "Platelet Aggregation (Plug formation)", can be noted from these 50 sex-dependent OA-responsive genes [44]. However, a more detailed dissection of the molecular events in the OA is needed to comprehensively uncover the sex-relative differences.

Over the years, several clinical studies have evaluated sex differences in patients with OA also in relation to anatomy and biomechanics (gait and kinematics), as well as pain, and functional outcomes after arthroplasty [45]. The results showed differences in the size of articular cartilage, trabecular bone, femur and tibia, with smaller values in women than in men [46-49]. This led to women having a higher risk of worsening OA than men. Sex also influenced joint motion and rotation, and stride length variability, in terms of increases in muscle volume, speed, and power in men compared to women [50-55]. In studies of sex differences in pain experience, it was found that women had a worse pain scenario, with increased Visual Analogue Scale (VAS), serum C-reactive Protein (CRP), inflammatory cytokines, and more impaired function than men. In addition, it was shown that, after arthroplasty, men achieved better and faster functional and physical recoveries, regardless of the joint replaced. Contrarily, women had worse final functional outcomes, probably due to a more advanced stage of disease, older age, and greater muscle wasting at the time of surgery [56]. Taking into consideration pain perception and pain measurement, it was found that women have also a lower sensitivity thresholds and pain thresholds than men [57,58]. However, it is important to underline that multiple biological, genetic, ethnic, emotional, environmental, and psychosocial factors contribute to pain and pain sensitivity in OA patients [59-62]. The real reasons for these differences are not yet fully understood, but understanding how and why OA occurs differently in men and women is critical to developing effective personalized strategies and refining/improving diagnostic techniques.

1.2.3.2 Age

Most individuals affected by OA are over the age of 65. OA has long been thought of as an inevitable part of aging. The relationship between aging and OA is complex. As joints age, their ability to withstand insult lessens. As a degenerative disease, cellular senescence has been proposed to be involved in OA. Cellular senescence is a cell state characterized by permanent cell cycle arrest, resistance to apoptosis, and the continuous secretion of senescence-associated secretory phenotype (SASP) factors. Chondrocyte senescence induced by a variety of harmful biomechanical factors is thought to induce stress and cause irreversible damage, leading to accumulation of reactive oxygen species (ROS) and cell death [63].The imbalance between ROS production and the ability to repair tissue damage through endogenous antioxidant defenses causes an increase in oxidative stress which induces negative effects on cellular function (damage to proteins, nucleic acids and lipids) and decreases the ability to repair of the tissues. Furthermore, the decline of mitochondrial function of cells is accompanied by reduced cellular autophagy, mechanism used by cells to compensate for stress without causing further damage, and increased apoptosis [64]. Autophagy declines in aging cells, and as a result, proteins are aggregated, misfolded proteins are accumulated, and dysfunctional mitochondria are formed, all culminating in oxidative stress. Furthermore, pro-inflammatory cytokines, catabolic state, elicit inflammatory responses and inhibit autophagy, leading to increased chondrocyte apoptosis [65]. Therefore, aging is associated with chronic systemic inflammation characterized by an imbalance between inflammatory and anti-inflammatory pathways. When the catabolism of aging cells is active, a remodeling of the cartilaginous matrix occurs with alterations of cell morphology and density; senescent cells decrease their proliferation and synthesis of matrix components [63,66]. However, the ability of cells to produce pro-inflammatory mediators and MMPs remains unchanged [67]. In the elderly, collagen fibers increase, while peptidoglycans and water decrease, therefore, cartilage is less responsive to mechanical stress and can undergo deformation. Cartilage undergoes a thinning process due to a decreased diffusion of nutrients among the ground substance, and loses its transparency and becomes opaque and yellow. Cartilage is not vascularized and cells undergo atrophy. Thus, in the joints of elderly patients, degradation of articular cartilage is observed due to the production of pro-inflammatory cytokines and catabolic factors that contribute to cartilage damage, thereby increasing the rate of inflammation and oxidative stress processes [68-70]. Even the synovial membrane, which in healthy conditions contributes to the maintenance of joint homeostasis and provides nourishment, protection and lubrication through the

production of hyaluronic acid to the joint cartilage, appears to be altered and this further activates the inflammatory pathway [68,69].

1.2.4 Primary mediators of the inflammatory response

The immune system plays a crucial role in the pathogenetic mechanisms of OA. Both humoral and cellular mediators contribute to cartilage destruction, abnormal bone remodeling, synovitis, and joint effusion [71]. The innate immune system plays a significant role in this process. Damage-associated molecular models (DAMPs) are endogenous molecules that include ECM damage products, free fatty acids, and dead cell debris. DAMPs activate the innate immune system by interacting with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), especially TLR2 and TLR4, which mediate catabolic responses, and the receptor for advanced glycation end products (RAGE) present on the surfaces of immune cells. The activation of the complement system is implicated in the early stages of OA. The released products induce apoptosis of chondrocytes or cause them to produce matrix-degrading enzymes, proinflammatory mediators, and other effectors that promote joint damage [72].

Several inflammatory cytokines such as IL (IL-1, IL-6 and IL-8) are involved in the progression of OA. These mediators act both autocrine and paracrine manners. They stimulate macrophages and chondrocytes to release proteases and eicosanoids, such as prostaglandins and leukotrienes, as well as nitric oxide (NO). Furthermore, this activity in cartilage results in the induction of catabolic processes, inhibition of matrix synthesis and promotion of apoptosis [73]. In normal adult cartilage, chondrocytes synthesize matrix components very slowly [74]. During OA, hypertrophic chondrocytes lose the ability to form new cartilage matrix. As a result, the subchondral bone undergoes abnormal remodeling at the interface between the bone and the calcified cartilage. The released MMPs degrade the matrix and, therefore, the articular cartilage. This leads to the formation of subchondral cysts and osteophytes. Abnormal bone remodeling also causes subchondral sclerosis, which can occur late in the disease process or cause OA [75]. The release of certain products, including other cytokines, such as IL-1, IL-4, IL-9, IL-13, and TNF-α, and degrading enzymes, such as ADAMTs and MMPs, from chondrocytes, osteoblasts and synoviocytes trigger these processes [75]. The production of IL-1 by activated chondrocytes induces the synthesis of MMPs, namely MMP-1, MMP-3 and MMP-13, and the simultaneous inhibition of the synthesis of key components of the ECM, such as proteoglycans, aggrecan and type II collagen. This is accompanied by the amplification of proinflammatory cytokines such as

TNF-α, IL-6 and IL-8, which enhance the degradation of the cartilage matrix in the catabolic cascade, further stimulating the destruction of joint chondrocytes [74]. The source of pain in OA is related to inflammation of the synovial membrane, called synovitis, which undergoes progressive fibrotic changes.

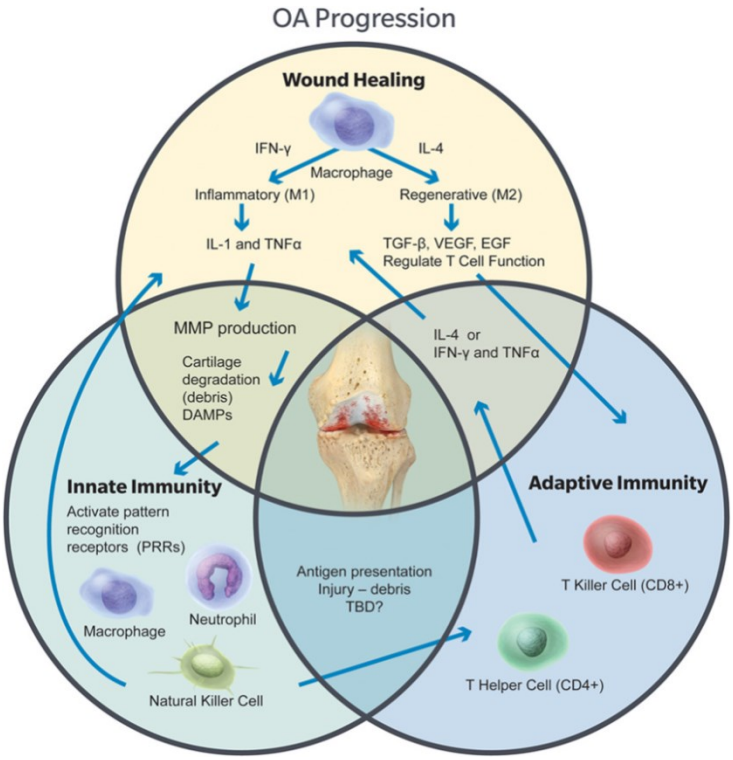


Figure 8. Role of inflammation and the immune system in the progression of osteoarthritis [76].

1.2.5 Components of cellular response and cells involved

Monocytes appear to be involved in the subchondral bone and synovium activation in the pathogenesis of OA. Cytokines and chemokines released by monocytes are found in increased concentrations in the synovial fluid during OA. In animal models, the depletion of synovial macrophages, derived from monocytes, decreases osteophyte formation and cartilage destruction. These data suggest that monocytes, monocyte-derived osteoclasts, and synovial macrophages participate in the pathogenesis of OA. Therefore, reductions in monocyte activation and joint recruitment may be beneficial in OA [71]. Osteoclasts derived from monocytes in the appropriate microenvironment can contribute to cartilage degradation. These cells release vascular endothelial growth factor (VEGF), TNF-α, IL-1β, IL-6, and chemokines, stimulating synovial vascularization and recruitment of circulating white blood cells. Thickening of the synovial membrane is involved in migrating monocytes,

which differentiate into synovial macrophages. Synovial fluid components can additionally activate subchondral bone through osteochondral lesions [71]. Chondrocyte hypertrophy has been described in OA. Hypertrophic changes trigger chondrocyte activity and play a key role in the development of OA. Cartilage destruction occurs through many mechanisms, most notably the increased production of proteases such as MMP-13. These alterations drive the strengthening cycle of the disease and result in tissue degradation, remodeling and ultimately calcification [77]. Neutrophils, present in the synovial fluid of patients with joint disorders, have the greatest impact on cartilage degradation. In joint inflammation, neutrophils invade the joint space and release several cartilage-damaging products, such as ROS and released proteolytic enzymes [78]. Furthermore, some neutrophil products contribute to the development of joint inflammation, pain, and neuropathy, suggesting their potential as a therapeutic target [79,80]. Macrophages are recognized as key factors in inflammatory joint disorders. Depending on their phenotypes, M1 or M2 macrophages can act as pro- or anti-inflammatory cells. In the presence of interferon (IFN), macrophages are polarized towards an M1 proinflammatory phenotype with release of TNF-α and IL-1β. This activity leads to exacerbation of the inflammatory process and destruction of cartilage.

1.3 Osteoarthritis models

1.3.1 *In vitro* models

In vitro models are able to provide reproducible data, thanks to the ability to precisely control the experimental parameters, and are fast and inexpensive [81]. Depending on the cell culture type and its interaction with the ECM, *in vitro* models can be divided into two-dimensional (2D) culture (monolayer or co-culture), three-dimensional (3D) culture (scaffold-free or scaffold-based) and explant (tissue)-based culture [82]. The advantages of *in vitro* studies are the ease of performing cultures which in turn allow the study of many aspects, from the basal expression of phenotypic markers to gene expression and secretion of cartilage matrix molecules or pro-inflammatory mediators or DNA differences and sex chromosomes, both as their intrinsic properties and in response to several stimuli [83]. Furthermore, the possibility of isolating cells from OA pathological tissues is relevant for the *in vitro* set-up characteristics typically expressed in the pathology in order to improve the basic knowledge of OA and test innovative regenerative/reparative therapeutic strategies.

Various biological and synthetic inflammatory stimuli can be used to induce OA-like conditions *in vitro*, which include, for example, cytokines, synovial fluid from OA patients,

conditioned media from joint tissues of OA patients, conditioned media from activated macrophages and others. Cytokines play a key role in the progression of OA pathogenesis. During OA, synoviocytes or chondrocytes may increase their expression of catabolic proteins following stimuli such as cytokine or chemokine exposure, including IL-1β and TNF-α, which are present in the joint following synovial inflammation [84,85]. Pro-inflammatory cytokines make ideal candidates for the induction of OA-like biological changes in articular cells, in which temporal and concentration effects can be explored. Models of OA where cytokines are the primary method of induction are common and are generally well understood, usually inexpensive and easily manipulated. Evidence for a role of IL-1β in OA is well established, and it has been used as a potential therapeutic target, for example through the design of vectors activated by IL-1 that protect against its catabolic effect or through the antagonism of the IL-1 receptor (IL-1R) [86,87]. Exposure to IL-1β stimulates chondrocytes and synoviocytes to produce catabolic proteases, with autocrine signalling further enhancing MMPs release and the resulting degradative cascade [83,88]. The catabolic response can be blocked by the inhibition of IL-1β through antagonism with the IL1-R antagonist (IL-1Ra) [89]. Inflammatory molecules produced by chondrocytes in response to IL-1β include prostaglandin (PG)E2, cyclooxygenase (COX)-2, IL-6 and IL-8. IL-1β also leads to the accumulation of ROS, through expression of inducible nitric oxide synthase (iNOS) by the transcription factor nuclear factor kappa B (NF-κB), ultimately leading to apoptosis. This mechanism can also be accelerated by IL-1β-mediated damage to mitochondrial DNA, leading to a further release of ROS and enhancing apoptosis [90]. TNF-α has also been used to induce OA-like changes in *in vitro* experiments, because it is found in diseased synovial fluid, and is able to induce catabolism and inhibit anabolic pathways in joint tissues and cartilage cells [91-93]. Cytokine-based models use a wide variety of concentrations and duration of stimulations. The choice of whether to use a monolayer, a cell scaffold or tissue influence the cells' response to the cytokine stimulus applied. Monolayer cultures allow the expansion of the cellular resource [94]. The ease of using chondrocytes in monolayer, combined with their rapid response to cytokine stimulation, has resulted in this being the most widely used model. Models that use cytokines added to cell culture medium have been shown to produce OA-like responses in chondrocytes in monolayer cultures, such as a decrease in the expression of type II collagen and aggrecan, and an increase in the expression of MMP-13 [95-97]. Since OA is recognized as a disease affecting and involving multiple tissues, co-culture experiments allow more study of these interactions *in vitro*. *In vitro* cartilage-synovia co-culture models were developed and characterized; both OA synovial tissue and cartilage from the same knee from an OA patient

have been used to prevent direct cell-to-cell contact [98]. Furthermore, 3D co-culture models were developed to study the interaction between chondrocytes and osteoblasts and to evaluate the influence of co-culture on the growth and maintenance of phenotyping of the mentioned cells [99]. Subchondral osteoblasts in the monolayer were cultured together with OA chondrocytes in alginate spheres, to study the effect of OA osteoblasts on bone sclerosis and cartilage degradation [100]. Finally, tissue explants have the advantage of native ECM and spatial organization, and their use for the assessment of cellular responses is a simple and convenient method because the cells are in a natural environment [81]. However, the availability of healthy clinical specimens is extremely limited. Clinical specimens are usually obtained from patients with advanced OA, but they can be contaminated with adipose tissue and are difficult to handle due to their irregular and deformed shape [101].

1.3.2 *In vivo* models

Over the years, several animal species, such as mice, rats, rabbits, guinea pigs, sheep, and horses, have been used as OA models. The main goal of animal models is to reproduce human pathology, as closely as possible. The choice of each animal depends on several factors, including the design and timing of the experiment, costs, ease of handling, and outcome measures [75]. The most used models are small animals [102]. Small animals (mice, rats, guinea pigs and rabbits) are used to evaluate the pathogenesis and pathophysiology of OA because they show more rapid disease progression and are relatively cheap and easy to handle. Large animals (goats/sheep and horses) are mainly used to study the process and treatment of OA [103]. Due to their anatomical similarity to humans, these large models allow the efficacy of a treatment to be confirmed before clinical use. The main OA preclinical *in vivo* models are spontaneous OA model, which in turn is subdivided into natural (primary OA) and generated by genetic abnormalities, and OA induced, either via a surgical procedure (secondary OA) or via intra-articular injection of chemicals [104]. Among the main advantages of the mouse as an animal model in OA studies is the ability to genetically modify specific strains, particularly those susceptible or resistant to OA. In the case of knockout mice, the lack of some proteases or the inactivation of the collagen type IX alpha-1 gene could make them resistant to developing OA. *STR/ort* mice can be used to show a correlation between OA and chondrocyte metabolism, whereas *Col2a1* knockout mice have a higher incidence of natural OA than wild-type mice [105]. Models of OA surgically induced via anterior cruciate ligament (ACLT) transection, meniscus destabilization, meniscectomy and collateral ligament transection, or chemically with monosodium

iodoacetate (MIA), papain, collagenase, and quinolones, can be used to evaluate the effects of different treatments of OA, the mechanism of pain and drug therapy. The typical animal model for studying naturally occurring OA is the Dunkin Hartley guinea pig, reflecting the human conditionof elderly [106]. This model allows to evaluate the pathogenetic mechanisms, the inflammatory process of the joint and the therapeutic applications. In some strains of mice, such as *STR/ort* and *C57BL/6*, the genetic predisposition to develop spontaneous OA has been confirmed. Concerning large animal models, horses are mainly used for the study of articular cartilage repair, osteochondral defects and bone remodeling, as their large size facilitates the observation of joint damage and cartilage thickness. Similarly, sheep are used to evaluate early cartilage changes during OA, to study meniscus changes and related surgical techniques due to its anatomical similarity to humans [75].

2. AIM OF THE STUDY

Sex affects the pathophysiology and severity of several diseases, including OA. Despite being understudied, sex is an important risk factor in the development of OA. Sex differences in OA are neither well addressed nor fully understood, and are still debated, especially from a cellular, molecular and mechanistic point of view due to the paucity of articles that have analyzed these aspects in both preclinical and clinical studies.

The aim of this study was to evaluate the behaviour of two cell phenotypes, human osteoarthritic synoviocytes and chondrocytes, and the role of sex differences in the presence of an inflammatory (typical of OA) and/or senescent (typical of aging) microenvironment, using a 2D *in vitro* model of monocultures and co-cultures.

For this purpose, cell cultures of both sexes were stimulated for 24 hours with the addition of two damaging factors: (1) inflammatory cytokine IL-1β, to reproduce an inflamed OA microenvironment, and/or (2) hydrogen peroxide H_2O_2, a factor that induces oxidative stress. The effect of the inflammatory cytokine and hydrogen peroxide, alone and in combination, was evaluated on the rate of proliferation, migration, gene expression of pro-inflammatory molecules, enzymes and markers of senescence and apoptosis in chondrocytes and synoviocytes through the use of biological and molecular techniques, mainly considering sex differences.

Furthermore, an *in vivo* model of aging and spontaneous OA in elderly animals (mouse strain *C57BL/6*), was investigated through histological and microtomographic analyses, to evaluate the possible presence of distinctive signs of the disease and any sex differences in the structure and morphology of the joints components (bone, articular cartilage and synovial membrane) and of the column.

From this *in vitro* study, limiting the complexity of the *in vivo* patophysiological microenvironment and condition, we expect to identify possible sex associated differences in the basic intrinsic cell behavior of the two cell phenotypes investigated as well as in their response to selected stimuli involved in OA.

3. MATERIALS AND METHODS

3.1 Cell cultures

3.1.1 Fibroblast-like synoviocytes

Human fibroblast-like synoviocytes (HFLSs), isolated from male (M) and female (F) donors with knee OA, were purchased from TebuBio (Cell Application, San Diego, CA, USA, n=5 for each sex). The donors were Caucasians ranging in age from 53 to 69 years and with a body mass index (BMI) between 25 and 30 for both sexes. The cells were cultured in synoviocyte growth medium (Cell Application, San Diego, CA, USA), with 10 % fetal bovine serum (FBS) (Euroclone, MI, Italy), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich, St. Louis, MO, USA), in standard conditions (37 °C, 5 % CO_2/95 % air, humidified atmosphere). When they were confluent, the cells were detached using Trypsin/ethylendiaminetetracetic acid (EDTA) (Sigma Aldrich, St. Louis, MO, USA) and counted in a Neubauer chambre using Erythrosin B vital dye (Alfa Aesar, Tewksbury, MA, USA). HFLSs were seeded at the bottom of 24-well plates at a density of $4x10^4/cm^2$ in synoviocyte growth medium [107].

3.1.2 Chondrocytes

Human chondrocytes (HCs), isolated from male (M) and female (F) donors with knee OA, were purchased from TebuBio (Cell Application, San Diego, CA, USA, n=5 for each sex). The subjects were Caucasians ranging in age from 54 to 65 years and with a BMI between 25 and 30 for both sexes. The cells were cultured in chondrocyte growth medium (Cell Application, San Diego, CA, USA), with 10 % fetal bovine serum (FBS) (Euroclone, MI, Italy), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich, St. Louis, MO, USA), in standard conditions (37 °C, 5 % CO_2/95 % air, humidified atmosphere). When they were confluent, the cells were detached using Trypsin/ethylendiaminetetracetic acid (EDTA) (Sigma Aldrich, St. Louis, MO, USA) and counted in a Neubauer chambre using Erythrosin B vital dye (Alfa Aesar, Tewksbury, MA, USA). HCs were seeded at the bottom of 24-well plates at a density of $5x10^4/cm^2$ to reach a hyperconfluent status in chondrocytes growth medium [107].

3.1.3 Co-culture model

To set-up the co-cultures, each cell type was seeded separately on proper supports and assembled in the final 24-well plate. Briefly, HFLSs were seeded at the bottom of well plates at a density of $4x10^4/cm^2$, while HCs were seeded onto the botton of a cell culture insert (0.4 μm pore-size, Millipore, Carrigtwohill, Country Cork, Ireland) at a density of $5x10^4/cm^2$. The medium was a mixture of each specific culture medium.

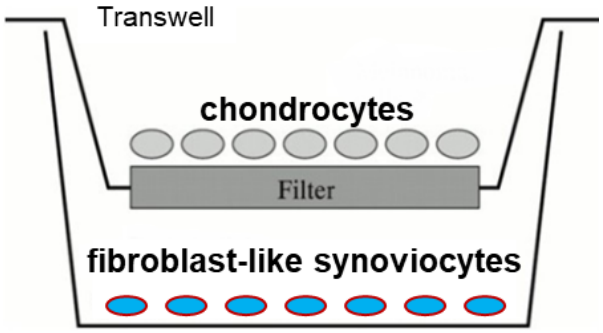


Figure 9. Schematic representation of the cell co-culture model.

3.2 Experimental set-up

Twenty-four hours after seeding, cell cultures were stimulated by setting up the following four experimental groups:

- Control group (CTR): the cells re-feed with synoviocyte or chondrocyte growth medium;
- Interleukin-1β (IL-1β) group: the cells cultured in synoviocyte or chondrocyte growth medium were supplemented with 10 ng/ml IL-1β (Cell Application, San Diego, CA, USA), to replicate the inflammatory microenvironment present in OA conditions and to maintain the OA phenotype;
- H_2O_2 group: the cells cultured in synoviocyte or chondrocyte growth medium were supplemented with 300 μM H_2O_2 (Farmac-Zabban, Bologna, Italy), for the induction of the oxidative stress that is typical of aging;
- IL-1β+H_2O_2 group: the cells cultured in synoviocyte or chondrocyte growth medium were supplemented with 10 ng/ml IL-1β and 300 μM H_2O_2 to evaluate the combined effect of inflammatory microenvironment and oxidative stress.

A concentration of 300 μM H_2O_2 was chosen based on previous studies and preliminary assays were performed to identify the best concentration able to stimulate oxidative stress without causing cell death [108,109]. In addition, as described in previous studies, a concentration of 10 ng/mL of IL-1β was used [107, 110-112].
All the experiments were performed in triplicate and evaluations were performed after 24 hours of cultures.

3.3 Cell viability assay

An Alamar Blue assay (AbDSerotec, Oxford, UK) was used to evaluate the cell viability 24 hours after the set-up of the monoculture and co-culture conditions of the 4 groups. The reagent was added to each culture well (1:10 v/v) and incubated for 4 hours at 37 °C. In particular for the co-cultures, the reagent was added both in the well containing HFLSs and in the transwell containing HCs, and medium was mixed for the analysis. The reagent is a dye that incorporates an oxidation-reduction (REDOX) indicator that changes colour in response to chemical reduction of the growth medium that is proportional to cell growth. The fluorescence was read at 530ex-590em nm wavelengths using a Micro Plate reader (VICTOR X2030, Perkin Elmer, Milan, Italy) and expressed as relative fluorescence units (RFU).

3.4 *In vitro* microwound healing model

At the point of confluence, single cultures of HFLSs and HCs cells were used in monolayer for the preparation of an *in vitro* microwound healing model to analyze the ability of the cellular migration and regenerative capacities in disease-related conditions. In detail, 24 hours after seeding, the cells were wounded by sliding a sterile 200 μm Eppendorf tip on the bottom of the well to create a cell free zone in the monolayer (at baseline, T0, wound dimension measured 654 ± 1 μm for HFLSs and 346 ± 1 μm for HCs). After the cellular scratch was performed, the cultures were incubated with the different media as above reported, and they were observed using an inverted microscope (Nikon Eclipse Ti-U, Nikon Italia, Italy) equipped with a digital camera (Sight DS-Fi2, Nikon Italia, Italy) at T0 and T24 hours. Each well was photographed at 4x magnification to cover the wounded width. The image acquisition software (NiSElements Advanced Research, Nikon Italia, Italy) was used to measure width of the cell free zone of the artificially created wound (10 measures for each

image), and the percentage (%) of healing rate was calculated through the following formula [112]:

$$Healing\ percentage\ (\%) = \frac{T0 - T24}{T0}\ width * 100$$

Figure 10. Representative microscopic image of the microwound. Magnification: 4x.

3.5 Gene expression analysis

After 24 hours from the set-up of monoculture and co-culture conditions, the total RNA was extracted using the RNeasy Mini Kit (Purelink™ RNA mini-Kit, Ambion by Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. In the co-cultures, the two cell types were solubilized together. The total RNA was eluted with RNase-free water, quantified by NanoDrop 2000 (NANODROP 2720, Thermal Cycler, Applied Biosystem, Waltham, MA, USA), reverse transcribed to cDNA using Super Script VILO cDNA Synthesis kit (Invitrogen, by Thermo Fisher Scientific, Waltham, MA, USA) and diluted to the final concentration of 5 ng/µl. The gene expression was evaluated by Real Time PCR analysis with SYBR green PCR master mix (QIAGEN GmbH, Hilden, Germany) in a Light Cycler 2.0 Instrument (Roche Diagnostics, GmbH, Manheim, Germany). The protocol included: a denaturation cycle at 95 °C for 15 min, 25 to 40 cycles of amplification (95 °C for 15 s, an appropriate annealing temperature for each target gene, as detailed in Table 1, for 20 s and 72 °C for 20 s) and a melting curve analysis to check for amplicon specificity. The mean threshold cycle was determined for each sample and used for the calculation of the relative expression using the Livak method ($2^{-\Delta\Delta Ct}$), and are present the

results in terms of the number of molecules of the gene of interest (GOI) per 100.000 molecules of the housekeeping gene GAPDH [113]. Each sample was tested in triplicate.

Table 1. Primers details used for gene expression analysis.

Gene	Primer forward	Primer reverse	Amplicon Lenght	Annealing Temperature
GAPDH	QuantiTect Primer Assay (Qiagen) Hs_GAPDH_1_SG		95 bp	55 °C
COL2A1	QuantiTect Primer Assay (Qiagen) Hs_COL2A1_1_SG		94 bp	55 °C
CASP3	QuantiTect Primer Assay (Qiagen) Hs_CASP3_1_SG		147 bp	55 °C
KLOTHO	QuantiTect Primer Assay (Qiagen) Hs_KL_1_SG		143 bp	55 °C
IL-6	QuantiTect Primer Assay (Qiagen) Hs_IL6_1_SG		107 bp	55 °C
ADAMTS-4	5'-CTGCCTACAACCACCG-3'	5'-GCAACCAGAACCGTCC-3'	293 bp	58 °C
ADAMTS-5	5'-GCACTTCAGCCACCATCAC-3'	5'-AGGCGAGCACAGACATCC-3'	187 bp	58 °C
MMP-13	QuantiTect Primer Assay (Qiagen) Hs_MMP13_1_SG		97 bp	55 °C

3.6 Immunoenzymatic analysis

After 24 hours, the cell monocultures and co-cultures supernatants of all the groups were collected and centrifuged to eliminate the particulates. Immunoenzymatic assays, an enzyme-linked immunosorbent assay (ELISA), were employed to quantify the amount of ROS (IU/ml) (MyBioSource, San Diego, CA, USA), according to the manufacturer's instructions.

3.7 *In vivo* model

In accordance with the principle of reduction established by Legislative Decree 26/2014 and Directive 2010/63/EU, tissues shared by other research groups were used for the purpose of this study. The tissues were recovered from animals used by other research groups for other experimental purposes, which otherwise would be disposed of at the end of the experiment. In detail, joints and vertebral columns were explanted from 32-month-old male (n=8) and female (n=8) *C57BL/6J* mice after standard protocol of general anesthesia and pharmacological euthanasia. In the study, it was used 32-month-old *C57BL/6J*

senescent/geriatric mice model, male and female untreated, to study joint aging and OA, as it is known that this is considered a strain predisposed to the development of spontaneous idiopathic OA, i.e. the animals spontaneously develop OA with aging. In addition, in commonly used *C57BL/6J* mice, spontaneous development of OA and thus OA-like changes become detectable starting at an age of 12-18 months [114-116]. Therefore, 32-month-old mice using in this study, definitely show OA development.

3.7.1 Histology

Intact and closed left hindlimb joints (femur, tibia) were fixed in 10 % formalin, decalcified in 5 % solution of formic and hydrochloric acid and processed for paraffin embedding procedures. The joints were then extensively rinsed in distilled water, dehydrated in graded alcohol solutions (70 %, twice in 95 % and three times in 100 %; 60 min for each step), cleared in xylene and finally paraffin embedded. Frontal sections (5 ± 1 μm) were taken through the entire joint by a semi-automated microtome (HM Leica microtome) and 5 sections were obtained from each joint. The slides were stained with Hematoxylin/Eosin (H&E) and Safranin O/Fast Green. For all sections, H&E staining was used to evaluate the morphology of cytoplasmic structures of connective tissue and intracellular substance, while Safranin O staining to assess proteoglycan content and pathological changes. Histological images were taken with a digital pathology slide scanner (AperioScanScope, Leica Biosystems, USA). The slides stained with H&E were scored by two blinded independent investigators, using a modified semi-quantitative grading score: the Kreen Modified Score evaluated at magnifications of 10x, 20x and 40x. The Kreen modified semi-quantitative score is based on cellular analysis of synovitis in order to quantify inflammation by scoring three major components of synovitis: lining layer hyperplasia, activation of resident cells and inflammatory infiltrate. All components are graded semi-quantitatively from 0 to 3 with a maximum score of 9 [117]. The slides stained with Safranin O/Fast Green were scored by two blinded independent investigators, using a semi-quantitative grading score: the Osteoarthritis Research Society International (OARSI) score, evaluated at magnifications of 10x, 20x and 40x. The OARSI score is based on six grades, which reflect depth of the lesion and four stages reflecting severity and extent of OA over the joint surface [118].

3.7.2 Microcomputed tomography

Vertebral column specimens, femur and tibia were fixed in 10 % formalin, rinsed in distilled water, and preserved in 70 % alcohol solution. Subsequently, the samples were scanned with a high resolution microtomography system (Skyscan 1172, Bruker Micro-CT, Belgium) at a source voltage of 70 kV and a source current of 100 μA, using an aluminum filter of 0.5 mm, a rotation step of 0.4 °, at a nominal resolution of 10 μm. The scan was performed on three consecutive and vertically connected fields of view (FOV) until the entire column segment was reached. The reconstruction of the obtained dataset was performed using the SkyScanNRecon software (version 1.7.4.6, BrukermicroCT) accelerated by GPUiii, applying corrections for specific misalignment and ring artifact reduction. Projections along the orthogonal planes (transversal, longitudinal and coronal) of the reconstructed images were obtained using the software DataViewer (version 1.5.6.2, BrukerMicrocT). Volume rendering of reconstructed column segments and joints was obtained using the CTvox software (version 3.3.1, BrukerMicroCT).

3.8 Statistical analysis

All data on *in vitro* experiments are presented as the mean ± standard deviation (SD), and they refer to five independent experiments carried out in triplicate. The statistical analysis of the data was conducted using IBM SPSS v 25 software. After analyzing the homogeneity of variances, the two-way ANOVA test followed by post hoc multiple comparison Tukey test were used to evaluate significant differences among groups; stimulus (CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2) and sex were considered as fixed factors; the significante level was set at $p < 0.05$, indicating the difference between stimuli with the symbol * (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$), and the difference between sexes with the symbol # (# $p < 0.05$; ## $p < 0.005$; ### $p < 0.0005$). Semi-quantitative grading scores data are reported as mean ± SD. The analysis it was carried out using the Student's t test to compare the differences between sexes.

4. RESULTS

4.1 Fibroblast-like synoviocytes

4.1.1 Cell viability

At 24 hours (T24), Alamar Blue assay showed that IL-1β+H_2O_2 group significantly reduced the cell viability in comparison to all other groups in both male and female cells.
Furthermore, male cells showed a significantly higher viability than female in all groups (Figure 11).

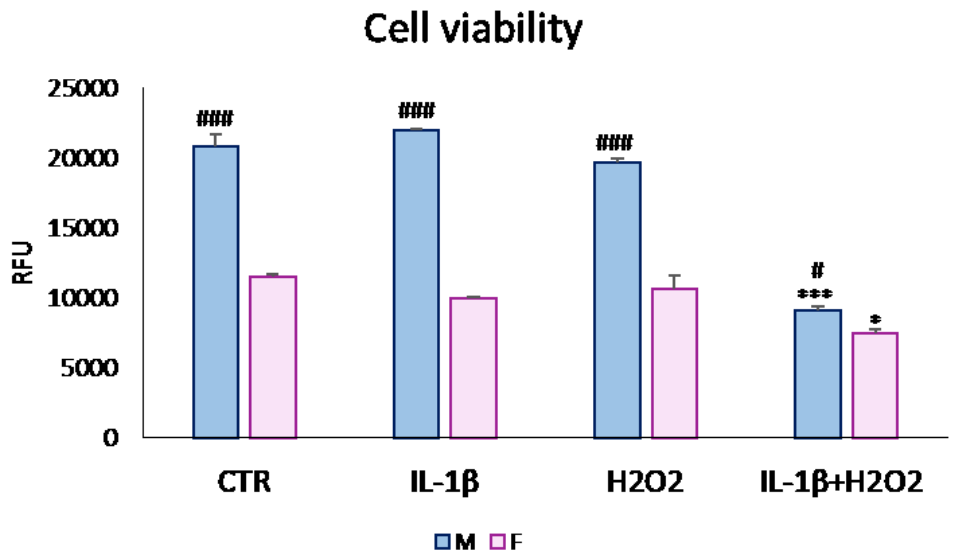


Figure 11. Cell viability (relative fluorescence units—RFU) in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in male (M) and female (F) HFLSs at 24 h (T24) (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

IL-1β+H_2O_2 *vs.* all other groups: *** $p < 0.0005$ for M; * $p < 0.05$ for F.

M *vs.* F: ### $p < 0.0005$ for CTR, IL-1β and H_2O_2; # $p < 0.05$ for IL-1β+H_2O_2.

4.1.2 Microwound healing

At T24, after the cellular scratch, IL-1β+H_2O_2 group significantly decreased the rate of cellular migration compared to all other groups in both male and female cells. In female cells, IL-1β and H_2O_2 groups also decreased migration than CTR.

Furthermore, male cells showed higher migration rate than female in both H_2O_2 and IL-1β+H_2O_2 groups (Figure 12).

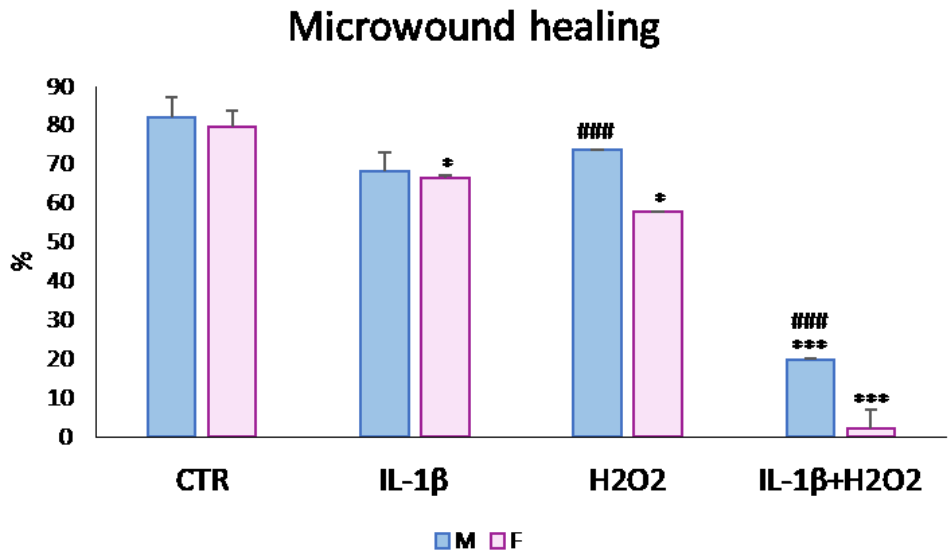


Figure 12. Microwound healing percentages (%) in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HFLSs at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

IL-1β+H_2O_2 *vs.* all other groups: *** $p < 0.0005$ for M and F.
IL-1β and H_2O_2 *vs.* CTR: * $p < 0.05$ for F.

M *vs.* F: ### $p < 0.0005$ for H_2O_2 and IL-1β+H_2O_2.

4.1.3 Gene expression

At T24, Caspase-3, IL-6, ADAMTS-4, ADAMTS-5, MMP-13, Klotho and COL2A1 expression was evaluated by Real Time PCR analysis (Figures 13 and 14).

The female compared to male cells showed higher Caspase-3 expression in all groups (Figure 13 a). Furthermore, females showed a significantly higher IL-6, ADAMTS-4 and ADAMTS-5 expression than male cells in all treated groups except CTR (Figure 13 b-d), while for MMP-13, female cells showed higher values in H_2O_2 and IL-1β+H_2O_2 groups (Figure 13 e).

Caspase-3 and ADAMTS-4 expression was significantly higher in both male and female cells of all treated groups than CTR (Figure 13 a, c). The IL-6 and ADAMTS-5 expression was significantly higher in female cells of treated groups than CTR (Figure 13 b, d). However, for male cells, IL-6 and ADAMTS-5 expression no significant difference showed between groups (Figure 13 b, d). Furthermore, MMP-13 expression was significantly higher in female and male cells treated with H_2O_2 and IL-1β+H_2O_2 than CTR and IL-1β groups (Figure 13 e).

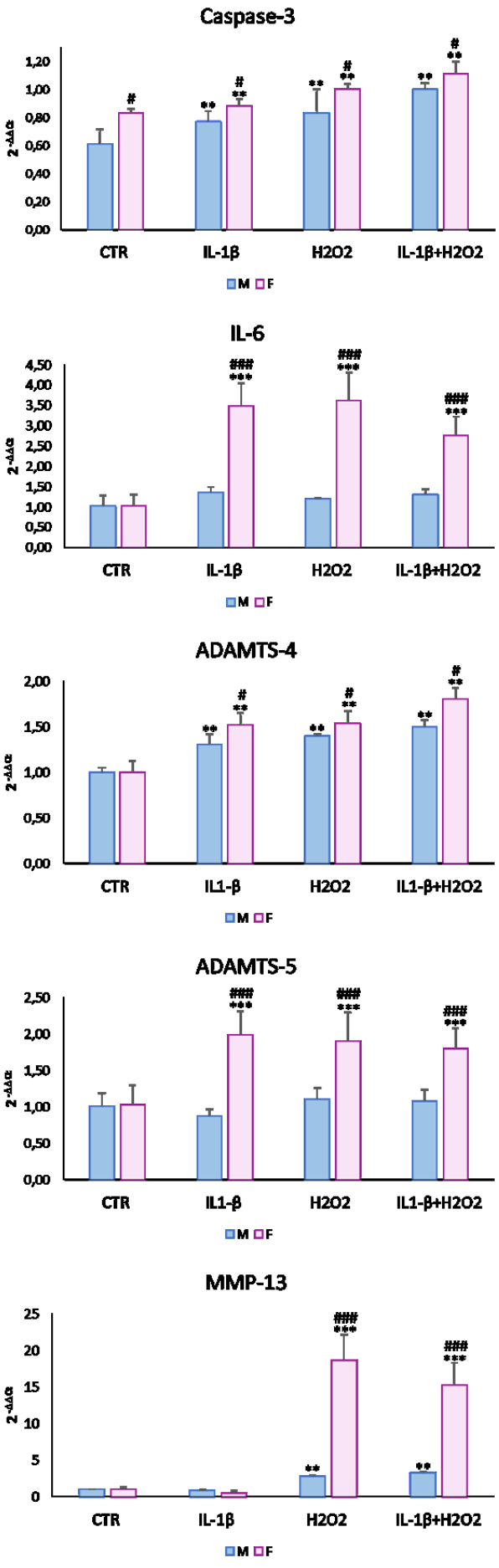


Figure 13 a-e. (a) Caspase-3, (b) IL-6, (c) ADAMTS-4, (d) ADAMTS-5 and (e) MMP-13 gene expression in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HFLSs at T24 (mean±SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

Treated groups *vs.* CTR: (a, c) ** $p <$ 0.005 for M and F; (b, d) *** $p <$ 0.0005 for F.
H_2O_2 and IL-1β+H_2O_2 *vs.* CTR and IL-1β: (e) *** $p < 0.0005$ for F; ** $p <$ 0.005 for M.

F *vs.* M: (a) ## $p < 0.005$ for all groups; (b, d) ### $p < 0.0005$ and (c) # $p < 0.05$ for all treated groups; (e) ### $p < 0.0005$ for H_2O_2 and IL-1β+H_2O_2.

Contrarly, Klotho and COL2A1 expression was significantly higher in male cells than female ones in all groups (Figure 14 a, b).

The male cells showed significantly higher Klotho expression in CTR compared to all other groups, while for female cells, expression was higher in CTR and IL-1β than H_2O_2 and IL-1β+H_2O_2 groups (Figure 14 a). Also, COL2A1 expression was significantly higher in female and male cells of CTR than all other groups (Figure 14 b).

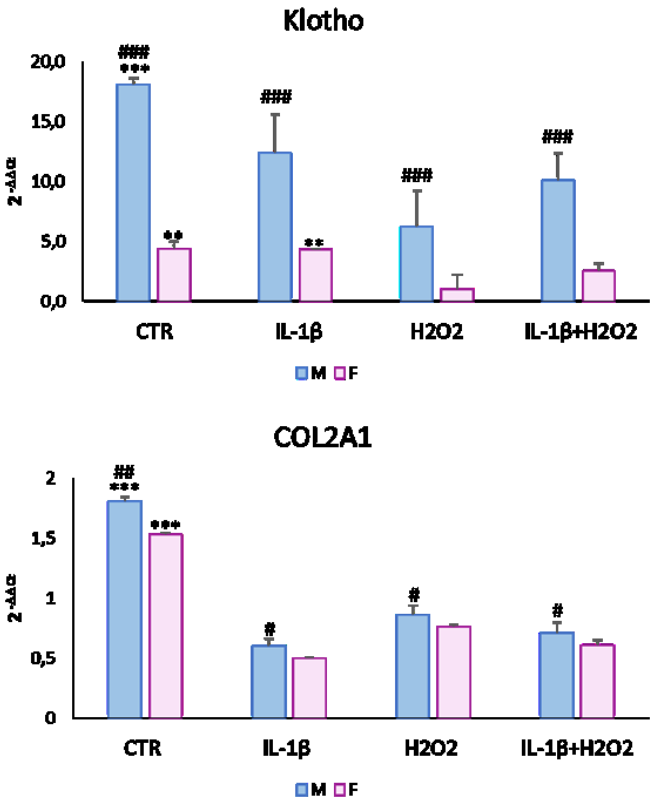


Figure 14 a, b. (a) Klotho and (b) COL2A1 gene expression in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HFLSs at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

CTR *vs.* all treated groups: (a) *** $p < 0.0005$ for M; (b) *** $p < 0.0005$ for M and F.
CTR and IL-1β *vs.* H_2O_2 and IL-1β+H_2O_2: (a) ** $p < 0.005$ for F.

M *vs.* F: (a) ### $p < 0.0005$ for all groups; (b) ## $p < 0.005$ for CTR and # $p < 0.05$ for all treated groups.

4.1.4 ROS level

At T24, the immunoenzymatic assays showed that, in both female and male cells, H_2O_2 and IL-1β+H_2O_2 groups significantly increased ROS production more than CTR and IL-1β groups did.

Furthermore, female cells showed higher ROS levels than males ones in H_2O_2 and IL-1β+H_2O_2 groups (Figure 15).

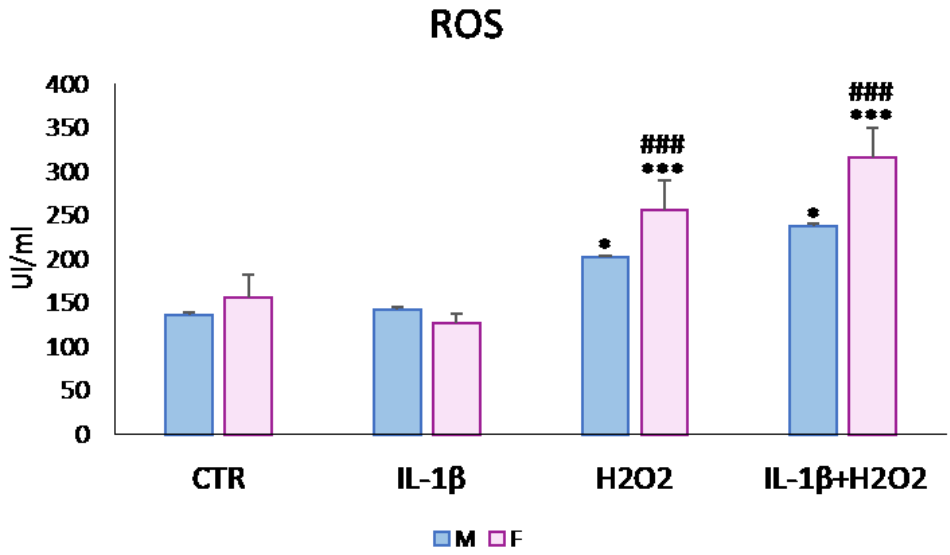


Figure 15. ROS (UI/mL) protein production in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HFLSs at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

H_2O_2 and IL-1β+H_2O_2 *vs.* CTR and IL-1β: *** $p < 0.0005$ for F; * $p < 0.05$ for M.

F *vs.* M: ### $p < 0.0005$ for H_2O_2 and IL-1β+H_2O_2.

4.2 Chondrocytes

4.2.1 Cell viability

At T24, Alamar Blue assay showed that IL-1β+H_2O_2 group significantly reduced cell viability in comparison to allother groups in male cells. In female cells, H_2O_2 and IL-1β+H_2O_2 decreased viability compared to CTR and IL-1β groups.
Furthermore, male cells showed a significantly higher viability than female ones in H_2O_2 and IL-1β+H_2O_2 groups (Figure 16).

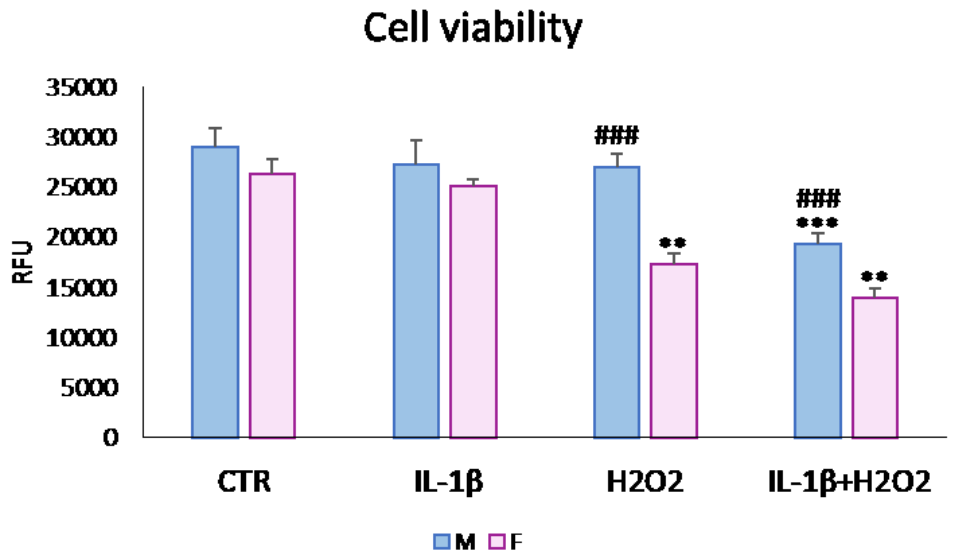


Figure 16. Cell viability (RFU) in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HCs at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

IL-1β+H_2O_2 *vs.* all other groups: *** $p < 0.0005$ for M.
H_2O_2 and IL-1β+H_2O_2 *vs.*CTR and IL-1β: ** $p < 0.005$ for F.

M *vs.* F: ### $p < 0.0005$ for H_2O_2 and IL-1β+H_2O_2.

4.2.2 Microwound healing

At T24, after the cellular scratch, it was observed that IL-1β+H_2O_2 group significantly decreased the rate of cellular migration compared to all other groups in both male and female cells. In female cells, H_2O_2 also decreased migration than CTR and IL-1β groups. Furthermore, male cells showed higher migration rate than female ones in CTR, H_2O_2 and IL-1β+H_2O_2 groups (Figure 17).

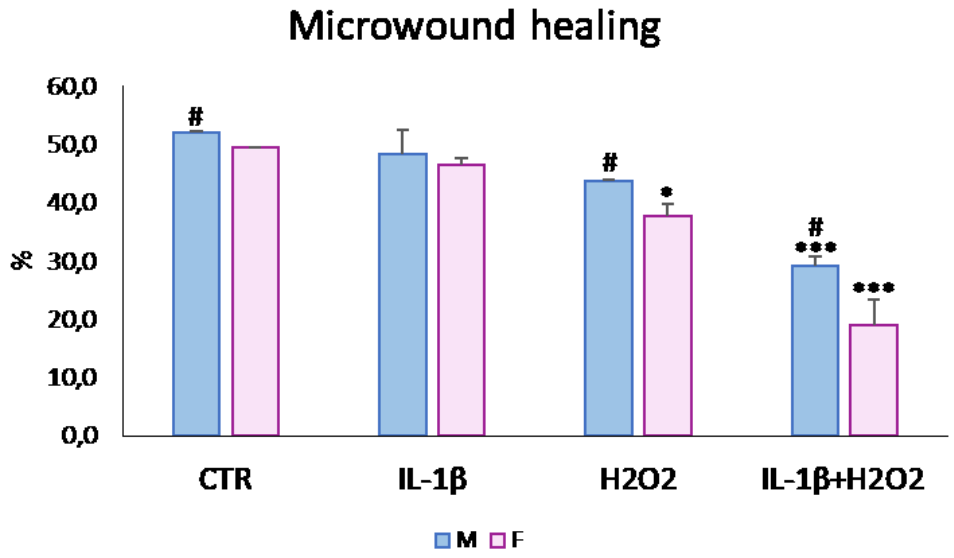


Figure 17. Microwound healing % in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HCs at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

IL-1β+H_2O_2 *vs.*all other groups: *** $p < 0.0005$ for M and F.
H_2O_2 *vs.* IL-1β and CTR: * $p < 0.05$ for F.

M *vs.* F: # $p < 0.05$ for CTR, H_2O_2 and IL-1β+H_2O_2.

4.2.3 Gene expression

At T24, Caspase-3, IL-6, ADAMTS-4, ADAMTS-5, MMP-13, Klotho and COL2A1 expression was evaluated by Real Time PCR analysis (Figures 18 and 19).

The females showed a significantly higher Caspase-3 and MMP-13 expression than male cells in H_2O_2 and IL-1β+H_2O_2 groups (Figure 18 a, e). Furthermore, the females showed a significantly higher IL-6, ADAMTS-4 and ADAMTS-5 expression than male cells in all treated groups except CTR (Figure 18 b-d).

The Caspase-3 and IL-6 expression was significantly higher in both male and female cells treated with IL-1β+H_2O_2 than all other groups (Figure 18 a, b). IL-6 expression also was significantly higher in female cells treated with IL-1β and H_2O_2 than CTR (Figure 18 b). The ADAMTS-4 and ADAMTS-5 expression was significantly higher in female cells treated with IL-1β+H_2O_2 than all other groups (Figure 18 c, d). ADAMTS-4 showed higher values in female cells treated with H_2O_2 than CTR and IL-1β, while the male cells showed higher values in IL-1β+H_2O_2 than IL-1β group (Figure 18 c). ADAMTS-5 showed higher values in female cells treated with H_2O_2 than IL-1β, while the male cells showed higher values in IL-1β+H_2O_2 than IL-1β and H_2O_2 (Figure 18 d). Furthermore, MMP-13 expression was significantly higher in female cells treated with H_2O_2 and IL-1β+H_2O_2 than CTR and IL-1β groups, while for male cells, IL-1β+H_2O_2 showed higher values than all other groups (Figure 18 e).

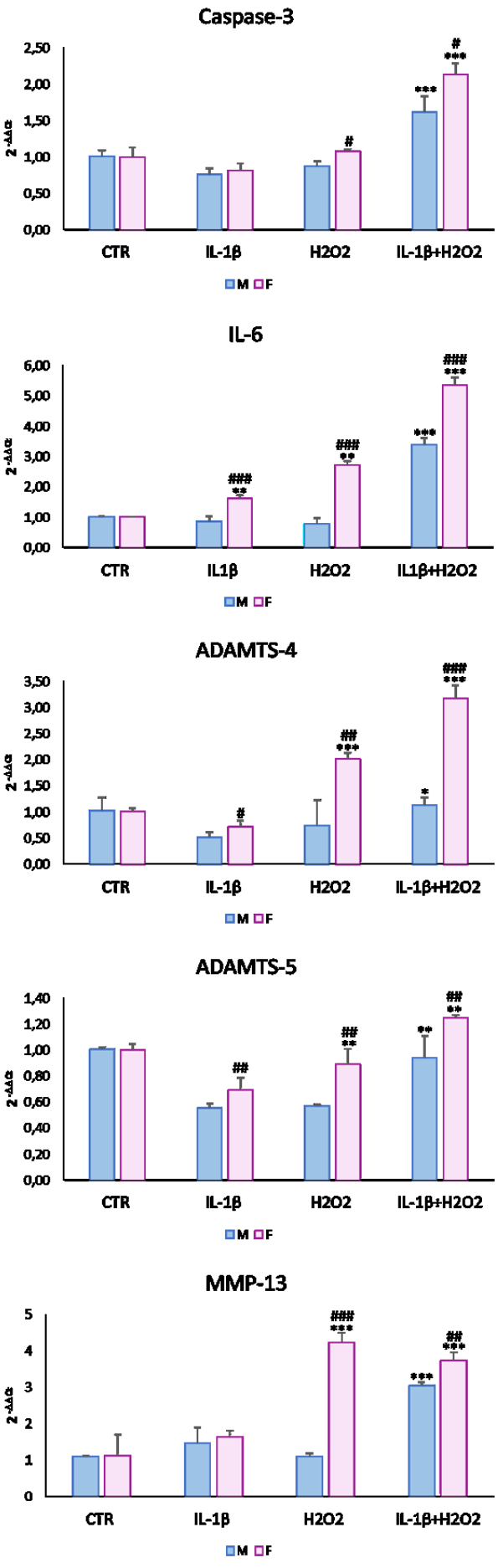


Figure 18 a-e. (a) Caspase-3, (b) IL-6, (c) ADAMTS-4, (d) ADAMTS-5 and (e) MMP-13 gene expression in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HCs at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

IL-1β+H_2O_2 *vs.* all other groups: (a, b) *** $p < 0.0005$ for M and F; (c) *** $p < 0.0005$ and (d) ** $p < 0.005$ for F; (e) *** $p < 0.0005$ for M.
IL-1β and H_2O_2 *vs.* CTR: (b) ** $p < 0.005$ for F.
H_2O_2 *vs.* CTR and IL-1β: (c) *** $p < 0.0005$ for F.
IL-1β+H_2O_2 *vs.* IL-1β: (c) * $p < 0.05$ for M.
H_2O_2 *vs.* IL-1β: (d) ** $p < 0.005$ for F.
IL-1β+H_2O_2 *vs.* IL-1β and H_2O_2: (d) ** $p < 0.005$ for M.
H_2O_2 and IL-1β+H_2O_2 *vs.* CTR and IL-1β: (e) *** $p < 0.0005$ for F.

F *vs.* M: (a) # $p < 0.05$ for H_2O_2 and IL-1β+H_2O_2; (b) ### $p < 0.0005$ and (d) ## $p < 0.005$ for all treated groups; (c) ### $p < 0.0005$ for IL-1β+H_2O_2, ## $p < 0.005$ for H_2O_2 and # $p < 0.05$ for IL-1β; (e) ### $p < 0.0005$ for H_2O_2 and ## $p < 0.005$ for IL-1β+H_2O_2.

Contrarly, Klotho expression was significantly higher in male cells than the female ones in CTR and IL-1β groups (Figure 19 a), while for COL2A1, the male than female cells showed higher values in all treated groups except CTR (Figure 19 b).

Both male and female cells showed a significantly higher Klotho expression in CTR than all other groups (Figure 19 a). In addition, male cells showed higher values in IL-1β than H_2O_2 and IL-1β+H_2O_2 groups, while female cells showed significantly higher values in IL-1β than H_2O_2 group (Figure 19 a). COL2A1 expression was also significantly higher in female and male cells of CTR than all other groups (Figure 19 b).

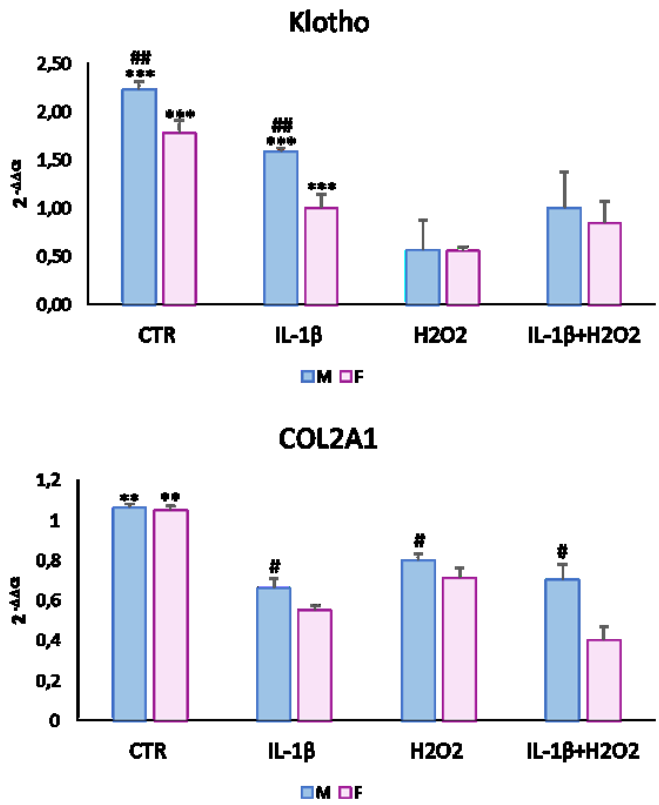


Figure 19 a, b. (a) Klotho and (b) COL2A1 gene expression in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HCs at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

IL-1β *vs.* H_2O_2 and IL-1β+H_2O_2: (a) *** $p < 0.0005$ for M.

CTR *vs.* all treated groups: (a) *** $p < 0.0005$ for F; (b) ** $p < 0.005$ for M and F.

IL-1β *vs.* H_2O_2: (a) *** $p < 0.0005$ for F.

M *vs.* F: (a) ## $p < 0.005$ for CTR and IL-1β and (b) # $p < 0.05$ for all treated groups.

4.2.4 ROS level

At T24, the immunoenzymatic assays showed that, in both female and male cells, H_2O_2 and IL-1β+H_2O_2 groups significantly increased ROS production more than CTR and IL-1β did. The female cells showed higher ROS levels than males ones in H_2O_2 and IL-1β+H_2O_2 groups (Figure 20).

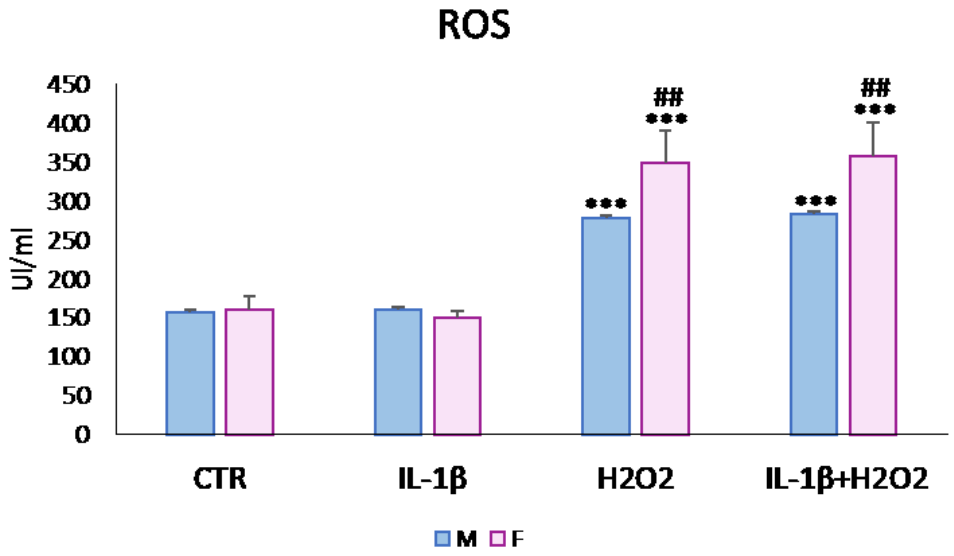


Figure 20. ROS (UI/mL) protein production in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F HCs at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

H_2O_2 and IL-1β+H_2O_2 *vs.* CTR and IL-1β: *** $p < 0.0005$ for M and F.

F *vs.* M: ## $p < 0.005$ for H_2O_2 and IL-1β+H_2O_2.

4.3 Co-coltures

4.3.1 Cell viability

At T24, Alamar Blue assay showed that all treated groups significantly reduced the cell viability of HFLSs and HCs co-cultured together, in comparison to CTR group in both female and male cells.

The male cells showed a significantly higher viability than the female ones in all groups (Figure 21).

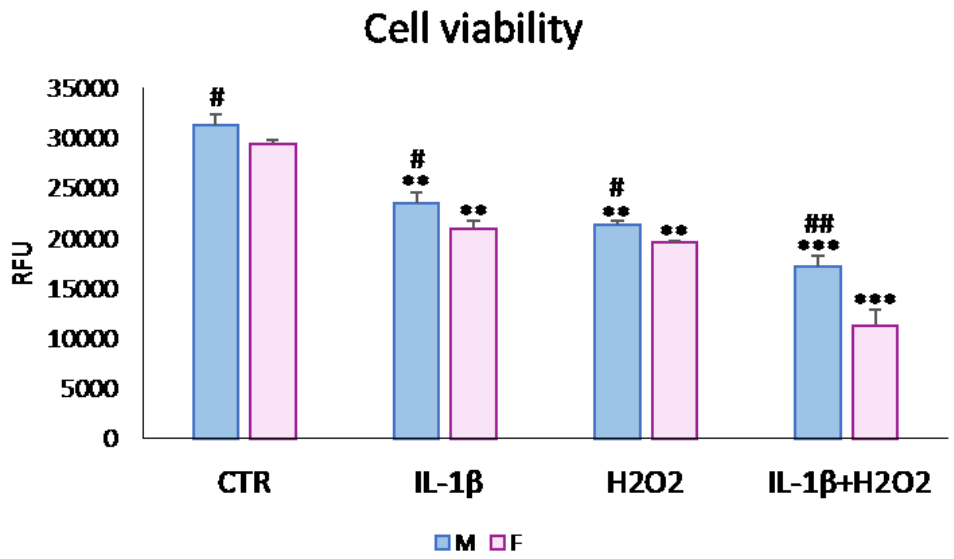


Figure 21. Cell viability (RFU) in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F co-culture at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

IL-1β+H_2O_2 *vs.* all other groups: *** $p < 0.0005$ for M and F.
IL-1β and H_2O_2 *vs.* CTR: ** $p < 0.005$ for M and F.

M *vs.* F: # $p < 0.05$ for CTR, IL-1β and H_2O_2 and ## $p < 0.005$ for IL-1β+H_2O_2.

4.3.2 Gene expression

At T24, Caspase-3, IL-6, AMATS-4, ADAMTS-5, MMP-13, Klotho and COL2A1 expression was evaluated by Real Time PCR analysis (Figures 22 and 23).

The female cells than male ones showed a significantly higher Caspase-3, IL-6, ADAMTS-5 and MMP-13 expression in all treated groups except CTR (Figure 22 a, b, d, e), while for ADAMTS-4, female cells showed significantly higher values in H_2O_2 and IL-1β+H_2O_2 groups (Figure 22 c).

The Caspase-3 and ADAMTS-5 expression was significantly higher in female cells treated with IL-1β+H_2O_2 than all other groups, while no significant difference was observed between the groups in males (Figure 22 a, d). H_2O_2 also increased Caspase-3 expression in comparison to CTR and IL-1β groups in females (Figure 22 a). In addition, IL-6 expression was significantly higher in both male and female cells treated with H_2O_2 than all other groups (Figure 22 b). IL-1β and IL-1β+H_2O_2 groups also increased IL-6 expression than CTR in female cells (Figure 22 b). Furthermore, ADAMTS-4 expression was significantly higher in female cells treated with H_2O_2 and IL-1β+H_2O_2 than CTR and IL-1β groups (Figure 22 c). However, no significant difference was observed between groups in males (Figure 22 c). MMP-13 expression was significantly higher in both male and female cells of all treated groups than CTR (Figure 22 e).

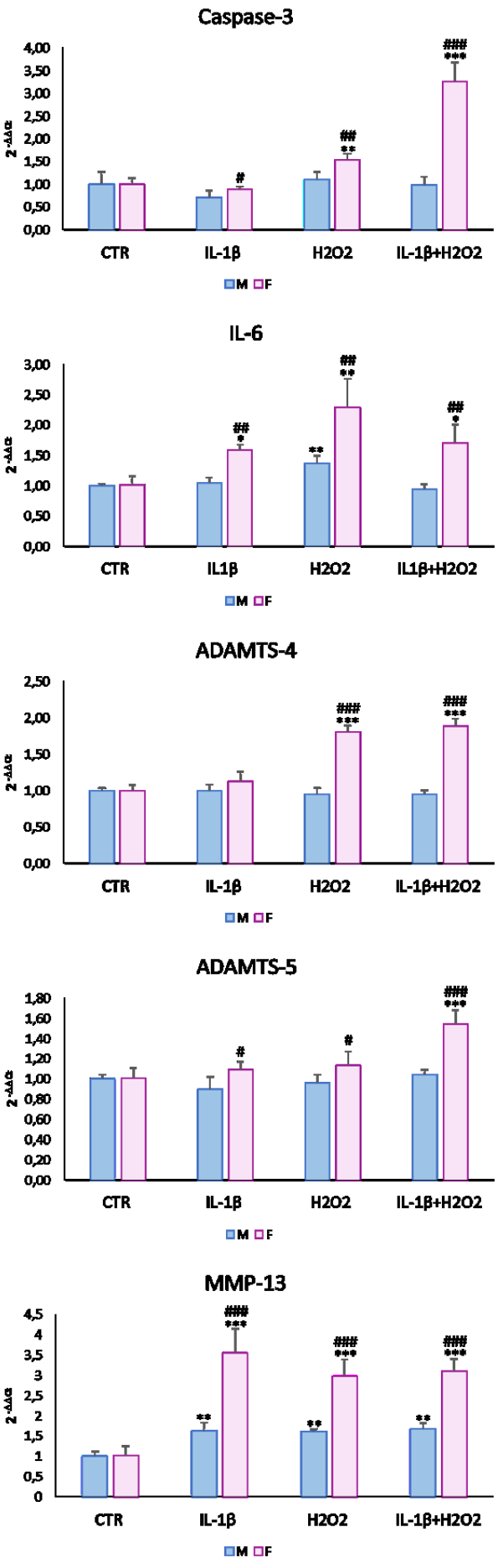


Figure 22 a-e. (a) Caspase-3, (b) IL-6, (c) ADAMTS-4, (d) ADAMTS-5 and (e) MMP-13 gene expression in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F co-culture at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

IL-1β+H_2O_2 *vs.* all the other groups: (a, d) *** $p < 0.0005$ for F.
H_2O_2 *vs.* CTR and IL-1β: (a) ** $p < 0.005$ for F.
H_2O_2 *vs.* all other groups: (b) ** $p < 0.005$ for M and F.
IL-1β and IL-1β+H_2O_2 *vs.* CTR: (b) * $p < 0.05$ for F.
H_2O_2 and IL-1β+H_2O_2 *vs.* CTR and IL-1β: (c) *** $p < 0.0005$ for F.
Treated groups *vs.* CTR: (e) *** $p < 0.0005$ for F and ** $p < 0.005$ for M.

F *vs.* M: (a) ### $p < 0.0005$ for IL-1β+H_2O_2, ## $p < 0.005$ for H_2O_2 and # $p < 0.05$ for IL-1β. (b) ## $p < 0.005$ and (e) ### $p < 0.0005$ for all treated groups. (c) ### $p < 0.0005$ for H_2O_2 and IL-1β+H_2O_2. (d) ### $p < 0.0005$ for IL-1β+H_2O_2 and # $p < 0.05$ for IL-1β and H_2O_2.

Contrarly, Klotho expression was significantly higher in male cells than the female ones in CTR, IL-1β and H_2O_2 groups (Figure 23 a), while for COL2A1, the males showed a significantly higher expression in all groups (Figure 23 b).

Furthermore, Klotho and COL2A1 expression was significantly higher in both female and male cells in CTR than all other groups (Figure 23 a, b).

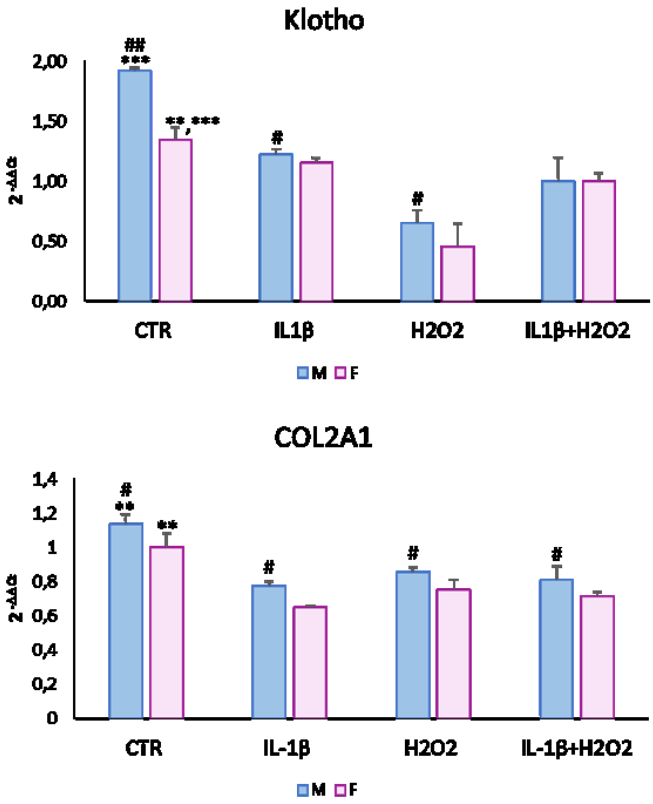


Figure 23 a, b. (a) Klotho and (b) COL2A1 gene expression in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F co-culture at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

CTR *vs.* all other groups: (a) *** $p <$ 0.0005 for M and (b) ** $p <$ 0.005 for M and F.
CTR *vs.* IL-1β and IL-1β+H_2O_2: (a) ** $p <$ 0.005 for F.
CTR *vs.* H_2O_2: (a) *** $p <$ 0.0005 for F.

M *vs.* F: (a) ## $p <$ 0.005 for CTR, IL-1β and H_2O_2; (b) # $p <$ 0.05 for all groups.

4.3.3 ROS level

At T24, the immunoenzymatic assays showed that, in both female and male cells, H_2O_2 and IL-1β+H_2O_2 groups significantly increased ROS production more than CTR and IL-1β did. Furthermore, female cells showed higher ROS levels than the males ones in H_2O_2 and IL-1β+H_2O_2 groups (Figure 24).

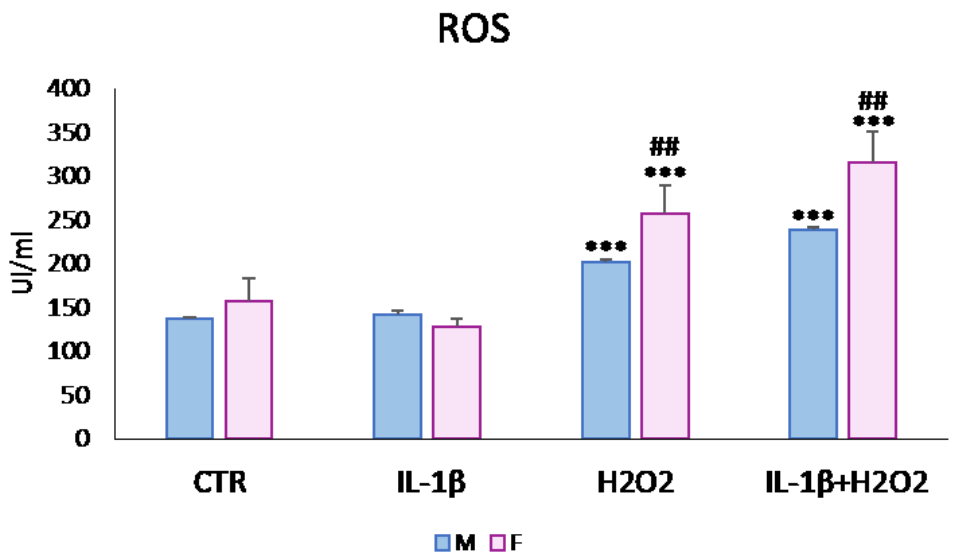


Figure 24. ROS (UI/mL) protein production in CTR, IL-1β, H_2O_2 and IL-1β+H_2O_2 groups in M and F co-cultures at T24 (mean ± SD, n=5). Two-way ANOVA followed by Tukey post hoc test:

H_2O_2 and IL-1β+H_2O_2 *vs.* CTR and IL-1β: *** $p < 0.0005$ for M and F.

F *vs.* M: ## $p < 0.005$ for H_2O_2 and IL-1β+H_2O_2.

4.4 *In vivo* model

4.4.1 Histology

As shown in the Figures 25 and 26, histological evaluation of the synovial membrane in joints of both sexes showed an enlargement of the synovial lining cell layer and an increased cell density (Figures 25 and 26). Cartilage structure loss was also evident in joints of both sexes. Joints articular cartilage of the male and female animals showed low Safranin O staining and severe extracellular matrix loss, indicative of focal lesions and degeneration. High number of chondrocytes grouped in cluster and hypertrophic chondrocytes were observed (Figures 27 and 28). In addition, fibrous tissue was present with mild fibrillation. Therefore, mice spontaneously developed OA characterized by severe degeneration of the articular cartilage and synovial membrane. However, the statistical analysis of Kreen (female: 5 ± 1; male: 4 ± 1) and OARSI (female: 20 ± 5; male: 19 ± 5) scores no showed significant differences between male and female joints (Figure 29).

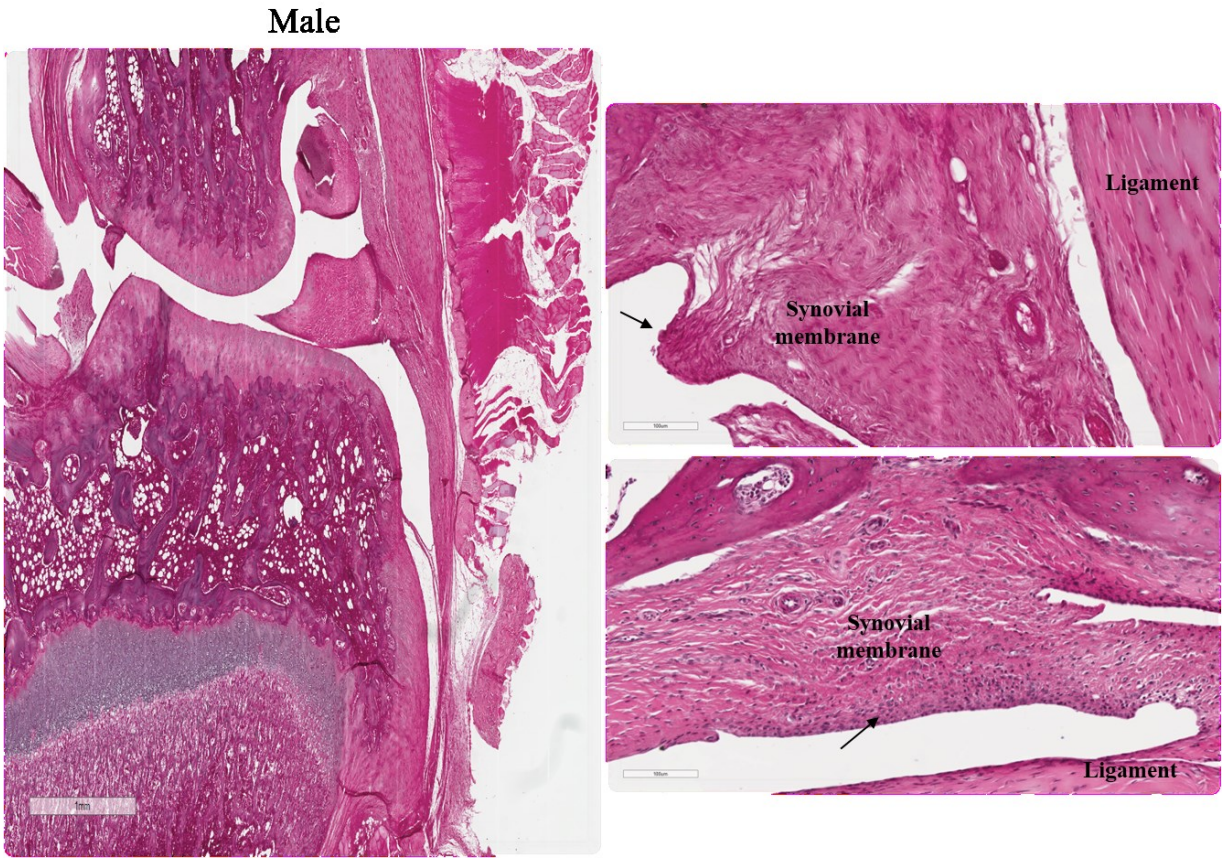


Figure 25. Synovial membrane of the joint of male animals. Hematoxylin/Eosin staining. Magnification: 4x (left) and 20x (right).

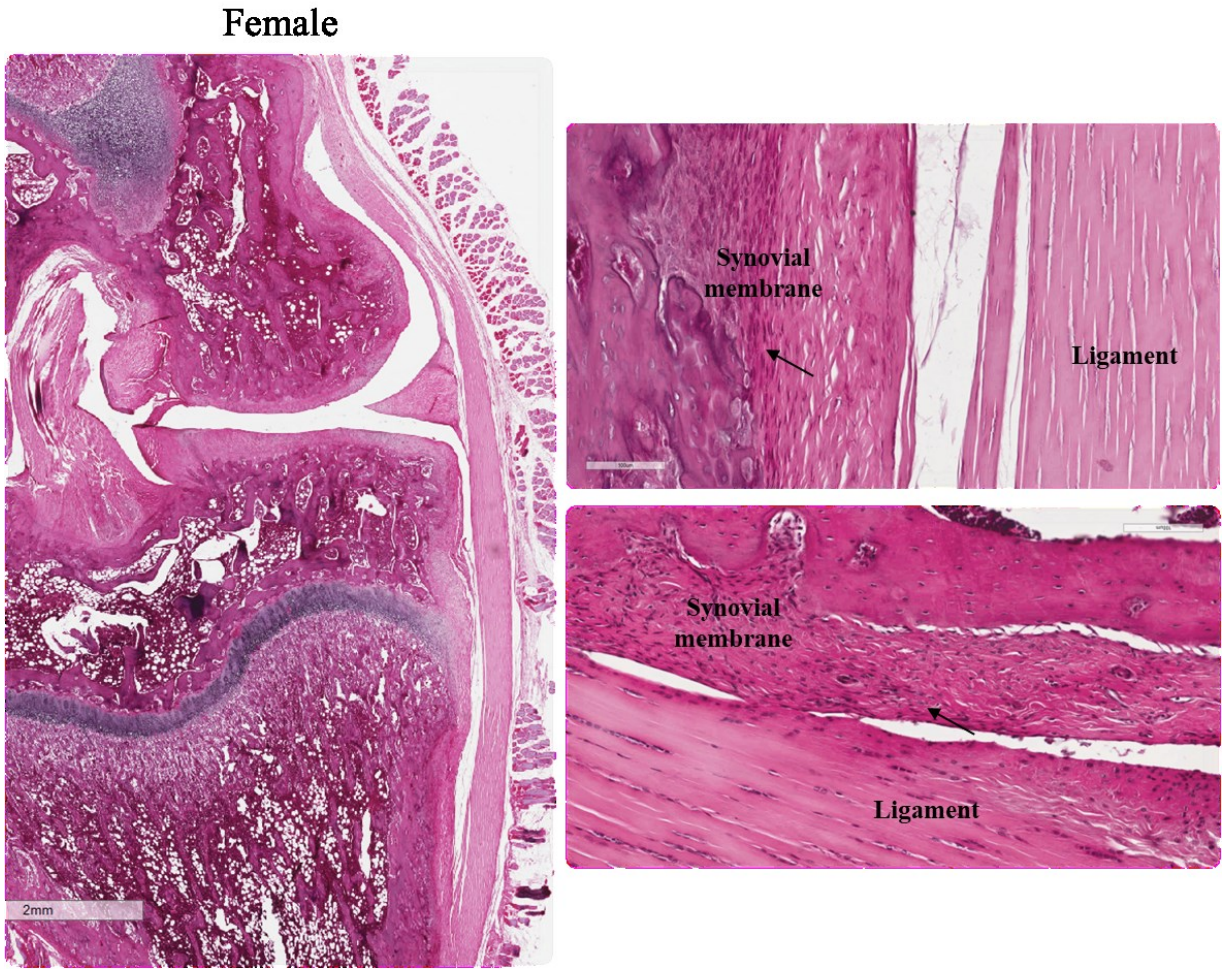


Figure 26. Synovial membrane of the joint of female animals. Hematoxylin/Eosin staining. Magnification: 4x (left) and 20x (right).

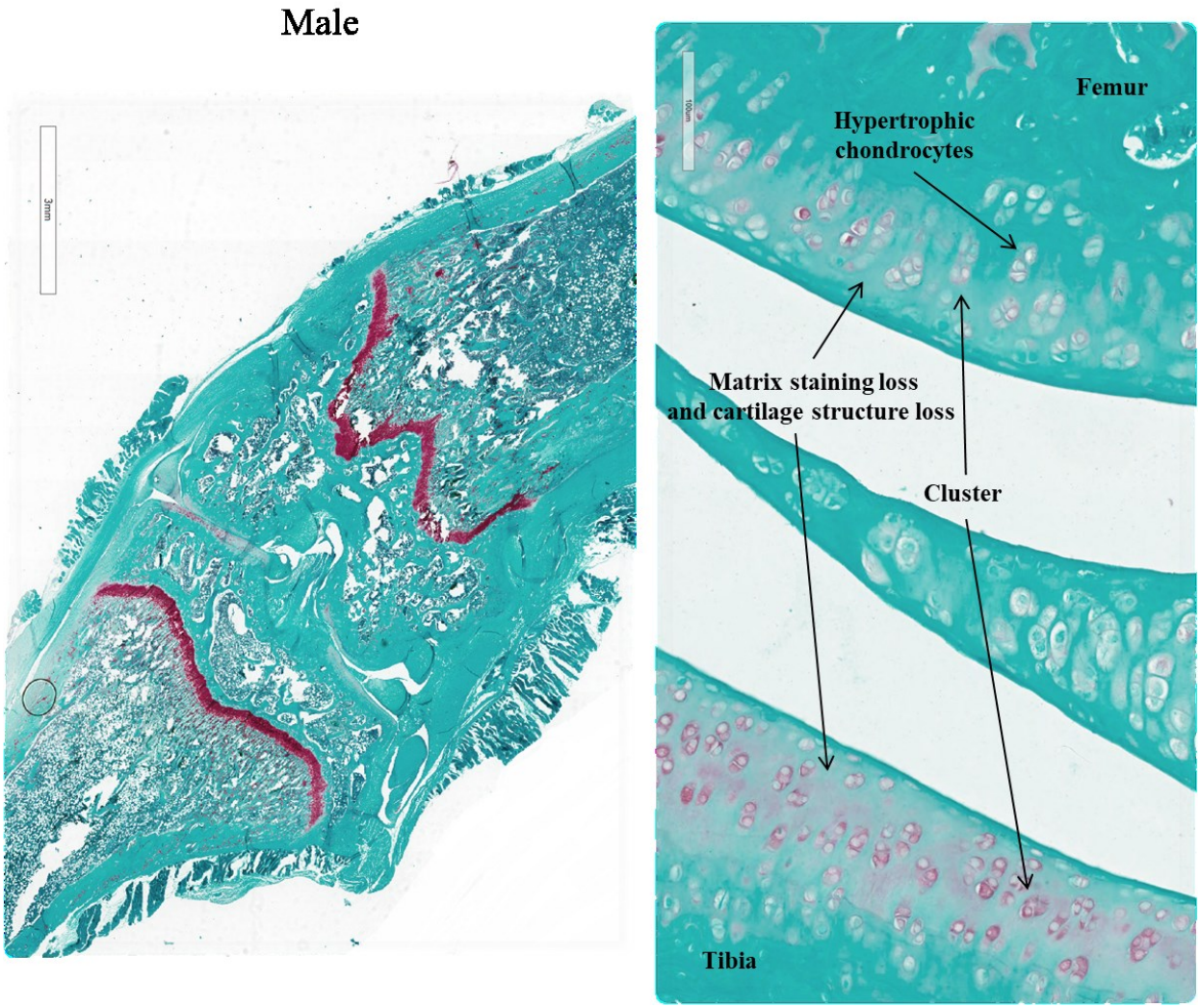


Figure 27. Articular cartilage of the joint of male animals. Safranin O/Fast Green staining. Magnification: 4x (left) and 20x (right).

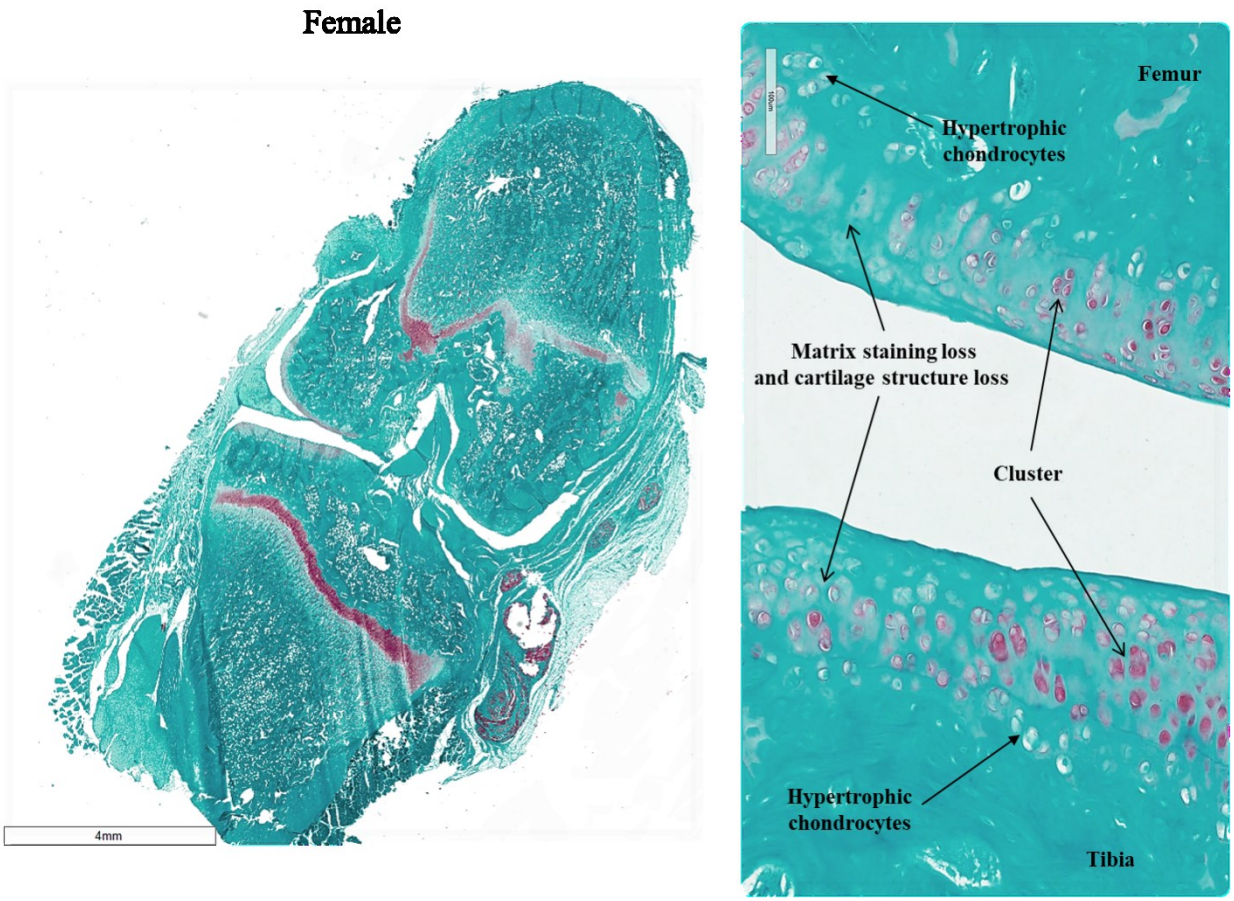
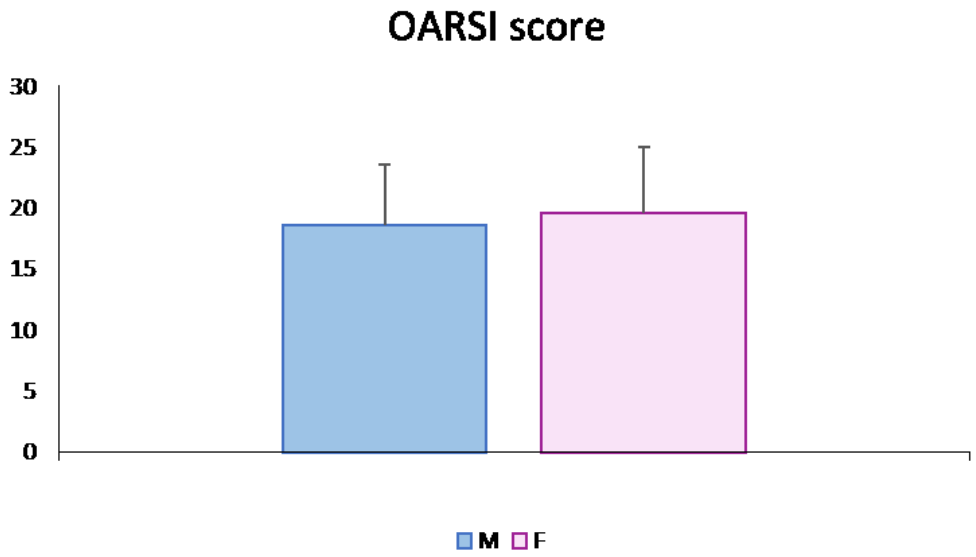


Figure 28. Articular cartilage of the joint of female animals. Safranin O/Fast Green staining. Magnification: 4x (left) and 20x (right).



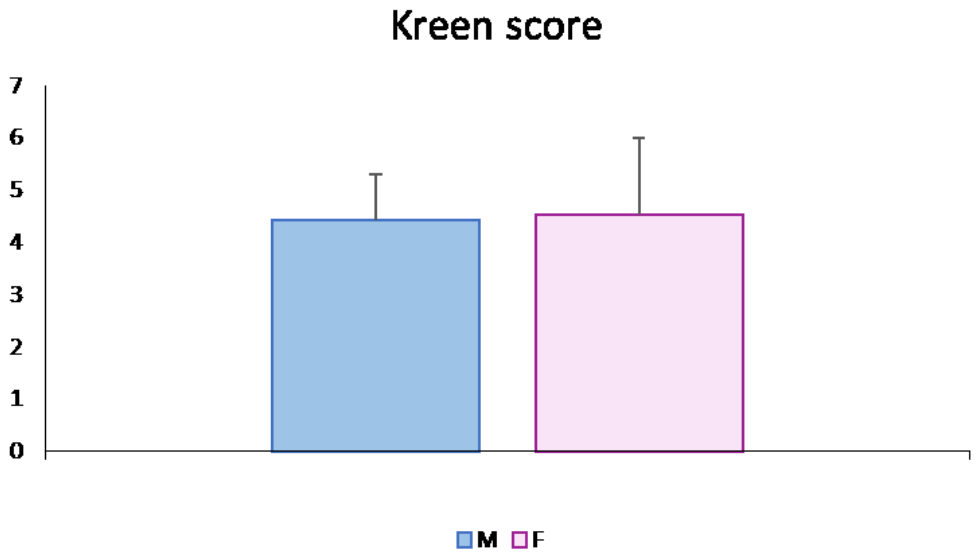


Figure 29. Kreen and OARSI scores.

4.4.2 Microcomputed tomography

2D and 3D images refer to microcomputed tomography (micro-CT) analysis of spinal column specimens (Figures 30 and 31) and joints (femur and tibia) (Figures 32 and 33) of both sexes. The images obtained by micro-CT scans showed alterations of vertebral segment microstructure and femur and tibia, with rarefaction of the bone tissue, reduced trabecular number, increased spacing among bone trabeculae and a decrease in the bone volume fraction (BV/TV) ratio in both sexes. The bone morphometrics of the spine and joints at trabecular level reveal classical aging pattern. Results appear to be consistent with analogue studies present in literature analysing age related bone alteration with high resolution imaging technique [119].

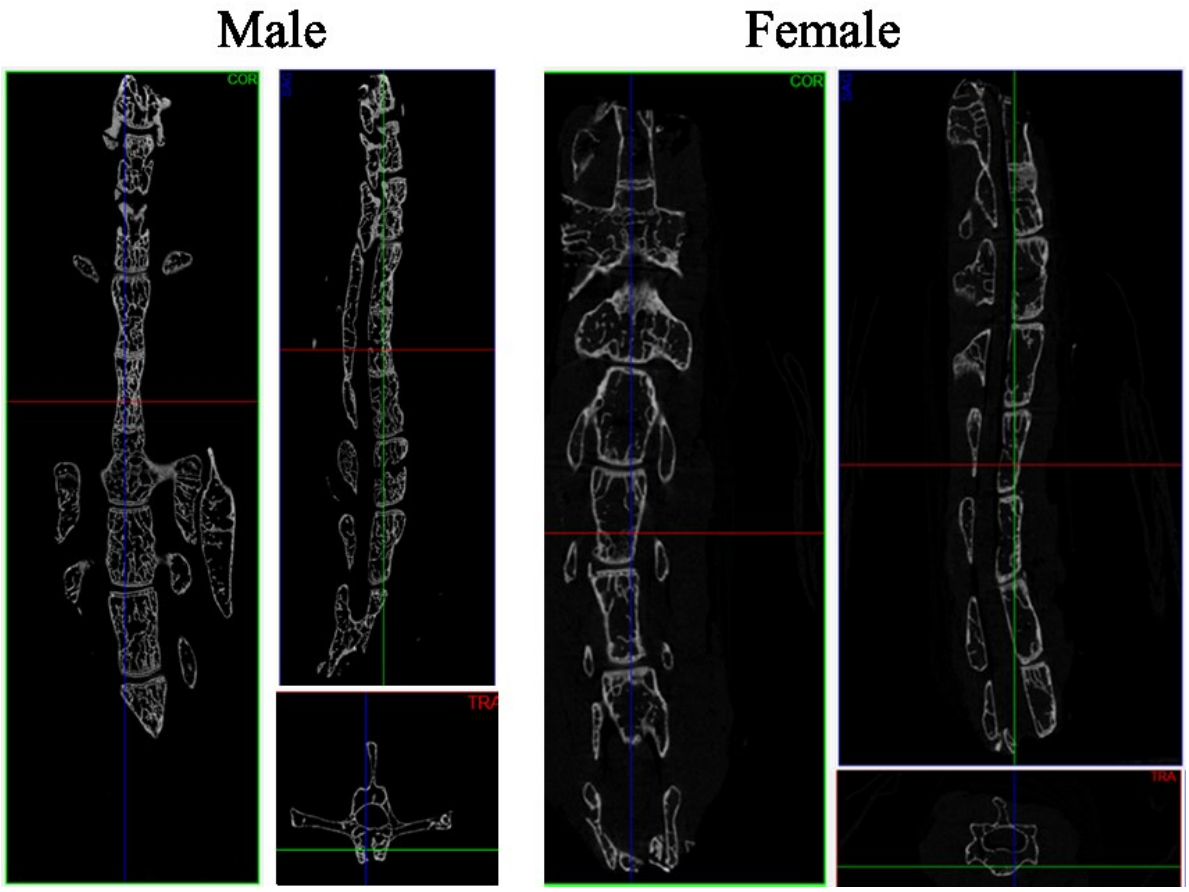


Figure 30. Visualization of the data set of images from two column samples in the orthogonal planes using Data Viewer software.

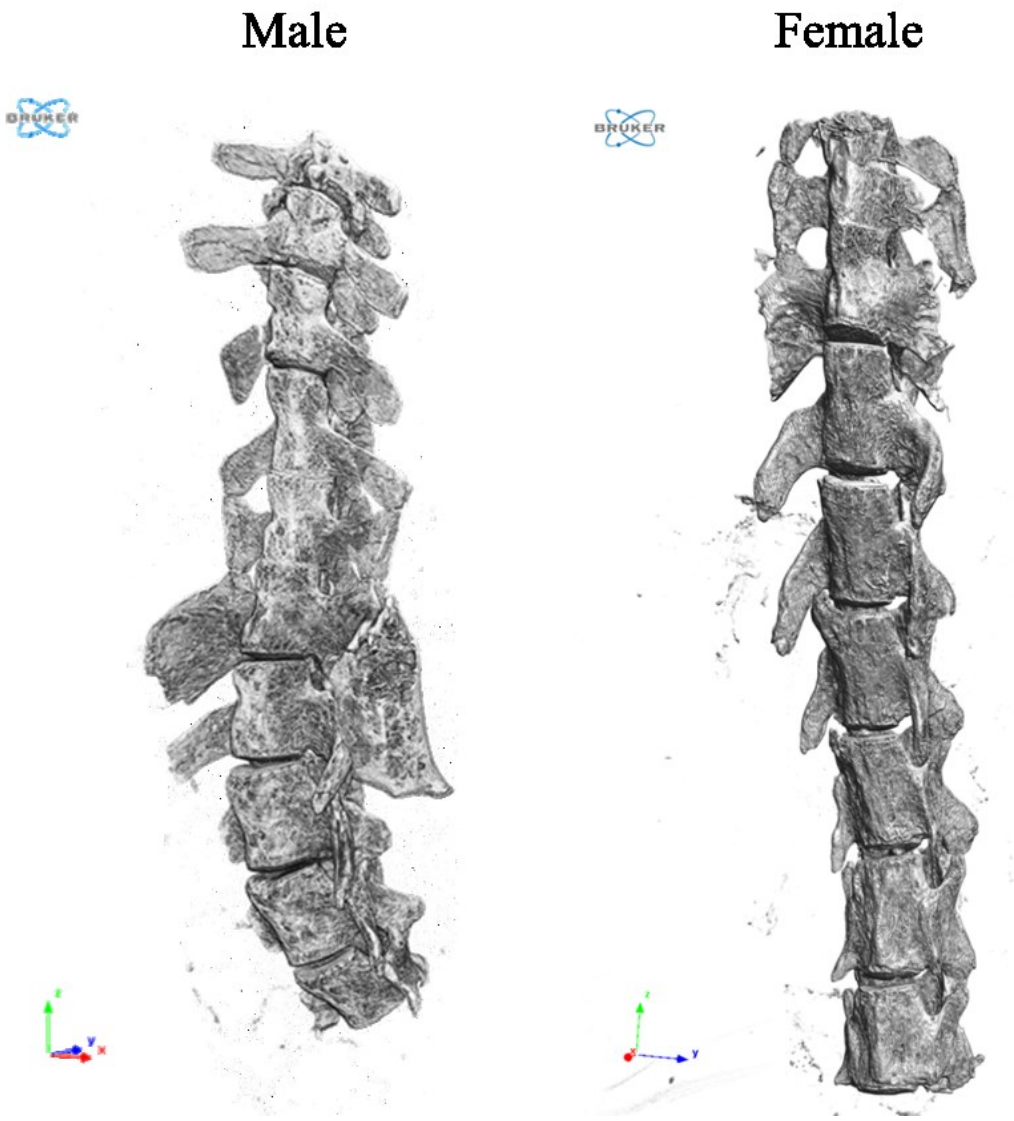


Figure 31. 3D visualization of volumetric data using volume rendering by CTVox software.

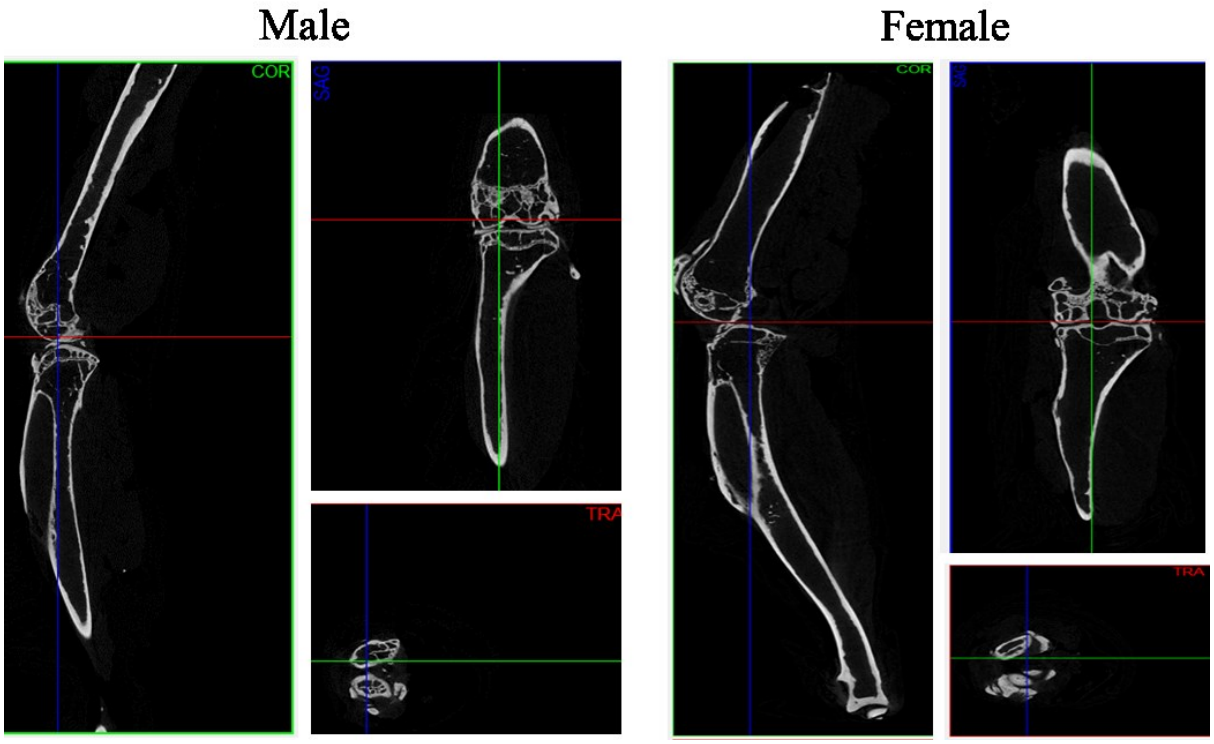


Figure 32. Visualization of the data set of images from two joint samples in the orthogonal planes using Data Viewer software.

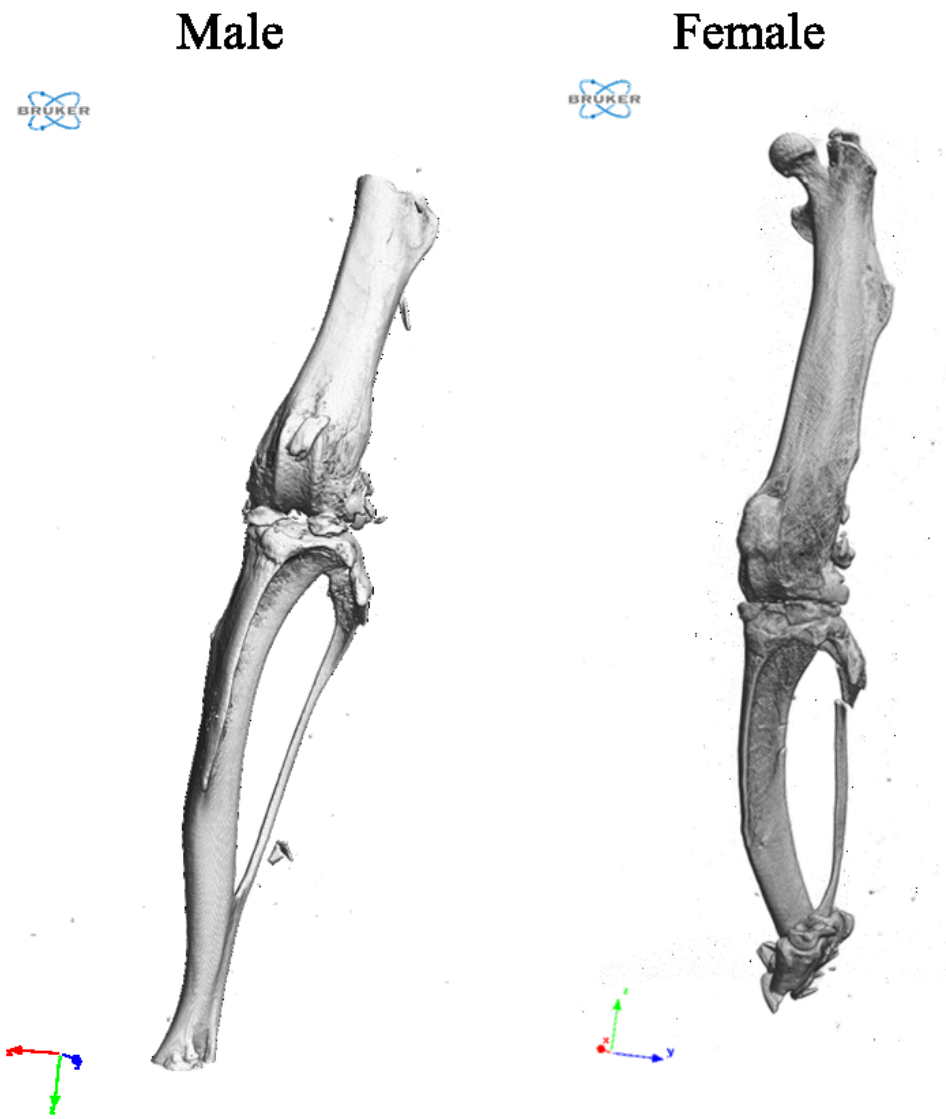


Figure 33. 3D visualization of volumetric data using volume rendering by CTVox software.

5. DISCUSSION

OA is one of the most common disorders of joints, mainly characterized by a progressive destruction of articular cartilage, synovial membrane inflammation and bone deformity, leading to pain and disability. OA is a complex disease with an unclear etiology and multiple risk factors, including sex and aging, but so far, the pathogenesis has not yet been fully understood. Recent studies suggest some critical events for OA initiation and disease progression: over activated catabolic activity primarily mediated by pro-inflammatory cytokines; deleterious stresses such as oxidative stress as well as the impaired defense mechanisms against these stress factors (i.e., oxidative stress); proteolytic enzymes which directly degrade the cartilage matrix such as MMPs. There is currently no cure for OA, and there are no therapies which prevent, slow, or arrest its progression [120].

Since OA is a multifactorial disorder and its pathogenesis is difficult to understand, there is no complex preclinical model which could recapitulate entirely the human pathology. However, different models have been frequently used by researchers for the study of OA development and progression, although with no consensus on the most appropriate model. Commonly *in vitro*, cell treatment with IL-1 has been used to mimic the joint inflammatory microenvironment [84]. IL-1 has been shown to inhibit anabolic activity of chondrocytes, including proteoglycan synthesis [121], and to stimulate catabolic activity through the production of MMPs and ADAMTSs, increasing the rate of inflammation [122]. In addition, IL-1 has also been shown to stimulate ROS expression, which results in increased oxidative damage [123]. Oxidative stress refers to the imbalance of oxidation and antioxidant activities in the body, producing a large amount of intracellular ROS, which is considered to be a major factor in aging and OA [124,125]. Many diseases are related to ROS. Under normal conditions, ROS are produced in the body and removed by the antioxidant system, maintaining a dynamic balance. Although most ROS are cleared, there are some escaped ROS that can cause cell and DNA damage [126,127]. Previous studies have shown that ROS-induced oxidative stress can lead to chronic inflammatory diseases, including arthritis [128,129]. Experimental animal models have also fully demonstrated that ROS play an important role in the development of OA [130,131]. H_2O_2 is considered a common form of ROS and H_2O_2 accumulation is involved in the progression of OA [132]. Therefore, H_2O_2 was chosen to stimulate human chondrocytes and synoviocytes by simulating the microenvironment of oxidative stress [133]. H_2O_2 induces chondrocytes and synoviocytes to produce iNOS, the key enzyme for NO synthesis. NO is a highly reactive cytotoxic free radical that can inhibit the synthesis and promote the degradation of type II collagen by

reducing the expression of COL2A1 [134]. NO also inhibits the secretion of ECM in chondrocytes, affecting the nutritional exchange of cartilage and creating a bad microenvironment that ultimately leads to the decline and progressive degeneration of cartilage quality [135].

Although it is known that differences in OA exist between males and females, to date they are understudied and poorly understood. The lack of knowledge on sex differences in the pathophysiology of OA is partly due to the paucity of studies evaluating and examining this aspect and the lack of relevant preclinical models. Indeed, a sex/gender imbalance is noted in the literature, where women remain under represented in biomedical research and there is a sexual bias in enrollment in clinical trials, with many more men being recruited than women [136]. Even preclinical research is preferentially conducted on male animals and *in vitro* tests and *in vivo* investigations that perform assays on cells and animals of both sexes are rare. This topic is still highly debated in the literature, indicating the need to perform more preclinical and clinical studies to elucidate sex-based determinants and mechanisms in view of developing sex-specific protocols and tailored drugs [37].

In the current study, human primary cells, FLSs and chondrocytes isolated from both male and female patients with knee OA were cultured and investigated. Further, a 2D *in vitro* co-culture model was selected to better mimic the environment of an OA joint, with cartilage-synovial crosstalk [137]. An *in vitro* inflammatory (typical of OA) and senescent (typical of aging) model was developed, stimulating the cells by the inflammatory cytokine, IL-1β, and H_2O_2. The results highlighted: (1) differences between applied stimuli and (2) sex differences in both chondrocytes and synoviocytes, with a very similar trend between monoculture and co-culture models. Although co-culture models allow for a more in-depth study of cell-cell interactions *in vitro*, in our experiments, they did not provide additional information compared to single cultures. Anyway, co-culture remains a system that more closely mimics the *in vivo* situation. In addition, this model of indirect co-culture, in the absence of cell contact, aims to evaluate the effect of soluble factor exchange between the two cell types and, as affirmed by Kraus et al., is applicable in translational research [138].

In detail, the association of the two stimuli (IL-1β+H_2O_2) significantly reduced cell viability of synoviocytes and chondrocytes, both in monoculture and co-culture. The percentage of microwound healing used to evaluate the migration capacity of synoviocytes and chondrocytes in monoculture significantly was also reduced by combination of the two stimuli. H_2O_2 alone did not cause cell death, but when associated with IL1-β, it reduced cell viability and microwound healing rate. Therefore, adopted model of treatment with IL-1β and H_2O_2 was found to be effective in inducing the inflammatory and oxidative stress

microenvironment, causing cell damage, inflammation, senescence, proliferation and altered cell activity. Caspase-3, involved in the aging and OA process, is one of the active executors of apoptosis; its activation and intrinsic apoptotic pathways are upregulated in an aged musculoskeletal system [139]. The increased production of cytokines and proteolytic enzymes, involved in cartilage degradation and synovial inflammation in OA pathology, increase Caspase-3, thus becoming a biomarker of OA [140]. In the study, the association of both stimuli (IL-1β+H_2O_2) induced a higher gene expression of Caspase-3 in both synoviocytes and chondrocytes monoculture. However, in co-culture, IL-1β+H_2O_2 but also H_2O_2 alone, induced a higher gene expression of Caspase-3. Generally, IL-6 is elevated in the synovial fluid and serum of OA patients. IL-6 increases the expression of MMPs that degrade cartilage and induce changes in subchondral bone and synovial tissue inflammation [141]. Furthermore, the elderly also show high systemic levels of IL-6 [142]. Compared to control group, IL-6 expression increases in synoviocytes and chondrocytes, in both monoculture and co-culture, after the single treatments with IL-1β and H_2O_2 and with a greater effect, after the combination of both (IL-1β+H_2O_2). In support of this result, extensive studies in the literature claim that IL-1β, present in a joint affected by OA, induces the production of IL-6, one of the most important biomarkers present in the synovial fluid [143]. The same trend was observed for ADAMTSs which play a key role in the pathological degradation of cartilage; they are capable of destroying the ECM independently of MMPs [144]. Both ADAMTS-4 and ADAMTS-5 were significantly more expressed in synoviocyte and chondrocyte, in both monoculture and co-culture, stimulated with IL-1β and H_2O_2 alone and in combination compared to control group. In chondrocytes, IL-1β can induce increased release of ADAMTS-4 and ADAMTS-5 [145]. Furthermore, direct evidence shows that inflammatory M1 secretion due to IFN-γ and TNF-α inhibits chondrogenic differentiation and cartilage repair by up-regulating IL-1β, IL-6, NO, MMP-13 and ADAMTS-5 and down-regulation of Aggrecan (ACAN) and COL2A1 [145]. Also MMP-13, involved in cartilage degradation, was more expressed in synoviocyte and chondrocyte, in both monoculture and co-culture, stimulated with IL-1β and H_2O_2, alone and in combination. In patients with OA, IL-1 is produced by certain stimulation of chondrocytes and synovial cells, which in turn activates MMPs in cartilage. IL-1 is expressed together with MMP-1, -3, and -13, among others, causing degradation of cartilage matrix [146]. The biomarker of aging Klotho which is an endogenous antagonist of the Wnt/β-catenin pathway that stimulates the degradation of articular cartilage, was found to have a protective effect in the development of OA [147]. For example, Klotho expression is higher in normal mouse cartilage than in the OA model, in which Wnt/β-catenin and its target genes are upregulated [148]. In addition, the serum

level of secreted Klotho decreases with aging in mice and humans [149]. Current study was in line with these data, in fact Klotho expression was higher in the control group and decreased following treatments, in synoviocyte and chondrocyte, in both monoculture and co-culture, especially after treatment with H_2O_2 and IL-1β+H_2O_2. The same trend was seen in synoviocyte and chondrocyte, both in monoculture and co-culture for COL2A1, a component of the extracellular matrix, more expressed in the control group, showing no differences between treatments. Finally, ROS production was higher in H_2O_2 and IL-1β+H_2O_2 groups for both synoviocytes and chondrocytes, both in monoculture and co-culture.

Regarding sex differences, female synoviocytes and chondrocytes showed significantly lower cell viability than male cells in all treated groups, especially when the two stimuli were combined. This indicates that cells from female patients have a worse ability to respond under unfavorable conditions of inflammation and oxidative stress than those from male patients. However, in synoviocytes, a baseline sex difference was also observed and this is probably due to the fact that the osteoarthritic cells still maintain the pathological condition after 24 hours of culture with a reduced proliferation for female cells. Also the microwound healing rate (i.e. migration capacity) was significantly lower in female synoviocyte and chondrocyte monoculture than male cells in treated groups with IL-1β+H_2O_2. Furthermore, in synoviocytes and chondrocytes, in both monoculture and co-culture, the gene expression of Caspase-3, IL-6, ADAMTS-4, ADAMTS-5 and MMP-13 was significantly higher in female than in male cells in all treated groups, while ROS production were significantly higher in female than in male cells in treated groups with H_2O_2 and IL-1β+H_2O_2. Contrarly, the expression of Klotho and COL2A1 was significantly higher in male than in female cells in all groups. This appears to partly reflect the condition observed in the clinic, with greater disability and higher inflammatory status for women than men [45]. One of the few *in vitro* studies using FLSs from female rats with temporomandibular OA and cultured in the presence of TNF-α, showed increased iNOS, IL-1β and MCP-1 compared to cells from male rats under the same conditions [150]. In agreement, our results showed that synoviocytes and chondrocytes of female subjects had lower cell viability, microwound healing, Klotho and COL2A1 expression, and higher ROS production, Caspase-3 expression, MMP-13, IL-6, ADAMTS-4 and ADAMTS-5 compared with those of male subjects.

When we investigated potential sex differences in OA in our *in vivo* model, histological and microtomographic evaluations was showed the morphology and structure of the bone, articular cartilage and synovial membrane representative of the pathological condition of OA and aging. However, no significant difference between the sexes was observed. Based

on the mouse life span, 3–6 months of age is referred as “matured adult”, 10–15 months of age is “middle-aged”, and 18–24 months of age is “old” [151]. From data in the literature, it emerges that age affects the progression of OA following destabilization of the medial meniscus (DMM) in female or male mice [152]. Aged male mice develop greater cartilage degeneration with subchondral bone changes than aged females [153,154]. Male *STR/ort* mice exhibiting spontaneous OA show a higher prevalence of OA with altered bone structure than females. In IL-6 deficient mice, which also develop spontaneous OA, more extensive cartilage loss is observed in males than in females with aging. Furthermore, *IL-6*$^{-/-}$ males show more extensive ECM depletion and subchondral bone sclerosis than females, and cartilage proteoglycan synthesis and bone mineral density decrease to a greater extent in males than females, suggesting a role protective of *IL-6*$^{-/-}$ in age-related OA in male mice [155]. Using genetic modifications in mice, such as the mutation targeting *Nov*, *Novdel3*$^{-/-}$ males were found to exhibit severe OA-like pathology at 12 months, affecting all joint tissue, including joint cartilage destruction, enlargement of the meniscus, osteophyte formation and fibrocartilage expansion compared to females [37].

In our study, the mice used in the *in vivo* model with such an advanced age (32 months) appear to be “very old” and with a very advanced state of OA and, no sex differences were found in the structure and morphology of the articular components (bone, cartilage joint and synovial membrane) and of the column; it is possible that sex differences are more evident in a less advanced state of OA and in less elderly animals, as reported in previous *in vivo* studies [156].

Indeed, the study has some limitations. Firstly, the low number of samples and the short selected experimental times. Secondly, as tissue harvesting in humans (articular cartilage or synovium) is complex due to several ethical and regulatory issues, including also long times for the request and the approval of the ethics committee, it was decided to set up this preliminary *in vitro* model by purchasing commercially available primary cells from male and female OA patients with the same age range, to obtain similar certified base characteristics and limit variability as much as possible between the patients (inter-variability), avoiding ethical implications. Conversely, this meant that the two cell types could not be isolated from the same patient. In the future, a separate analysis of the two cell phenotypes could be useful to study the mutual influence of co-cultured cells. Finally, the analysis of a data greater number (eg, additional factors and markers released by cells) should be investigated to elucidate the complex mechanisms of this pathology associated with age and sex differences.

Indeed, our *in vitro* data are in line with *in vivo* data reported by others, indicating a different behaviour of cells derived female and male. If the differences may depend on the genetic background or from changes in cell behaviour occurring during OA pathology remains to be clarified. Thus, there remains the need to reduce experimental variability and improve data reliability by increasing sample sizes, adopting advanced *in vitro* models, and revealing the underlying data and molecular aspects involved that may lead to a better understanding of how cellular processes are differentially regulated in males and females.

6. CONCLUSIONS

OA is a disease with a multifactorial etiology characterized by degenerative, oxidative and inflammatory phenomena that contribute to the progression of the pathological event. In conclusion, this study has developed culture conditions to mimic aging and OA microenvironment found in the knee joint, to evaluate the behaviour of chondrocytes and synoviocytes, the cells that play a fundamental role in the homeostasis of the joint. In addition, relevance has been attributed to sex differences, by testing both synoviocytes and chondrocytes from OA male and female patients.

The study evaluated sex differences indicating that synoviocytes and chondrocytes from female patients demonstrated a worse response capacity in unfavorable conditions of inflammation and oxidative stress than those from male patients.

Differently from *in vitro* data, the *in vivo* model confirmed the presence of OA in old mice without showing differences between the sexes. For this reason, it remains necessary to better understand the mechanisms of OA pathology and the potential sex-related differences through adequate preclinical models, to evaluate new hypotheses, concepts and therapeutic treatments able to halt or reverse the progression of the disease in a personalized approach.

7. BIBLIOGRAPHY

[1] Principles of bone biology, Second Edition, Volume 1, Edited by John P. Bilezikian, Lawrence G. Raisz, Gideon A. Rodan.

[2] Istologia di V. Monesi. IV Edizione, Piccin, Molinaro, Rizzoli, Siracusa, Stefanini.

[3] Smith MD. The Normal Synovium. Open Rheumatol J. 2011;5:100–6.

[4] Singh JA, Arayssi T, Duray P, Schumacher HR. Immunohistochemistry of Normal Human Knee Synovium: A Quantitative Study. Ann Rheum Dis. 2004;63(7):785–90.

[5] Orr C, Vieira-Sousa E, Boyle DL, Buch MH, Buckley CD, Canete JD, et al. Synovial Tissue Research: A State-of-the-Art Review. Nat Rev Rheumatol. 2017;13(8):463–75.

[6] Abramoff B, Caldera FE. Osteoarthritis: Pathology, Diagnosis, and Treatment Options. Med Clin North Am. 2020;104(2):293-311.

[7] Hawker GA. Osteoarthritis is a serious disease. Clin. Exp. Rheumatol. 2019;37:3–6.

[8] Horváth G, Koroknai G, Ács B, Than P, Bellyei A, Illés T. Prevalence of radiographic primary hip and knee osteoarthritis in a representative Central European population. Int Orthop. 2011;35(7):971-5.

[9] Madry H, Luyten FP, Facchini A. Biological aspects of early osteoarthritis. Knee Surg Sports Traumatol Arthrosc. 2012;20(3):407-22.

[10] Saito M, Sasho T, Yamaguchi S, Ikegawa N, Akagi R, Muramatsu Y, et al. Angiogenic activity of subchondral bone during the progression of osteoarthritis in a rabbit anterior cruciate ligament transection model. Osteoarthritis Cartilage. 2012;20(12):1574-82.

[11] Liu-Bryan R, Terkeltaub R. Emerging regulators of the inflammatory process in osteoarthritis. Nat. Rev. Rheumatol. 2015;11:35–44.

[12] Carlesso LC, Hawker GA, Torner J, Lewis CE, Nevitt M, Neogi T, et al. Association of Intermittent and Constant Knee Pain Patterns With Knee Pain Severity and With Radiographic Knee Osteoarthritis Duration and Severity. Arthritis Care Res (Hoboken). 2021;73(6):788-793.

[13] Safiri S, Kolahi AA, Smith E, Hill C, Bettampadi D, Mansournia MA. Global, regional and national burden of osteoarthritis 1990-2017: a systematic analysis of the Global Burden of Disease Study 2017. Ann Rheum Dis. 2020;79(6), 819-828.

[14] Glyn-Jones S, Palmer AJR, Agricola R, Price AJ, Vincent TL, Weinans H, et al. Osteoarthritis. Lancet. 2015;386(9991):376-87.

[15] GBD 2019 Diseases and Injuries Collaborators. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2020;396(10258):1204-1222.

[16] Kennedy S, Tambiah JRS, Lane NE. Osteoarthritis today: Lost in translation? Best Pract Res Clin Rheumatol. 2023;101810. Online ahead of print.

[17] Chaney S, Vergara R, Qiryaqoz Z, Suggs K, Akkouch A. The Involvement of Neutrophils in the Pathophysiology and Treatment of Osteoarthritis. Biomedicines. 2022;10:1604.

[18] Goldring MB, Goldring SR. Articular Cartilage and Subchondral Bone in the Pathogenesis of Osteoarthritis. Ann. N. Y. Acad. Sci. 2010;1192:230–237.

[19] Chow YY, Chin KY. The Role of Inflammation in the Pathogenesis of Osteoarthritis. Mediat. Inflamm. 2020;2020:8293921.

[20] Akkiraju H, Nohe A. Role of Chondrocytes in Cartilage Formation, Progression of Osteoarthritis and Cartilage Regeneration. J. Dev. Biol. 2015;3:177–192.

[21] Xia B, Chen D, Zhang J, Hu S, Jin H, Tong P. Osteoarthritis Pathogenesis: A Review of Molecular Mechanisms. Calcif Tissue Int. 2014;95:495–505.

[22] Manivong S, Cullier A, Audigié F, Banquy X, Moldovan F, DemoorM, et al. New trends for osteoarthritis: Biomaterials, models and modeling. Drug Discov Today. 2023;28(3):103488.

[23] Nakasone A, Guang Y, Wise A, Kim L, Babbin J, Rathod S, et al. Structural features of subchondral bone cysts and adjacent tissues in hip osteoarthritis. Osteoarthritis Cartilage. 2022;30(8):1130-1139.

[24] Smith MD, Triantafillou S, Parker A, Youssef PP, Coleman M. Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. J Rheumatol. 1997;24(2):365-71.

[25] Felson DT. Clinical practice. Osteoarthritis of the knee. N Engl J Med. 2006;354(8):841-8.

[26] Kellgren JH, Lawrence JS. Radiological assessment of osteothritis. Ann Rheum Dis 1957;16:494-502.

[27] Lotz M, Martel-Pelletier J, Christiansen C, Brandi ML, Bruyère O, Chapurlat R, et al. Value of biomarkers in osteoarthritis: current status and perspectives. Ann Rheum Dis. 2013;72(11):1756-63.

[28] Wick MC, Kastlunger M, Weiss RJ. Clinical imaging assessments of knee osteoarthritis in the elderly: a mini-review. Gerontology. 2014;60(5):386-94.

[29] Emami A, Namdari H, Parvizpour F, Arabpour Z. Challenges in osteoarthritis treatment. Tissue Cell. 2023;80:101992.

[30] Salamanna F, Giavaresi G, Parrilli A, Martini L, Nicoli Aldini N, Abatangelo G, et al. Effects of intra-articular hyaluronic acid associated to Chitlac (arty-duo®) in a rat knee osteoarthritis model. J Orthop Res. 2019;37(4):867-76.

[31] Garbin LC, Olver CS. Platelet-rich products and their application to osteoarthritis. J Equine Vet Sci. 2020;86:102820.

[32] Zhang W,Ouyang H, Dass CR, Xu J. Current Research on Pharmacologic and Regenerative Therapies for Osteoarthritis. Bone Res. 2016;4:15040.

[33] Maleki-Fischbach M, Jordan JM. New developments in osteoarthritis. Sex differences in magnetic resonance imaging-based biomarkers and in those of joint metabolism. Arthritis Res. Ther. 2010;12:212.

[34] Stumm M, Boger E, Gaissmaier CG, Oßwald C, Blankenburg M, Wegner RD, et al. Genomic chondrocyte culture profiling by array-CGH, interphase-FISH and RT-PCR. Osteoarthr. Cartil. 2012;20:1039–1045.

[35] Pan Q, O'Connor MI, Coutts RD, Hyzy SL, Olivares-Navarrete R, Schwartz Z, et al. Characterization of osteoarthritic human knees indicates potential sex differences. Biol. Sex. Differ. 2016;7:27.

[36] Koelling S, Miosge N. Sex differences of chondrogenic progenitor cells in late stages of osteoarthritis. Arthritis Rheum. 2010;62:1077–1087.

[37] Contartese D, Tschon M, De Mattei M, Fini M. Sex Specific Determinants in Osteoarthritis: A Systematic Review of Preclinical Studies. Int. J. Mol. Sci. 2020;21:3696.

[38] O'Connor MI. Sex differences in osteoarthritis of the hip and knee. J Am Acad Orthop Surg 2007;15(1):S22–S25.

[39] Stevens-Lapsley JE, Kohrt WM. Osteoarthritis in Women: Effects of Estrogen, Obesity and Physical Activity. Women's Health. 2010;6:601–615.

[40] Richette P, Corvol M, Bardin T. Estrogens, cartilage, and osteoarthritis. Joint Bone Spine. 2003;70(4):257-62.

[41] Nevitt MC, Cummings SR, Lane NE, Genant HK, Pressman AR. Current use of oral estrogen is associated with a decreased prevalence of radiographic hip osteoarthritis in elderly white women. Arch Intern M. 1996;156:2073-2080.

[42] Zhang Y, McAlindon TE, Hannan MT, Chaisson CE, Klein R, Wilson PW, et al. Estrogen replacement therapy and worsening of radiographic knee osteoarthritis: the Framingham study. Arthritis Rheum. 1998;41:1867-1873.

[43] De Klerk BM, Schiphof D, Groeneveld FP, Koes BW, van Osch GJ, van Meurs JB, et al. No clear association between female hormonal aspects and osteoarthritis of the hand, hip and knee: A systematic review. Rheumatology. 2009;48:1160–1165.

[44] Li C, Zheng Z. Males and Females Have Distinct Molecular Events in the Articular Cartilage during Knee Osteoarthritis. Int J Mol Sci. 2021;22(15):7876.

[45] Tschon M, Contartese D, Pagani S, Borsari V, Fini M. Gender and Sex Are Key Determinants in Osteoarthritis Not Only Confounding Variables. A Systematic Review of Clinical Data. J Clin Med. 2021;10(14):3178.

[46] Boissonneault A, Lynch J, Wise B, Segal N, Gross K, Murray D, et al. Association of hip and pelvic geometry with tibiofemoral osteoarthritis: Multicenter Osteoarthritis Study (MOST) Osteoarthr. Cartil. 2014;22:1129–1135.

[47] Mochizuki T, Tanifuji O, Koga Y, Sato T, Kobayashi K, Nishino K, et al. Sex differences in femoral deformity determined using three-dimensional assessment for osteo-arthritic knees. Knee Surg. Sports Traumatol. Arthrosc. 2017;25:468–476.

[48] Yang B, Yu JK, Zheng ZZ, Lu ZH, Zhang JY, Cheng JH. Computed Tomography Morphometric Study of Gender Differences in Osteoarthritis Proximal Tibias. J. Arthroplast. 2013;28:1117–1120.

[49] Nelson A, Stiller J, Shi X, Leyland K, Renner J, Schwartz T, et al. Measures of hip morphology are related to development of worsening radiographic hip osteoarthritis over 6

to 13 year follow-up: The Johnston County Osteoarthritis Project. Osteoarthr. Cartil. 2016;24:443–450.

[50] Phinyomark A, Osis ST, Hettinga BA, Kobsar D, Ferber R. Gender differences in gait kinematics for patients with knee osteoarthritis. BMC Musculoskelet. Disord. 2016;17:157.

[51] Allison K, Hall M, Wrigley TV, Pua YH, Metcalf B, Bennell KL. Sex-specific walking kinematics and kinetics in individuals with unilateral, symptomatic hip osteoarthritis: A cross sectional study. Gait Posture. 2018;65:234–239.

[52] Foucher KC. Sex-specific hip osteoarthritis-associated gait abnormalities: Alterations in dynamic hip abductor function differ in men and women. Clin. Biomech. 2017;48:24–29.

[53] Debi R, Mor A, Segal G, Debbi EM, Cohen MS, Igolnikov I, et al. Differences in gait pattern parameters between medial and anterior knee pain in patients with osteoarthritis of the knee. Clin. Biomech. 2012;27:584–587.

[54] Paterson K, Sosdian L, Hinman R, Wrigley T, Kasza J, Dowsey M, et al. The influence of sex and obesity on gait biomechanics in people with severe knee osteoarthritis scheduled for arthroplasty. Clin. Biomech. 2017;49:72–77.

[55] Kiss RM. Effect of severity of knee osteoarthritis on the variability of gait parameters. J. Electromyogr. Kinesiol. 2011;21:695–703.

[56] Lim JBT, Chi CH, Lo LE, Lo WT, Chia SL, Yeo SJ, et al. Gender Difference in Outcome after Total Knee Replacement. J. Orthop. Surg. 2015;23:194–197.

[57] Sorge RE, Mapplebeck JCS, Rosen S, Beggs S, Taves S, Alexander JK, et al. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. Nat. Neurosci. 2015;18:1081–1083.

[58] Bartley EJ, Fillingim R. Sex differences in pain: A brief review of clinical and experimental findings. Br. J. Anaesth. 2013;111:52–58.

[59] Edwards CL, Fillingim R, Keefe F. Race, ethnicity and pain. Pain. 2001;94:133–137.

[60] Diatchenko L, Slade GD, Nackley AG, Bhalang K, Sigurdsson A, Belfer I, et al. Genetic basis for individual variations in pain perception and the development of a chronic pain condition. Hum. Mol. Genet. 2005;14:135–143.

[61] Green CR, Anderson KO, Baker TA, Campbell LC, Decker S, Fillingim R, et al. The Unequal Burden of Pain: Confronting Racial and Ethnic Disparities in Pain. Pain Med. 2003;4:277–294.

[62] Ahn H, Weaver M, Lyon DE, Kim J, Choi E, Staud R, et al. Differences in Clinical Pain and Experimental Pain Sensitivity Between Asian Americans and Whites with Knee Osteoarthritis. Clin. J. Pain. 2017;33:174–180.

[63] Viña J, Borrás C, Miquel J. Theories of ageing. IUBMB Life. 2007;59:249–254.

[64] Blanco FJ, Valdes AM, Rego-Pérez I. Mitochondrial DNA variation and the pathogenesis of osteoarthritis phenotypes. Nat. Rev. Rheumatol. 2018;14:327–340.

[65] Aigner T, Soeder S, Haag J. IL-1beta and BMPs--interactive players of cartilage matrix degradation and regeneration. Eur Cell Mater. 2006;12:49-56.

[66] Li Z, Huang Z, Bai L. Cell Interplay in Osteoarthritis. Front Cell Dev Biol. 2021;9:720477.

[67] Loeser RF. Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. Osteoarthritis Cartilage. 2009;17(8):971-9.

[68] Chen YJ, Chan DC, Chiang CK, Wang CC, Yang TH, Lan KC, et al. Advanced Glycation End-Products Induced VEGF Production and Inflammatory Responses in Human Synoviocytes Via RAGE-NF-kB Pathway Activation. J. Orthop. Res. 2016;34:791–800.

[69] Nakamura H, Shimamura S, Yasuda S, Kono M, Kono M, Fujieda Y, et al. Ectopic RASGRP2 (CalDAG-GEFI) expression in rheumatoid synovium contributes to the development of destructive arthritis. Ann. Rheum. Dis. 2018;77:1765–1772.

[70] Xu R, Liu Z, Hou J, Huang T, Yang M. Osthole improves collagen-induced arthritis in a rat model through inhibiting inflammation and cellular stress. Cell. Mol. Biol. Lett. 2018;23:19.

[71] Loukov D, Karampatos S, Maly MR, Bowdish DME. Monocyte Activation Is Elevated in Women with Knee-Osteoarthritis and Associated with Inflammation, BMI and Pain. Osteoarthr. Cartil. 2018;26:255–263.

[72] Hached F, Vinatier C, Le Visage C, Gondé H, Guicheux J, Grimandi G, et al. Biomaterial-Assisted Cell Therapy in Osteoarthritis: From Mesenchymal Stem Cells to Cell Encapsulation. Best Pract. Res. Clin. Rheumatol. 2017;31:730–745.

[73] Abramson SB, Attur M. Developments in the Scientific Understanding of Osteoarthritis. Arthritis Res. Ther. 2009;11:227.

[74] Yunus MHM, Nordin A, Kamal H. Pathophysiological Perspective of Osteoarthritis. Medicina 2020;56:E614.

[75] Kuyinu EL, Narayanan G, Nair LS, Laurencin CT. Animal models of osteoarthritis: classification, update, and measurement of outcomes. J Orthop Surg Res. 2016;11:19.

[76] Woodell-May JE, Sommerfeld SD. Role of Inflammation and the Immune System in the Progression of Osteoarthritis. J Orthop Res. 2020;38(2):253-257.

[77] van der Kraan PM, van den Berg WB. Chondrocyte Hypertrophy and Osteoarthritis: Role in Initiation and Progression of Cartilage Degeneration? Osteoarthr. Cartil. 2012;20:223–232.

[78] Schiller J, Benard S, Reichl S, Arnhold J, Arnold K. Cartilage Degradation by Stimulated Human Neutrophils: Reactive Oxygen Species Decrease Markedly the Activity of Proteolytic Enzymes. Chem. Biol. 2000;7:557–568.

[79] Wessely-Szponder J, Michalska J, Szponder T, Zyli´nska B, Tarczy´nska M, Szubstarski M. The Role of Antimicrobial ˙Neutrophil Extract in Modification of the Inflammatory Response During Osteochondral Autograft and Allograft Transplantation in Rabbits. J. Comp. Pathol. 2020;175:49–63.

[80] Muley MM, Krustev E, Reid AR, McDougall JJ. Prophylactic Inhibition of Neutrophil Elastase Prevents the Development of Chronic Neuropathic Pain in Osteoarthritic Mice. J. Neuroinflamm. 2017;14:168.

[81] Johnson CI, Argyle DJ, Clements DN. In vitro models for the study of osteoarthritis. Vet J. 2016;209:40-9.

[82] Singh YP, Moses JC, Bhardwaj N, Mandal BB. Overcoming the Dependence on Animal Models for Osteoarthritis Therapeutics—The Promises and Prospects of In Vitro Models. Adv. Healthc. Mater. 2021;10:2100961.

[83] Harvanova D, Matejova J, Slovinska L, Lacko M, Gulova S, Fecskeova LK, et al. The Role of Synovial Membrane in the Development of a Potential In Vitro Model of Osteoarthritis. Int J Mol Sci. 2022 ;23(5):2475.

[84] Fernandes JC, Martel-Pelletier J, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. Biorheology 2002;39:237–246.

[85] Sohn DH, Sokolove J, Sharpe O, Erhart JC, Chandra PE, Lahey LJ, et al. Plasma proteins present in osteoarthritic synovial fluid can stimulate cytokine production via Toll-like receptor 4. Arthritis Res Ther. 2012;14(1):R7.

[86] Campbell SE, Bennett D, Nasir L, Gault EA, Argyle DJ. Disease- and cell-type-specific transcriptional targeting of vectors for osteoarthritis gene therapy: Further development of a clinical canine model. Rheumatology 2005;44:735–743.

[87] Chevalier X, Goupille P, Beaulieu AD, Burch FX, Bensen WG, Conrozier T, et al. Intraarticular injection of anakinra in osteoarthritis of the knee: A multicenter, randomized, double-blind, placebo-controlled study. Arthritis and Rheumatism. 2009;61:344–352.

[88] Maccoux LJ, Salway F, Day PJR, Clements DN. Expression profiling of select cytokines in canine osteoarthritis tissues. Veterinary Immunology and Immunopathology. 2007;118:59–67.

[89] Bujak M, Frangogiannis NG. The role of IL-1 in the pathogenesis of heart disease. Archivum Immunologiae et Therapiae Experimentalis. 2009;57:165–176.

[90] Loeser RF. Aging and osteoarthritis. Current Opinion in Rheumatology. 2011;23:492–496.

[91] Horiuchi T, Yoshida T, Koshihara Y, Sakamoto H, Kanai H, Yamamoto S, et al. The increase of parathyroid hormone-related peptide and cytokine levels in synovial fluid of elderly rheumatoid arthritis and osteoarthritis. Endocrine Journal. 1999;46:643–649.

[92] Fujita Y, Hara Y, Nezu Y, Yamaguchi S, Schulz KS, Tagawa M. Direct and indirect markers of cartilage metabolism in synovial fluid obtained from dogs with hip dysplasia and correlation with clinical and radiographic variables. American Journal of Veterinary Research. 2005;66:2028–2033.

[93] Liacini A, Sylvester J, Li WQ, Huang W, Dehnade F, Ahmad M, et al. Induction of matrix metalloproteinase-13 gene expression by TNF-alpha is mediated by MAP kinases, AP-1, and NF-kappaB transcription factors in articular chondrocytes. Experimental Cell Research. 2003;288:208–217.

[94]Nicholson IP, Gault EA, Foote CG, Nasir L, Bennett D. Human telomerase reverse transcriptase (hTERT) extends the lifespan of canine chondrocytes in vitro without inducing neoplastic transformation. The Veterinary Journal. 2007;174:570–576.

[95] Miyaki S, Nakasa T, Otsuki S, Grogan SP, Higashiyama R, Inoue A, et al. MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. Arthritis and Rheumatism. 2009;60:2723–2730.

[96] Novakofski KD, Torre CJ, Fortier LA. Interleukin-1α, -6, and -8 decrease Cdc42 activity resulting in loss of articular chondrocyte phenotype. Journal of Orthopaedic Research. 2012;30:246–251.

[97] Yang B, Kang X, Xing Y, Dou C, Kang F, Li J, et al. Effect of microRNA-145 on IL-1β-induced cartilage degradation in human chondrocytes. FEBS Letters 2014;588:2344–2352.

[98] BeekhuizenM, Bastiaansen-Jenniskens YM, Koevoet W, Saris DBF, Dhert WJA, Creemers LB, et al. Osteoarthritic synovial tissue inhibition of proteoglycan production in human osteoarthritic knee cartilage: establishment and characterization of a long-term cartilage-synoviumcoculture. Arthritis Rheum. 2011;63(7):1918-27.

[99] Jiang J, Nicoll SB, Lu HH. Co-culture of osteoblasts and chondrocytes modulates cellular differentiation in vitro. Biochem Biophys Res Commun. 2005;338(2):762-70.

[100] Sanchez C, Deberg MA, Piccardi N, MsikaP, Reginster JYL, Henrotin YE. Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1 beta and oncostatin M pre-treated non-sclerotic osteoblasts. Osteoarthritis Cartilage. 2005;13(11):979-87.

[101] Stefani RM, Halder SS, Estell EG, Lee AJ, Silverstein AM, SobczakE, et al. A Functional Tissue-Engineered Synovium Model to Study Osteoarthritis Progression and Treatment. Tissue Eng Part A. 2019;25(7-8):538-553.

[102] Cohen-Solal M, Funck-Brentano T, Hay E. Animal models of osteoarthritis for the understanding of the bone contribution. Bonekey Rep. 2013;2:422.

[103] Serra CI, Soler C. Animal Models of Osteoarthritis in Small Mammals. Vet. Clin. N. Am. Exot. Anim. Pract. 2019;22:211–221.

[104] Bapat S, Hubbard D, Munjal A, Hunter M, Fulzele S. Pros and cons of mouse models for studying osteoarthritis. Clin. Transl. Med. 2018;7:36.

[105] Esdaille CJ, Ude CC, Laurencin CT. Regenerative Engineering Animal Models for Knee Osteoarthritis. Regen Eng Transl Med. 2022;8(2):284-297.

[106] Veronesi F, Salamanna F, Martini L, Fini M. Naturally Occurring Osteoarthritis Features and Treatments: Systematic Review on the Aged Guinea Pig Model. Int J Mol Sci. 2022;23(13):7309.

[107] Pagani S, Minguzzi M, Sicuro L, Veronesi F, Santi S, Scotto D'Abusco A, et al. The N-Acetyl Phenylalanine Glucosamine Derivative Attenuates the Inflammatory/Catabolic Environment in a Chondrocyte-Synoviocyte Co-Culture System. Sci. Rep. 2019;9:13603.

[108] Forte L, Sarda S, Torricelli P, Combes C, Brouillet F, Marsan O, et al. Multifunctionalization Modulates Hydroxyapatite Surface Interaction with Bisphosphonate: Antiosteoporotic and Antioxidative Stress Materials. ACS Biomater. Sci. Eng. 2019;5:3429–3439.

[109] Forte L, Torricelli P, Boanini E, Rubini K, Fini M, Bigi A. Quercetin and alendronate multi-functionalized materials as tools to hinder oxidative stress damage. J. Biomed. Mater. Res. A. 2017;105:3293–3303.

[110] Veronesi F, Contartese D, Borsari V, Pagani S, Fini M, De Mattei M, et al. Ageing and Osteoarthritis Synergically Affect Human Synoviocyte Cells: An In Vitro Study on Sex Differences. J Clin Med. 2022;11(23):7125.

[111] Pagani S, Borsari V, Veronesi F, Ferrari A, Cepollaro S, Torricelli P, et al. Increased Chondrogenic Potential of Mesenchymal Cells From Adipose Tissue Versus Bone Marrow-Derived Cells in Osteoarthritic In Vitro Models. J. Cell. Physiol. 2017;232:1478–1488.

[112] Veronesi F, Della Bella E, Torricelli P, Pagani S, Fini M. Effect of adipose-derived mesenchymal stromal cells on tendon healing in aging and estrogen deficiency: An in vitro co-culture model. Cytotherapy. 2015;17:1536–1544.

[113] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 2008;3:1101–1108.

[114] Rios JL, Sapède D, Djouad F, Rapp AE, Lang A, Larkin J, et al. Animal Models of Osteoarthritis Part 1-Preclinical Small Animal Models: Challenges and Opportunities for Drug Development. Curr Protoc. 2022;2(11):e596.

[115] Kwok J, Onuma H, Olmer M, Lotz MK, Grogan SP, D'Lima DD. Histopathological analyses of murine menisci: implications for joint aging and osteoarthritis. Osteoarthritis Cartilage. 2016;24(4):709-18.

[116] Sanada Y, Ikuta Y, Ding C, Shinohara M, Yimiti D, Ishitobi H, et al. Senescence-accelerated mice prone 8 (SAMP8) in male as a spontaneous osteoarthritis model. Arthritis Res Ther. 2022;24(1):235.

[117] Krenn V, Morawietz L, Burmester GR, Kinne RW, Mueller-Ladner U, Muller B, et al. Synovitis score: discrimination between chronic low-grade and high-grade synovitis. Histopathology. 2006;49(4):358-64.

[118] Pritzker KPH, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, et al. 2006. Osteoarthritis cartilage histopathology: grading and staging. Osteoarthritis Cartilage 14:13–29.

[119] Shim J, Iwaya C, Ambrose CG, Suzuki A, Iwata J. Micro-computed tomography assessment of bone structure in aging mice. Sci Rep. 2022;12(1):8117.

[120] Le Graverand-Gastineau MPH. Disease modifying osteoarthritis drugs: facing development challenges and choosing molecular targets. Curr Drug Targets. 2010;11(5):528-35.

[121] Benton HP, Tyler JA. Inhibition of cartilage proteoglycan synthesis by interleukin I. Biochem Biophys Res Commun 1988;154:421–8.

[122] Gowen M, Wood DD, Ihrie EJ, Meats JE, Russell RG. Stimulation by human interleukin 1 of cartilage breakdown and production of collagenase and proteoglycanase by human chondrocytes but not by human osteoblasts in vitro. Biochim Biophys Acta 1984;797:186–93.

[123] Stadler J, Stefanovic-Racic M, Billiar TR, Curran RD, McIntyre LA, Georgescu HI, et al. Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. J Immunol 1991;147:3915–20.39.

[124] Dai X, Li Y, Zhang R, Kou Y , Mo X, Cao J, et al. Effects of sodium selenite on c-Jun N-terminal kinase signaling pathway induced by oxidative stress in human chondrocytes and c-Jun N-terminal kinase expression in patients with Kashin-Beck disease, an endemic osteoarthritis. Br. J. Nutr. 2016;115:1547–1555.

[125] Hsu DZ, Chu PY, Wu PT, Shen PC, Jou IM. Oxidative stress participates in quadriceps muscle dysfunction during the initiation of osteoarthritis in rats. Int J Clin Exp Pathol. 2015;8(10):12491-9.

[126] Jeong CH, Joo SHJ. Downregulation of Reactive Oxygen Species in Apoptosis. Cancer Prev. 2016;21:13–20.

[127] Roberts JS, Yilmaz O. Dangerous Liaisons: Caspase-11 and Reactive Oxygen Species Crosstalk in Pathogen Elimination. Int. J. Mol. Sci. 2015;16:23337–23354.

[128] Oyinloye BE, Adenowo AF, Kappo AP. Reactive oxygen species, apoptosis, antimicrobial peptides and human inflammatory diseases. Pharmaceuticals 2015;8:151–175.

[129] Mishra R, Singh A, Chandra V, Negi MP, Tripathy BC, Prakash J, et al. A comparative analysis of serological parameters and oxidative stress in osteoarthritis and rheumatoid arthritis. Rheumatol. Int. 2012;32:2377–2382.

[130] Kyostio-Moore S, Bangari DS, Ewing P, Nambiar B, Berthelette P, Sookdeo C, et al. Local gene delivery of heme oxygenase-1 by adeno-associated virus into osteoarthritic mouse joints exhibiting synovial oxidative stress. Osteoarthritis. Cartil. 2013;21:358–367.

[131] Kishimoto H, Akagi M, Zushi S, Teramura T, Onodera Y, Sawamura T, et al. Induction of hypertrophic chondrocyte-like phenotypes by oxidized LDL in cultured bovine articular chondrocytes through increase in oxidative stress. Osteoarthritis. Cartil. 2010;18:1284–1290.

[132] Li D, Xie G, Wang W. Reactive oxygen species: the 2-edged sword of osteoarthritis. Am J Med Sci. 2012;344:486–490.

[133] Zhuang C, Xu NW, Gao GM, Ni S, Miao KS, Li CK, et al. Polysaccharide from Angelica sinensis protects chondrocytes from H2O2-induced apoptosis through its antioxidant effects in vitro. Int. J. Biol. Macromol. 2016;87:322–328.

[134] Zhou Y, Liu SQ, Yu L, He B, Wu SH, Zhao Q, et al. Berberine prevents nitric oxide-induced rat chondrocyte apoptosis and cartilage degeneration in a rat osteoarthritis model via AMPK and p38 MAPK signaling. Apoptosis. 2015;20:1187–1199.

[135] Santoro A, Conde J, Scotece M, Abella V, Lopez V, Pino J, et al. Choosing the right chondrocyte cell line: Focus on nitric oxide. J. Orthop. Res. 2015;33:1784–1788.

[136] Prakash VS, Mansukhani NA, Helenowski IB, Woodruff TK, Kibbe MR. Sex bias in interventional clinical trials. J. Women's Health. 2018;27:1342–1348.

[137] Nefla M, Holzinger D, Berenbaum F, Jacques C. The danger from within: alarmins in arthritis. Nat Rev Rheumatol. 2016;12:669–683.

[138] Kraus A, Woon C, Raghavan S, Megerle K, Pham H, Chang J. Co-culture of human adipose-derived stem cells with tenocytes increases proliferation and induces differentiation into a tenogenic lineage. Plast Reconstr Surg. 2013;132:754e-766e.

[139] Alm-Eldeen A, Khamis A, Elfiky N, Ahmad R. Quercetin modulates age-induced changes in the transcript levels of some apoptosis related genes in the skeletal muscles of male rats. Braz. J. Pharm. Sci. 2020;56:e18861.

[140] Zharova TA, Kogan EA, Makarov VI, Smorchkov MM, Lychagin AV, Ivannikov SV, et al. Correlation of synovial caspase-3 concentration and the photodynamic effectiveness in osteoarthritis treatment. Photodiagnosis Photodyn. Ther. 2020;30:101669.

[141] Liu S, Deng Z, Chen K, Jian S, Zhou F, Yang Y, et al. Cartilage tissue engineering: From proinflammatory and anti-inflammatory cytokines to osteoarthritis treatments (Review) Mol. Med. Rep. 2022;25:99.

[142] Xu YS, Wang MM, Chen D, Jiang X, Xiong ZF. Inflammatory biomarkers in older adults with frailty: A systematic review and meta-analysis of cross-sectional studies. Aging Clin. Exp. Res. 2022;34:971–987.

[143] Wang L, He C. Nrf2-mediated anti-inflammatory polarization of macrophages as therapeutic targets for osteoarthritis. Front. Immunol. 2022;13:967193.

[144] Stanton H, Rogerson FM, East CJ, Golub SB, Lawlor KE, Meeker CT, et al. ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. Nature. 2005;434(7033):648–52.

[145] Cortial D, Gouttenoire J, Rousseau CF, Ronzière MC, Piccardi N, Msika P, et al. Activation by IL-1 of bovine articular chondrocytes in culture within a 3D collagen-based scaffold. An in vitro model to address the effect of compounds with therapeutic potential in osteoarthritis. Osteoarthritis Cartilage. 2006;14(7):631-40.

[146] Wang X, Li F, Fan C, Wang C, Ruan H. Effects and relationship of ERK1 and ERK2 in interleukin-1beta-induced alterations in MMP3, MMP13, type II collagen and aggrecan expression in human chondrocytes. Int. J. Mol. Med. 2011;27(4):583-589.

[147] Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature. 1997;390:45–51.

[148] Yao Y, He GY, Wu XJ, Wang CP, Luo XB, Zhao Y, et al. Association between environmental exposure to perchlorate, nitrate, and thiocyanate and serum α-Klotho levels

among adults from the National Health and nutrition examination survey (2007–2014) BMC Geriatr. 2022;22:740.

[149] Yamazaki Y, Imura A, Urakawa I, Shimada T, Murakami J, Aono Y, et al. Establishment of sandwich ELISA for soluble alpha-Klotho measurement: age-dependent change of soluble alpha-Klotho levels in healthy subjects. BiochemBiophys Res Commun. 2010;398:513–18.

[150] Xue XT, Zhang T, Cui SJ, He DQ, Wang XD, Yang RL, et al. Sexual dimorphism of estrogen-sensitized synoviocytes contributes to gender difference in temporomandibular joint osteoarthritis. Oral Dis. 2018;24:1503–1513.

[151] Flurkey K, Currer JM, Harrison DE. 2007. The Mouse in Aging Research. In The Mouse in Biomedical Research 2nd Edition. Fox JG, et al. editors. American College Laboratory Animal Medicine (Elsevier), Burlington, MA. pp. 637–672.

[152] Huang H, Skelly JD, Ayers DC, Song J. Age-dependent Changes in the Articular Cartilage and Subchondral Bone of C57BL/6 Mice after Surgical Destabilization of Medial Meniscus. Sci. Rep. 2017;7:42294.

[153] Javaheri B, Razi H, Piles M, de Souza R, Chang YM, Maric-Mur I, et al. Sexually dimorphic tibia shape is linked to natural osteoarthritis in STR/Ort mice. Osteoarthr. Cartil. 2018;26:807–817.

[154] Uchida K, Urabe K, Naruse K, Kozai Y, Onuma K, Mikuni-Takagaki Y, et al. Differential age-related bone architecture changes between female and male STR/Ort mice. Exp. Anim. 2012;61:59–66.

[155] De Hooge AS, van de Loo FA, Bennink MB, Arntz OJ, de Hooge P, van den Berg WB. Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging. Osteoarthr. Cartil. 2005;13:66–73.

[156] Kim JR, Kim HA. Molecular Mechanisms of Sex-Related Differences in Arthritis and Associated Pain. Int J Mol Sci. 2020;21(21):7938.

8. ACKNOWLEDGEMENTS

I would like to express my gratitude to Professor Monica De Mattei, for availability, guidance, support and advice she has provided me throughout my PhD.

I thank Professor Paolo Pinton, coordinator of the doctoral course in Biomedical and Biotechnological Sciences at the University of Ferrara.

I would like to thank Dr. Milena Fini, Scientific Director of the IRCCS Rizzoli Orthopedic Institute of Bologna and Dr. Gianluca Giavaresi, Director of the Laboratory Surgical Sciences and Technologies, where I work and have conducted my research activity.

I thank all my colleagues for the support, Dr. Lucia Martini, Dr. Francesca Veronesi, Dr. Melania Maglio, Dr. Silvia Brogini. In particular, Dr. Matilde Tschon, Dr. Veronica Borsari and Dr. Stefania Pagani for practical help in carrying out the tests envisaged by the thesis project, Dr. Maria Sartori for practical suggestions in developing the statistical data, and special thanks to Dr. Francesca Salamanna, always present in difficult moments of the PhD course but also of life.

Finally, I thank my family who have always believed in me.