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COORDINATORE Prof. Bernardi Francesco

"EFFECT OF GROWTH HORMONE WAS MODULATED BY HYALURONAN AMIDE DERIVATIVE ON HUMAN OSTEOARTHRITIC CHONDROCYTES"

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Dottorando
Dott. Paolella Francesca

Tutore Interno
Prof. Gambari Roberto

Tutore Esterno
Dott.ssa Lisignoli Gina

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1.1 <u>Pathology</u>

Osteoarthritis (OA) is the most common form of arthritis and a major cause of pain and disability in older adults (over the age 65) (Blumenkrantz *et al.*, 2004). The prevalence of OA in most joints is higher, before 50 years, in men than in women and after this age, women are more often affected than men. (Felson *et al.*, 2000)

 Often OA is referred to as degenerative joint disease "(DJD)" and it is characterized by joint pain and limited function of the articulation. This is a misnomer because OA is not simply a process of wear and tear but rather abnormal remodelling of joint tissues driven by a host of inflammatory mediators within the affected joint. (Loeser *et al.*, 2012).

Nowadays OA is considered a disease of the whole joint as an organ, the articular cartilage is altered to some extent in all affected joints with OA. In addition to the development of cartilage changes with aging, cartilage degeneration may occur in response to inappropriate mechanical stress and low-grade local systemic inflammation associated with trauma, metabolic syndrome, and genetic predisposition, which are major risk factors of OA development and progression. However, strong functional interactions among the cartilage, synovium, and subchondral bone impact on cartilage function in such a way that it is difficult to know where and when pathological changes begin. (Houard *et al*., 2013)

Bone, cartilage, synovial fluid, ligaments and also the muscles around the joint are tissues that change with OA and affect the function of the joint. (Andriacchi *et al*., 2009; Buckwalter *et al*., 2004) Several tissues might be a starting point for pathways that lead to OA. Cartilage might be the tissue where the pathophysiological process of OA starts, but biochemical and imaging studies have shown that synovium and bone could be also good starting points. However, it remains unclear which of these three types of tissue, or some combination thereof, might serve as the key tissue for OA. (Samuels *et al*., 2008)

One of the most affected joints is the knee, in this joint osteoarthritis is a chronic, indolent disease that will affect an ever increasing number of patients, especially the elderly and the obese. It is characterized by degeneration of the cartilage substance inside the knee which leads to pain, stiffness and tenderness. By some estimations in 2030, only in the United States, this medical condition will burden 67 million people. (Uth and Trifonov, 2014)

A diagnosis of OA is mainly based on symptoms. A patient that has reached a certain age and has joint pain, limitation of movement, crepitus and, sometimes, effusion in the joint might get the diagnosis of OA. Recommendations for the diagnosis of knee OA were published in 2010. (Zhang *et al*., 2010) The treatment of OA is mainly symptom driven and based on the use of anti-inflammatory medication in combination with exercise treatment and lifestyle changes. However such treatment cannot prevent or cure OA and often fails to provide satisfactory pain relief. Joint replacement may be possible in developed countries for patients with severe OA and significant disability. Research efforts during the past decades have focused on the search for disease-modifying treatments. Most of these disease-modifying treatments were directed towards regeneration of the cartilage and were tested in patients with evident OA. However, so far, these efforts have not been very successful and have not had a significant influence on the symptoms of OA. (Hunter 2011)

A more comprehensive definition summarizing the clinical, phatophysiological, biochemical and biomechanical changes that occur in OA was summarized in Table 1.

Table 1 Features of OA	
Clinical	· Joint pain
	· Joint tenderness
	· Limitation of movement
	· Crepitus
	· Occasional effusion
	· Variable local inflammation without sistemi side effects
Pathological	· Irregular loss of cartilage, especially in areas of increate load
	· Sclerosis of subchondral bone
	· Subchondral cysts
	· Marginal osteophytes
	· Increased metaphyseal bood flow
	· Variable synovial inflammation
Histological	· Fragmentation of cartilage surface
	· Cloning of chondrocytes
	· Vertical clefts in the cartilage
	· Variable crystal deposition
	· Remodelling and violation of the tidemark by blood vessels
	· Osteophytes
	· Loss cartilage
	· Sclerosis of subchondral bone
	· Focal osteonecrosis of subchondral bone
Biomechanical	· Alteration in tensile, comprensive and shear properties
	· Altered cartilage hydraulic permeability
	·Excessive cartilage swelling
	· Increased stiffness of subchondral bone
Biochemical	· Reduction in proteoglycan concentration
	·Possible alteration in size and aggregation of proteogycans
	·Alteration in collagen fibril size and weave
	· Increased synthesis and degradation of matrix macromolecules

1.2 Classification

Criteria for classification of symptomatic OA have been developed by a subcommittee of the American College of Rheumatology (ACR). The ACR OA criteria were developed to standardise the definition of hip, knee and hand OA and are comprised of joint symptoms, exclusion of inflammatory conditions and positive radiography.

The Kellgren-Lawrence (Kellgren and Lawrence, 1957) system for radiographic grading of OA has been the standard for several decades and is based upon the presence and severity of certain defined radiographic features including osteophytosis, joint space narrowing, joint line sclerosis and subchondral cysts. These radiographic features are used to grade the severity of OA from 0 (normal joint) to 4 (complete joint space loss). (Shane Anderson and Loeser, 2010)

There are two main types of osteoarthritis, which have differing causes.

1) **Idiopathic osteoarthritis** — Idiopathic OA has no identifiable cause. It may be localized (confined to one or two joints) or generalized (present in three or more joints).

2) **Secondary osteoarthritis** — Secondary OA is caused by an underlying condition, such as a joint injury, accumulation of calcium inside the joint, other bone and joint conditions (e.g. rheumatoid arthritis), or a medical condition, such as diabetes. It can be divided into four main categories (Table 2):

- metabolic disorders such as ochronosis, which lead to joint damage that can be indistinguishable from OA;
- anatomic derangements such as a slipped epiphysis, which can lead to OA of the one affected joint only;
- major trauma or surgery to a joint, such as a meniscectomy;
- a previous inflammatory arthropathy, such as RA, resulting in a secondary OA process in some of the affected joints.

However, the distinction between primary and secondary OA is not always clear.

Table 2 Classification of OA
Classification into primary and secondary forms of OA
• Primary = idiopathic
• Secondary indicates that a likely cause can be identified Causes of secondary OA: 1. **Metabolic:** ochronosis, acromegaly, hemochromatosis, calcium crystal deposition; 2. **Anatomic:** slipped femoral epiphysis, epiphyseal dysplasias, blount's disease, legg-perthes disease, congenital dislocation of the hip, leg-lenght inequality, hypermobility syndromes; 3. **Traumatic:** major joint trauma, fracture through a joint or osteonecrosis, joint surgery (e.g. meniscectomy), chronic injury; 4. **Inflammatory:** any inflammation arthropathy, septic arthritis.
Classification by the presence of specific features • Inflammation • Erosive OA • Artrophic or destructive OA • OA with chondrocalcinosis

1.3 <u>Epidemiology</u>

OA is the most common rheumatic disease, and it represents 72.6% of rheumatic diseases in our country. (De Filippis *et al.*, 2004) In regards to the epidemiology of knee OA is the most common joint disorder in the world and one of the most common sources of pain and disability. In the elderly studies indicate that knee osteoarthritis in men aged 60 to 64 is usually found in the right knee (23%) than in the left knee (16.3%), while distribution seems to be more evenly balanced in women of the same age (right knee, 24.2%; left knee, 24.7%). (Michael *et al.*, 2010) A variety of endogenous (e.g., age, sex) and exogenous (obesity, patient's lifestyle) risk factors for OA have also been defined. (Uth and Trifonov, 2014)

While there remains considerable heterogeneity in defining OA among epidemiological studies, the evidence is conclusive that age remains the greatest risk factor for the development of OA in susceptible joints. Radiographic changes, in particular osteophytosis, are very common in the aging population and when used alone may provide an overestimation of the true prevalence of symptomatic OA. Defining OA solely as joint pain occurring in an older adult without evidence for another form of arthritis is also inaccurate in fact there are many causes of non articular-pain, such as bursitis, that are common in older adults. (Shane Anderson and Loeser, 2010, Li Y *et al.*, 2013)

The aging however is not the only cause of the disease, but other causes, later discussed, also contribute to OA progression. (De Filippis *et al.*, 2004)

1.4 <u>Risk Factors</u>

OA is a disease characterized by chronic multifactorial etiology, that includes different factors. These factors can increase the risk of developing osteoarthritis; most people with OA have one or more of these factors.

Multiple components of the joint are adversely affected by OA, including the peri-articular bone, synovial joint lining, and adjacent supporting connective tissue elements furthermore multiple factors, including joint instability and/or malalignment, obesity, increasing age, associated intra-articular crystal deposition, muscle weakness and peripheral neuropathy, are known to affect the progression of OA. These factors can be segregated into categories that include hereditary contributions, mechanical factors, and the effects of aging. (Goldring MB and Goldring SR, 2007)

1. <u>Intrinsic factors:</u>

- **Age:** Advancing age is one of the strongest risk factors for OA. The condition rarely occurs in people younger than age 40, but at least 80% of people over age 55 have some x-ray evidence of the disorder. However, not all people with arthritis on an x-ray have joint pain or other joint problems. The effects of age might by mediated through excess joint loading from obesity over time, impaired muscle function and neurological responses that otherwise protect the joint (Newman *et al.*, 2003) and increased joint instability due to ligamentous laxity. Aging may also cause a change in the material properties of the tissue involved, in fact aging make the cartilage more prone to failure. The repair capacity of the joint is also believed to diminish with increasing age.

- **Gender:** For unknown reasons, women are between two and three times more likely than men to develop OA. Before age 50, the joint involvement is slightly higher in men; while after that age women have a higher incidence, probably due to decreased estrogenic activity.

- **Obesity:** People who are obese are at high risk of developing OA. Obesity leads to an overload of the joints and to accumulation of cholesterol therefore weight loss may reduce this risk. (De Filippis *et al.*, 2004)

- **Ereditariety:** some studies have found that OA has an hereditary components, in fact some locus have been mapped on different chromosomes. (De Filippis *et al.*, 2004)

- **Ethnicity:** Some data indicate a higher prevalence of OA of the hip in Caucasians compared to people of color, while other works indicate a higher prevalence of knee OA in a Japanese population compared to a Caucasian. (Nevitt *et al*., 2002)

- **Genetic factors:** Genetics of OA has been the subject of several studies. There is now good evidence indicating a genetic contribution to about half the population variability in susceptibility to hip and knee OA. A number of susceptibility loci have been identified but no single genetic variant has been found that has a strong association with OA. (Valdes *et al*., 2008)

- **Metabolic factors:** nutritional factors play a role to increase OA. Low vitamin C intake has been associated with accelerated progression of OA, similar data have been found with vitamin D. (De Filippis *et al.*, 2004)

2. **Local factors:**

- **Occupation:** OA of the knee has been linked to certain occupations that require frequent squatting and kneeling, including cotton processing, dock work, shipyard work, and carpentry. OA of the hip has been linked to farm work, construction work, and other activities that require heavy lifting, prolonged standing, or walking several miles each day.

- **Sports** : The risk of OA is increased in people who participate in some specific sports, including wrestling, boxing, pitching in baseball, cycling, parachuting, cricket, gymnastics, ballet dancing, soccer, and football; in contrast, running does not appear to increase the risk of OA.

- **Alterations joint:** produced by diseases of inflammatory nature, post-traumatic epiphyseal necrosis, etc. (Litwic *et* al., 2003)

1.5 <u>Sintomatology</u>

The onset of this disease generally is often gradual and usually affects one or a few joints. Pain, functional limitation and morning stiffness are the most clinical characteristic. (Chaganti and Lane, 2011)

Furthermore, as the cartilage substance decreases, the bone surface may also become affected. This results in development of ostephytes (bone spurs) and direct bone-bone contact. In addition to the stiffness of the joint, the patient tries to avoid pain by minimizing joint movement, which leads to muscle atrophy and laxity of the ligaments. (Uth and Trifonov, 2014)

The symptoms of OA usually begin after age 40 and can vary considerably from one person to another.

- **Pain** — The main symptom of OA is joint pain that is worse with activity and is relieved by rest. In severe cases, the pain may also occur at rest or at night. The pain usually occurs near the affected joint; however, in some cases, the pain may be referred to other areas. For example, the pain of OA of the hip may actually be felt in the knee. Joints affected by OA may be tender to the touch. The level of pain is typically constant over time. Any sudden increases in the level of pain may indicate recent injury or an underlying condition such as gout.

- **Stiffness** — Morning stiffness is a common symptom of osteoarthritis. This stiffness usually resolves within 30 minutes of rising, but it may recur throughout the day during periods of inactivity. Some people note a change in symptoms related to the weather.

- **Swelling** (effusion) — Osteoarthritis may cause a type of joint swelling called an effusion, which results from the accumulation of excess fluid in the joint.

- **Crackling or grating sensation (crepitus)** — Movement of a joint affected by osteoarthritis may cause a crackling or grating sensation called crepitus. This sensation likely occurs because of roughening of the normally smooth surfaces inside the joint.

- **Bony outgrowths (osteophytes)** — Osteoarthritis often causes outgrowths of bone called osteophytes or bone spurs. These bony protuberances can be felt under the skin near joints and typically enlarge over time.

- **Symptoms in specific joints** — Osteoarthritis does not affect all joints equally. The condition most commonly affects the fingers, knees, hips, and spine; it rarely affects the elbow, wrist, and ankle. Furthermore, it often affects joints on one side of the body differently than on the other side. (Khanna *et al.*, 2012)

1.6 <u>Physiological modification of the OA joints</u>

OA is considered a "whole joint" disease that mainly affects: articular cartilage, subchondral bone and synovium.

1.6.1 <u>Articular cartilage</u>

Articular cartilage is avascularized, aneural tissue, and is highly specialized tissue constituted by an extensive extracellular matrix (ECM), which is mainly composed of water, collagen type 2, collagen type 9, collagen type 11 and proteoglycan (aggrecan). (Figure 1)

The biophysical properties of cartilage derive from this highly organized fibrillar framework, that supplies shape, strength, tensile stiffness and compressive resistance to the tissue. The synthesis, maintenance and degradation of ECM proteins are coordinated by chondrocytes, the only resident cell type in cartilage. (Goldring and Marcu, 2009; Wang *et al.*, 2011)
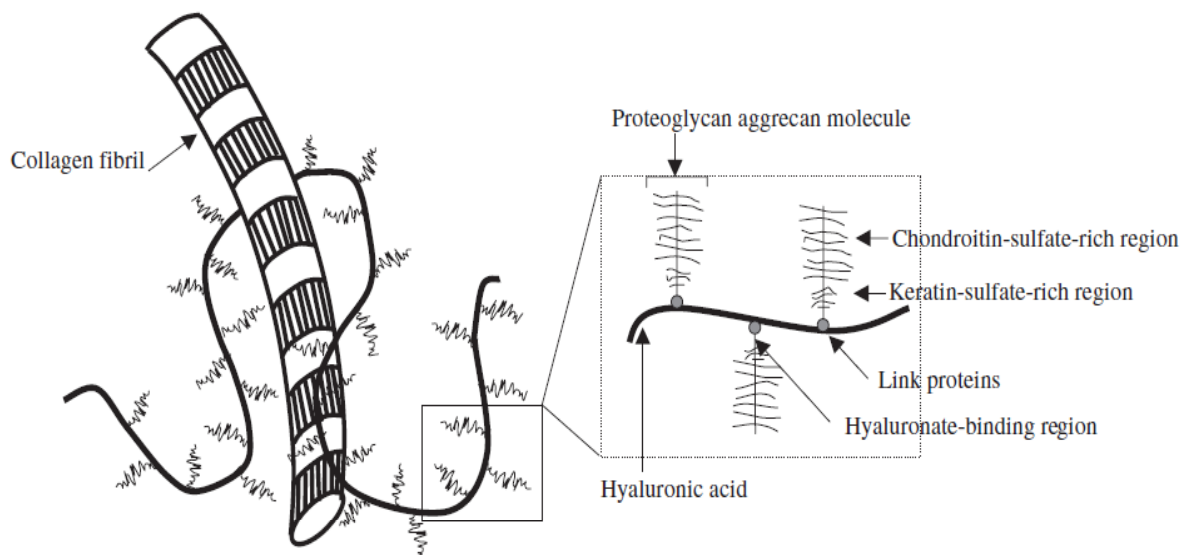
Figure 1: The extracellular matrix of cartilage is composed of proteoglycans attached to a backbone of hyaluronic acid that is intertwined among collagen fibrils. Proteoglycans have both chondroitin-sulfate- and keratin-sulfate-rich regions, and link proteins facilitate binding of aggrecan to hyaluronic acid.

Chondrocytes are the only cells type of the cartilage, encapsulated within a lacuna, which hinders their ability to migrate to the site of injury. Chondrocytes do not have the capacity for renewal, proliferation, or repair the damage tissue. (Wang *et al*., 2010)

In normal cartilage, there are four zones of chondrocytes: resting cells orientating within the collagen fibers in the superficial zone, large and randomly distributed cells in the middle zone, columns of chondrocytes in the deep zone, and hypertrophic cells in the calcified zone (Figure 2). (Salminen *et* al.,2002)

Under normal conditions, articular chondrocytes maintain a dynamic equilibrium between synthesis and degradation of ECM components, including collagen type 2 and aggrecan, the most abundant proteoglycan in articular cartilage. (Sandell *et al*., 2001)
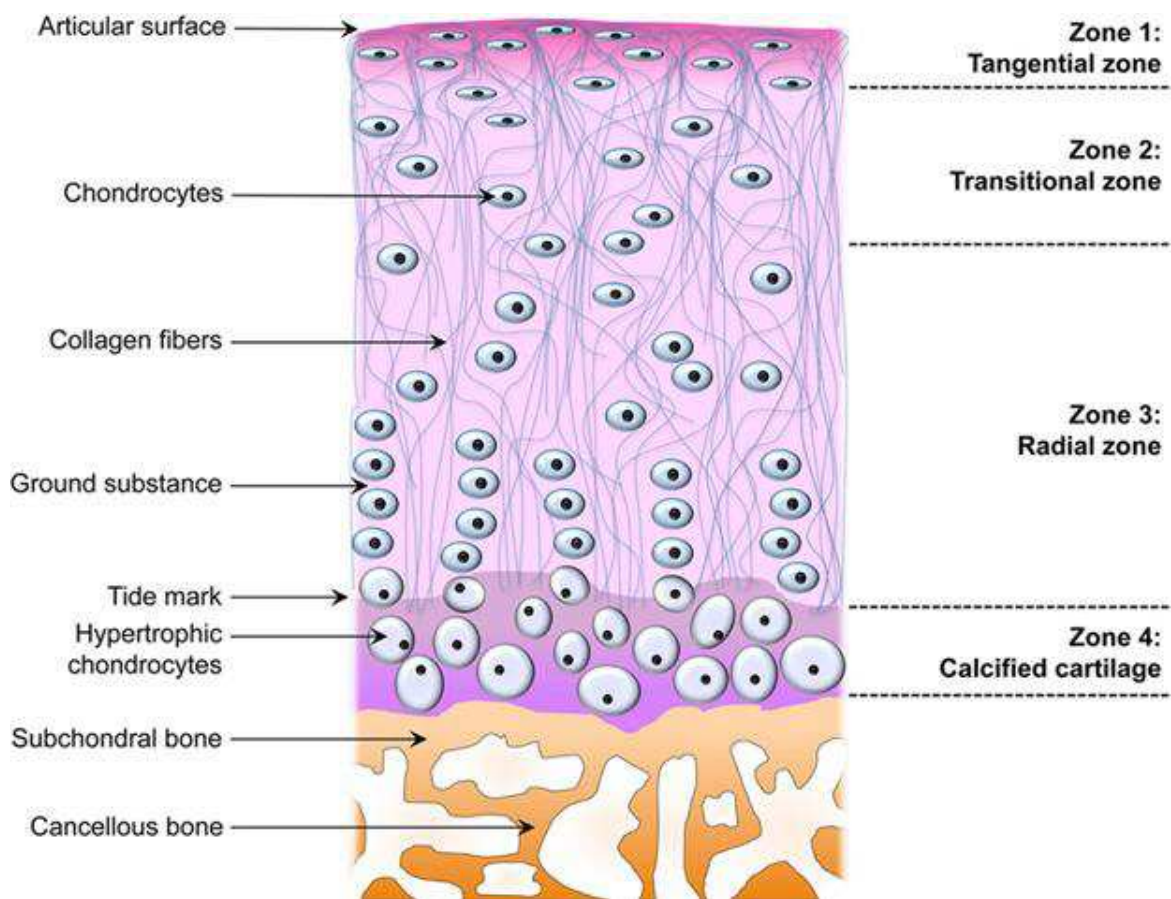
Figure 2: Zonal architecture in normal articular cartilage.

A series of catabolic and anabolic mediators have been found to play key roles in articular cartilage homeostasis. The balance between synthesis and degradation is affected by age and is regulated by several factors produced by the synovium and chondrocytes, including cytokines, growth factors, aggrecanases, and metalloproteinases (MMPs).

The pathogenesis of knee OA have been linked to biomechanical and biochemical changes in the cartilage of the joint mainly associated to inability to withstand normal mechanical stresses, limited supply of nutrients and oxygen, inadequate synthesis of extracellular matrix components, increased synthesis of tissue-destructive proteinases (matrix metalloproteinases and aggrecanases) and overall apoptosis of chondrocytes (Figure 3). (Buja and Krüger, 2014)
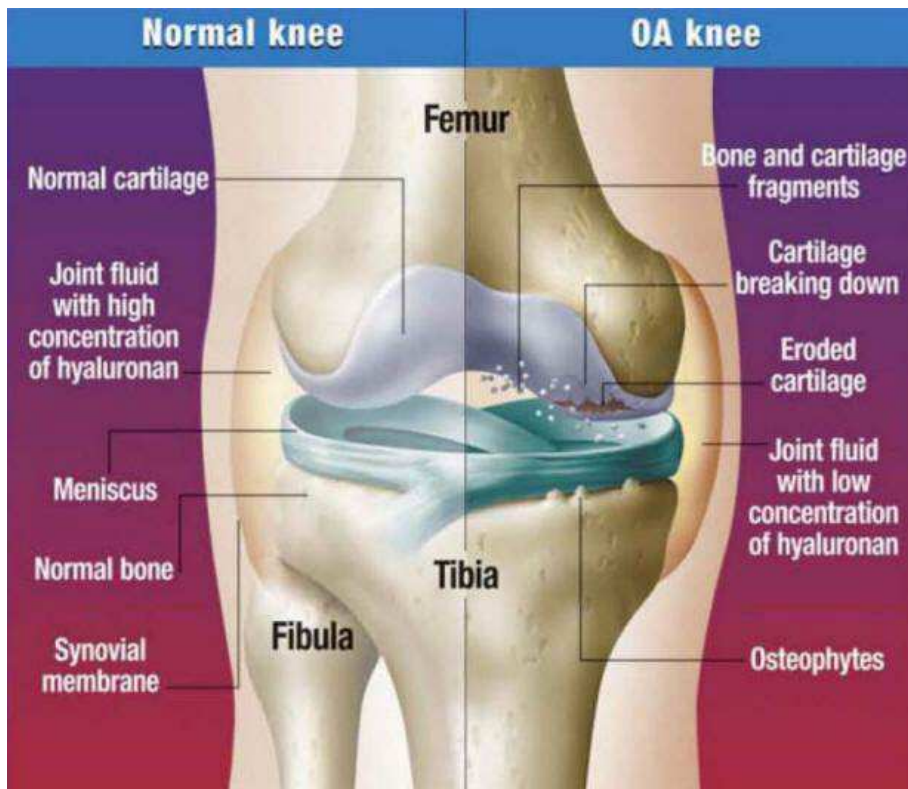
Figure 3: Main characteristics of normal and OA knee joint

Chondrocytes can respond to direct biomechanical perturbation by up-regulating synthetic activity or by increasing the production of inflammatory cytokines (e.g. IL-1β, IL-6, IL-8, TNF-α) , which are also produced by other joint tissues. This process causes depletion of proteoglycans and damage to the collagen network and decreases the synthesis of cartilage matrix proteins, whereas dynamic compression increases matrix synthetic activity. (Guilak *et al.*, 2004) In response to traumatic injury, global gene expression is activated, resulting in increased expression of inflammatory mediators, cartilage-degrading proteinases, and stress response factors (Figure 4). (Goldring and Otero, 2011)
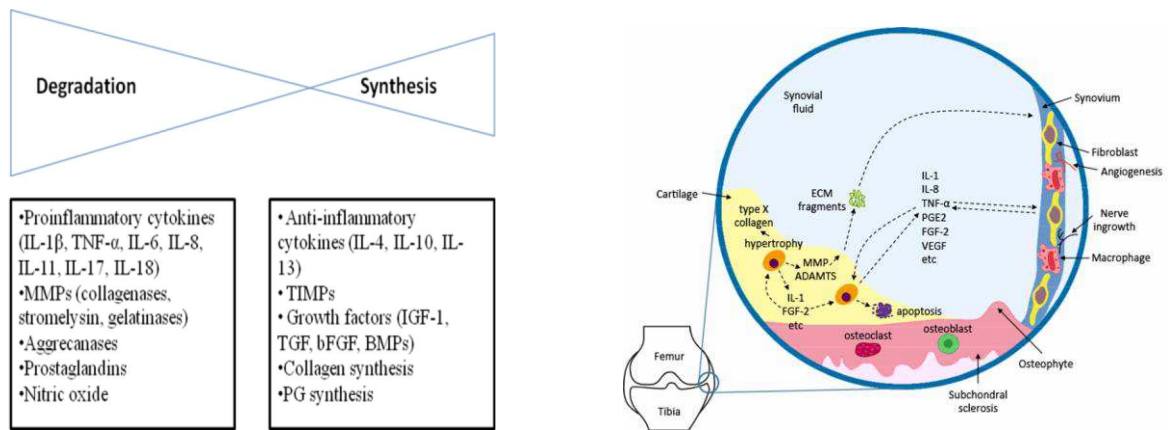
Figure 4: In osteoarthritis (OA), the balance between cartilage degradation and synthesis leans toward degradation. In osteoarthritic state, aberrantly activated chondrocytes produce ECM-degrading proteases (MMPs, aggrecanases) and pro-inflammatory cytokines (e.g. IL-1). Fragments derived from ECM degradation are also present in the synovial fluid as catabolic inducers. In OA, a subpopulation of chondrocytes undergoes hypertrophic changes, as manifested by their expression of type X collagen. Chondrocytes may also upregulate apoptosis, resulting in diminished local cellularity. In response to cartilage loss, pathological remodeling of subchondral bone gives rise to sclerosis and osteophyte formation.

Chondrocytes have receptors for responding to mechanical stimulation, many of which are also receptors for ECM components. (Millward-Sadler and Salter, 2004) The activation of these receptors can stimulate the production of matrix-degrading proteinases and inflammatory cytokines and chemokines. (Goldring MB and Goldring SR, 2007; Pulai *et al.*, 2005)

The pathological changes of OA in articular cartilage are mainly due to an increase of catabolic factors, that cause the loss of ECM and cell apoptosis. Cartilage matrix degradation products like collagen type 2, proteoglycans, and fibronectin seem to favour cartilage destruction. (Santos *et al.*, 2011)

A further hallmark of degenerated cartilage is the modification of chondrocyte differentiation stage, that switches them toward a hypertrophic phenotype (Goldring and Marcu, 2009; Fosang and Beier 2011), thus recapitulating some of the physiological differentiation steps occurring in growth plates and endochondral ossification.

Hypertrophic chondrocytes are characterized by the expression of terminal differentiation markers,

including Runt-related transcription factor 2 (RUNX-2), Collagen 10, MMP-13. (Van der Kraan and Van den Berg, 2012)

In fact, when OA occurs and proceeds, chondrocytes start to express several proteins such as biglycan, decorin, perlecan, and collagen type 1 and 10, so forming repaired fibrocartilage. (Moreland, 2003; Lee *et* al.,2013)

1.6.2 **Subchondral bone**

Subchondral bone consists of the subchondral bone plate and the underlying trabecular bone and bone marrow space. The subchondral bone plate consists of cortical bone and is separated from the articular cartilage by the zone of calcified cartilage.

Subchondral bone properties are modified through the cell mediated process of remodeling and modelling. Bone remodeling includes the coupling of mechanisms that reabsorb bone and form new bone on a previously reabsorbed surface, whereas bone modeling is a mechanism that drives changes in the architecture and volume of bone via direct apposition to existing bone surfaces. (Man and Mologhianu, 2014)

During OA process all of these mechanisms may be altered so resulting in subchondral bone structure changes.

Subchondral sclerosis is commonly considered an indisputable sign of OA. However, some studies suggest that different microarchitectural alterations of subchondral bone occur during different stages of OA; subchondral sclerosis may be observed only during more advanced stages of OA.

In early stages of OA, elevated bone remodeling and subchondral bone loss was observed, and was considered as a determinant of OA progression. (Li *et al*., 2013)

An incidence of microdamage (associated with subchondral bone stiffening and cartilage degeneration) was also reported in the subchondral bone of patients with early OA. Although the underlying mechanism for the increased bone turnover and structural deterioration in the early phase of OA is not completely understood, several factors have been implicated, including microdamage repair, increased vascularity stimulated by angiogenic factors and enhanced bone-cartilage crosstalk via increased subchondral plate pores. (Verborgt *et* al., 2000)

In the late stage of OA, subchondral bone microarchitectural is characterized by elevated apparent density, increased bone volume, thickening of subchondral bone plate, increased trabecular thickness, decrease of trabecular separation and bone marrow spacing. (Ding, 2010)

The overlying calcified cartilage is also thickened, with advancement and duplication of the tidemark, which contributes to articular cartilage thinning and deterioration. Despite increased bone volume density in the sclerotic subchondral bone, its mineralization is reduced and lower than that in normal or even osteoporotic joints. In OA subchondral

bone, collagen synthesis is elevated (especially collagen type 1), the deposited collagen is abnormal and this leads to abnormal mineralization. (Man and Mologhianu, 2014)

Moreover, in areas of the joint that not support the weight, usually develop further bone and cartilaginous tissue, better known as osteophytes.

The osteophytes are covered with a layer of hyaline and fibrous cartilage and blends with adjacent synovium.

The shape and position of osteophytes depends on:

- the instability of the joint;

- the degree of subluxation.

However, the presence of osteophytes is not absolute proof of the OA development.

Summarizing features of OA in the subchondral bone are fibrillation, sclerosis, even collapse, together with bone cysts, thickened cortical plate, extensive remodelled trabeculae and osteophyte formation surrounding articular margins.

1.6.3 Synovial membrane

Clinical evidence shows that OA and synovitis (inflammation of the synovium) are closely related.

Abnormalities of the synovium are detectable in 50% of patients with OA; synovitis is detected by some osteoarthritic symptoms that characterize the disease, such as swelling, effusion, redness and pain.

The synovial inflammation is certainly more evident in patients who have pain, and the severity of the pain is associated with thickening of the synovial membrane. It remains unclear whether the morphological changes that occur in the osteoarthritic synovial membrane are primary or whether they are the result of joint inflammation, cartilage degradation and lesions of the subchondral bone. (Sutton *et al.*, 2009)

 Histologically, the synovial membrane of osteoarthritic joints commonly exhibits hyperplasia of the lining cell layer occasionally accompanied by focal infiltration of lymphocytes and monocytes in sublining layers. (Brandt *et al.*, 2009)

Synovitis is believed to be induced at first by the cartilage matrix proteolytic degradation products that produce wear particles and soluble cartilage-specific neo-antigens, as well as other factors including microcrystals and abnormal mechanical stress. These components are released into the synovial fluid and are phagocyted by synovial lining macrophages,

perpetuating the inflammation of the synovial membrane through the synthesis of mediators, which in turn diffuse through the synovial fluid into the cartilage, and create a vicious circle, with increased cartilage degradation, and subsequently produce more inflammation. This also explains the increase in the amount of CD68-positive type A synoviocytes (macrophage-like), which have phagocytic capacity, in the synovial lining layer. (Martel-Pelletier and Pelletier, 2010)

The synovium produces some of the chemokines and metalloproteinases that degrade cartilage, even though the cartilage itself produces most of these destructive molecules. In turn, cartilage breakdown products, resulting from mechanical or enzymatic destruction, can induce the release of collagenase and other hydrolytic enzymes from synovial cells and lead to vascular hyperplasia in osteoarthritic synovial membranes. (Man and Mologhianu, 2014)

Synovial neovascularization may be largely driven by synovitis as inflammatory cells such as macrophages that can themselves secrete pro-angiogenic factors. These cells also secrete factors that stimulate other cells, such as endothelial cells and fibroblasts, to produce vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and other factors that further promote angiogenesis.

Angiogenesis in the synovium is closely associated with chronic synovitis and may occur at all stages of osteoarthritis.

1.7 <u>Molecules involved in OA pathophysiology</u>

Numerous mediators contribute to the progression of OA. Inflammatory stimuli initiate a cascade of events, including the release of cytokines by chondrocytes, leading to complex biochemical and mechanical interplay with other biological mediators to induce OA and promote pain. Particular catabolic mediators include pro-inflammatory members from the interleukin family (IL-1, IL-6, and IL-17), tumor necrosis factor-α (TNF-α), and prostaglandin E2 (PGE2). Each of these mediators not only stimulates the production of cartilage-degrading proteases to induce ECM degradation, but also contributes to OA-associated pain pathways. (Bauer *et al*., 2006)

On the other hand a number of growth and differentiation factors that regulate cartilage development and homeostasis of mature articular cartilage have been identified. The most characterized factors which stimulate the anabolic activity in cartilage include transforming growth factor (TGF-β), bone morphogenetic protein (BMP), fibroblast growth factors (FGF), insulin growth factor 1 (IGF-1). (Vinatier *et* al., 2009)

Moreover, since articular cartilage is an avascular tissue and, consequently, chondrocytes receive oxygen and nutrients via a passive diffusion from the synovial fluid, and their adaptation to low oxygen tension is mediated by transcription factors, such as hypoxia inducible factor (HIF). (Schipani *et al*., 2001)

1.7.1 <u>Interleukin-1 (IL-1)</u>

IL-1 is one of the most well-studied cytokines involved in OA.

IL-1 demonstrates potent bioactivities in inhibiting ECM synthesis and promoting cartilage breakdown, represses the expression of essential ECM components (e.g. aggrecan and collagen type II) in chondrocytes and induces proteolytic enzymes such as collagenases (MMP-1 and MMP-13) and ADAMTS-4, in both chondrocytes and synovial fibroblasts. In addition to these direct effects, IL-1β induces a variety of other cytokines, including IL-6, IL-8, and leukemia inducing factor (LIF), which interact to induce additive or synergistic effects in the catabolic cascade. (Goldring *et* al., 1988)

1.7.2 <u>Interleukin-6 (IL-6)</u>

IL-6 is a pro-inflammatory cytokine that is involved in cartilage degradation, and it has also been associated with hyperalgesia and hypersensitivity in joint tissues. IL-6 plays an important role in the pathogenesis of arthritis diseases, and its concentration is elevated in the serum and synovial fluid of arthritic patients. (Lee *et* al., 2013)

The production of IL-6 in the tissues of the affected joint is usually in response to IL-1β and TNFα and is mainly implemented by chondrocytes, osteoblasts, fibroblast-like synoviocytes, macrophages, and adipocytes. (Wojdasiewicz *et al*., 2014)

The effect of IL-6 on joint cartilage is not different from other cytokines and, in synergy with them, causes a decrease in the production of type II collagen and increases the production of enzymes from the MMPs group. (Porée *et* al., 2008)

IL-6 is considered to be the key cytokine, which causes changes in the subchondral bone layer. Its effect is largely based on promoting the formation of osteoclasts and thus bone resorption while showing synergism with IL-1β and TNFα. Osteoblasts stimulated by IL-1β, TNFα, and IL-6 become a source thereof and may also produce MMPs by adversely affecting the cartilage located near it. (Wojdasiewicz *et al*., 2014)

1.7.3 <u>Tumor necrosis factor-α (TNF-α)</u>

TNF-α is known for its powerful catabolic effects in the pathophysiology of OA.

In OA patients, TNF-α levels are increased in synovial fluid, synovial membrane, subchondral bone and cartilage. TNF-α inhibits synthesis of the major extracellular matrix (ECM) components: proteoglycans, collagen type II and cartilage link protein. Moreover, TNF-α up-regulates expression and release of various cartilage-destructive MMPs, like MMP-1, MMP-3 and MMP-13.

It is an extremely potent pro-inflammatory cytokine that interacts with chondrocytes by binding to receptors on their surface. (Haseeb and Haqqi, 2013)

In fact, TNF-α has an exacerbating effect on inflammation by inducing production of the pro-inflammatory cytokines and chemokines: IL-6, IL-8, IL-1 via the receptors TNFR1 and TNFR2. (Wojdasiewicz *et al*., 2014)

Systemic TNF-α levels are associated with increased prevalence of knee cartilage loss, indicating that systemic low-grade inflammation indeed plays a role in OA pathogenesis.

1.7.4 **Prostaglandin E2 (PGE2)**

During pro-inflammatory states in articular cartilage, numerous enzyme products are produced and released, including PGE2, that is considered to be the major contributor to inflammatory pain in arthritic conditions. PGE2 exerts its effects via a variety of E prostanoid (EP) receptors (EP1, EP2, EP3, EP4), which are present in both peripheral sensory neurons and the spinal cord. Activation of these receptors induces a variety of effects, ranging from calcium influx to cAMP activation or inhibition

It has been shown that IL-1β stimulates and produces high levels of PGE2 that may induce pain and the degeneration in OA. Further, PGE2, when combined with the catabolic cytokine IL-1, synergistically up-regulates IL-6 mRNA levels. (Li *et al*., 2009)

1.7.5 **Fibroblast growth factor (FGF-2)**

FGF-2, a potent catabolic and anti-anabolic factor, play an important role in human cartilage homeostasis. FGF-2 is released in higher amounts during loading and/or injury of the cartilage matrix and activates multiple transduction signal pathways (MAPKs), such as ERK, p38, and JNK. These kinases in turn phosphorylate a set of transcription factors to regulate gene expression and modify cellular function, resulting in a decrease in proteoglycans synthesis and antagonism against anabolic growth factors, such as insulin-like growth factor 1 (IGF-1) and bone morphogenetic protein (BMP-7) in articular cartilage. FGF-2 potently stimulates MMP-13 expression, which is the major type II collagen-degrading enzyme. (Im *et al*., 2008)

However anabolic activity of FGF-2 in articular cartilage has been reported. (Ellman *et al.,* 2008)

In adult cells, the chondrogenic effect of FGF has been confirmed in some studies. In adult chondrocytes, it has been shown that FGF2 has various property, in fact it has mainly a mitogenic, proliferative and chondrogenic factor. Furthermore, the contradictory results for the potential role of various FGFs in chondrogenesis highlight the need for a better characterization of the signalling pathways that are activated by FGFs to be able to fully understand how they affect FGF activity. (Vinatier *et* al., 2009)

1.7.6   Insulin-like growth factor (IGF)

The insulin-like growth factor (IGF) family comprises the ligands IGF-1 and IGF-2, the receptors IGF1R and IGF2R, at least six different IGF-binding proteins (IGFBPs) and multiple IGFBP proteases, which regulate IGF activity. IGF-2 mainly has a role in embryonic and foetal development, whereas IGF-1 is more relevant for cartilage repair.

In adults, IGF-1 and IGF1R are expressed by chondrocytes, osteoblasts and osteoclasts. IGF-1 is considered an essential mediator of cartilage homeostasis through its capacity to stimulate proteoglycan synthesis and to promote chondrocyte survival and proliferation. IGF-1 was also able to induce migration of chondrocytes and, moreover, the combined use of chondrocytes and IGF-1 seemed to improve the overall consistency of the repair tissue. (Vinatier *et* al., 2009)

However, the ability of chondrocytes to respond to IGF-I decreases with age and in OA. Evidence suggests an uncoupling of IGF-I responsiveness in OA, indicating that, in OA cartilage IGF-I is able to robustly stimulate proteoglycan synthesis, but it is unable to modulate proteoglycan catabolism. (Martin *et* al., 1997)

1.7.7   Hypoxia inducible factor (HIF)

HIF is a heterodimer that consists of the subunit HIF-1α or -2α and the aryl hydrogen receptor nuclear translocator (ARNT) subunit, also known as HIF-1β.

Whereas HIF-1β is stable in normoxic conditions, HIF- 1α and -2α are unstable and are rapidly degraded through the ubiquitin proteasome pathway. Under hypoxic conditions, HIF-1α and -2α are stabilized and translocate from the cytoplasm to the nucleus, where they heterodimerize with ARNT to bind to the hypoxic responsive element (HRE), thereby initiating the transcription of hypoxia-specific genes. HIF-1α has been shown to be essential for growth arrest and survival of chondrocytes.

Hypoxia has also been shown to increase the synthesis of ECM proteins in cultured chondrocytes in vitro. These data suggest that low oxygen tension is a key regulatory factor of proliferation, differentiation and activity of chondrogenic cells. Interestingly, hypoxia has also recently been suggested to inhibit the expression of collagen type X, which is the major marker of chondrocyte hypertrophy. (Vinatier *et* al., 2009)

Furthermore, HIF-1α contributes to the maintenance of ECM homeostasis, inducing the

gene expression of two main matrix components: collagen II and aggrecan. (Duval *et al.*, 2009)

An increasing transcription of HIF-1α in OA cartilage compared to normal samples was shown, particularly in the late-stage of the disease. Consistent with this evidence, subsequent studies reported a growing number of HIF-1α-positive chondrocytes during OA progression and a higher expression of HIF-1α mRNA in degenerated cartilage compared to uninjured cartilage.

In addition to hypoxic conditions, HIF-1α expression can be up-regulated by other factors, including inflammatory cytokines (IL-1 and TNF-β), reactive oxygen species and mechanical loading, which are all recognized as key players in cartilage damage. (Yudoh *et* al.,2005)

Since HIF-1α has a pivotal role in supporting chondrocyte survival and cartilage homeostasis, these characterize HIF-1α as a key factor for chondrocyte survival promoting compensatory mechanisms in response to catabolic modifications of OA cartilage.

2. <u>Therapeutic treatments for OA</u>

Treatments aim at educating the patient about OA, reducing pain, optimizing and maintaining physical function, and preventing or retarding progression of adverse structural damage affecting the joint tissues (cartilage, bone, ligament, muscle). Actually available treatments include a wide range of non-pharmacologic, pharmacologic and surgical modalities.

The basic premise for the management of patients with symptomatic OA is that it involves a combination of both non-pharmacologic and pharmacologic modalities that are individualized to the patient's value and preferences. Firstly it will be discussed the non-pharmacologic modalities that serve as the basis for the patient's treatment and then the pharmacologic modalities that can be added as necessary for additional symptom control, in some cases require the surgery. (Gossec *et* al., 2007)

In addition to non-surgical treatments (e.g. physiotherapy, diet rich in vitamin D and supportive sport, like swimming), there are several medicinal and homeopathic products on the market, which promise pain relief and a decrease in symptoms. However, researchers have a keen interest in investigating new treatments to cure OA of the knee. (Uth and Trifonov, 2014)

Currently available pharmacological therapies target palliation of pain and include analgesics (e.g., acetaminophen, cyclooxygenase-2-specific inhibitors, non-selective non-steroidal anti-inflammatory drugs, tramadol, opioids), intra-articular therapies (glucocorticoids and/or hyaluronic acid, HA), and topical treatments (i.e. capsaicin, methylsalicylate). (Moreland, 2003)

Treatments are individual for each patient and they are dependent by severity of pain and stiffness of affected joints and by specific response to the treatment. It is important to work with a healthcare provider to create an effective and acceptable plan in the long term for living with arthritis.

2.1 <u>Non-pharmacological therapy of osteoarthritis</u>

Non-pharmacological treatments can substantially improve OA symptoms, and they are usually the first treatments recommended.

They include:

- **Rest:** OA symptoms are typically worsened by activity and are improved with rest. However, a complete lack of activity can lead to a loss of muscle and joint stiffness. If arthritis causes significant pain and inflammation, healthcare provider may recommend rest for 12 to 24 hours, followed by a return to usual activities. It would seem sensible if something hurts to rest it. This may only be true in acute situations and may not hold for chronic conditions.

  Muscle loss is a feature of both rheumatoid arthritis and OA, and so pain is not an indicator of musculoskeletal damage. (Hochberg *et al.,* 2012)

- **Weight loss**: Obesity is strongly linked to the development of arthritis of the knee. Weight loss, even modest weight loss, appears to lower this risk. It is not known if weight loss slows the worsening of arthritis in joints that are already affected. However, weight loss may reduce joint pain in weight bearing joints, such as the hips and knees. (Bliddal *et* al., 2011)

  Physical therapy and exercise programs: Physical therapy and exercise improve flexibility and strengthen the muscles surrounding the joints. People who exercise regularly despite their arthritis will typically have less pain and better function than those who are inactive. Canes, walkers, electric-powered seat lifts, raised toilet seats, and tub and shower bars can reduce the stress on joints and can make it easier to perform daily tasks. A physical therapist may suggest these and other assistive devices, depending upon the severity and location of arthritis. (Fransen *et al*., 2001)

- **Vitamins**: Studies have linked certain vitamins to joint health, but the role of vitamins in arthritis treatment is uncertain. OA is less likely to worsen in people who have a high dietary intake of vitamin C (ascorbic acid) and a high dietary intake and high blood levels of vitamin D. However, it is unknown if supplementation with these vitamins has the same effects or if high dietary intakes of vitamins can prevent the onset of OA.

- **Thermoterapy**: Thermotherapy has for many years been advocated as a useful adjunct to pharmacological therapies. Ice is used for acute injuries and warmth is

used for sprains and strains. It seems appropriate to use hot and cold packs in osteoarthritis knee.

Applying heat and cold to arthritic joints can help to control arthritis symptoms such as pain and stiffness.

1) *Heat therapy*: Heat relieves pain and stiffness in arthritic joints. Heat can be applied to the joints with hot packs, hot water bottles, heating pads, or electrically heated mittens. It is important to avoid burning the skin with heat therapy. To avoid burns, hot water bottles should be filled with warm, not boiling, water. Heating pads should be set on a timer and used for no more than 20 minutes at a time. The heating pad can be reapplied after 20 minutes of no use.

2) *Cold therapy*: Cold relieves pain in arthritic joints and reduces muscle spasms. Cold can be applied for short periods using ice packs or coolant sprays. People with certain medical conditions, such as the Raynaud phenomenon, should not use cold therapy. (Brosseau *et al.*, 2003)

- **Transcutaneous electrical nerve stimulation (TENS)**: A TENS unit delivers a mild electrical current to the skin, stimulating nerve fibers in the skin that may interfere with the transmission of pain signals from the arthritic joint. The use of TENS as an arthritis treatment is controversial. Some studies have found that those who use TENS for arthritis of the knee have reduced knee pain, a greater ability to bend the knee, and a reduced duration of morning stiffness. However, another study found that TENS was no more effective for relieving pain than the drug naproxen (Aleve, Anaprox) or a placebo. (Hochberg *et al.,* 2012)

- **Dietary supplements** — Glucosamine and chondroitin are dietary supplements that have received a lot of attention for their potential benefit in reducing pain and in slowing the progression of arthritis.

  1) *Glucosamine*: Glucosamine was no more effective in relieving arthritis pain or in improving function than placebo in a well-designed, controlled trial; it is possible that other formulations may be effective. Glucosamine does not appear to slow the worsening of arthritis over the long term. There are few side effects of glucosamine; it should not be used by patients who are allergic to shellfish.

  2) *Chondroitin*: Chondroitin used alone appears to provide little benefit for people with OA. There are no significant side effects of chondroitin.

The combination of glucosamine and chondroitin sulfate has not proven to be better than placebo for pain relief or for functional improvement in patients with OA of the knee. (Clegg *et al.*, 2006)

- **Traditional Chinese medicine**: Several components of traditional Chinese medicine, including herbs and acupuncture, may help control the arthritis symptoms in some people, although the benefits of these therapies have not been confirmed in large, well-designed clinical studies. Reumalex, willow bark, stinging nettle, Articulin-F, devil's claw, extract of soybean and avocado unsaponifiables (ASU), and Phytodolor may improve arthritis pain, while other herbs and combinations such as Eazmov, Gitadyl, or ginger extract are probably ineffective. (Vickers *et* al., 2012)

2.2 Pharmachological treatments of osteoarthritis

An appropriate pharmacological treatment forms is the main key for treating osteoarthritis when non-pharmacological therapy on its own is insufficient. The use of such analgesia may be use to cure different kind of pain, including night pain or exercise-associated pain. Oral analgesics, especially paracetamol, have been used for many years, with increasing use of opioid analgesics in recent years, partly fuelled by fears over the safety of NSAIDs. (NICE, 2014)

Pharmacologic modalities could potentially be divided into topical, intra-articular and oral (systemic) agents. Furthermore, those that relieve symptoms may have a rapid or slow onset of effect.

- **Topical agents:** Some people experience relief of arthritis pain when they apply creams containing capsaicin, the active substance in hot chili peppers. Capsaicin depletes a pain-causing substance in nerve endings and lessens the arthritis pain by about 30 percent in some people. Forty percent of people experience side effects when using capsaicin cream, including burning, stinging, and redness of the skin and especially the eye. (Vaile and Davis, 1998) There are a variety of non-steroidal anti-inflammatory drug (NSAID) preparation that are available for topical application. Analgesics relieve pain but do not have any effect on inflammation. These drugs are often recommended when arthritis pain does not respond to non-pharmacologic measures. Drugs in this class include acetaminophen and opioid (narcotic) analgesics. Acetaminophen (Tylenol and others) can relieve mild to moderate arthritis pain. To avoid the serious but rare side effects of kidney and/or liver damage due to acetaminophen, it is important to follow dosing instructions and to avoid drinking excessive amounts of alcohol. The sudden pain, severe arthritis exacerbations may require treatment with narcotic analgesics such as codeine. Narcotics should be taken for only short periods of time because they can be addictive.

- **Intra-articular therapy:** Two types of injections are used for people with arthritis pain: corticosteroid injections or hyaluronic acid injections.

1. *Corticosteroid injections*: Corticosteroid can suppress inflammation and can relieve arthritis symptoms when injected into arthritic joints. corticosteroid injections may be recommended for people who have OA confined to a few joints and who still have pain despite the use of NSAIDs. Corticosteroid injections may also be recommended for people with OA who cannot take NSAIDs. Joint injections have few side effects, but some people experience a brief flare of arthritis symptoms after an injection. There is also a small risk of joint infection. Glucocorticoids may damage certain joints when injected frequently. Therefore, clinicians recommend no more than three to four injections per year for each particular weight bearing joint such as a knee. Corticosteroids have not only anti-inflammatory effects but also immunosuppressive effect. The presence of an effusion is not in itself an indication for corticosteroid injection, unless there is significant restriction of function associated with the swelling. Rather, the indication should be based on severity of pain and disability. (Jevsevar, 2013)

2. *Hyaluronic acid (HA) injections* — Normal joint fluid contains a large amount of hyaluronic acid, which allows the joint fluid to be slippery. Synthetic HA may be injected into the knee to treat arthritis. After the injection, pain relief may last for several months. HA is generally injected in the knee, but their use in other joints is being studied. Joint inflammation can occasionally occur after this type of injection and as with steroids, it is possible a small risk of infection. HA injections are generally reserved for people with OA who cannot take NSAIDs or who do not achieve adequate pain relief with them. People awaiting joint surgery may benefit from these injections. HA is administered as 3-5 intra-articular weekly injections and is generally well tolerated, with a low incidence of local adverse events (from 0% to 13% of patients) that was similar to that found with placebo.

Because the residence time of exogenously administered HA in the joint is relatively short (approximately less than 26 hours), a variety of chemical modifications of the molecule, such as coupling or cross-linking, have been tested

as a means of increasing both residence time and viscoelastic properties. (Borzacchiello *et al*., 2010; Larsen *et al*., 2008)

HA is a large, linear glycosaminoglycan and is a major non-structural component of both the synovial and cartilage extracellular matrix. It is also found in synovial fluid and is produced by the lining layer cells of the joint. HA is removed from the joint via the lymphatic circulation and degraded by hepatic endothelial cells. Its key functions in the joint are to confer viscoelasticity, lubrication and help maintain tissue hydration and protein homeostasis by preventing large fluid movements and by acting as an osmotic buffer. Synthetic HA was isolated from roosters' comb and umbilical cord tissue and developed for clinical use in ophthalmic surgery and arthritis in the 1960s. The beneficial effects in ophthalmic surgery were followed by the use of HA in osteoarthritis: the rationale was to replace the properties lost by reduced HA production and quality as occurs in osteoarthritis joints, a concept known as viscosupplementation. Commercial preparations of HA have the same structure as endogenous HA although cross-linked HA molecules (known as hylans) were later engineered by linking HA molecules in order to obtain greater elastoviscosity and intra-articular dwell-time. However, the mechanism by which HA exerts its therapeutic effect, if any, is currently unknown, and evidence for restoration of rheological properties is lacking. It has been suggested that two stages might be involved; an initial biomechanical stage followed by a physiological stage. It is suggested that biomechanical mechanisms initially come into effect when the synovial fluid in the osteoarthritic joint is replaced by the higher molecular weight exogenous HA. Clinical studies report that exogenous HA contribute in restoring the elastoviscosity, and the lubricating and shock absorbing abilities, of synovial fluid. It is noted that physiological mechanisms may account for the clinical benefits of intra-articular administration of HA that persist beyond the residence time of HA, although evidence has largely been obtained from preclinical studies. Given the relatively short intra-articular residency (hours to days), any hypothesis for its mechanism of action must account for the sometime reported long-duration of clinical efficacy (months). (Lohmander *et* al., 1996; Altman and Moskowitz, 1998)

Nowadays the development of pharmacological treatments with the potential for structure-modifying activity in OA joint treatment has become a major focus in the field of OA research. Such compounds retard or stabilize the progression of

established OA by altering the underlying pathological processes. There is a growing body of preclinical and clinical data, which suggests that intra-articular injection of hyaluronan (HA) has a disease-modifying effect, in addition to its proven efficacy and safety in treating the OA patients, aimed at relieving pain and regaining function. (Grishko *et al.*, 2009; Elron-Gross *et* al., 2008)

- **Oral pharmacological agents:** This group of agents includes analgesics such as non-opioid analgesics (e.g. paracetamol) and opioid analgesics, or anti-inflammatory agents such as NSAIDs.

1. *Non-opioid analgesics:* for many patients with OA, relief of mild to moderate joint pain can be achieved with paracetamol. However, in several trials, NSAIDs have been more efficacious, particularly in those with moderate to severe symptoms from their OA. (Altman and Moskowitz 1998)

2. *Opioid analgesics:* there is limited published clinical research, weak opioid analgesics have been commonly administered for OA. Stronger narcotics or narcotic derivatives are useful in selected patients with OA. Narcotics are used most often as rescue medications for severe pain

3. *Non- steroidal anti-inflammatory drugs*: NSAIDs relieve pain and reduce inflammation. Many of the nonprescription products that are available for treating arthritis pain are NSAIDs. These drugs are often recommended before analgesics for people who have osteoarthritis and evidence of inflammation. They are also recommended for some people with non-inflammatory OA who do not get adequate pain relief with simple analgesics. Non-steroidal anti-inflammatory drugs (NSAIDs) have been available for many years and are thought to work by reducing the production of pro-inflammatory and pain-related prostaglandins. The discovery of different cyclooxygenase (COX) enzymes with different physiological actions brought with it the concept that differential blockade of COX-1 (important in normal regulation of the gastro-intestinal (GI) mucosa) and COX-2 (up-regulated at sites of inflammation amongst other functions and thought responsible for pro-inflammatory mediator production) may provide effective analgesic/anti-inflammatory actions without the common GI complications of traditional NSAIDs. These GI complications are well known to clinicians and include a spectrum of problems from

dyspepsia and ulcers to life-threatening ulcer perforations and bleeds. However the blocking of COX-2 always carried the potential for a pro-thrombotic effect, by changing the balance of pro- and anti-thrombotic mediators. (NICE, 2014; Camu *et* al., 2002)

2.3 <u>Differents agents</u>

Although research continues into symptom-modifying medications for OA, there is a new emphasis on the development of structure modifying agents. Structure modifying medications are intended to retard, prevent or reverse the progression of OA.

Several groups of agents are under investigation, mostly directed at cartilage repair:
- Growth factors and cytokine
- Sulphated and non-sulfated sugars
- Hormones and other steroids
- Enzyme inhibitors
- Chondrocytes/stem cell transplantation
- Hyaluronan with structure-modifying

2.4 <u>Surgery</u>

Surgery is usually reserved for severe arthritis that significantly limits your activities and that does not respond to other arthritis treatments.

Furthermore, those who undergo surgery should be in the best possible physical condition and should be prepared for rehabilitation after surgery.

Only 5% of OA patients need surgical treatment when conservative treatment displays no satisfactory effect. There are four categories of surgical procedures: osteotomy, arthroscopy, arthrodesis, and arthroplasty.

The purpose of the osteotomy is to transfer the load bearing from the pathologic to the normal compartments of the knee. Thirteen studies involving over 693 people indicated that valgus high tibial osteotomy is effective in improving knee function and relief of pain. However, it is uncertain which treatment, osteotomies or conservative treatment is more effective. Furthermore, a successful result of the osteotomy (about 60.3%) depends on

proper patient selection, stage of osteoarthritis, and achievement and maintenance of adequate operative correction.

Arthroscopy is an alternative procedure to osteotomy. It is not only useful for the treatment of the same symptoms, but also for diagnosis of the disease. Although arthroscopic methods are widely used, they are not suitable for patients who have displayed OA symptoms for more than 2 years, or who display tibial osteophytes and joint space narrowing of less than 5 mm. Arthroscopy is effective only temporarily in reducing the pain of mild to moderate hip OA.

Arthrodesis is an efficient procedure for OA of the hands, feet, ankles, and spine, but usually not for the hip and knees.

Arthroplasty refers to the insertion of an artificial joint in order to restore the integrity and the function of the joint withered by OA. Joints commonly deteriorate after more than ten years, depending on the composition. The perioperative morbidity of unicompartmental knee arthroplasty would be less than total knee arthroplasty; furthermore, this arthroplasty has been reported to be used in very elderly patients (79-94 years) with tricompartment OA. Therefore, age is not a limiting factor for this surgical treatment.

3. <u>Hyaluronic acid (hyaluronan)</u>

Hyaluronic acid, also called hyaluronan (HA) is a carbohydrate, more specifically a mucopolysaccharide, occurring naturally in all living organisms. It can be several thousands of sugars (carbohydrates) long. When not bound to other molecules, it binds to water giving it a stiff viscous quality similar to "Jello". HA is found primarily in the extracellular matrix and pericellular matrix, but has also been shown to occur intracellularly. The biological functions of HA include maintenance of the elastoviscosity of liquid connective tissues such as joint synovial and eye vitreous fluid, control of tissue hydration and water transport, supramolecular assembly of proteoglycans in the extracellular matrix, and numerous receptor-mediated roles in cell detachment, mitosis, migration, tumor development and metastasis, and inflammation. (Toole and Hascall 2002; Turley *et* al., 2002)

The unique viscoelastic nature of HA along with its biocompatibility and non-immunogenicity has led to its use in a number of clinical applications, including the supplementation of joint fluid in arthritis (Medina *et al*., 2006), as a surgical aid in eye surgery, and to facilitate the healing and regeneration of surgical wounds. (Necas *et* al., 2008)

In addition the high exclusion properties of HA molecules restrict the entry of plasma proteins into the aqueous phase of synovial fluid. This exclusion effect is dependent on the molecular weight (MW) of the proteins.

By contrast HA facilitates the transport of water and smalls solutes through synovial fluid to articurlar cartilage from capillaries in the synovium and reduces fluid loss as intra-articular (IA) pressure is raised during joint flexion. These properties of HA are important for the nutrition of articular cartilage as well as for the elimination of metabolites and noxious substance from the joint cavity. (Gosh and Guidolin, 2002)

3.1 <u>Biochemistry of hyaluronan</u>

HA is a linear polysaccharide composed of repeating disaccharide units, 1,4-glucuronic acid (GlcUA) and 1,3-N-acetylglucosamine (GlcNAC). HA belongs to the group of glycosaminoglycans, but unlike chondroitin sulfate or keratan sulfate, HA is not sulfated. HA is synthesized by bioactivity of hyaluronan synthase (HAS), which has been reported to have three isoforms (HAS1, HAS2, and HAS3) in humans. (Masuko *et* al., 2009)

HA is characterizes by an high molecular weight (Figure 5) and its relatively simple structure is conserved throughout all mammals, suggesting that HA is a biomolecule of considerable importance. (Necas *et* al., 2008)
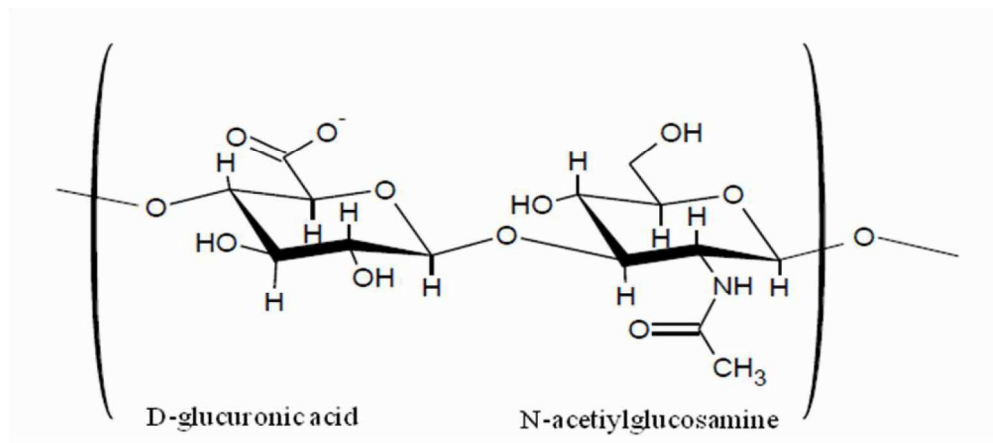

Figure 5: Chemical structure of HA

3.2 <u>HA receptors</u>

HA plays several important organizational roles in the extracellular matrix (ECM) by binding with cells and other components through specific and nonspecific interactions. Receptors are constituents of the extracellular matrix, and stabilize its integrity. Hyaluronan receptors are involved in cellular signal transduction. It is now widely accepted that HA binds to specific receptors, CD44, ICAM-1, LYVE-1 and RHAMM, that are expressed by a wide range of cells, including those implicated in the pathology of OA (e.g. inflammatory cells, synoviocytes and chondrocytes). (Banerji *et* al., 1999) The finding of specific cell receptors for HA, notably CD44, support a pharmacological mechanism of action for this GAG in OA, and because these receptors are widely distributed on the surfaces of many types of cells, it also accounts for the diversity of its effects.

CD44 is a structurally variable and multifunctional cell surface glycoprotein expressed on most cell types, including macrophages and hepatocytes, and has been implicated in many biological process. (Kang *et al*., 2013) CD44 has been reported to regulate a variety of inflammatory responses, including the induction of pro-inflammatory cytokines and the migration of macrophages and neutrophils. (Hollingsworth *et al*., 2007)

ICAM: also known as CD54 (Cluster of Differentiation 54) is a protein that in humans is encoded by the ICAM1 gene. This gene encodes a cell surface glycoprotein which is typically expressed on endothelial cells and cells of the immune system.

LYVE-1: the first identification of an HA receptor that is almost exclusively expressed on lymph vessels and is absent from blood vessels. (Banerji *et* al., 1999)

RHAMM (Receptor for HA-Mediated Mobility), has been found on cell surfaces, as well as in the cytosol and nucleus. (Leach *et al*., 2004) It has been implicated in regulating cellular responses to growth factors and plays a role in cell migration, particularly for fibroblasts and smooth cells. (Necas *et* al., 2008)

The binding of HA to CD44 and RHAMM and their various isoforms has been reported to trigger a variety of intracellular signal events, such as the protein phosphorylation cascades, cytokine release and stimulation of cell cycle proteins. (Gosh and Guidolin, 2002)

3.3 <u>Mechanism of action</u>

HA is highly hygroscopic and this property is believed to be important for modulating tissue hydration and osmotic balance. (Dechert *et al*., 2006) In addition to its function as a passive structural molecule, HA also acts as a signalling molecule by interacting with cell surface receptors and regulating cell proliferation, migration, and differentiation. (Necas *et al*., 2008)

Synovial cells, fibroblasts and chondrocytes synthesize and secrete HA into the joint, contributing to enhance viscosity and elastic nature of synovial fluid. In the osteoarthritic joint, synovial inflammation leads to increased permeability of the synovial membrane for HA. Also, the elevated synovial fluid levels of free radicals, inflammatory cytokines, and proteolytic enzymes in osteoarthritic knees impair HA function and contribute to the progression of OA. Therefore in OA, both the molecular weight and the concentration of HA are decreased. (Moreland, 2003)

The intra articular injection of HA is thought to restore normal viscoelastic properties of the pathologically altered synovial fluid, which explains why it was defined the term of the "viscosupplementation". (Balazs and Denlinger, 1993)

Moreover, several studies suggest that viscosupplements also have disease modifying effects, such as reduction of synovial inflammation, protection against cartilage erosion (Amiel *et al*., 2003), and promotion of intra-articular HA production. (Ayhan *et al*., 2014) HA possesses a number of functions that may provide some additional chondroprotective effects and may explain its longer term effects on articular cartilage.

3.3.1 <u>Condroprotective effects</u>

The physical properties of HA are important but there is evidence to suggest that HA may provide both physiochemical and pharmacological advantages. Chondrocytes express the glycoprotein CD44 on their cell surface. This has the capacity to function as a HA receptor and so may be involved in biochemical interactions with chondrocytes. The effect of a HA injection may be mediated via CD44 interactions. (Necas *et* al., 2008)

Therefore, HA has important chondroprotective effects, indeed covers the articular surface and exerts mechanical protection on synoviocytes of the articular cartilage, preventing cell damage by mechanical stress. In articular cartilage HA allows the organization of proteoglycans in huge aggregates and act as a filter against the free diffusion of molecules through the synovial membrane. Maintenance of normal structure of HA is essential for the homeostasis of the environment and any event that could change the physiological characteristics of this polysaccharide can cause serious repercussions.

3.3.2 <u>Effects of hyaluronan on the extracellular matrix</u>

Beneficial effects on proteoglycans synthesis have also been demonstrated *in vitro* with HA. This glycosaminoglycan has been shown to increase proteoglycans synthesis in equine articular cartilage, rabbit chondrocytes, and bovine articular cartilage treated with IL-1, which has been shown to reduce proteoglycans synthesis *in vitro*. An increase in high-MW proteoglycans production was also demonstrated with HA in cells of rabbit ligament. (Ghosh *et* al., 1995)

HA also reduced the expression of IL-1β and stromelysin (MMP-3), two mediators known to play a role in cartilage degradation. (Takahashi *et al*., 1999)

A reduction in collagen gene expression induced by IL-1β in rabbit articular chondrocytes has also been suppressed by HA. In an *in vivo* model of canine OA, a reduced amount of glycosaminoglycan release was found in HA-treated joints compared with an increased release in untreated joints.

HA has also been shown to suppress cartilage damage by fibronectin fragments *in vitro* and *in vivo*. This protective effect was associated with its coating of the articular surface, suppression of fibronectin-fragment-enhanced stromelysin-1 release, increased

proteoglycan synthesis, and restoration of proteoglycans in damaged cartilage. (Moreland, 2003)

3.4 <u>Viscosupplementation</u>

HA plays a vital role in the development of cartilage, the maintenance of the sinovial fluid and the regeneration of tendons. High concentrations of HA have been found in the ECM of all adult joint tissues, including the sinovial fluid and the outer layer cartilage. (Leach *et al*., 2004)

HA has viscoelastic nature and ability to form highly hydrated matrices, and acts in the joint as a lubricant and shock absorber. The pathologic changes of synovial fluid hyaluronic acid, with its decreased molecular weight and concentration, led to the concept of viscosupplementation.

The concept of viscosupplementation with hyaluronic acid, namely the intra-articular injection of the same for the treatment of osteoarthritis, was introduced for the first time by Balazs and Denlinger 70s (Balazs and Denlinger, 1993) and in 1997 that practice has been approved by the Food and Drug Administration (FDA).

It is a novel, safe, and possibly effective form of local treatment for osteoarthritis. (Uthman *et al*., 2003) Viscosupplementation with HA products helps to improve the physiological environment in an osteoarthritic joint by supplementing the shock absorption and lubrication properties of osteoarthritic synovial fluid. The rationale for using viscosupplementation is to restore the protective viscoelasticity of synovial HA, decrease pain, and improve mobility. The immediate benefits of viscosupplementation are the relief of pain. Longer-term benefits are believed to include the return of joint mobility by the restoration of trans-synovial flow and, ultimately, the metabolic and rheologic homeostasis of the joint. (Wang *et* al., 2004)

Viscosupplementation came into clinical use in Japan and Italy in 1987, in Canada in 1992, in Europe in 1995, and in the United States in 1997. (Necas *et* al., 2008)

The mechanism of action of intra-articular injection of hyaluronic acid is greatly influenced by the molecular weight, in fact low molecular weight HA prevails the biological activity, while high molecular weight HA is better in performing rheological and analgesic activities. (Maneiro *et* al., 2004)

Currently in the market there are several formulations of hyaluronic acid injectable approved for the treatment of osteoarthritis in the knee, These preparations are different in

origin, production methods, molecular weight, half-life in the synovial fluid, rheological properties, pharmacodynamics and costs.

3.5 HYADD®4-G

Intra-articular (IA) sodium hyaluronate has proven efficacious and well tolerated for the treatment of pain associated with knee OA, but optimal molecular weight of HA and dosing regimen is still not known. Hyaluronic Acid alone is not suitable for a prolonged release of a drug or a protein in the joint, due to its biodegradability and to the immediate dilution effect with synovial fluid.

It has been synthesized a new product: a hyaluronan amide derivative of 500–730 kDa (HYADD®4-G), that it has been produced via amidation of the polymer carboxyl groups by long chain alkyl amine to enhance the rheological properties and longer residence time in the joint cavity compared with native HA. (Finelli *et al.*, 2009)

HYADD®4-G is a physical hydrogels based on HA modification, of interest to the biomedical industry.

The high molecular weight of HA together with its high degree of hydration allows multiple chains to organize themselves to form a dynamic structure of reticular type (Figure 6) with two main functions in the physiological concentration (3 mg / ml):

1) Create molecular scaffold with internal architecture to maintain the shape and tone of the tissue,
2) Function as a filter against the free diffusion in the tissue of particular substances, bacteria, infectious agents.
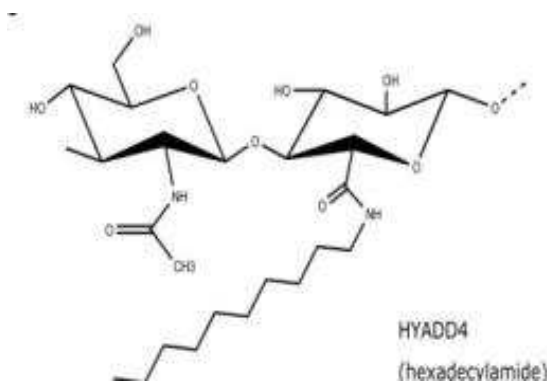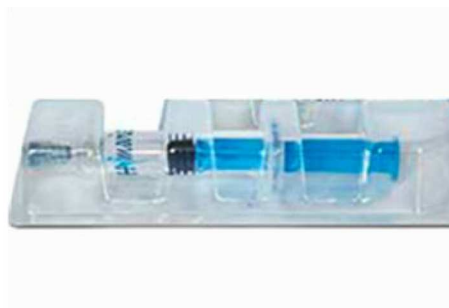

Figure 6: Biochemical structure of HYADD®4-G

The chemical modification of HA has been used in medicine as a main component of polymer scaffolds for tissue engineering and as a material used for drug delivery.

As shown in Figure 7, the hydrogel HYADD®4-G is a physical hydrogel clear and transparent, free of macroscopic in homogeneity.

Figure 7: Syringe of HYADD®4-G

In this hydrogel modification consists in the introduction of hydrophobic aliphatic chains C16 glucuronica unit of the polysaccharide with a low degree of substitution (~ 2 mol% replacement / mol of repeating unit), produced on an experimental basis by the *Fidia Farmaceutici S.p.A.*

The result of this substitution is a physical hydrogel injection stable even at very low polymer concentrations (0.3-0.8% w / w). In these systems the amount of saline solution is 99.2-99.7% of the total mass. The structural and dynamic characterization of this system showed that the formation of the gel is due to hydrophobic interactions between the side chains on the main polymer chain aliphatic introduced, despite the low degree of substitution. (Finelli *et al.*, 2009)

Hydrogels are a class of compounds that are used in more applications in the food industry or in the pharmaceutical and biomedical field. Physical gels of polysaccharides are used in large scale as food additives or as thickeners. Also the chemical gels have many applications, particularly in the biomedical and pharmaceutical fields, with the synthesis of biocompatible materials and can respond selectively and reversibly to external stimuli (such as changes in pH, temperature, ionic strength) by varying their properties.

This HYADD®4-G biopolymer has been shown to exhibit on OA chondrocytes and synoviocytes a reduction in inflammatory and matrix degradative factors. (Brun *et* al., 2012; Smith *et al.*, 2013)

Moreover, another study performed in an ovine model of OA showed that HA treatment decreased synovial vascularity and fibrosis as this HYADD®4-G biopolymer, which also significantly increased synthesis of high MW HA by synovial fibroblasts. (Smith *et* al., 2008)

4. <u>Growth hormone</u>

Growth hormone (GH), commonly known as somatotropin, is the major growth stimulating hormone of the body. It is a single-chain polypeptide and produced in pulsatile manner by the anterior lobe of the pituitary gland as a 22 kDa molecule. It is a polypeptide chain containing about 191 amino acid residues, and is responsible for a number of anabolic processes (Figure 8). (Denko and Malemud 2005)

In children as well as adults, GH is secreted in short-term episodic and pulsatile patterns, suggesting that the physiological effects of GH are tightly regulated by its release from the pituitary gland. Moreover, episodic GH release is affected by gender, age and sleep. (Cornford *et al.*, 2012)

Figure 8: GH amino acid sequence with disulfide bridge.

It is found that human growth hormone (hGH) has a three-dimensional structure containing a four-helical bundle with unusual linkage (Figure 9); the helices run up-up-down-down instead of the more common up-down-up-down topology. α-Helix 1 and 4 (the NH2- and COOH-terminal helices) are also longer than the other two helices. Helices 1-2 and 3-4 are connected by a long crossover connection whilst helices 2 and 3 are connected by a much

shorter segment. At the NH2-terminus, eight amino acid residues extend away from the molecule whereas the COOH-terminus is linked to helix 4 by a disulfide bond. A second disulfide bond can be found between the first crossover connection and helix 4. Like most other peptides, the core of the 4-helical bundle consists of more hydrophobic residues. (Cunningham and Wells, 1991)
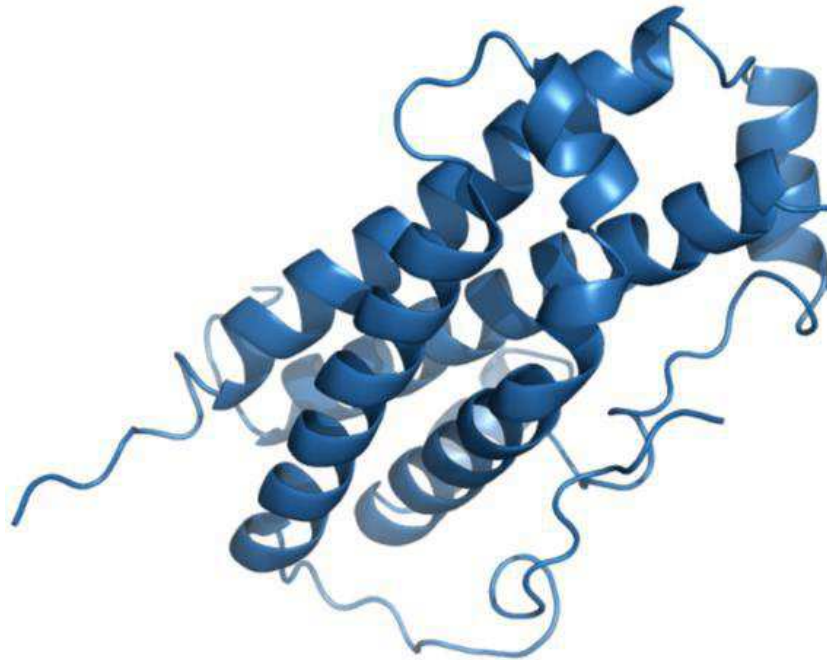
*Figure 9: Three-Dimensional structure of human Growth Hormone*

4.1 <u>Physiologic Effects of Growth Hormone</u>

Growth hormone is a major participant in control of several complex physiologic processes, including growth and metabolism. Growth hormone is also of considerable interest as a drug used in both humans and animals.

A critical concept in understanding growth hormone activity is that it has two distinct types of effects (Figure 10):

- **Direct effects** are the result of growth hormone binding its receptor on target cells.
- **Indirect effects** are mediated primarily by an insulin-like growth factor 1 (IGF-1), a factor that is secreted from the liver and other tissues in response to growth hormone. A majority of the growth promoting effects of growth hormone is actually due to IGF-1 acting on its target cells.
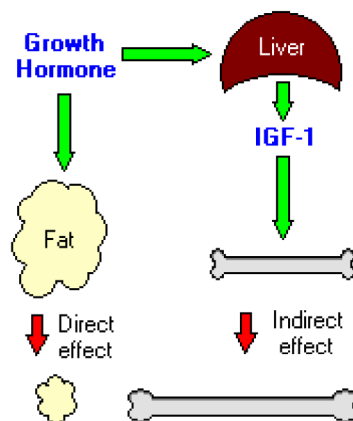
Figure 10: Schematic effects of growth hormone

Keeping this distinction in mind, we can discuss two major roles of growth hormone and of IGF-1 in physiology.

• *Effects on Growth*

Growth is a very complex process, and requires the coordinated action of different hormones. The major role of growth hormone in stimulating body growth is to stimulate the liver and other tissues to secrete IGF-1.

IGF-1 enhances cell proliferation, maturation and differentiation of many tissues including bone and skeletal muscle, for example, IGF-I stimulates proliferation of chondrocytes (cartilage cells), resulting in bone growth. Growth hormone does seem to have a direct effect on bone growth in stimulating differentiation of chondrocytes.

IGF-I also appears to be the key player in muscle growth. It stimulates both the differentiation and proliferation of myoblasts. It also stimulates amino acid uptake and protein synthesis in muscle and other tissues. (Alvarez-Garcia *et* al., 2012)

• *Metabolic Effects*

Growth hormone has important effects on protein, lipid and carbohydrate metabolism. These effects can be directly mediated by growth hormone or it can exert indirect effect through the critical mediator IGF-1.

- **Protein metabolism:** In general, growth hormone stimulates protein anabolism in many tissues. This effect reflects increased amino acid uptake, increased protein synthesis and decreased oxidation of proteins. (Chikani and Ho 2013)

- **Fat metabolism:** Growth hormone enhances the utilization of fat by stimulating triglyceride breakdown and oxidation in adipocytes.

- **Carbohydrate metabolism:** Growth hormone is one of a battery of hormones that serves to maintain blood glucose within a normal range. Growth hormone is often said to have anti-insulin activity, because it supresses the abilities of insulin to stimulate uptake of glucose in peripheral tissues and enhance glucose synthesis in the liver. Somewhat paradoxically, administration of growth hormone stimulates insulin secretion, leading to hyperinsulinemia. (Vijayakumar, *et al.,* 2011)

4.2 Secretion and regulation of Growth Hormone

Production of growth hormone is modulated by many factors, including stress, exercise, nutrition, sleep and growth hormone itself. However, its primary controllers are two hypothalamic hormones and one hormone from the stomach:

- **Growth hormone-releasing hormone** (GHRH) is a hypothalamic peptide that stimulates both the synthesis and secretion of growth hormone.
- **Somatostatin** (SS) is a peptide produced by several tissues in the body, including the hypothalamus. Somatostatin inhibits growth hormone release in response to GHRH and to other stimulatory factors such as low blood glucose concentration.
- **Ghrelin** is a peptide hormone secreted from the stomach. Ghrelin binds to receptors on somatotrophs and potently stimulates secretion of growth hormone.

Secretion occurs in a pulsatile manner under both positive and negative control of the hypothalamus and under the influence of these important hypothalamic hormones together with peptides such as ghrelin through positive and negative feedback mechanisms, it is also an age-dependent secretion, in fact decrease with age. (Denko and Malemud, 2005)

Secretion of hGH is highest within one hour after the onset of sleep. Other conditions during which hGH release is amplified are physical stress, exercise and fasting, while an excess of glucose and lipids inhibits hGH release in a healthy individual. (Ritsche *et* al., 2014)

Basal GH levels are low, usually less than 5 ng/mL for most of the day and night In children and young adults, the most intense period of growth hormone release is shortly after the onset of deep sleep (Figure 11). **(**Takahashi *et* al., 1968)
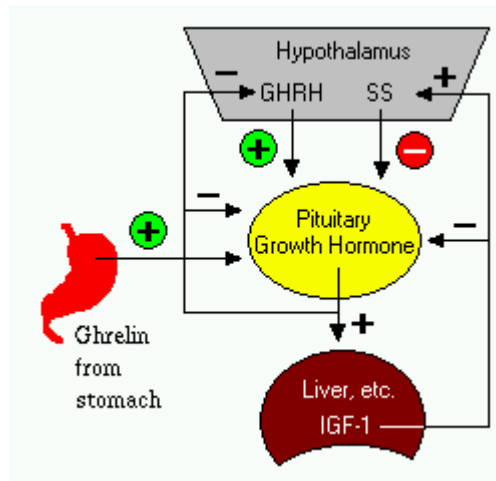
Figure 11:Schematic presentation of growth hormone regulation

4.3 The growth hormone receptor and signal transduction

GH exerts its actions on tissues through the GH receptor (GHR). GHR is a key regulator of postnatal growth and has important actions on metabolism, reproductive, gastrointestinal, cardiovascular, hepatobiliary, and renal systems, so it is required for normal human growth and development. (Tsukazaki *et* al., 1994)

The receptor is a member of the cytokine receptor superfamily that are found on various cell types generally involved with cell growth, proliferation, apoptosis and differentiation. (Rico-Bautista *et al*., 2005)

The GH receptor is a 620 amino-acid, single chain glycoprotein with a single transmembrane domain and an extracellular domain involved in GH signaling. This extracellular part, known as the human growth hormone binding protein (GHBP) of which there are two classes, also occurs separately in the blood circulation in nanomolar concentrations, where they will bind with a substantial part of plasma hGH. (Kato *et al*., 2002)

GHR has a half-life of around 1 hour and is continuously degraded even the absence of GH.

The majority of GHR resided in the endoplasmatic reticulum and cell surface, however there was pronounced nuclear localization in many cells, and was demonstrated that nuclear GHR is a common feature of tissue and in cells high proliferative status. (Conway-Campbell *et al*., 2008)

Upon binding its receptor, GH can activate several different signaling pathways including: mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK); Insulin receptor substrate 1 (IRS-1)/phosphatidylinositol 3-kinase (PI3K); phospholipase C/protein kinase C/Ca^{2+}. However, the majority of signaling occurs through the best characterized pathway Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling. (Frank, 2008; Gevers *et al.,* 2009)

The best characterized is the JAK-STAT5b pathway, in which GH-induced JAK2 activation phosphorilates Stat5b, which dimerizes and translocates to the nucleus, to bind response elements to regulate transcription of GH target genes. The transcription of specific genes leading to the production of other peptides like IGF-1. (Rico-Bautista *et al*., 2005)

Although GH signalling through STAT proteins is the primary signaling pathway (Figure 12).

The transcription factor STAT5 acts to induce expression of the key growth mediator (Waters and Brooks, 2012)

GH may regulate the phosphorylation of a range of STATs and this may depend on the cell type;

for example, STAT5 is activated by GH in adipocytes but not in adherent epithelial cells. (Han *et al*., 1996)

 To date, the specific STAT family member(s) involved in mediating GH signaling in chondrocytes has focused on STAT5b is, as yet, unknown. It has been shown that both GHR and STAT5 are localized to the resting, proliferating and pre-hypertrophic chondrocytes, and thus STAT5 phosphorylation in response to GH is likely to occur predominantly in these zones. (Gevers *et al.,* 2009)

Different studies suggest GH actions on chondrocytes have STAT5 independent effects. (Sims *et* al.,2000; Giustina *et* al., 2008) Interestingly, growth retardation and reduced circulating IGF-1 are observed in STAT5b null mice but not STAT5a null mice, suggesting that STAT5b may be the important isoform in GH signaling in bones, so STAT5b seem to be the crucial mediator of GH-induced IGF-1 transcription. (Herrington *et* al.,2000)

In humans, mutations of GHR or STAT5b that result in the inhibition of STAT5b signaling but maintain STAT1 and STAT3 and MAPK-ERK signaling result in severe short stature. (Kofoed *et* al.,2003)
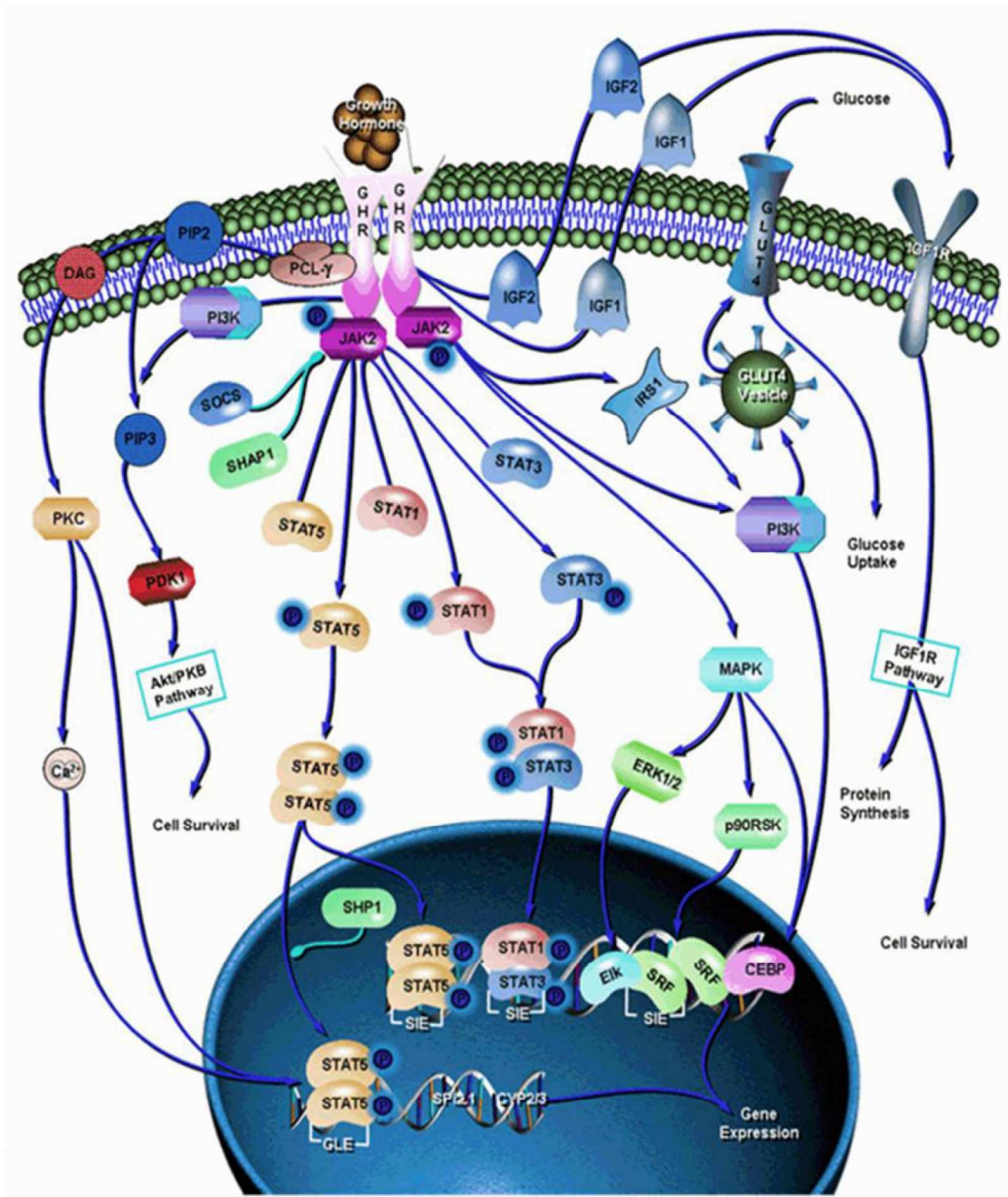
Figure 12: Growth hormone signalling pathway

4.4 Negative regulator of GH: Suppressor of Cytokine Signalling-2 (SOCS2)

Cytokine signaling is negatively controlled by a variety of proteins, including protein tyrosine phosphatases and the SOCS proteins. There have been eight SOCS molecules identified, CIS and SOCS1-7, all of which are involved in negatively regulating cytokine signaling. SOCS proteins consist of a conserved C-terminal motif named the SOCS box, a central SH2 domain and a variable N-terminal domain. They can bind through their SH2 domains to phosphorylated tyrosines within the cytokine receptor-JAK complex and inhibit JAK signaling and downstream STAT activation.

Expression of SOCS is normally stimulated by the very cytokines they inhibit, thereby creating a negative feedback loop. (Pass *et al.*, 2012)

SOCS1 and SOCS3 have been shown to inhibit signaling by IL-2, IL-3, IL-4, IL-6, GH, prolactin, erythropoietin, IFN-γ, IFN-α, oncostatin M (OSM), thymic stromal lymphopoietin (TSLP), thrombopoietin (TPO), thyrotropin and IGF-1. It can directly bind phosphorylated JAK proteins via its SH2 domain. (Greenhalgh *et al.*, 2005; Piessevaux *et al.*, 2006)

CIS and SOCS2 can inhibit signaling by IL-2, IL-3, prolactin, erythropoietin, IGF-1 and GH and inhibits STAT signaling by interacting with the cytokine receptor to block STAT binding sites. (Krebs and Hilton, 2001)

Inhibition of GH signaling by SOCS1 and 3 is complete whereas SOCS2 and CIS only cause partial inhibition and it is difficult to reconcile these actions with the observed growth of the transgenic mice. (Lee *et* al., 2008)

Clearly, other interactions are important possibly involving the other SOCS proteins.

It has been proposed that at physiological levels, SOCS2 inhibits GH signalling by blocking sites of STAT activation on the GHR, but at higher doses it inhibits signalling of other, more potent GH inhibiting SOCS (SOCS1 and 3). (Greenhalgh *et al.*, 2005)

Expression of SOCS is usually stimulated by the cytokines that they can inhibit, so that they create negative feedback loops. GH signalling is inhibited by CIS, SOCS1, SOCS2 and SOCS3, but this work in chondrocytes will focus on SOCS2.

Incidentally, these four SOCS proteins (CIS, SOCS1, 2 and 3) are the only ones that have been widely studied and it is possible other SOCS family members may also inhibit GH signalling. (Inaba *et al.*, 2005)

It has been well documented that GH signalling stimulates SOCS2 expression, furthermore, it is thought that SOCS2 production is regulated by GH signalling through STAT5b. (Vidal *et* al.,2007)

This confirms the hypothesis that SOCS2 acts in a negative feedback loop to control and regulate GH signalling under physiological conditions.

The precise mechanism by which SOCS2 regulates GH signalling is unclear.

There are still many aspects on the actions of SOCS2 that have yet to be investigated. The precise mechanism by which SOCS2 alters GH/IGF-I signalling have yet to be fully determined as are the resultant cellular events that occur at the growth plate and are responsible for normal growth. It is also unclear if SOCS2 mediates the deleterious effects of inflammatory cytokines on linear bone growth.

Moreover it has been shown that SOCS2 and CIS-1 expression are reduced in OA chondrocytes compared to healthy chondrocytes. These results indicate a potential positive feedback mechanism in OA chondrocytes, with significant implications for OA pathology. Further understanding of the role of SOCS proteins in chondrocytes will be needed to determine their therapeutic potential for OA. (de Andrès *et al.,* 2011)

4.5 GH and signalling molecules in normal and OA cartilage

A number of signalling molecules involved in chondrocyte activity, like proliferation or differentiation, in growth cartilage were also shown to play a regulative role in OA articular cartilage (Table 3).

Table 3: Different signalling factors involved in both chondrocyte differentiation processes during endochondral ossification and in osteoarthritis

Signaling factor	Effects on growth plate chondrocytes	Role in OA
Bone morphogenic proteins	Induce proliferation Inhibit hypertrophy	Stimulation of MMP-13
Fibroblast growth factors	Decrease proliferation Decrease hypertrophy Decrease matrix production	Stimulation of ADAMTS-5
TGFβ	Variable effects depending on species and concentration	Stimulation of MMP-13
Wnt/β-catenin	Positive regulator of hypertrophy and ossification	Activation of maturational genes Induction of matrix degradation Induction of MMPs and aggrecanases mediated by WISP-1
Indian hedgehog	Stimulates proliferation Inhibits hypertrophy via parathyroid hormone-related peptide Directly induces hypertrophy in vitro	Induction of ADAMTS-5 via Runx2
Retinoic acid	Positive regulator of hypertrophy and matrix mineralization	Induction of MMP-13 and aggrecanases
Growth hormone/IGF-I	Stimulate proliferation Initiate hypertrophy	Growth hormone is a beneficial factor in OA IGF-I signaling is antagonized by IGF-binding proteins
Collagen IX	Stimulates chondrocyte proliferation Essential for columnar organization of growth plate chondrocytes	Essential for tissue integrity, loss of collagen IX induces OA
β₁ Integrins	Mediate adhesion to surrounding matrix and motility Essential for proliferation	Essential for normal knee joint development Minor influence on cartilage homeostasis
Discoidin domain receptors	Regulate cell proliferation, adhesion and motility Control matrix remodeling	Induction of MMP-13 and MMP-derived type II collagen fragments
MMPs/ADAMTSs	Essential for matrix remodeling Influence bioavailability of VEGF	Key factors in matrix degradation during OA Matrix degradation is accompanied by terminal chondrocyte differentiation, positive feedback mechanism?
Sox 9	Regulate proliferation and hypertrophic differentiation	Involved in MMP-13 expression
Runx2/3	Positive regulation of chondrocyte hypertrophy Influence on angiogenesis by upregulation of VEGF	Induction of chondrocyte hypertrophy Induction of MMP-13 expression
CCAAT/enhancer binding protein beta	Inhibition of proliferation Stimulation of hypertrophy Activation of collagen X expression	Mediates cartilage destruction

Chondrocyte differentiation in growth cartilage is subject to positive and negative control elements that interact within a signalling network to regulate the rate and progression of the process. (Kronenberg, 2003)

In addition to locally produced growth factors (e.g. BMPs, FGFs, TGFβ) , systemic hormones, such as GH and IGFs, tightly regulate longitudinal bone growth. Local and systemic agents control the rate and extent of chondrocyte proliferation and differentiation at several checkpoints. This endocrine control enables longitudinal bone growth in healthy individuals and leads to increased growth rates and subsequent growth plate closure around puberty. GH and IGFs are potent stimulators of longitudinal bone growth, both factors stimulate proliferation of resting zone chondrocytes and initiate chondrocyte hypertrophy. (Hunziker *et al.*, 1994)

GH exerts its effects on the growth plate predominantly through stimulation of secretion of insulin-like growth factor 1 (IGF1), both by liver cells and by growth plate chondrocytes. (Nilsson *et* al.,2005; Pass *et* al., 2009)

Under normal conditions, the cells remain in a resting state and do not undergo proliferation or differentiation. In a diseased state, however, some articular chondrocytes lose their differentiated phenotype, and activate proliferation and hypertrophic differentiation, inducing specific markers. (Tallheden *et* al., 2005)

More information were obtained studying small animals, which better reflect human OA pathophysiology.

With regard to the GH/IGF-I axis, however, it was shown in a rat model of OA that chronic GH deficiency causes an increased severity of articular cartilage lesions of OA, although the IGF-I expression is increased. (Ekenstedt *et al.*, 2006)

IGF-1 is considered one of the factors responsible for preservation or loss of the articular cartilage matrix. It has been shown that this factor enhance chondrocyte proliferation, proteoglycan and collagen synthesis by chondrocytes in normal cartilage, also during cytokine exposure, and drives most predominating catabolic process in cartilage. (Yammani and Loeser, 2012; Claessens, 2011) IGF-1 is also involved in the differentiation and hypertrophy of chondrocytes. (Repudi *et al.*, 2013)

Anabolic IGF-I signaling is antagonized by increased occurrence of IGF-binding proteins, which then negatively regulate IGF-I signaling in chondrocytes during OA. (Martel-Pelletier *et al.*, 1998)

Cytokines, like IL-1, inhibit cartilage matrix production, stimulate catabolic activity in cartilage, and appear to play a role in the development of OA, so IGF-1can inhibit the

activity of IL-1 and increasing the activity of IGF-1 in cartilage, would be of potential benefit in preventing cartilage, degeneration and the progression to OA. (Ekenstedt *et al*., 2006)

Studies have shown that in OA there is a decrease of GH, this could also be due to a decrease of circulating hormones, besides data on GH actions on chondrocyte proliferation have so far been largely conflicting, some authors show strong proliferative effects of GH while others show little or none. (Madsen *et* al., 1983; Hutchison *et al.,* 2007) Another functions of GH is that it could also increase cartilage metabolism. (Dunn, 2002)

An another important aspect is that patients with inflammatory conditions can have variable levels of GH but generally reduced levels of circulating IGF-1, indicating GH resistance. (Wong *et al.,* 2010)

IL-6, in combination with IL-6 receptor, has been shown to inhibit articular chondrocyte differentiation via the JAK/STAT pathway decreasing IGF-1 levels. (Legendre *et al.,* 2003)

It is also possible that inflammatory cytokines act on GH signalling but to date little knowledge of the effects of inflammatory cytokines on STAT signalling are available on chondrocytes. (Dreier, 2010)

4.6 GH in OA treatment

The administration of growth hormone has been well documented to be useful in the treatment of statural impairment in Turner syndrome, intrauterine growth restriction, chronic renal failure and above all in GH deficiency or insufficiency. (Brooks *et* al., 2008)

Therapy with recombinant human GH has also been used in humans with chronic inflammatory diseases and has shown variable extent of improvement in growth and even disease. (Mauras *et al.,* 2002; Wong *et* al., 2007)

Scientists suggested that GH dysfunction was part of OA pathogenesis, but the relationship between GH levels and OA is poorly understood.

Why is it important study GH in OA?

There are different data demonstrating an important GH role in OA disease. In particular, in several animals studies it has been shown that GH:

1. Stimulates IGF-1 production and induces chondrocytes proliferation; (Tsukazaki *et al.*, 1994; Madsen *et al.,* 1983)

2. Stimulates the extracellular matrix synthesis; (Smith *et al.,* 1975)

3. Stimulates the formation of bone and cartilage; (Mankin *et al.,* 1978)

4. Modulates the proliferation and apoptosis of chondrocytes, through the stimulation of NF-kβ p65;

but it has also been demonstrated that in animals it could increase collagen type 10. (Wu *et al.,* 2011)

Several studies using GH in animals have demonstrated that hGH intra-articular administration is a potential anabolic treatment for OA. Interestingly some preclinical data have suggested that GH has potential regenerative property in the joint. However, the short half-life (16 hours) of the native protein in the joints limits its utility as a therapeutic agent, but a study has demonstrated that exogenous hGH exhibits longer half-lives in joint than native hGH. (Nemirovskiy *et al.,* 2010)

In a OA rat knees model, it appears that the exogenous GH, for not clear reason, seem to exert a more protective function than endogenous GH.

GH and IGF-1 are required in the adult rat to maintain articular cartilage integrity and a deficiency of these proteins leads to OA-like lesions. The age-related reduction in GH and IGF-1 levels in humans may therefore contribute to the development of OA. (Ekenstedt *et al.*, 2006)

The effects of GH in human fetal epiphyseal and osteoarthritic chondrocytes not induce IGF-1 production even if the basal levels of IGF-1 and GH are higher in the synovial fluid and serum of OA patients. (Fernandez-Cancio *et al.,* 2009; Dore *et al.,* 1995; Denko and Malemud, 2005; Denko *et al.,* 1996)

Private practices in USA already use hGH as an OA intra-articular therapy to regenerate articular cartilage, and a study of 66 patients with advanced OA of the knee treated with GH shown excellent results, in fact the patients did not need total knee arthroplasty. (Dunn, 2012)

moreover, a clinical X-ray study document that OA patients treated with GH shown signifying articular cartilage repair. (Hauser, 2009)

5. <u>Aim</u>

Osteoarthritis (OA) is the most common joint disease, and is characterized by progressive loss of articular cartilage, subchondral bone sclerosis, osteophyte formation, synovial membrane changes, and an increase in synovial fluid with decreased viscosity and lubrication properties. Mechanical, biochemical, and genetic factors are all involved in pathogenesis of OA.

Chondrocytes are considered active protagonists in cartilage damage: while in healthy cartilage these cells exert a strict control over the turnover of the extracellular matrix (ECM). In OA cartilage catabolic and anabolic processes are enhanced with the overall result of the loss of functional tissue and ECM degradation, so articular cartilage lesions, with their inherent limited healing potential, are hard to treat and remain a challenging problem.

In this context, it is trying to develop new drugs that allow to treat OA reducing pain, maintaining joint function and preventing disability.

Intra-articular injection into osteoarthritic joints may play an important role in the therapeutic plan.

Injection should be contemplated as complementary to the overall treatment plan-never as the only component of therapy. At this time, no targeted treatments for osteoarthritis have been developed. Therefore, preclinical and clinical research studies using other pharmacologic agents that might provide additional benefit are currently underway.

In this context, the pharmaceutical industry Fidia Farmaceutici SpA (Abano Terme, PD, Italy) has developed a new chemically modified hyaluronic acid (HYADD®4-G) and commissioned a study aimed to evaluate the effects *in vitro* on human osteoarthritic chondrocytes of the interaction between different concentration of hGH alone or in combination with HYADD®4-G, to define their effect for developing a new drug therapy for intra-articular OA treatment.

To reach this aim, the study was divided in the following sections:

1) Analysis of growth hormone receptor and of hyaluronan receptor on normal and OA chondrocytes;

2) Analysis of hGH and HYADD®4-G effect on chondrocytes: cellular migration, morphology, cell viability and metabolic activity of OA treated chondrocytes;

3) Analysis of the effect of hGH and HYADD®4-G on CD44, GHR, chondrocytic markers, release of IGF-1, FGF-2 and IL-6;

4) Analysis of factors involved in the GH mechanism of action (SOCS2).

6. <u>Materials and Methods</u>

6.1 <u>Patient characteristics</u>

Cartilage tissue was obtained from the femoral condyles of OA patients (n=8) undergoing total knee replacement with a Kellegren/Lawrence grade 3-4 (Kellgren and Lawrence, 1957) (Table 4).

Table. 4 OA patients characteristics

Patients	Age	Sex	BMI	Disease duration (year)	Oral drugs
#1	46	F	25,6	1	NSAIMs*
#2	55	F	23,2	2	
#3	56	F	23,4	10	NSAIMs*
#4	65	M	26	5	
#5	67	F	27,5	2	NSAIMs*
#6	68	F	30,8	8	NSAIMs*
#7	70	F	33,4	2	NSAIMs*
#8	78	F	24,8	4	NSAIMs*
Mean	63		27	7	
SD	±9,98		±3,60	±7,54	

*Nonsteroidal anti-inflammatory drugs

Normal cartilage biopsies (n=3) were obtained at autopsy from donating organ subjects (Table 5).

Table. 5 Normal donor characteristics

Patients	Age	Sex	BMI
#1	20	F	26
#2	28	M	23
#3	16	M	19
Mean	21,3		22,7
SD	±6,1		±3,5

The study was approved by the local ethics committee and all patients provided their informed consent.

6.2 Immunoistochemical analysis

Cartilage tissue from normal subjects and OA patients were fixed in a freshly prepared 9:1 mixture of B5 solution (mercuric-chloride containing fixative)/40% formaldehyde at room temperature for 2 hours, decalcified in 0.1M ethylenediaminetetraacetic acid (EDTA), dehydrated and embedded in paraffin.

Embedded-samples were cut at 5μm using a microtome. Slides were then deparaffined in xylene and hydrated through a series of alcohol steps (from ethanol 100° to 70°).

Slides were washed in distilled water for 5 minutes, then the sections were re-equilibrated in Tris-buffered saline (TBS: NaCl, Trizma, HCl 37%) containing 1% bovine serum albumin (BSA) for 10 minutes at room temperature.

The slides were incubated with monoclonal anti-human GHR (Santa Cruz Biothechnology, Santa Cruz, CA, USA) diluted 1:200 or monoclonal anti-human CD44 (R&D Systems, McKinley Place NE, Minneapolis, U.S.A) diluted 1:50 , in TBS containing 0.1% BSA overnight at 4°C, and then sequentially incubated with multilinker biotinilated secondary antibody (Biocare Medical, Concord, CA, USA) and alkaline phosphatase-conjugated streptavidin (Biocare Medical) for 20 minutes at room temperature.

The reactions were developed using fast red substrate (Biocare), counterstained with hematoxylin and mounted in glycerol gel. The sections were evaluated with a bright field microscope (Nikon Instruments Europe BV, Amstelveen, the Netherlands). Negative and isotype-matched controls sections were performed either by omitting the primary antibodies or using an isotype-matched control.

6.3 <u>Cellular culture</u>

6.3.1 <u>Chondrocytes isolation</u>

Chondrocytes were isolated following a standardized procedure. (Grigolo *et al.,* 2002)

Briefly, the cartilaginous fragments were weighed and then placed in culture medium Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) containing penicillin / streptomycin (100 U/ml, Gibco), gentamycin (0.05 mg/ml, Eurobio), and 10% of fetal bovine serum (FBS, Gibco), (defined as COMPLETE DMEM). The fragments were incubated in a solution with protease (13,25 U/ml, Sigma-Aldrich) to digest the cartilage matrix for 60 minutes at 37°C.

After incubation, the samples were washed twice in COMPLETE DMEM for 5 minutes each.

Subsequently, the cartilaginous fragments were further cut with a scalpel to reduce the tissue to a consistency of homogenate, and transferred to a sterile bottle containing DMEM with collagenase type II (545 U/ml, Sigma-Aldrich). The homogenate was transferred in a bottle containing a magnetic stirrer and incubated in a water-bath at 37 °C until complete digestion of cartilaginous fragments, for not longer than 2 hours.

After incubation, cells suspension was filtered sequentially using three different cells strainer (100 μm, 70 μm and 40 μm), and the effects of collagenase enzymatic action was blocked adding FBS.

These steps are performed to remove tissue residues and possible cellular aggregates in order to obtain a cell population as homogeneous as possible.

The sample was centrifuged for 7 minutes at 677 x g and the resulting pellet resuspended in an appropriate volume of COMPLETE DMEM.

The cells were then counted using the vital dye Eosin in Neubauer counting chamber. Cells isolated were seeded (passage 0, p0) in COMPLETE DMEM at the density of $1,8x10^6$, in flasks having an area of 150 cm^2 and incubated in a humidified atmosphere (37°C, 5% CO_2).

The cultured cells were checked daily and the culture medium was changed twice a week. After reaching confluence, cells were washed with a buffer solution of Hank supplemented with $NaHCO_3$ and glucose. Chondrocytes were detached from the flask using a trypsin solution at 0,05% EDTA at 37°C for 6-8 minutes.

Trypsin activity was blocked adding FBS and cells suspension was transferred into sterile tubes and centrifuged for 5 minutes at 410 x g.

The pellet was resuspended in culture medium for chondrocytes (COMPLETE DMEM), counted and use for the experiments.

6.3.2 <u>Synoviocytes isolation</u>

As control cells we isolated human synoviocytes from synovial tissue following standardized procedure. (Haubeck, 1995)

Briefly, synovial tissue was dissected from underlying connective tissue and minced fragments (approximately 1×1 mm) were transferred into a petri 3003 and cultured in 12 ml of Optimem-1 (Life Technologies Italia Srl, San Giuliano Milanese, Italy) supplemented with 15% FBS, 50 µg/ml of gentamicin (complete medium). After 7–10 days of culture, tissue fragments were removed and synovial cells outgrown from the synovial tissue were mantained in culture. Cells from the second passage were used for the experiments.

6.4 <u>Experimental culture conditions</u>

Chondrocytes were seeded in monolayers at two-cell densities (12.500 cells/cm^2 and 24.000 cells/cm^2) and cultured in COMPLETE DMEM medium at 37°C in 5% CO_2. Twenty-four hours later, the medium was removed and chondrocytes were treated with: 1) fresh medium (basal condition); 2) human Growth Hormone (hGH, kindly provided by Fidia Farmaceutici s.p.a., Abano Terme, PD, Italy) at different concentrations (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml); 3) hyaluronan amide derivative (HYADD®4-G kindly provided by FIDIA Farmaceutici, Abano Terme, PD, USA patent N.7863256) at 1 mg/ml or 4) HYADD®4-G in combination with all different concentrations of hGH.

HYADD®4-G stock solution (8 mg/ml) was diluted 1:2 (4 mg/ml) the day before and let gently rotating overnight at 37°C, to ensure a homogenous and fluid solution. This procedure is necessary due to the high viscosity of this solution.

24 hours later the solution was diluted 1:2 (2 mg/ml) and then mixed (50.50) with the different concentrations of hGH to have a final concentration of HYADD®4-G at 1 mg/ml.

Cell cultures were analyzed at different time points (24, 48, 72 hours and 7 days) for: morphology, viability, cell proliferation, metabolic activity, cell migration, CD44 and

GHR expression, molecular biology analysis, protein assays evaluation, and analysis of GH and HA pathways.

6.5 <u>Cellular morphology</u>

Cellular morphology was analyzed using toluidine blue, a metachromatic dye that stains the nucleus blue and the cytoplasm light blue/purple.

Chondrocytes were fixed, after 7 days of culture, in 10% formaldehyde for 15 minutes at room temperature. Slides were washed twice and incubated with 1% solution of toluidine blue for 10 seconds, and then washed 4 times with distilled water under agitation for 5 minutes each.

6.6 <u>Cellular viability</u>

LIVE & DEAD viability kit (Molecular Probes[TM] Invitrogen, Paisley, UK) was used to evaluate chondrocyte viability.

Chondrocytes were seeded in 24-well plates at the highest cell concentration, 48 hours and 7 days later, cells were washed three times in 1 ml of buffer solution (D-PBS: 200 mg/l KCl + 8 g/l NaCl + 1.15 g/l Na_2HPO_4 + 200 mg/l KH_2PO_4) and then incubated with 1 ml of ethidium/calcein solution (4µM EthD/ 2 µM calcein AM) at 37°C, in 5%CO_2 for 45 minutes. The cells were washed twice with D-PBS and the cell fluorescence evaluated by fluorescence microscopy.

Cells were visualised using 2 filters, one at 495 nm to evidence live cells (stained by green fluorescent calcein) and one at 510 nm to evidence dead cells (stained by red fluorescent ethidium).

6.7 Cellular metabolic activity

To evaluate cellular metabolic activity, the chondrocytes (48 x 10^3 cells) were seeded in a 24-well plates at all the conditions previously described. At each time point the chondrocytes were washed three times and incubated with 10% Alamar Blue$^®$ solution (AbD Serotec, Oxford, UK) in a humidified atmosphere at 37 °C in 5% CO_2. After 6 h, 100 µl of medium was collected and transferred to 96-well plates and absorbance read at 570 nm and 600 nm. (Figure 13)

The percentage of Alamar Blue$^®$ reduction was calculated according to the manufacturer's instructions:

$$\text{Percentage reduction of alamarBlue} = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

Where: O1 = molar extinction coefficient of oxidized alamarBlue (Blue) at 570nm (80586)

O2 = molar extinction coefficient of oxidized alamarBlue at 600nm (117216)

R1 = molar extinction coefficient of reduced alamarBlue (Red) at 570nm (155677)

R2 = molar extinction coefficient of reduced alamarBlue at 600nm (14652)

A1 = absorbance of test wells at 570nm

A2 = absorbance of test wells at 600nm

N1 = absorbance of negative control well (media plus alamarBlue but no cells) at 570nm

N2 = absorbance of negative control well (media plus alamarBlue but no cells) at 600nm
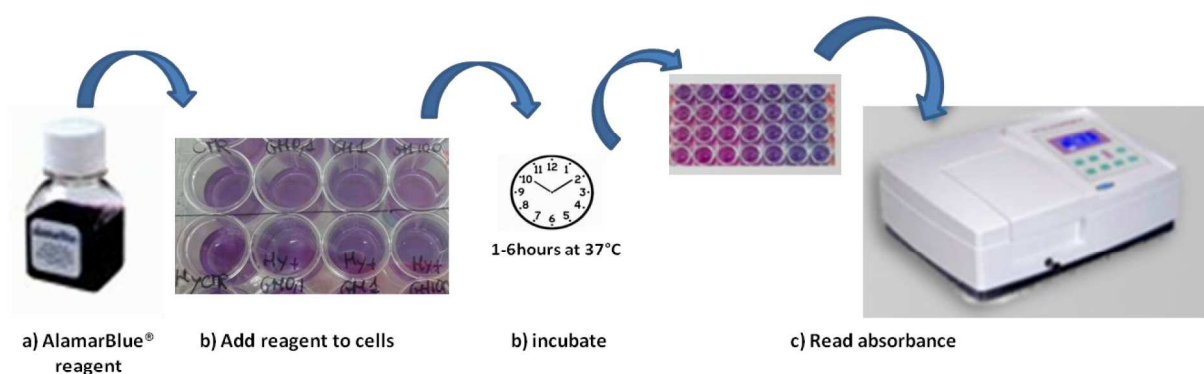
Figure 13: AlamarBlue® assay converts resazurin to resorufin

6.8 Cellular migration

To evaluate the migration of cells, chondrocytes and control synoviocytes were seeded in a 24-well plates (48 x 10^3 cells).

After 24 hours in each well was done a scratch in the center using a tip and then the cells were washed 2 times with medium.

Basal cells and cells treated with HYADD®4-G 1mg/ml were exposed to normoxia (at 37°C in 20% O_2) and in hypoxia (at37°C in 5% O_2). After 24 hours medium was eliminate, cells were fixed with 10% formaldehyde for 15 minutes at room temperature, stained with Toluidine Blue and observed under a bright field microscope (Nikon Instruments Europe BV, Amstelveen, the Netherlands).

6.9 Immunocychemical analysis

Cells were plated in eight-well chamber slides at a density of 10.000 cells/well. The day after cells were treated with hGH or HYADD®4-G as previously describe and after 48 hours the chamber slides were washed twice with PBS and fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Then slides were incubated with TBS + 1% BSA to block non-specific staining for 10 minutes.

Cells were then incubated with the following antibodies: monoclonal anti-human GHR (Santa Cruz Biothechnology, Santa Cruz, CA, USA) diluted 1:200, or anti-human CD44 diluted 1:20 (clone NIH44 ibridoma obtained from ATCC, Manassas, VA, USA), or anti-human SOCS2 diluted 1:150 (Cell Signaling Technology, Inc., Danvers, MA USA). All the primary antibody were diluted in TBS containing 0.1% BSA and incubated at 4°C over night. Slides were washed 3 times with TBS and then incubated with multilinker biotinilated secondary antibody (Biocare Medical, Concord, CA, USA) and alkaline phosphatase-conjugated streptavidin (Biocare Medical) at room temperature for 30 minutes.

The reactions were developed using fast red substrate (Biocare), counterstained with hematoxylin and mounted in glycerol gel. The sections were evaluated with a bright field microscope. Negative and isotype-matched controls were performed either by omitting the primary antibodies or using an isotype-matched control.

6.10   Molecular biology analysis

6.10.1 RNA extraction

At the experimental times (24h, 48h and 72h), all wells were lysed by adding 1 ml of reagent EUROGOLD RNA per well (Euroclone).

Total RNA was extracted from the cell lysate by the addition of chloroform, which allows to separate the RNA from the cellular components and interfering substances. After centrifugation al 12.000 x g two phases were obtained:

· aqueous phase containing the nucleic acid (upper);

· organic phase containing proteins (lower).

The aqueous phase was collected, treated with isopropanol and and centrifuged at 12.000 x g to allow the RNA precipitation. The RNA pellet was then washed with 75% ethanol.

Subsequently, the pellet was treated with DNase I (DNA-free DNase Kit, Ambion) to remove

any DNA contamination.

The extracted RNA was analyzed by UV spectrophotometer (NanoDrop) at 260 and 280 nm. Absorbance at 260 nm was measured to determinate RNA concentration and 260/280 nm ratio to evaluate RNA purity.

6.10.2 Reverse transcription

Reverse transcription was performed using "VILO SuperScript cDNA Synthesis Kit" (Invitrogen). Each sample (0,5 μg) was diluted in a reaction mixture (20 μl final volume) consisting of:

- 5X VILO Reaction Mix (4 uL);

- 10X SuperScript Enzime Mix (2 uL);

- RNase-free H_2O to a total volume of 20 μl.

Reverse transcription was performed as follow: 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 85°C.

At the end, all the samples were diluted with H_2O to obtain a concentration of 10 ng/μl and 2μl of cDNA were used for the RealTime PCR.

6.10.3 <u>Real Time PCR</u>

Primers for PCR amplification were generated from GenBank sequences using the Primer3 Software (Rozen and Skaletsky, 2000). BLASTN searches were conducted on all oligonucleotide sequences to ensure pairing specificity. The housekeeping gene ribosomal protein subunit 9 (RPS9) was used as an endogenous control to normalize for intersample variations in the amount of total RNA. (Table 6).

Table. 6 Oligonucleotides sequences

TARGET GENE	PRIMERS	PRODUCT SIZE (bp)	GENE BANK ACCESSION No.
RPS9	Forward: GATTACATCCTGGGCCTGAA Reverse: ATGAAGGACGGGATGTTCAC	161	NM_001013
COL1A1	Forward: CCTGGATGCCATCAAAGTCT Reverse: CGCCATACTCGAACTGGAAT	170	NM_000088
COL2A1	Forward: GACAATCTGGCTCCCAAC Reverse: ACAGTCTTGCCCCACTTAC	257	NM_001844 NM_033150
COL10A1	Forward: TGCTGCCACAAATACCCTTT Reverse: GTGGACCAGGAGTACCTTGC	192	NM_000493
SOCS2	Forward: CTCGCATTCAGACTACCTAC Reverse: CTGTCCGCTTATCCTTG	191	NM_001270471.1

Real time PCR was run in a LightCycler Instrument (Roche Molecular Biochemicals, Mannheim, Germany) using the SYBR Premix Ex Taq (TaKaRa Biomedicals, Tokyo, Japan) with the following protocol: activation of Taq DNA polymerase at 95°C for 10 sec, followed by 45 cycles of 94°C for 5 sec and 60°C for 20 sec. The increase in PCR product was monitored for each amplification cycle by measuring the increase in fluorescence caused by the binding of SYBR Green I Dye to dsDNA. The crossing point (CT) values (i.e. the cycle number at which the detected fluorescence exceeds the threshold value) were determined for each sample and specificity of the amplicons was confirmed by melting curve analysis.

For each target gene mRNA levels were calculated from the CT values using the quantitative relative method ($2^{-\Delta Ct}$) and expressed as percentage of the housekeeping gene.

6.11   Soluble factors analysis

6.11.1 Multiplex system

Soluble factors were evaluated by "Milliplex® MAP Kit" (Millipore, St. Charles, Missouri, U.S.A).

The principle of this assay is based on color-coded magnetic beads, which covalently binds the biomarker of interest. Bead coloration is achieved by utilizing different concentrations of red and infrared fluorophore dyes to create 100 uniquely-colored bead sets. Considering that these bead sets contain a unique color / fluorophore signature, they can then be combined within the same assay well.

As shown in Figure 14 each analyte analyzed bind to the captured antibody. Then a secondary biotinylated antibody specific for a different epitope of the markers of interest is used as detection antibody, and finally the complex Streptavidin-Phycoerythrin (SA-PE) conjugate is responsible for the fluorescence signal.
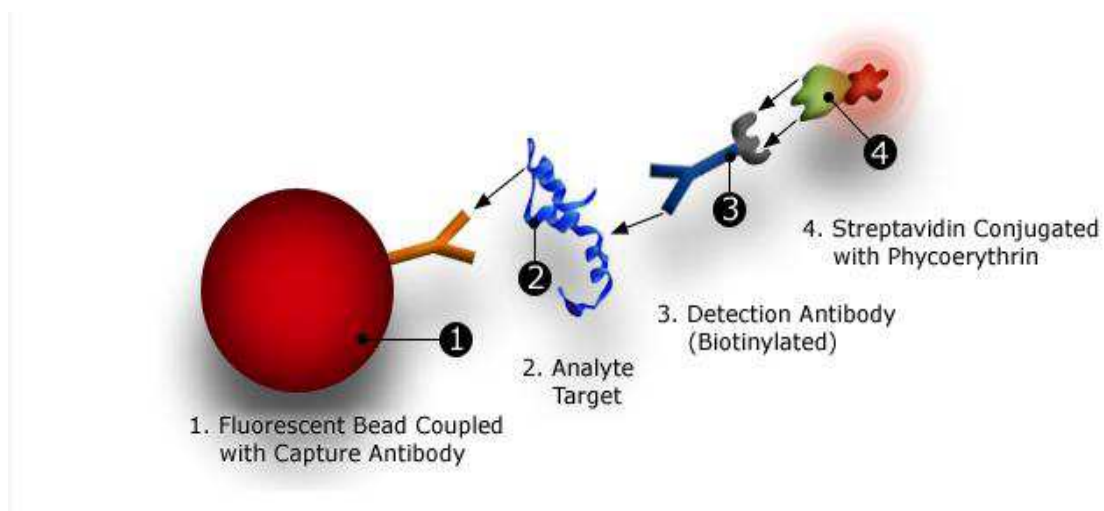
Figure 14: Assay Steps in Detecting Fluorescent Intensities

This system was used to evaluate the expression profile of the cytokines IL-6 and FGF-2 in the supernatant of chondrocytes.

Specifically, the plate containing the filter in the bottom is prewet adding 200 µl of Assay Buffer and then the liquid was removed by vacuum. Diluted standards and sample (50 µl) were added to each well and then were added the mixed beads (25 µl). The plate was incubated under rotation on a plate shaker for 1 hour at room temperature. Fluid was then removed gently by vacuum and the plate washed twice with wash buffer. Biotinylated secondary antibody was added to wells and plate incubated under rotation on a plate shaker for 1 hour at room temperature. Plates were each washed twice and and Streptavidin-PE added to each well and incubated for 5 minutes. The plate was read with the fluorimeter. In particular, the suspension was sucked in a test instrument (Bio-Plex Array System), which uses a system of microfluidics to align the microspheres in a single row in a column of liquid, which are intercepted by two laser beams with wavelengths appropriate. The first illuminates the fluorophores inside the ball (color code) and the second excite the fluorochrome bound to the 'complex on the surface of the microsphere. Subsequently, an advanced optical system captures fluorescence signals.

6.11.2 Test ELISA : Enzyme-linked immunosorbent assays

The most popular application for immunological detection is the Enzyme-Linked Immunosorbent Assay (Quantikine® ELISA, R&D Systems, McKinley Place NE, Minneapolis, U.S.A).

This assay employs the quantitative sandwich enzyme immunoassay technique. This system was used to quantized IGF-1 in supernatants.

The plate is pre-coated with a monoclonal antibody anti-IGF-1. Standards, control and samples are added into the well and incubated for 2 hours at 4°C.

The plate was washed to remove unbound substances, and enzyme-linked polyclonal antibody, specific for a different epitopes, IGF-1 was added to the wells and incubated for 1 hour at 4°C. The plate was washed, a substrate solution was added to each well and incubated for 30 minutes at room temperature. The colour in the well should change from blue to yellow in proportion to the amount of IGF-1 bound. The colour development was stopped adding 50 µl of stop solution in each well and the intensity of the colour was measured by a spectrophotometer. The absorbance was read at 450 nm and 540 nm. (Figure 15)
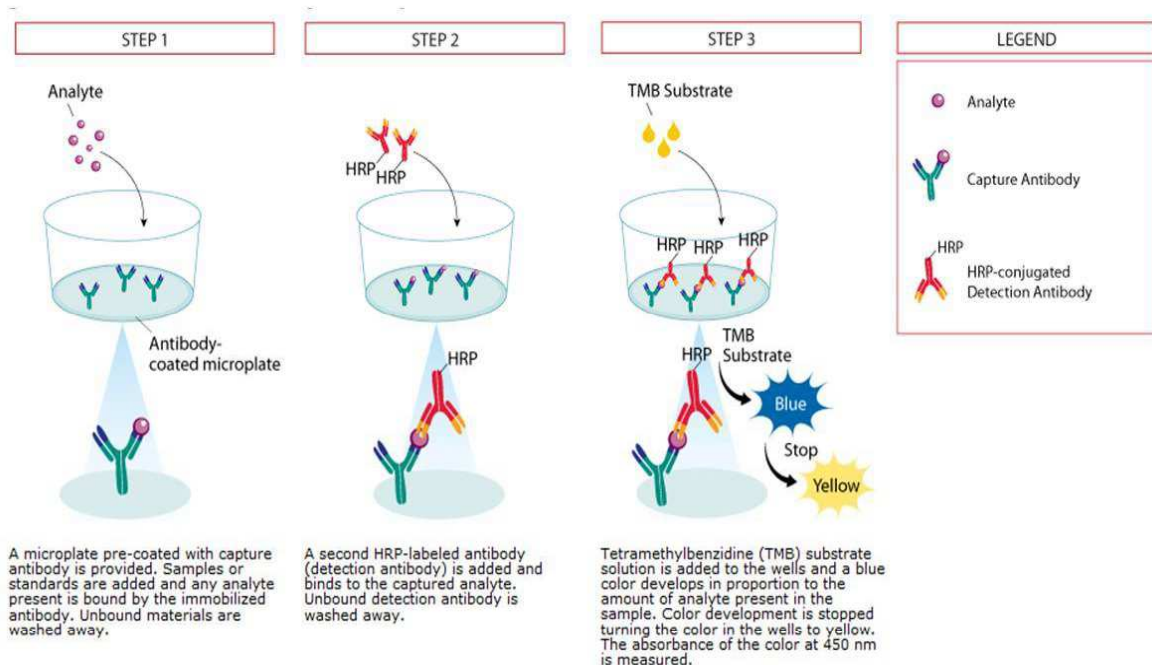
Figure 15: Quantikine ELISA Assay Principle

6.12 Intracellular proteins analysis by Western Blotting

6.12.1 Protein extraction

Primary chondrocytes were washed in PBS to remove excess medium and cells were scraped in presence of 120 µl lysis buffer containing inhibitor cocktail mix: 2 µl/ml phenylmethanesulfonylfluoride (PMSF), 5 µl/ml protease inhibitor cocktail mix (PIC), 2 µl/ml sodium fluoride (NaF), 4µl/ml sodium orthovanadate and 991 µl Tris-HCl-NaCl-Nonidet P-40-SDS (Bio-Rad Laboratories)

Samples were stored for 15 minutes in ice and then centrifuged 10.000 rpm for 15 minutes to recover the protein samples. Samples were then stored at -20°C.

Protein content was determined using a Pierce™ BCA Protein Assay Kit (Thermo scientific, Rockford, USA), which is a colorimetric assay for detecting protein concentration. Serial dilutions of BSA was used as standard ranging from 2 to 0.025 mg/ml.

96-well plate was used for the assay, and each well contained 10µl sample/standard and 200 µl of the working reagent (ratio 50:1, reagent A : reagent B). The plate was incubated 37°C for 30 minutes, then protein levels were measured by absorbance at 562 nm.

The protein concentration of each sample was calculated from the standard curve.

6.12.2 <u>Western Blot</u>

30 μg of total protein from each sample was loaded in the wells of NuPAGE® Novex® precast 4-12% polyacrylamide gel (Invitrogen, Life Technologies) which was subsequently transferred onto polyvinylidene fluoride
membranes by a dry electroblotting method using IBlot® Gel Transfer Device (Invitrogen, Life Technologies).

The membrane was blocked with 0,25% BSA in TBS for 10 minutes at room temperaturean then incubated with polyclonal anti-human SOCS2 diluted 1:200 (Cell Signaling Technology, Inc., Danvers, MA USA) in 0,25% BSA (Fraction V) at 4°C overnight. Sequentially incubated with an anti-mouse IgG HRP (211-032-171,115-035-174, Jackson ImmunoResearch, West Grove, PA, USA). Signal detection was revealed using Amersham ECL Select kit (RPN2235, GE Healthcare Italia, Milan, Italy) and acquired through KODAK Image Station 4000R Digital Imaging System (Kodak, Rochester, NY, USA). and Carestream Molecular Imaging Software 5.0. (Carestream Health, Inc.). β-actin (Sigma) was used as loading controls.

6.13   <u>Statistical analysis</u>

The experimental design considered for each patient the following conditions: 1. two different treatments (hGH and HYADD®4-G), six different concentrations (0, 0.01, 0.1, 1, 10, 100 μg/ml) and their combinations at four different time points (24h, 48h, 72h and 7 days). Therefore, the statistical units included in the analysis were 384 (8 patients x 2 treatments x 6 concentrations x 4 time points) or less (depending on marker analyzed) and the statistician selected the multivariate analysis to increase the power of the study. Specifically was used General Linear model (GLM) for repeated measures with the following parameters: the age of the patients as covariates and treatments (hGH and HYADD®4-G), concentrations (0, 0.01, 0.1, 1, 10, 100 μg/ml) and cell numbers and their combinations as fixed effects. This statistical model permit to calculate also the correlation among two parameters (e.g. age and collagen type 2 etc.) considering more than one independent variable (e.g. the time course). P-value <0.05 was considered statistically significant. All graphs were performed using the program GraphPad Prism and data express as mean and 95% confidence interval or as mean ± SD.

7. RESULTS

7.1 GHR and CD44 expression on cartilage biopsies and isolated chondrocytes

We firstly analyzed the receptors of growth hormone (GHR) and hyaluronic acid (CD44) at protein level by immunohistochemistry on cartilage biopsies from healthy subjects and OA patients.

As shown in **Figure 16A-B**, GHR was positive in chondrocyte clusters of OA cartilage and only in a few cells in normal cartilage.
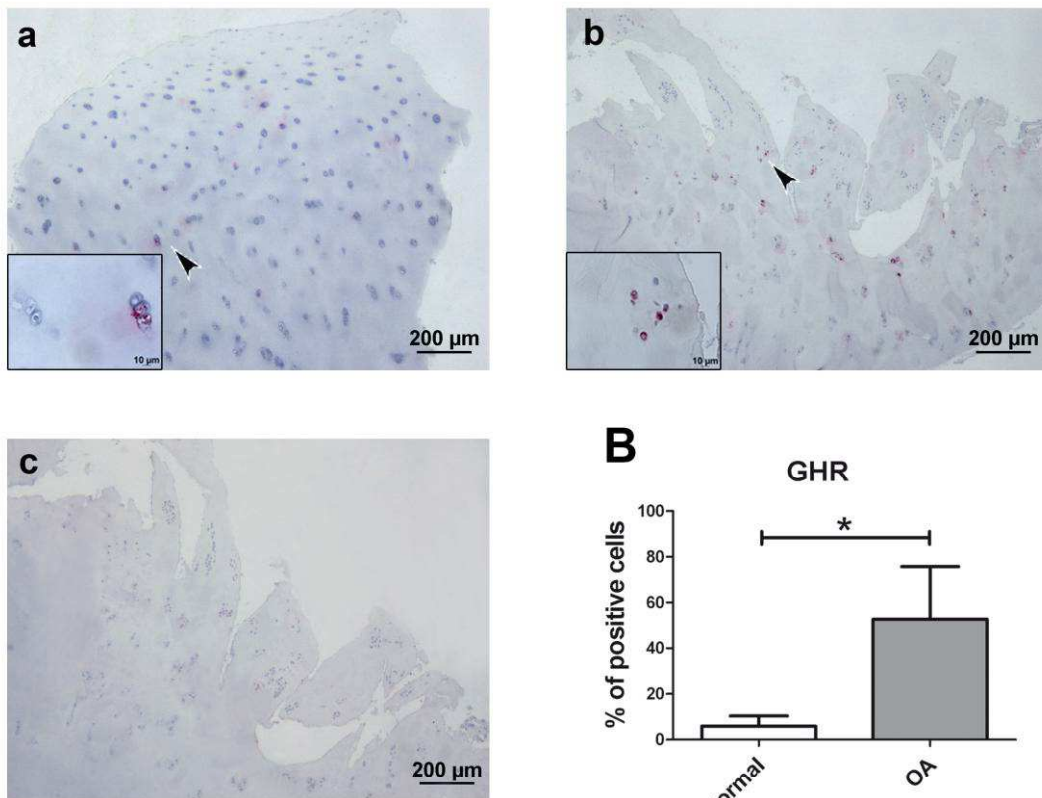
Figure 16A-B: GHR expression on cartilage biopsies. **A:** Immunohistochemical analysis of GHR on healthy cartilage (a) and OA (b). Isotype-matched negative control (c). Bars: a, b, c, = 200μm. Inset bars = 10 μm. **B:** Data of the immunocytochemical are expressed as mean ± SD. Significant differences are highlighted by the asterisk *.

CD44 was positive in few cells both in OA and in normal cartilage. **(Figure 17)**
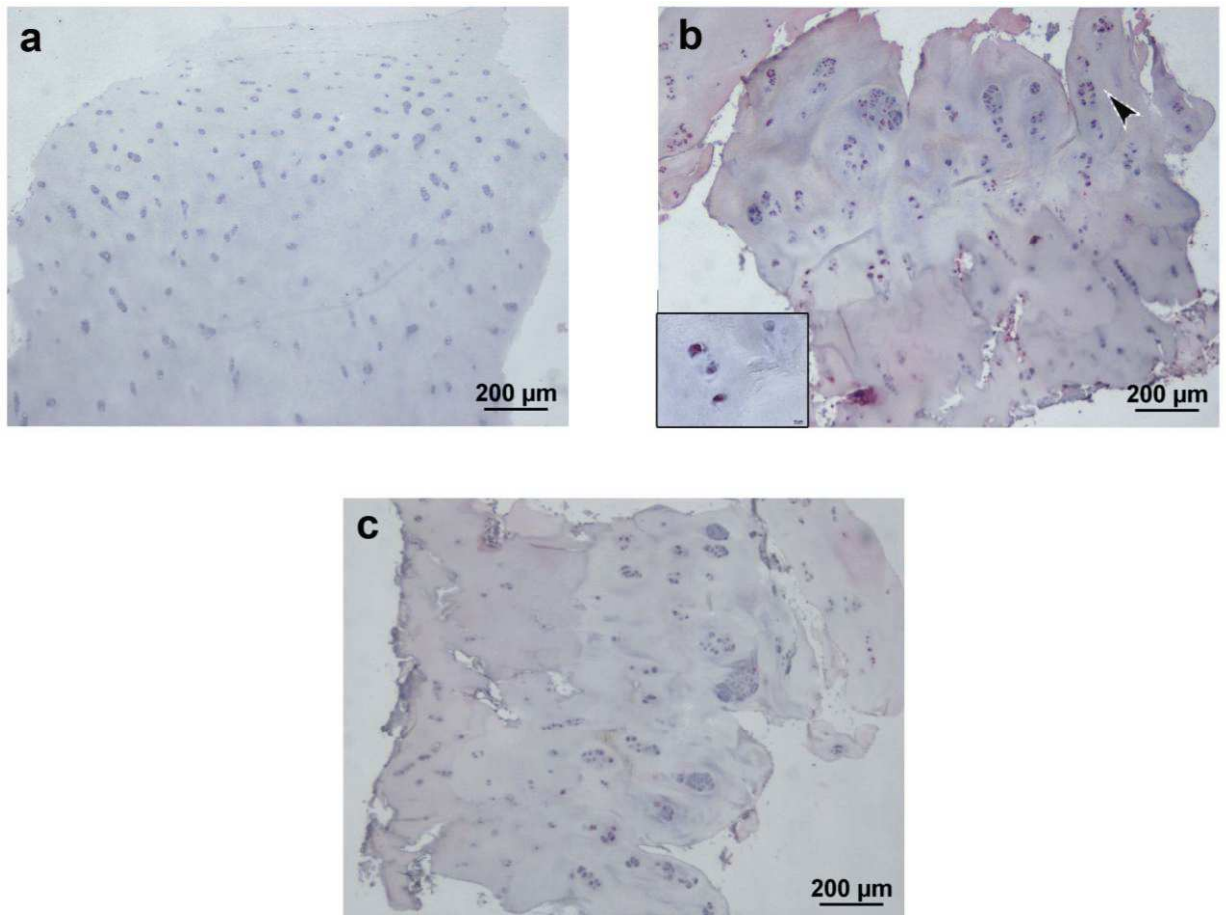
Figure 17: CD44 expression on cartilage biopsies. Immunohistochemical analysis of CD44 on healthy cartilage (a) and OA (b). Isotype-matched negative control (c). Bars: a, b, c, = 200µm. Inset bars = 10 µm.

Subsequently we analyzed these receptors in OA isolated chondrocytes by immunocytochemical analysis. As shown in **Figure 18** GHR and CD44 were both expressed in OA isolated chondrocytes.
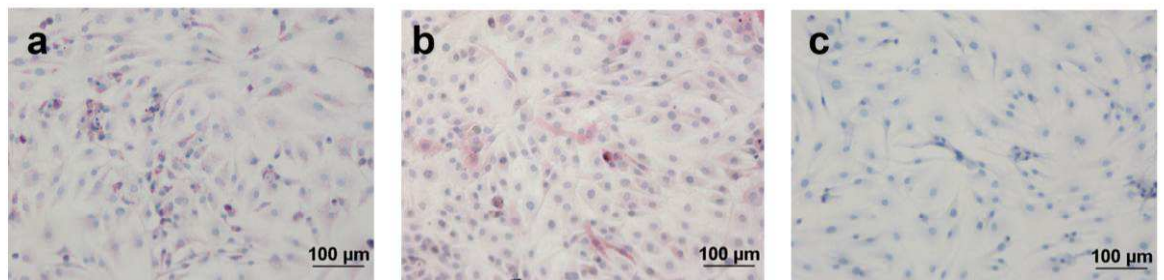
Figure 18: GHR and CD44 expression on OA isolated chondrocytes. Immunocytochemical evaluation of GHR (a) and CD44 (b) on isolated chondrocytes, isotype-matched negative control (c). Bars: a, b, c = 100µm.

7.2 HYADD®4-G effect on chondrocytes

We noted that at 24 hours HYADD®4-G, added to the wells, forms a solid plug that covered all the area of the well and does not permit the analysis of cell morphology by contrast microscope. HYADD®4-G became more soluble at 7 days and by contrast microscope was possible to observe adherent cells (**Figure 19**).
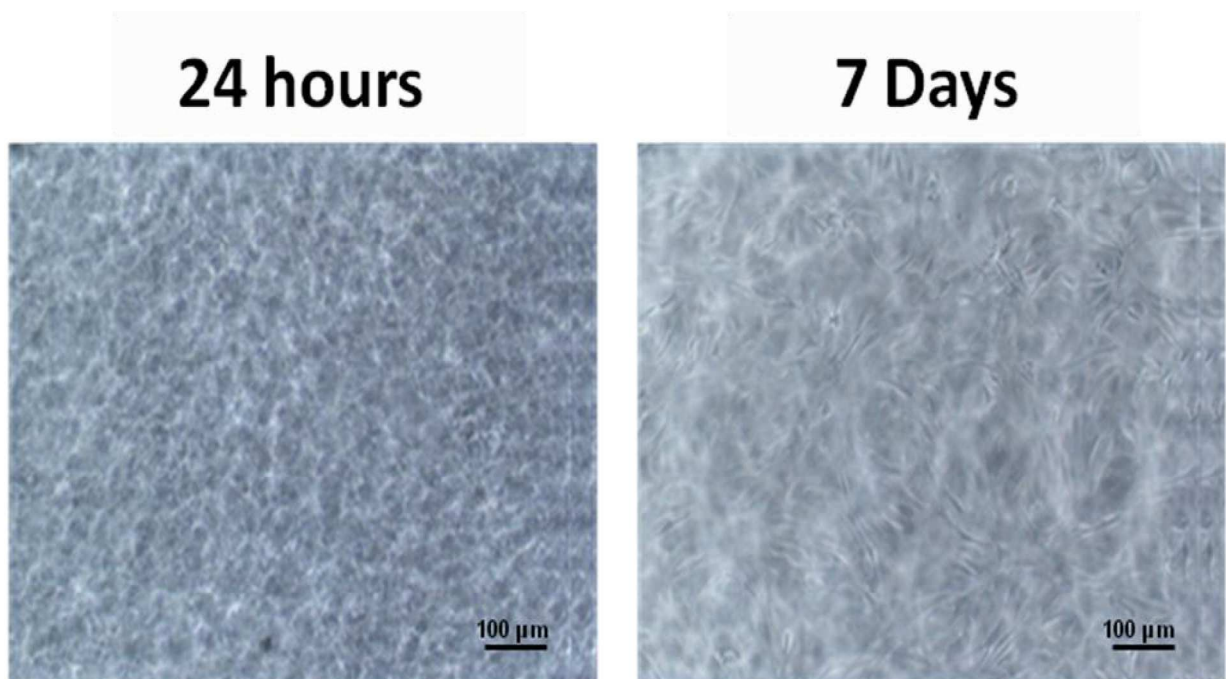
Figure 19: Evaluation of HYADD®4-G characteristic in culture treated with HYADD®4-G for 24 hours and 7 days. Bars = 100µm.

Therefore, we decided to assess if HYADD®4-G was able to mimic in vivo joint environment creating an hypoxic condition for the cells. A scratch assay was performed on chondrocytes and control migrating cells (synovial fibroblast) cells cultured under normoxic (20%O_2) and hypoxic (5%O_2) conditions were also tested. The **Figure 20A** shows representative images of chondrocytes and synoviocytes under normoxic conditions, while in **Figure 20B** are shown the cells under hypoxic condition.
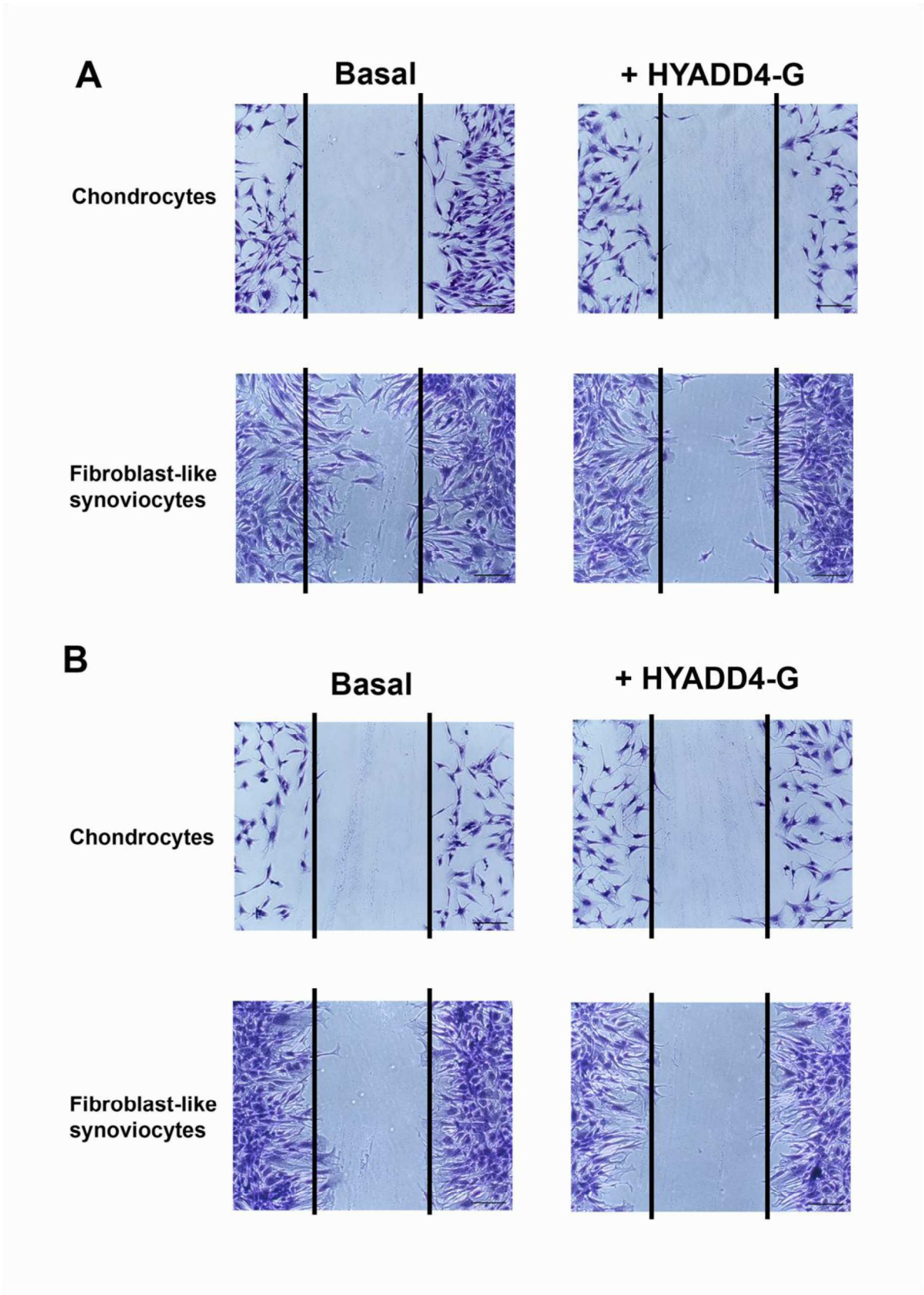
Figure 20: Scrath assay on chondrocytes and fibroblast-like synoviocytes treated with or without HYADD®4-G **A:** under normoxic condition (20% O_2) **B:** under hypoxic condition (5% O_2). Bars = 100μm

In normoxia, after 24 h from the scratch, chondrocytes do not migrate to fill the wound both in basal conditions and in presence of HYADD®4-G, while fibroblast-like synoviocytes in basal condition start to migrate to cover the scratch, instead with HYADD®4-G did not migrate. By contrast in hypoxia condition, after 24 hours from the scratch, in basal condition and treated with HYADD®4-G, both chondrocytes and fibroblast-like synoviocytes did not migrate to cover the scratch confirming that HYADD®4-G contribute to maintain hypoxic condition.

7.3 Morphology and viability of chondrocytes treated with hGH and HYADD®4-G

Morphology was evaluated by Toluidine Blue staining and viability by live and dead assay, respectively. In basal conditions Toluidine Blue stained chondrocytes showed a rounded, polygonal morphology, whereas after hGH treatment we observed an increase in mitosis and cells with rounded shape independently from the different hGH concentrations tested (**Figure 21**).
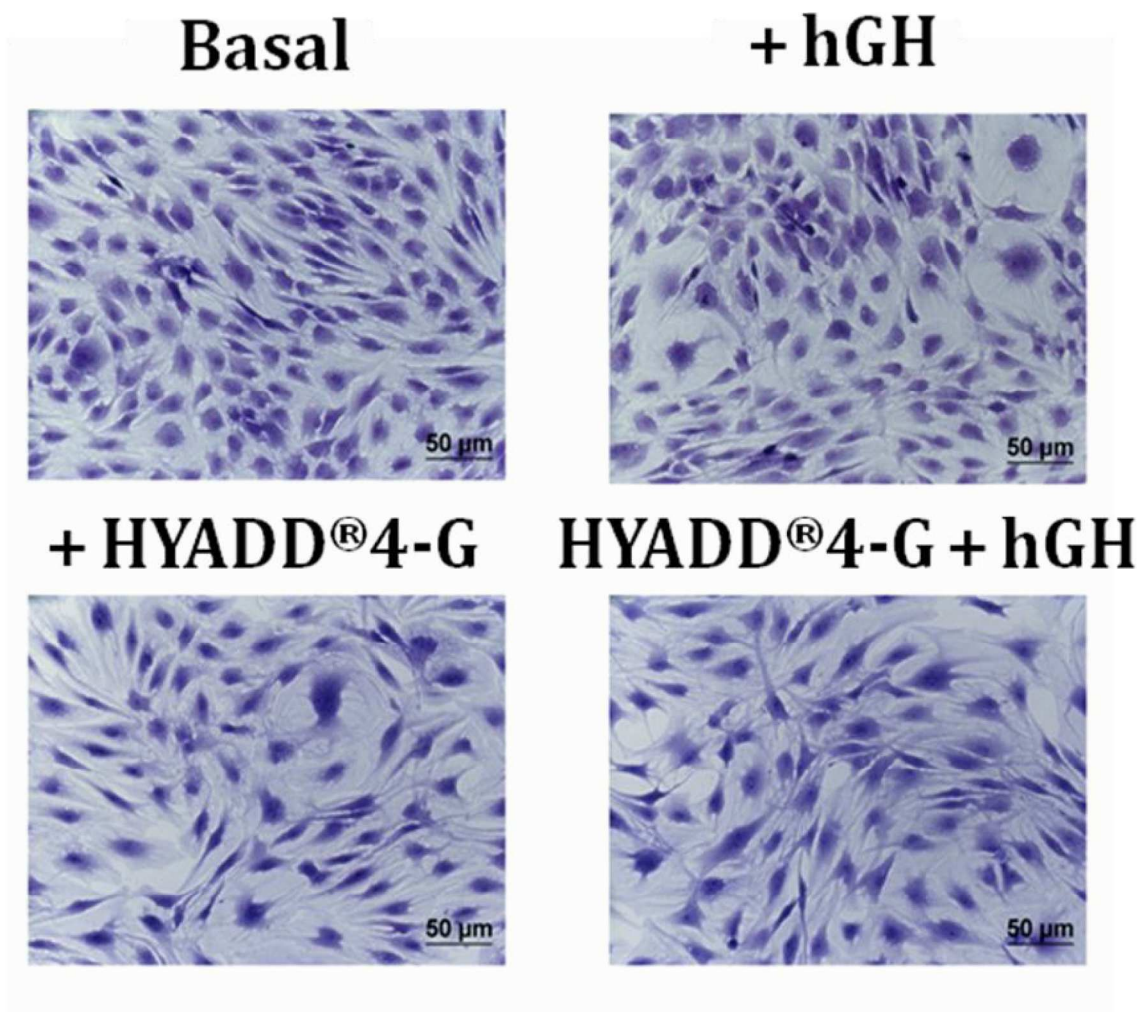
Figure 21: Morphological characteristics of OA chondrocytes treated with hGH and HYADD®4-G alone or in combination. Morphological characteristics of isolated OA chondrocytes stained with Toluidine Blue in basal conditions (Basal), treated with hGH 1μg/ml (+hGH), HYADD®4-G 1mg/ml (+HYADD®4-G) or their combination (HYADD®4-G +hGH). Bars = 50μm.

Conversely, HYADD®4-G-treated chondrocytes still showed mitosis but their shape appeared more as fibroblast-like cells.

The viability test confirmed that the chondrocytes were viable at all time points analyzed. In particular, the different hGH concentrations used with or without HYADD®4-G did not induce cell death from 48 hours until 7 days (**Figure 22**).
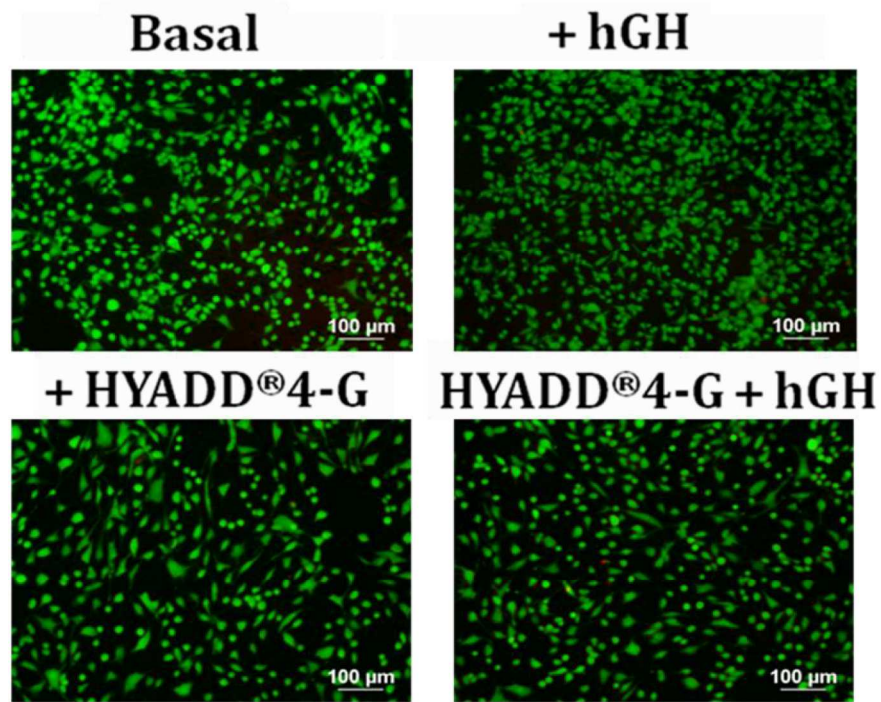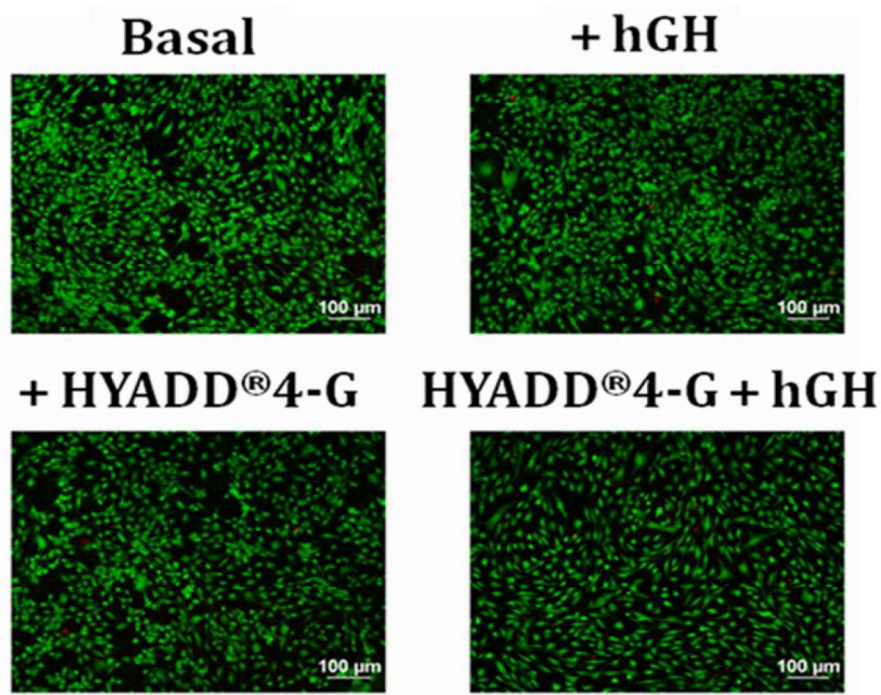
Figure 22: Analysis of chondrocyte viability in basal conditions (Basal), after treatment with hGH 1μg/ml (+hGH), HYADD®4-G 1mg/ml (+HYADD®4-G) or their combination (HYADD®4-G +hGH), using the live and dead test at 48 hours (**A**) and at 7 days (**B**).

Calcein-AM positive cells (green) are alive; propidium iodide cells (red) are dead. Bars = 100μm.

7.4 Evaluation of chondrocyte metabolic activity

We investigated chondrocyte metabolic activity using Alamar Blue assay. In basal conditions chondrocyte metabolic activity showed the same values and trend as those of hGH (independently of the concentration used) treated cells, while this activity was decreased by the treatment with HYADD®4-G both on basal and hGH treated cells (**Figure 23A**). GLM repeated measures statistical analysis showed that the metabolic activity, independently from the treatment used, proportionally decreased with the increasing age of the patients ($\beta = -0.17$) (**Figure 23B**). Moreover, the statistical analysis confirmed that none of the concentrations of hGH (0.01-10μg/ml) used both individually or in combination with HYADD®4-G affected the metabolic activity. However, as shown in **Figure 23C** HYADD®4-G treatment significantly decreased this activity at all the time points analyzed ($p < 0.05$).
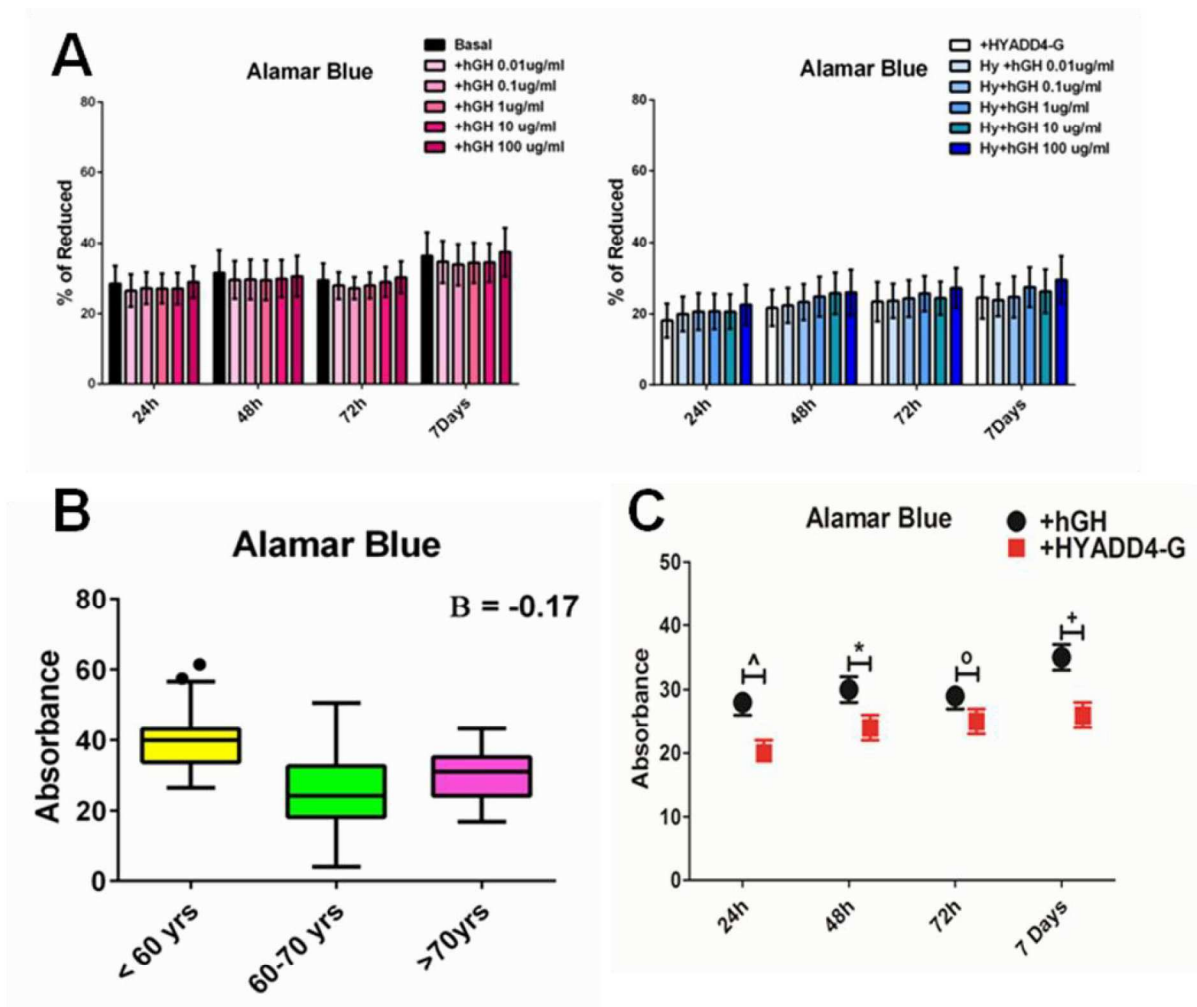
Figure 23: Analysis of chondrocytes metabolic activity treated with hGH or HYADD®4-G. **A.** Evaluation of chondrocytes metabolic activity at different time points (24h, 48h, 72h and 7 days) after treatment with hGH (0.01-10μg/ml) or HYADD®4-G (1mg/ml) alone or in combination. Data are expressed as absorbance (mean and 95% confidence interval). **B.** GLM statistical analysis showed that the metabolic activity was proportionally decreased with the increasing the age of the patients ($\beta = -0.17$). Age ranges: <60 years, 60-70 years, >70 years). **C.** GLM statistical analysis showed significant differences, highlighted by asterisks (^ for 24h, * for 48h, **o** for 72h and + for day 7), between treatments (hGH versus HYADD®4-G), independently by the concentrations used.

7.5 Effect of hGH and HYADD®4-G on GHR and CD44

Immunocytochemical analysis was performed to evaluate GHR and hyaluronan receptor CD44 on treated chondrocytes. GHR, was not modulated by different treatment performed (data not shown).

As shown in **Figure 24A-B**, in basal conditions CD44 was positive on a low percentage of chondrocytes and the treatments with hGH 1 μg/ml or HYADD®4-G increased the number of positive cells after 24h. Interestingly, the combination of HYADD®4-G with hGH induced a synergic increase of positive cells.
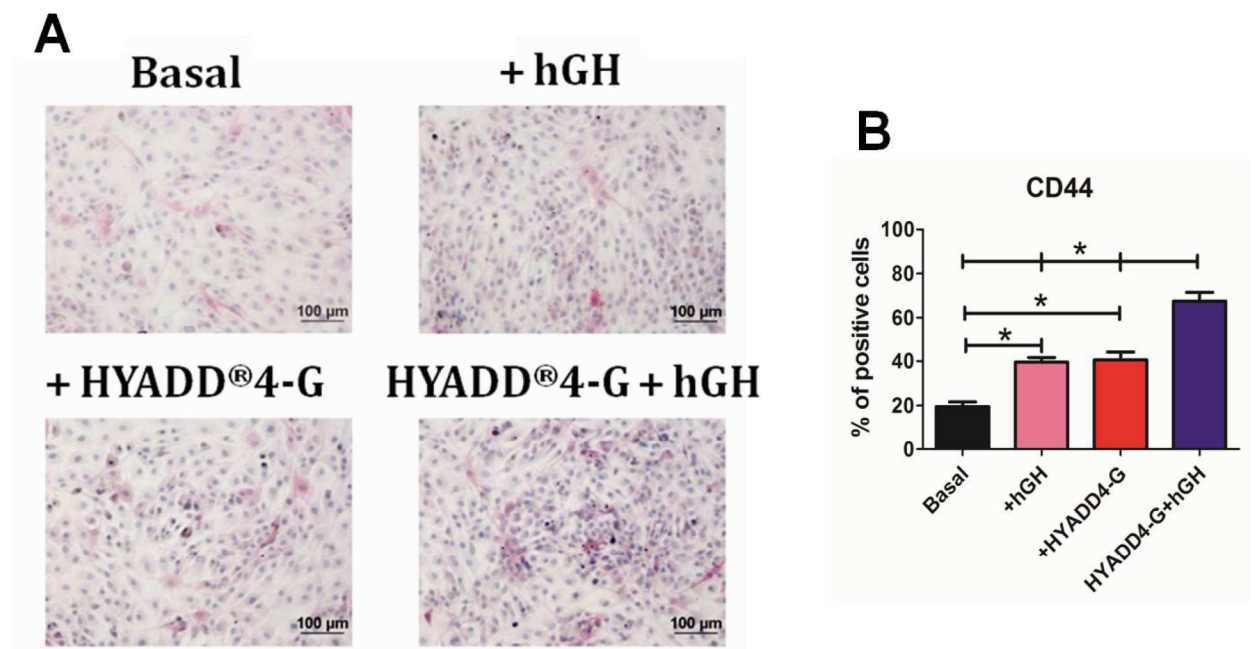
Figure 24: Analysis of CD44 receptor in chondrocytes treated with hGH or with HYADD®4G±hGH. **A.** Immunocytochemical evaluation of CD44 on isolated chondrocytes (Basal), after treatment with hGH 1μg/ml (+hGH) or HYADD®4-G or HYADD®4-G + hGH 1μg/ml. Bars = 100μm. **B.** Data of the immunocytochemical are expressed as mean ± SD. Significant differences in all condition tested (Basal, +hGH, + HYADD®4-G, HYADD®4-G +hGH) are highlighted by the asterisk *.

7.6 Effects of hGH and HAD on chondrocyte markers

Collagen type 2 a specific positive marker of chondrocyte and collagen type 1 and 10 markers of chondrocyte fibrosis and hypertrophy, respectively, were evaluated in basal condition, after treatment with hGH (0.01-100μg/ml) or with HYADD®4-G with or without all hGH concentrations. Collagen type 2, collagen type 1 and collagen type 10 (**Figure 25**) expression was unaffected by none of the concentrations of hGH-treatments alone or in combination with HYADD®4-G at all time points tested.
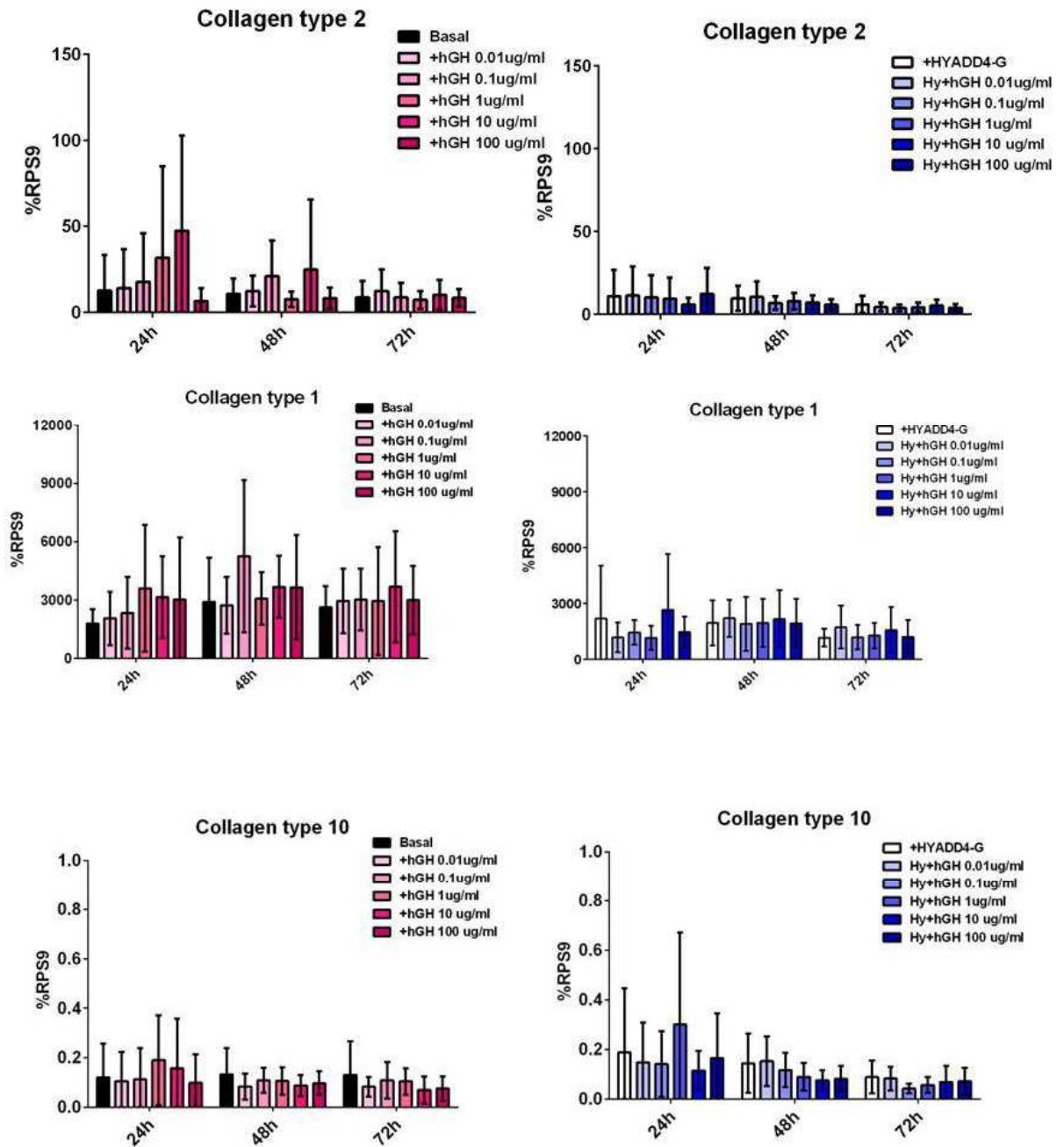
Figure 25: Analysis of chondrocyte markers expression over time. Collagen type 2, collagen
type 1, collagen type 10 expression was evaluated at different time points (24h, 48h
and72h) in basal condition and after treatment with hGH (0.01-100μg/ml) or HYADD®4-G
(1 mg/ml) alone or in combination. Data are expressed as % RPS9 (mean and 95%
confidence interval).

GLM repeated measure statistical analysis evidenced that the expression level of all these markers, independently from the treatment used (hGH or HYADD®4-G), progressively decreased with the increasing age of the patients (β = -0.4 , -80, - 0.003, respectively) (**Figure 26**).
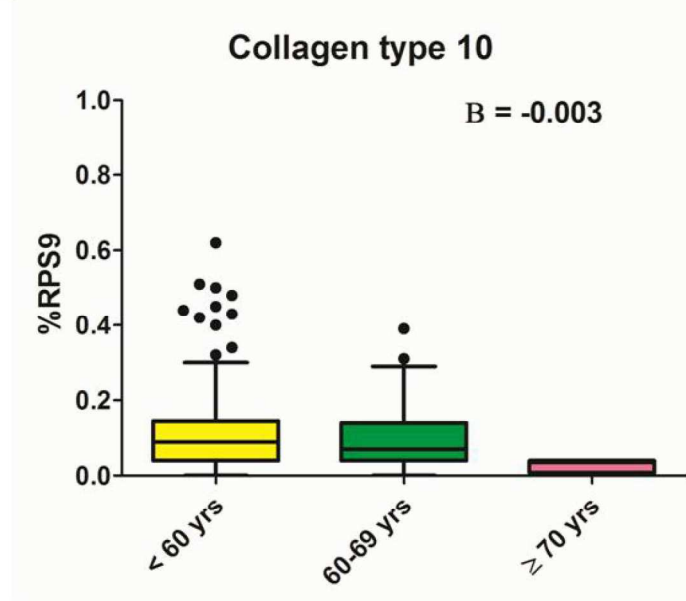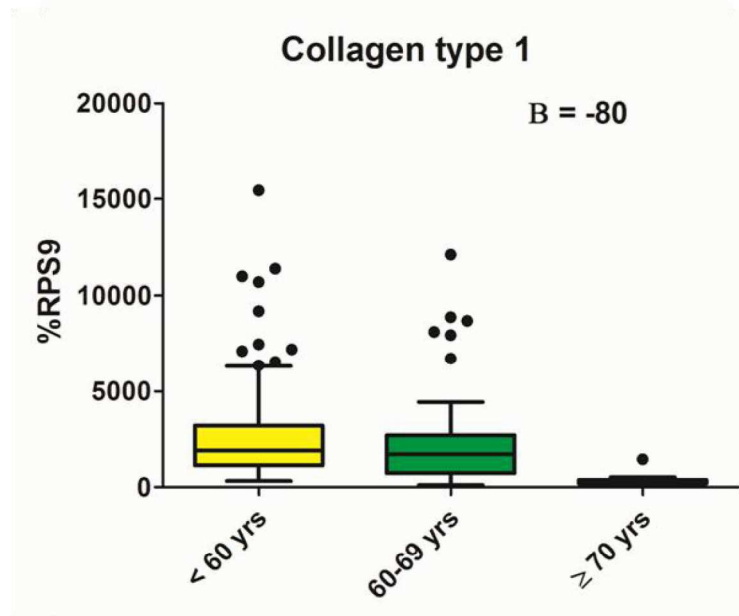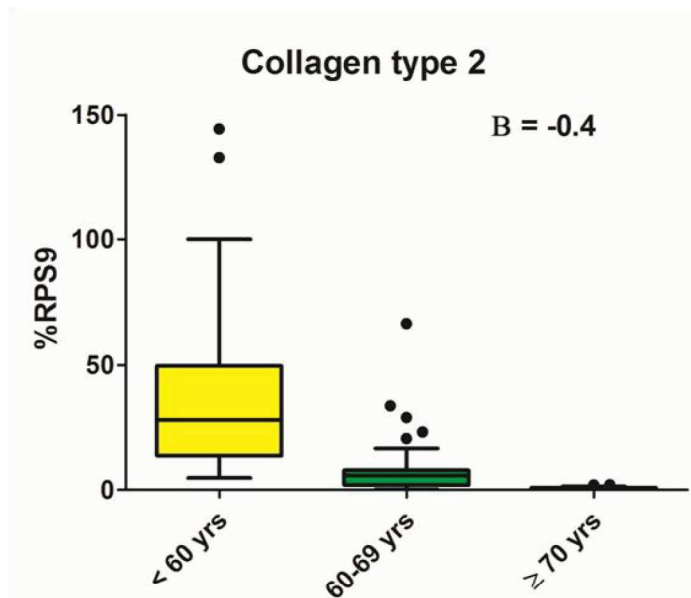
Figure 26: Chondrocyte markers expression in patients with different age and treatments. GLM statistical analysis showed that collagen type 2, collagen type 1 and collagen type 10 expression was proportionally decreased with the increasing the age of the patients (β = -0.4, -80, -0.003, respectively). Age ranges (<60 years, 60-70 years, >70 years).

Both the treatments applied (hGH or HYADD®4-G) showed a significant decrease (p<0.01) from 24h to 72h of the expression of all markers analyzed (**Figure 27**). Moreover, HYADD®4-G treatment significantly (p<0.05) decreased collagen type 2 and 1 expression and partially affected collagen type 10. In particular, HYADD®4-G significantly decreased collagen type 10 only at 72h.

Collagen type 2

Collagen type 1
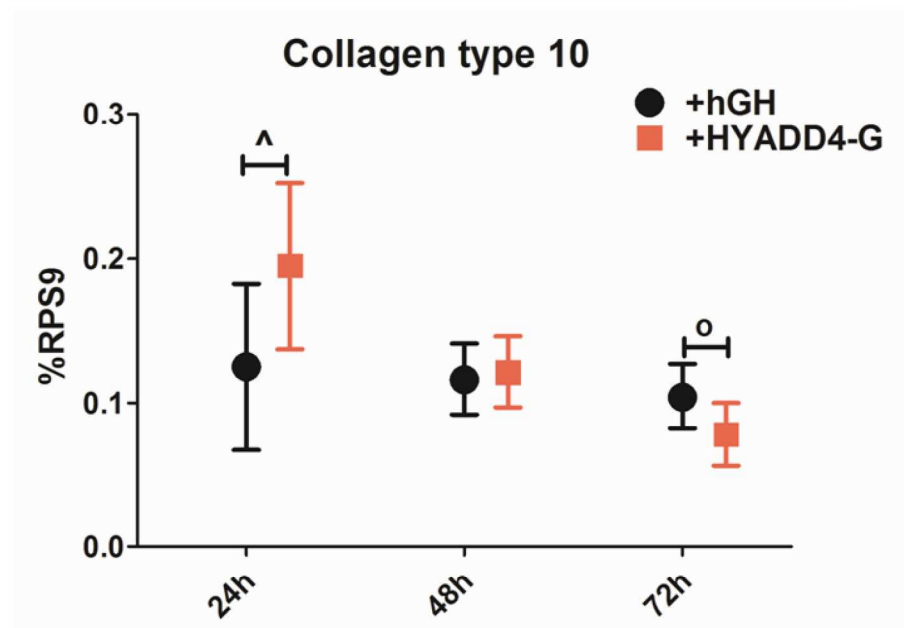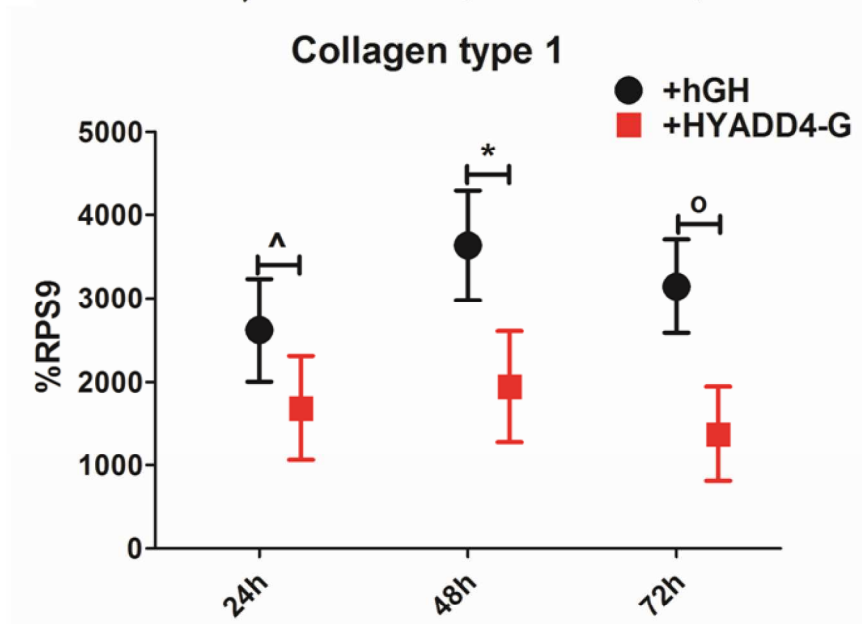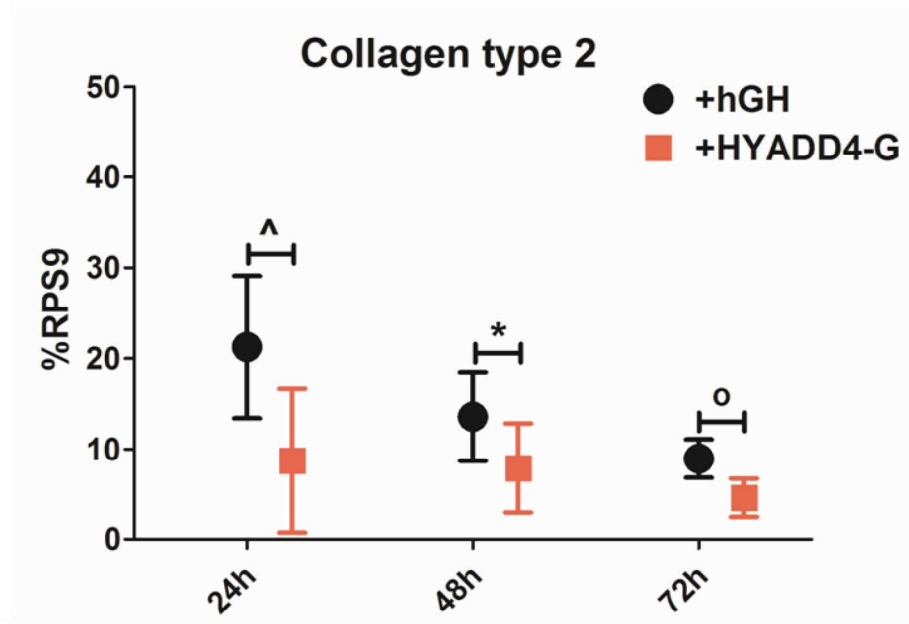
Collagen type 10

Figure 27: Chondrocyte markers expression in patients with different age and treatments. Comparison between treatments (hGH versus HYADD®4-G) for collagen type 2, collagen type 1 and collagen type 10 expression, showed significant differences, highlighted by asterisks (^ for 24h, * for 48h and o for 72h), between hGH versus HYADD®4-G, independently by the concentrations used.

7.7 Release of IGF-1, FGF-2 and IL6 by treated chondrocytes

Human OA chondrocytes did not release IGF-1 and FGF-2 at any of the time points analyzed or conditions tested. IL6 release was significantly induced only by hGH 100 µg/ml from 48 h to 7 days (**Figure 28**).
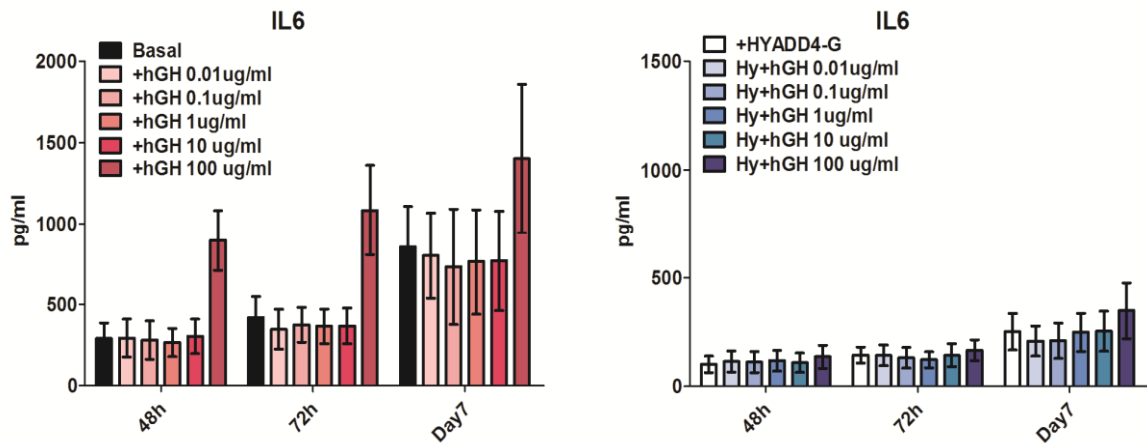
Figure 28: IL6 release of chondrocytes treated with hGH or HYADD®4-G alone or in combination. IL6 release was evaluated at different time points (48h, 72h and 7 days) on chondrocytes in basal condition and treated with hGH (0.01-100 µg/ml) alone or combined with HAD (1mg/ml). Data are expressed as pg/ml (mean and 95% confidence interval).

Conversely, when hGH (independently of the concentration used) was combined with HYADD®4-G, IL6 release was significantly down-modulated GLM statistical analysis indicated that the release of IL6 significantly increased from 48 h to 7 days independently from the treatment applied (hGH or HYADD®4-G), but it was not age-related (**Figure 29**).
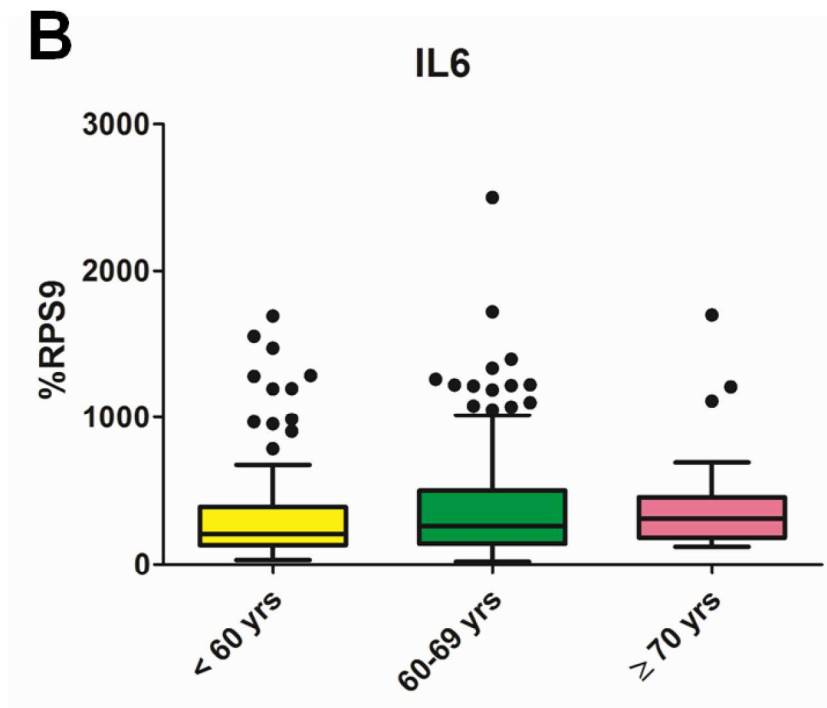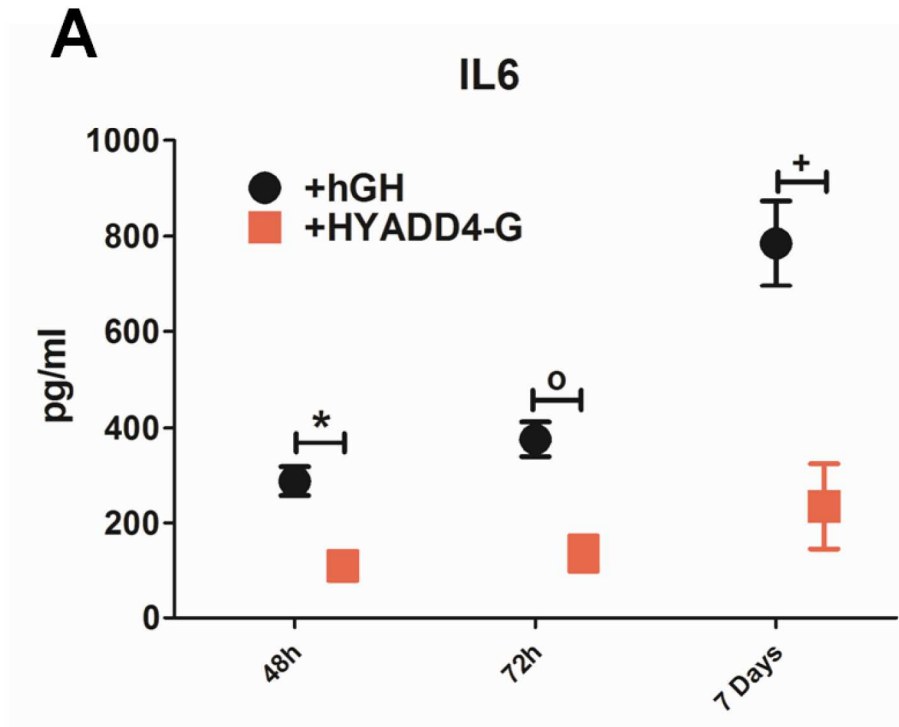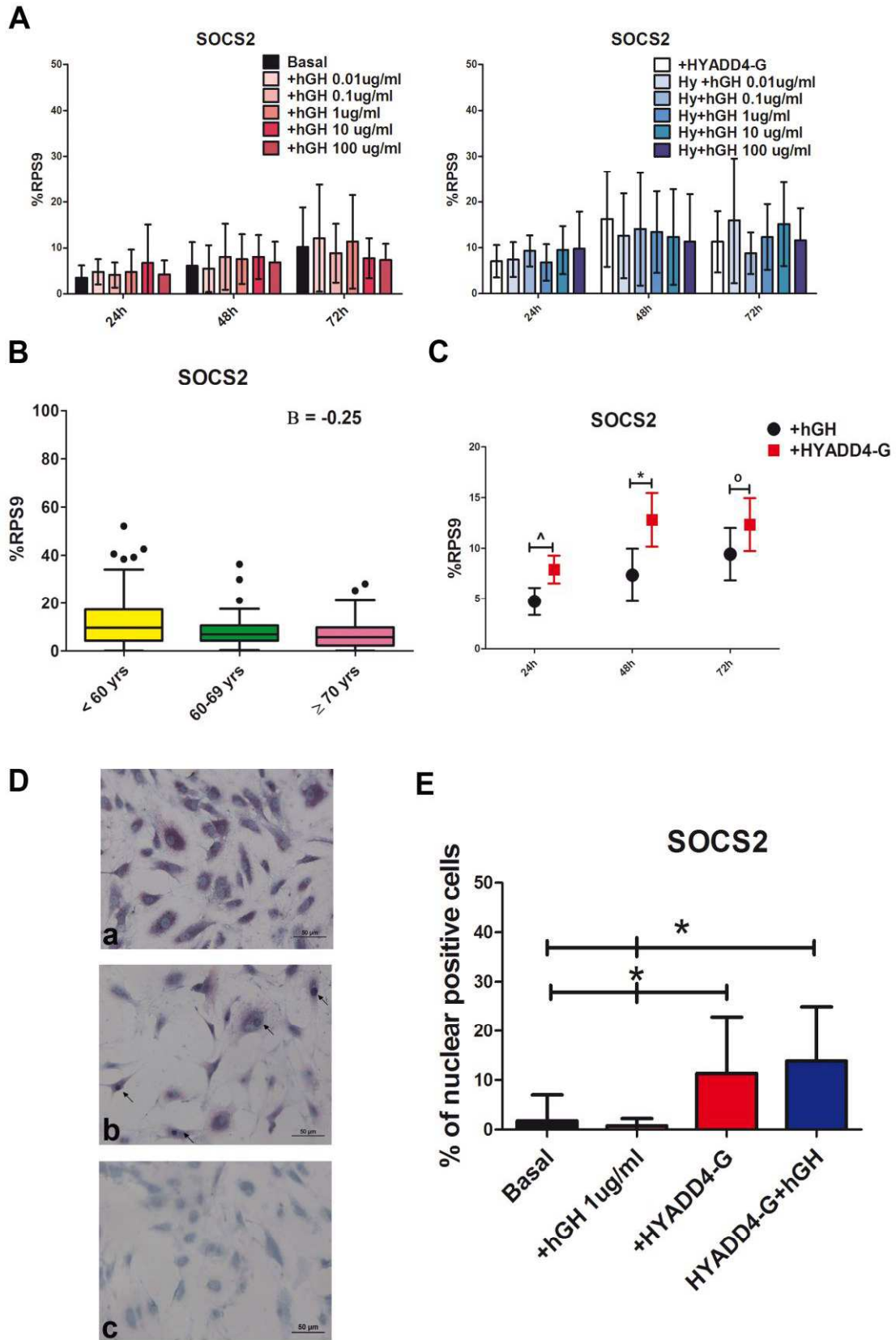
Figure 29: A. IL6 release of chondrocytes treated with hGH or HYADD®4-G alone or in combination. GLM statistical analysis showed significant differences, highlighted by asterisks (* for 48h, **o** for 72h and + for day 7), between treatments (hGH versus HYADD®4-G), independently by the concentrations used. **B.** IL6 release in patients with different age and treatments. GLM statistical analysis showed that IL6 was not age-related

7.8 Evaluation of SOCS2

The expression of SOCS2, a negative regulator of GH signaling, was also analyzed on basal and treated chondrocytes. SOCS2 expression showed the same levels and trend in basal condition, after treatment with hGH (0.01-100μg/ml) or with HYADD®4-G, with or without all hGH concentrations (**Figure 30A**). GLM statistical analysis showed that SOCS2 expression, independently from the treatment (hGH or HYADD®4-G) used proportionally decreased with the increasing age of the patients (β = -0.25) (**Figure 30B**). SOCS2 expression was not induced by any concentrations of hGH alone or in combination with HYADD®4-G. However, as shown in **Figure 30C**, statistical analysis evidenced that HYADD®4-G treatment significantly induced SOCS2 expression at all time points tested. Western blotting for SOCS2 was unsuccessful since HYADD®4-G, that has an high viscosity, interferes with the protein analysis (data not shown). Therefore, we decided to analyze the protein expression of SOCS2 with the immunocytochemical analysis.

As shown in **Figure 30D-E**, we found that HYADD®4-G induced a significant translocation of the protein into the nucleus compared to basal condition.

Figure 30: Analysis of SOCS2 in chondrocytes treated with hGH or HYADD®4-G alone or in combination. **A.** SOCS2 expression was evaluated on chondrocytes at different time points (24h, 48h and 72h) in basal condition and after treatment with hGH (0.01-10µg/ml) or HYADD®4-G (1 mg/ml) alone or in combination. Data are expressed as %RPS9 (mean and 95% confidence interval). **B.** GLM statistical analysis showed that SOCS2 expression was proportionally decreased with the increasing the age of the patients (β = -0.25). Age ranges (<60 years, 60-70 years, >70 years). **C.** GLM statistical analysis showed significant differences, highlighted by asterisks (^ for 24h, * for 48h and **o** for 72h), between treatments (hGH versus HYADD®4-G), independently by the concentrations used. **D.** Immunocytochemical analysis of SOCS2 on OA isolated chondrocytes. Representative pictures of basal (**a**), HAD treated condition (**b**) and isotype-matched negative controls (**c**). Bars: **a**, **b**, **c** = 50µm. **E.** Immunocytochemical quantification of SOCS2 on isolated chondrocytes (Basal), after treatment with hGH 1µg/ml or HYADD®4-G (1mg/ml) or HYADD®4-G + hGH 1µg/ml. Data are expressed as mean ± SD. Significant differences in all condition tested (Basal, +hGH, + HYADD®4-G, HYADD®4-G +hGH) are highlighted by the asterisk *.

8. <u>DISCUSSION</u>

Available drugs for treating OA mainly contribute to reducing pain, but only in part counteract the progression of the disease. Therefore, much effort is aimed at finding new drugs with structure-modifying activity to test if their potential and efficacy was influenced by the age of OA patients. (Grishko *et al.,* 2009; Fukui *et al.,* 2014; Yatabe *et al.,* 2009; Peng *et al.,* 2010; Kataoka *et al.,* 2013) Based on recent data showing the positive effects of GH on cartilage in an animal model (Tsukazaki *et al.,* 1994; Wu *et al.,* 2011; Alvarez-Garcia *et al.,* 2012; Hunziker *et al.,* 1994), and knowing the efficacy of HA in preventing OA (Gomis *et al.,* 2009; Mainil-Varlet *et al.*, 2013) we evaluated the potentiality of hGH and HAD on human OA chondrocytes of patients ranging from 46 to 78 years old.

First, we found that GHR was positive in only a few chondrocytes of healthy cartilage and it was highly positive on OA cartilage and isolated OA chondrocytes, thus suggesting its potential involvement in articular cartilage maturation and pathological processes.

Its expression becomes quiescent in mature articular cartilage and is re-expressed in the pathological state of OA, thus indicating a different involvement in cellular processes both in normal and OA cartilage as found for vascular endothelial growth factor receptor. (Lingaraj *et al.*, 2010) When isolated OA chondrocytes were treated with hGH, we showed that some of them changed their morphology from a spindle to a round shape, instead, HYADD®4-G treatment showed a fibroblast-like shape. However, hGH and HYADD®4-G treatment for 7 days induced the death of only a few cells, thus indicating that even at high hGH concentrations (100 µg/ml) the cell viability remained very high.

The metabolic activity of chondrocytes did not reveal any effect of different hGH concentrations tested on OA chondrocytes, whereas this activity was significantly reduced by HYADD®4-G alone and in combination with hGH at all the time points considered, thus confirming that HYADD®4-G interfere in some way with GH or modulate its pathways. Our data are not in line with a previous paper (Brun *et al.*, 2012) that reported HAD-induced metabolic activity; this difference was probably due to either the low number of OA patients analyzed (three) or to different *in vitro* tests and conditions as demonstrated by others. (Li *et al.*, 2013; Loeser, 2013)

Hyaluronan CD44 receptor is expressed in lower amount in normal cartilage and is increased in OA mainly in chondrocyte clusters. (Ostergaard *et al.*, 1997) It has been shown that hGH from 0.01 µg/ml induces the hyaluronan receptor CD44 on human arterial

smooth muscle cells (Schultz *et al.*, 2005), as we also found on human OA chondrocytes, thus suggesting that a combination of hGH with HYADD®4-G increased CD44 receptor, that as reported is essential for HYADD®4-G effects, so contributing to potentiate the effects of HYADD®4-G.

Interestigly, we demonstrate that HYADD®4-G not stimulates cellular migration as occur in hypoxic conditions tested suggesting that HYADD®4-G is probably able to mimic an ideal environment for chondrocytes creating an hypoxic condition close to joint in vivo.

The other aspect that we evaluated concerns the analysis of the major chondrocyte markers. We found that neither collagen type 2, nor collagen type 1 and 10 were affected by the different hGH concentrations tested. It has been shown on chondrocytes from rats that GH at low concentrations induce collagen type 10 expression (Wu *et al.*, 2011), highlighting a differential effect with respect to human OA chondrocytes. When hGH was combined with HYADD®4-G did not affect the expression of typical chondrocytic marker (collagen type 2), or of typical fibrotic (collagen type 1) and hypertrophic (collagen type 10) chondrocytic markers, as previously reported using the same HYADD®4-G concentration. (Smith *et al.*, 2013).

In line with another report (Dore et al., 1995) we found that hGH, alone or in combination with HYADD®4-G, was unable, at all different concentrations tested, to induce the release of IGF-1 or FGF2 by chondrocytes, thus suggesting that STATs signaling, strictly dependent on SOCS2, was not triggered by GH in these cells. IGF-1 in basal conditions is not released by human OA chondrocytes or human epiphyseal chondrocytes (Fernandez-Cancio *et al.*, 2009), thus indicating that GH and IGF-1 do not act in concert as usually occurs in animal proliferating chondrocytes (Hunziker et al., 1994). In fact, although IGF-1 and GH are released in the synovial fluid and serum of OA patients (Denko *et al.*, 1996), our data indicate that it is not GH that trigger GH release.

Our data also evidenced that OA chondrocytes after hGH treatment at high concentration (100 µg/ml)induce the release of IL6 , whereas when in combination with HYADD®4-G its production was significantly reduced. These data were in line with a recent study (Smith *et al.*, 2013) that showed that HYADD®4-G alone was already able to reduce dose-dependently IL6 expression.

To define the role of these two molecules better, we focused our attention on the suppressor of cytokine signaling SOCS2 a negative regulator of GH signaling. It was shown that SOCS2 mRNA was reduced approximately eight fold in OA chondrocytes compared to controls (de Andres et al., 2011). Our data show that SOCS2 mRNA

expression was not induced by all the hGH concentrations tested, suggesting that GH/STAT/IGF-1 signaling pathway is blocked and explaining the absence of IGF-1 release by treated chondrocytes. Conversely, our data highlighted for the first time that HYADD®4-G treatment was able to induce SOCS2 expression at all the time points tested, and increased nuclear protein translocation, thus indicating a direct effect of HYADD®4-G on a negative regulator of hGH. This finding confirms that HYADD®4-G modulates SOCS2 and might also explain the reduced metabolic activity found after treating human chondrocytes with HYADD®4-G.

Together, all these findings, evidence that hGH and HYADD®4-G combination is biologically effective on OA chondrocytes since these factors act on the respective receptors so balancing their effects.

Moreover, our data demonstrate a proportional decrease of some of the parameters tested (metabolic activity, collagen type 1, 2, 10 and SOCS2 expression) was found with the increasing the age of the patients, thus supporting an age-dependent response to the targets analyzed. According to our data, this age-dependent response was previously reported also for other chondrocytes parameters such as matrix synthesis (Bolton *et al.*, 1999), reduced responsiveness to growth factors (van der Kraan *et al.*, 2010).

These data also suggests the importance to define an age-range for OA clinical treatments, where the effects of intra-articular treatment could be more efficacious for its use in patients. Our data indicated that, even though, the treatment tested were independent to the age, suggesting that their use could be advantageous for OA treatment independently than the age of the patients.

In conclusion, our data show that hGH was unable to induce human OA chondrocyte metabolic activity, IL6 or IGF1 or FGF2 release or chondrocytic markers but increased the expression of CD44. The effects of hGH on human chondrocytes were down-modulated when used in combination with HYADD®4-G, which significantly induced SOCS2 expression and translocation into the nucleus. However, these treatments did not affect the response of the OA chondrocytes demonstrating that the cellular response was age-independent and suggesting that their efficacy was not related to the age of the OA patients.

However, further experiments are necessary to evaluate the pathways involved in this regulation process and focusing on chondrocytes in 3D culture systems using a defined GH and HYADD®4-G concentration; to confirm the efficacy of this treatment on the other tissues that compose the knee joint (e.g. synovium and bone).

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