Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Biomediche e Neuromotorie

Ciclo XXXIV

Settore Concorsuale: 05/E1

Settore Scientifico Disciplinare: BIO/10

NOTCH-1 SIGNALLING AND OTHER MOLECULAR MECHANISMS IN OSTEOARTHRITIS: POTENTIAL THERAPEUTIC ROLE OF OLIVE-DERIVED POLYPHENOLS

Presentata da: Veronica Panichi

Coordinatore Dottorato

Prof.ssa Matilde Yung Follo

Supervisore

Prof. Flavio Flamigni

Co-supervisore

Prof.ssa Silvia Cetrullo

Prof.ssa Paola Taddei

Esame finale anno 2021

Abstract

Age-related diseases have become in recent years a progressive issue due to aging of the population. Among these, osteoarthritis (OA) is the most common joint disease worldwide and the primary cause of disability in the elderly. Chondrocytes, the only cell type in articular cartilage, are primarily involved in the progression of the disease. The molecular mechanisms underlying osteoarthritic modifications in cartilage have not been elucidated entirely but it is clear that onset of the disease triggers the maturation of chondrocytes, which are blocked in a maturational resting state in healthy cartilage, through hypertrophy and terminal differentiation. These steps replicate what happens during endochondral ossification in embryogenesis and lead to progressive loss of tissue homeostasis, remodelling and degeneration of extracellular matrix and subsequent calcification of cartilage. These processes are sustained by a low-grade inflammation, which further aggravates chondrocyte response to damages. Finally, mineralization of cartilage allows vascularisation and innervation of a tissue that is, normally, avascular and aneural. From a clinical point of view these changes translate with progressive stiffness, reduced mobility and pain. Due to its multifactorial nature, there are no disease-modifying therapies available for OA to date, and treatment is addressed to pain management. Current research in the field is focused on finding new targets for therapy and novel therapeutic approaches. Among these, nutraceuticals (including food-derived bioactive compounds) present an interesting option. Our study aims to investigate the molecular mechanisms involved in the onset and progression of OA and, in particular, to understand the role of NOTCH-1 signalling, a novel target of interest, in the disease. This signalling pathway is a major regulator of endochondral ossification and is important to maintain homeostasis of chondrocytes in mature cartilage. However, it was found to be deregulated in OA therefore suggesting a potential role in the pathogenesis.

We used two *in vitro* model of OA: primary chondrocytes derived from patients with OA undergoing total knee arthroplasty and C28/I2 cells, a human chondrocyte cell line. Both 2-D and 3-D cultures were used to assess the role of NOTCH-1 in OA. Protein and gene expression of pivotal genes was investigated along with matrix degradation after transient silencing of NOTCH-1. Our results indicate that this signalling pathway exerts a pivotal role in the progression of OA and especially in chondrocyte differentiation and matrix remodelling by the regulation of key pathways in OA, such as those of MMP-13, the main metalloprotease involved in matrix degradation, RUNX-2, a differentiation factor expressed by hypertrophic chondrocytes, VEGFA, NFKB1 and its regulator IKKα. NOTCH-1 silencing also prevented cartilage calcification and reduced cell death in

micromasses. Furthermore, we investigated the potential therapeutic and chondroprotective role of two olive-derived nutraceuticals, oleuropein (OE) and hydroxytyrosol (HT), *in vitro*. Our data showed an antioxidant effect of both compounds against LPS-induced ROS production in C28/I2 cells. In addition to that, OE and HT were effective in decreasing the expression of inflammation markers, as well as of NOTCH-1 and its downstream targets. The blocking of its active domain NICD with γ-secretase inhibitor DAPT also supported the role of NOTCH-1 in LPS-induced effects, showed by the decreased expression of MMP-13. Finally, we investigated the crosslink between NOTCH-1 and potential upstream regulators of its activation. In particular, our preliminary data suggested that the downregulation of SIRT-1 leads to increased expression of NOTCH-1 and its putative target MMP-13. Another possible pathway involved in LPS-mediated effects in NOTCH-1 signalling is that of JNK, which we found to be more present in the phosphorylated, active form in LPS-stimulated C28/I2 cells, but decreased by nutraceutical pre-treatment.
Taken together our results identify specific mechanisms of NOTCH-1 involved in OA and demonstrated that its transient silencing delays the progression of OA in *in vitro* models. Furthermore, we proved that olive-derived compounds, OE and HT, exert a significant antioxidant and chondroprotective effect in chondrocytes and are able to modulate the expression of NOTCH-1 as well as OA markers that are regulated by this pathway. This evidence suggests a potential therapeutic use of these compounds in OA management.

Index

Osteoarthritis 5
Epidemiology 5
Anatomy of the human knee joint 5
Articular cartilage morphology and endochondral ossification 6
Extracellular matrix structure 8
Endochondral ossification 11
Risk factors associated with OA 13
Osteoarthritic modifications in articular cartilage 16
Therapy and management of osteoarthritis 20
Notch signalling pathway 23
Canonical activation of Notch 26
Notch in articular cartilage and osteoarthritis 29
Nutraceuticals 31
Material and methods 36
Cell isolation and culture 36
Cell treatment 37
RNA interference mediated NOTCH-1 gene silencing 37
RNA interference mediated SIRT-1 gene silencing 38
3-D cultures (micromasses) set up 38
Proliferation assay (PicoGreen) 39
RNA extraction and real time RT-PCR 39
Western Blot 40
MMPs quantitative assessment 41
Viability assay in 3-D constructs 42
Alizarin-RED staining 42
Toluidine Blue staining 43
Immunoistochemical and immunofluorescent staining 43
DCFDA Cellular ROS Assay 44
Statistical analysis 44
Aim of the study 45
Results 45
Validation of NOTCH-1 silencing 45
NOTCH-1 silencing interferes with chondrocytes proliferation 46
NOTCH-1 knock down delays primary chondrocytes differentiation in 3-D cultures 47
NOTCH-1 transient silencing ameliorates matrix remodelling in micromasses 50
Chondrocytes viability increases after NOTCH-1 KD 51
Lipopolysaccharide stimulates inflammation markers in C28/I2 and primary chondrocytes 52
Olive-derived nutraceuticals are efficient in limiting the effects of LPS treatment 53

OE and HT show antioxidant properties against LPS-induced ROS production 54
OE and HT exert chondroprotective effects against markers of inflammation 56
OE and HT reduce LPS-induced expression of NOTCH-1 57
Olive-derived nutraceuticals modulate OA-related and inflammation markers 59
Discussion.. 63
Conclusions 65
References 66

Osteoarthritis

Epidemiology

Osteoarthritis (OA) is the most common joint disease in the elderly. With an average 15% of the population over 60 years of age suffering from this pathology, it is also becoming a significant concern in terms of expense for public health in developed countries [1]. OA can affect any joint of the body but, considering the prevalence among patients, the most affected cartilages are those of the knee, the hip, the hand, the spine and feet [2], with knees and hips largely more prevalent than the others.

The clinical definition of OA differentiates between pathological OA, determined by radiographic evidence, and symptomatic OA, which is characterized by onset of symptoms, such as pain and stiffness, as well as radiographic modifications [3]. Criteria defined by the American College of Rheumatology are the most used for assessment of clinical OA [4]. Anyway, thanks to the standardised methods used, radiography is considered the golden standard for OA diagnosis and severity grading based on the Kellgren and Lawrence (KL) system [5] with a grade higher than or equal to 2 for radiographic assessment of tibiofemoral knee OA [6, 7]. The Osteoarthritis Research Society International atlas defines semi-quantitative specific radiographic features for OA diagnosis, such as presence of osteophytes (growths at the level of the joint) and joint-space narrowing [8]. The World Health Organisation (WHO) adopted these criteria to define OA in clinical studies [9]. Another factor that has to be taken into account in OA studies is the socioeconomic burden linked to its treatment, although it usually underestimates the most relevant morbidity of this pathology, which is disability due to motor limitation in severe or end-stage disease [10, 11]. Population studies also suggest an increased risk of mortality due to cardiovascular diseases and dementia among patients with clinical and radiographic diagnosis of OA compared to the general population [12, 13], but evidence of shortened lifespan due to OA is still limited.

Anatomy of the human knee joint

From a functional point of view, the knee consists of two joints: the patellofemoral (between femur, patella and rotula) and the tibiofemoral (between femur and tibia). The patella articulates with the femur through the medial and lateral facets of the condyles, which, in turn, through their convex shape, articulate the concave surface of the tibial plate. The interaction of these surfaces allows extensor mechanism and knee flexion. The patella is contained by the articular capsule, a sleeve shaped structure that covers the joint limiting damages due to attrition of knee bones. Articular

cartilage, located at the epiphysis of tibia and femur, is the tissue responsible for this fundamental role. Articular cartilage allows a balanced distribution of loads thus limiting friction between long bones. The degeneration of this thin layer is the primary reason of OA onset. The articular capsule is surrounded by the synovia, whose structure delimits a cavity in which the synovial fluid allows lubrication of the joint while also supplying articular cartilage with nutrients. Other structures such as the lateral menisci, tendons and cruciate and collateral ligament contribute to the correct distribution of loads and the integrity of the articulation [14]. The anterior cruciate ligament is particularly important in the context of OA, since its rupture has been linked to potential increased risk of developing a subtype of OA known as post-traumatic OA. Breaking of subsidiary structures necessary for the integrity of the joint, such as the menisci, is also a common method for inducing OA-like features in *in vivo* experimental models, as in the case of destabilization of the medial meniscus (DMM) [15].

Articular cartilage morphology and endochondral ossification

Healthy articular cartilage has the unique role of allowing nearly frictionless movement of the joint thanks to the structure and mechanical properties shortly described previously. But, compared to other tissues, cartilage has very limited capacity of self-repair. To date, no artificial material has been able to efficiently reproduce its functions [16] meaning the ability to tolerate mechanical stress and revert its morphology in response to deformation thanks to the plasticity of the matrix [17]. Articular cartilage consists of a thin layer, generally between 3-4 mm and spacing up to 6-8 mm in thickness in the knee [18, 19]. It is an avascular and aneural connective tissue where chondrocytes, the only cellular element, are embedded in a complex extracellular matrix (ECM) consisting of collagen, glycosaminoglycans, proteoglycans and other non-collagenous proteins. In normal adult cartilage, chondrocytes are in a state of proliferative and differentiative quiescence known as resting state. Despite lacking of proliferative activity, in physiological conditions, they are responsible for the extremely slow turn-over of the ECM components through the expression of anabolic and catabolic factors. It was calculated that, in normal condition, the half-life of collagen type II (COLII), an important component of the ECM, is around 120 years, and 24 years for aggrecan while other elements, for instance glycosaminoglycans, only require 120 days for complete turnover [20].

From a morphological point of view, chondrocytes are arranged in four different zones depending on the grade of differentiation and the expression of ECM components. The thickest part of cartilage consists of non-mineralized tissue, comprising for up to 90% of the total volume as shown in Fig. 1, it can be divided in three zones: the superficial, the middle and the deep zone [16, 21].

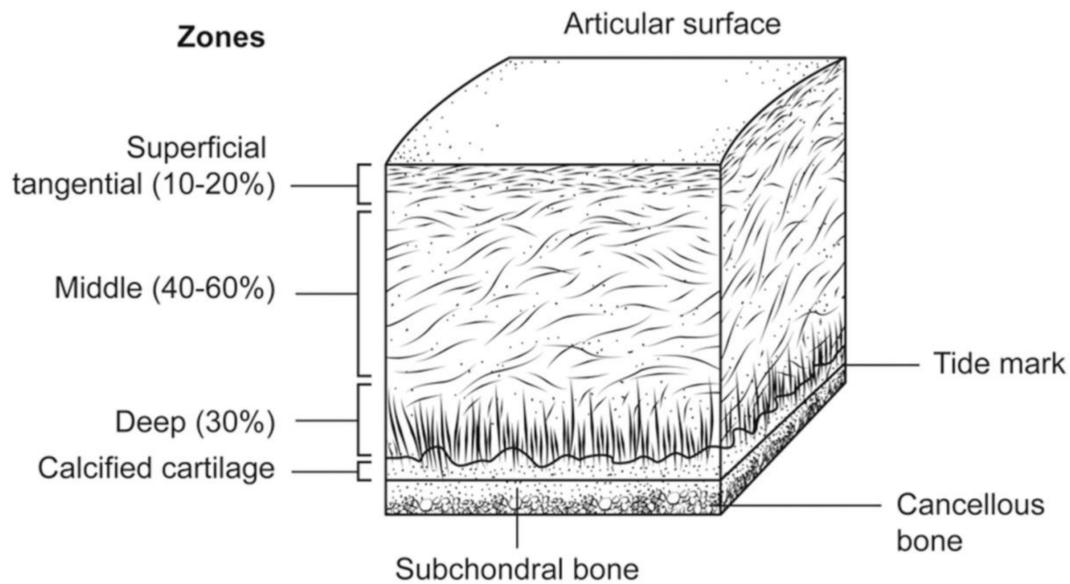


Figure 1. Articular cartilage is organized in four different layers depending. From the superficial to the deep zone different layers are defined by specific structural organization and morphological changes in chondrocytes reflecting the maturational process throughout terminal differentiation occurring in endochondral ossification [16].

The superficial or tangential zone (SZ), as the name suggests, is the outermost layer of non-mineralized cartilage and mostly responsible for resisting rotational and arthrokinematic forces [16]. This zone is the richest of cells and fibers, chondrocytes are elongated and fibers, rich in decorin and low in aggrecan expression [20], are organized in parallel to the surface and covered by a structure known as the *lamina splendens* that helps increasing resistance to shear forces [22]. In spite of this, chondrocytes in this layer show very low metabolic activity and repair capacity therefore making the SZ the most susceptible to damages and degeneration that subsequently expose underlying layers to shear forces [16].

The middle or transitional zone (MZ) contains fibers with larger diameter interspersed randomly along with chondrocytes in the layer. Higher presence of metabolically active organelles (mitochondria, endoplasmic reticulum and Golgi membranes) also grants higher reparative capability [16].

Finally, the deep or radial zone (DZ) below the MZ shows even larger fibers which, along with chondrocytes, are disposed perpendicularly in respect to the underlying surface. In this layer water content is minimal while proteoglycan expression increases significantly compared to the overlying zones. This structure allows resistance to compressive and tensile forces [16].

The fourth layer of articular cartilage consists of the calcified zone, in which the tidemark delineates the border between the DZ and the subchondral bone, that we can define as the interface between the thin layer of calcified cartilage and the trabecular bone. The tidemark also defines the source of

nutrition in adult cartilage: above the tidemark, diffusion of nutrients from the synovial fluids serves chondrocytes, while, under the tidemark, vascularization provides necessary nutrients to the subchondral bone [16, 22]. The presence of this lonely source of nutrition in adult chondrocytes is the primary reason for impaired recovery capability and low rate metabolism, but is also necessary to keep cartilage properly functional.

Hypoxia is another pivotal feature of cartilage tissue in consequence of the absence of vascularization. The maintenance of a hypoxic environment is necessary for chondrocytes homeostasis and *in vitro* evidence suggests that it allows higher synthesis and accumulation of COLII and aggrecan. Hypoxia has also a protective role since matrix degrading protein expression is lower compared to normoxia conditions [23]. In the hypoxic status, cells are maintained by the expression of the hypoxia inducible factor 1 alpha (HIF-1α), which stimulates the activation of proteic transporters GLUT3 and GLUT1 [24] that support chondrocytes with adequate intake of glucose, therefore promoting their function and survival [25].

Across the MZ and the DZ we incur into a progressive loss of cellular density and increase in cell and fibers volume. Morphological changes in chondrocytes reflect the difference in the matrix suggesting that the expression pattern of differentiation markers expressed by the microenvironment is responsible for these changes [20].

Chondrocytes are flat in the SZ and assume the typical round shape in the MZ, while in the DZ they are grouped in clusters [20]. Resting chondrocytes are located in the DZ; these chondrocytes remain in an arrested maturation throughout adult life in healthy cartilage but, undergo a differentiation process in OA that leads to the proliferative phenotype. Proliferating chondrocytes are adjacent to the resting ones. These cells undergo cytological changes losing the typical round morphology for a flat one to pass in the transitional stage of pre-hypertrophic chondrocytes. This differentiation of chondrocytes is normally blocked in adult cartilage, but active in embryogenesis during the process of endochondral ossification when these cells will undergo terminal maturation and cell death to be replaced by bone cells [26].

Extracellular matrix structure

Contrarily to most tissues, functional properties of articular cartilage are not directly due to the cellular component but rely mostly on the ECM. Comprising collagen fibrils and proteoglycans, the structure of healthy ECM allows resistance to compression by dissipating loads through its complex structure. Collagen is the main fiber of ECM, it is synthetized by chondrocytes and contributes to more than half of the weight of dry cartilage. COLII is the prevalent type in ECM, although others, like collagen IX, X, and XI are expressed depending on the layer and differentiation phase of

chondrocytes [16, 27]. Cross-link between COLII fibers creates a net that allows resistance to compression [19]. The second components of the ECM are proteoglycans. They are also synthetized from chondrocytes and consist of glycosaminoglycans (GAG) attached to a core protein. GAG are long polysaccharide chains with repeating disaccharides [28] with at least one negatively charged sulphate. GAG subunits found in articular cartilage are: chondroitin sulphate, keratan sulphate and dermatan sulphate. The presence of negative charges is fundamental to allow GAG interaction with water and positively charged ions (Ca^{++}, Na^{+}) [19]. In addition to that, the interplay between opposite GAG chains, showing negative subunits, keeps the fibers expanded and maintains the ECM mechanically functional. Another essential component of the ECM is hyaluronic acid, or hyaluronan, which is not attached to the core protein but aggregates proteoglycans by means of a link protein. When a single hyaluronan molecule attaches to multiple proteoglycans, the main complexes of cartilage ECM, known as aggrecans, are formed. These complexes, shown in Fig.2, can link hundreds of GAG molecules together, measuring up to 10,000 nm in length [29] and are indispensable to maintain cartilage function.

Although chondrocytes just made up to the 10% of the total volume of the tissue, they are responsible for the maintenance of the remaining 90% complex structure previously described. Therefore, any loss in volume or function impairs cartilage health [16]. Aging tissue shows a progressive loss of chondrocyte functionality that impacts indirectly the ECM capacity of protecting chondrocytes from mechanical stress and creates a vicious cycle that leads to onset of OA.

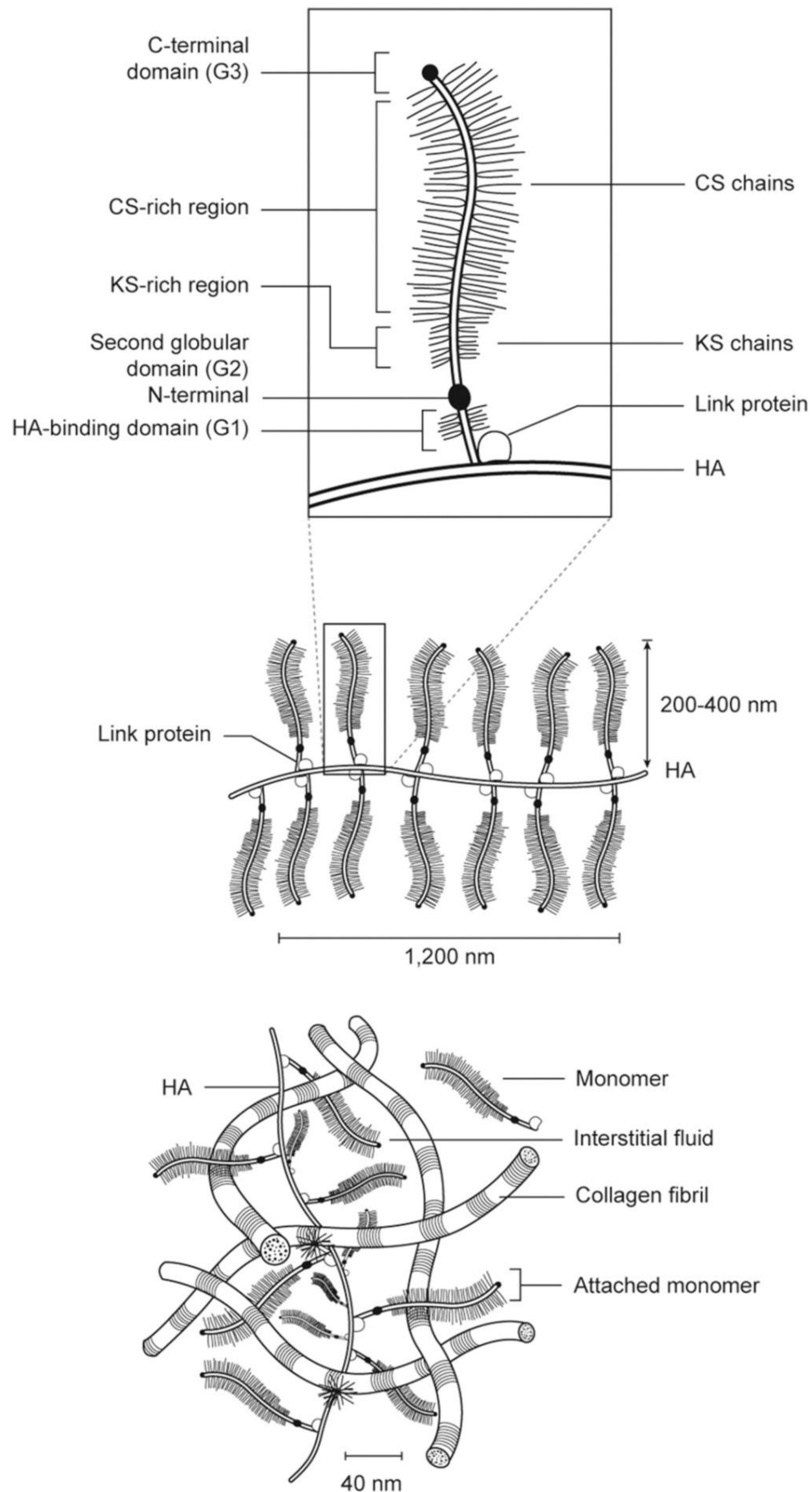


Figure 2. Structure of aggrecan[16]. HA, hyaluronic acid; CS, chondroitin sulphate; KS, keratan sulphate.

Endochondral ossification

Cartilage tissue is a necessary intermediate for bone formation during embryogenesis. Two processes regulate the formation of the skeleton in mammalians: intramembranous bone formation leads to the production of flat bones directly from mesenchymal progenitors, while endochondral ossification allows the formation of the axial skeleton from a cartilage template (anlagen) [21]. Bone formation requires several steps starting from chondrogenesis, the process of maturation and terminal differentiation of chondrocytes, followed by their substitution with osteoblasts. This process is finely regulated by changes in the pattern of expression of few essential factors through three phases: mesenchymal condensation, differentiation and maturation of chondrocytes [30].

Mesenchymal condensation is a process first described in 1925 by Fell [31] that starts around the fourth week of embryogenesis when, in humans, limb buds begin to develop into the appendicular skeleton [32]. The growth plate and the articular-epiphyseal cartilage are the two locations at each end of the developing bone where cartilage differentiates [26]. The first step in endochondral ossification, mesenchymal condensation, consists in the recruitment of mesenchymal cells from three sources: craniofacial bones form from progenitors of the neural crest in the ectoderm, the axial skeleton derives instead from the somites of the mesoderm and finally progenitors of limb bones are found in the somatopleure of the lateral mesoderm [33]. Common mesenchymal precursors during the phase of condensation develop into myogenic lineage that will later form muscles and chondrogenic lineage that will lead to the formation of cartilage [21]. The molecular mechanisms that control this first phase are not completely understood but currently it is believed that several systems, finely regulated, work sequentially through the process [34]. Bone morphogenetic proteins (BMPs), SOX9 (SRY box transcription factor 9), fibroblast growth factor (FGF) and Wnt pathways seem to be essential for the maintenance of mesenchymal cell aggregates and guide the following stage of chondrogenic differentiation [30, 35]. Correct development is directed by two signalling centers: the apical ectodermal ridge produces signals responsible for the proximal-distal outgrowth, and the zone of polarizing activity for anterior-posterior patterning [33, 36]. In order to increase cell adhesion to form precursor clusters, an increase in hyaluronidase, neural cell adhesion molecule (N-CAM), and neural cadherin (N-cadherin) is also observed during condensation. These molecules are instead not expressed during chondrocyte differentiation [21]. Following condensation, chondrogenesis, i.e. the differentiation of cells in chondrocytes, begins. Cartilage matrix, characterized by the expression of COLII, IX, and XI and aggrecan, is deposited under the control of SOX9 [37]. BMP and FGF expression seems to be in large part responsible of the proliferation rate of chondrocytes during their differentiation process [38], but multiple signals, expressed at

different stages, cooperate with them to regulate chondrogenesis. Once they reach the pre-hypertrophic phase, chondrocytes can either maintain their chondrogenic lineage or proceed through endochondral ossification to form long bones. In this regard, chondrocytes forming the anlagen start terminal differentiation first acquiring the hypertrophic phenotype, then increasing in cellular volume up to 20 times (Fig. 3). Hypertrophic chondrocytes express Rant related transcriptor factor 2 (RUNX-2, also known as core binding factor 1 or Cbfa1) and Indian hedgehog (Ihh) signalling [21, 39]. Wnt/β-catenin signalling is also necessary to inhibit mesenchymal condensation thus allowing differentiation of hypertrophic chondrocytes [40, 41]. Another signalling pathway, which will be discussed more in detail later, which plays a pivotal role in the regulation of chondrogenesis, especially in chondrocyte hypertrophy and differentiation is NOTCH.

Evidence suggests that an increase of NOTCH activation and its primary targets HES-1 and HEY-1 inhibits the expression of Sox-9-mediated COLII activation by binding the col2a1 enhancer [42, 43]. These results hint the capacity of NOTCH of blocking chondrocyte differentiation. NOTCH-1, the most well studied of the four receptors, is expressed in cell clusters during mesenchymal condensation and later in several layers of differentiating chondrocytes, specifically the pre-hypertrophic and hypertrophic layers of articular cartilage [44]. Other two receptors of the family, NOTCH-2 and NOTCH-3, are instead mostly expressed in proliferating, pre-hypertrophic and hypertrophic chondrocytes across the different layers of the growth plate and articular cartilage [45], suggesting different activities during early or late phases of chondrocyte differentiation.

Differentiation is followed by rapid proliferation of chondrocytes, leading to the expansion of the growth plate and linear ossification starting from this location. As previously described, final maturation of chondrocytes leads to a significant increase in volume and release of components from the matrix. Mineralization of these, along with expression of collagen X will lead to the production of hydroxyapatite (calcium and phosphate crystals) that forms the bone [26].

Evidence also suggests that MMP-13 is important in this phase [46, 47]. ECM remodelling, also one of the main features of OA, proceeds simultaneously to terminal differentiation, controlling the time-rate that leads to cartilage calcification [48] and forming the thin layer that will remain in the adult as the interface with the subchondral bone. Further regulation of MMP-13 is led by expression of RUNX-2 and NOTCH-1 [30]. Signals from the ECM are also necessary for vascular invasion of the hypertrophic zone and the perichondrium is the last step required for ossification. Angiogenesis is led by stimuli, particularly from the vascular endothelial growth factor (VEGF) [49], which is released in the ECM under the influence of remodelling factors such as MMP-9 and MMP-13 [50]. This sequence of events allows the recruitment of osteoclasts and osteoblasts that replace mineralized cartilage with bone tissue [21].

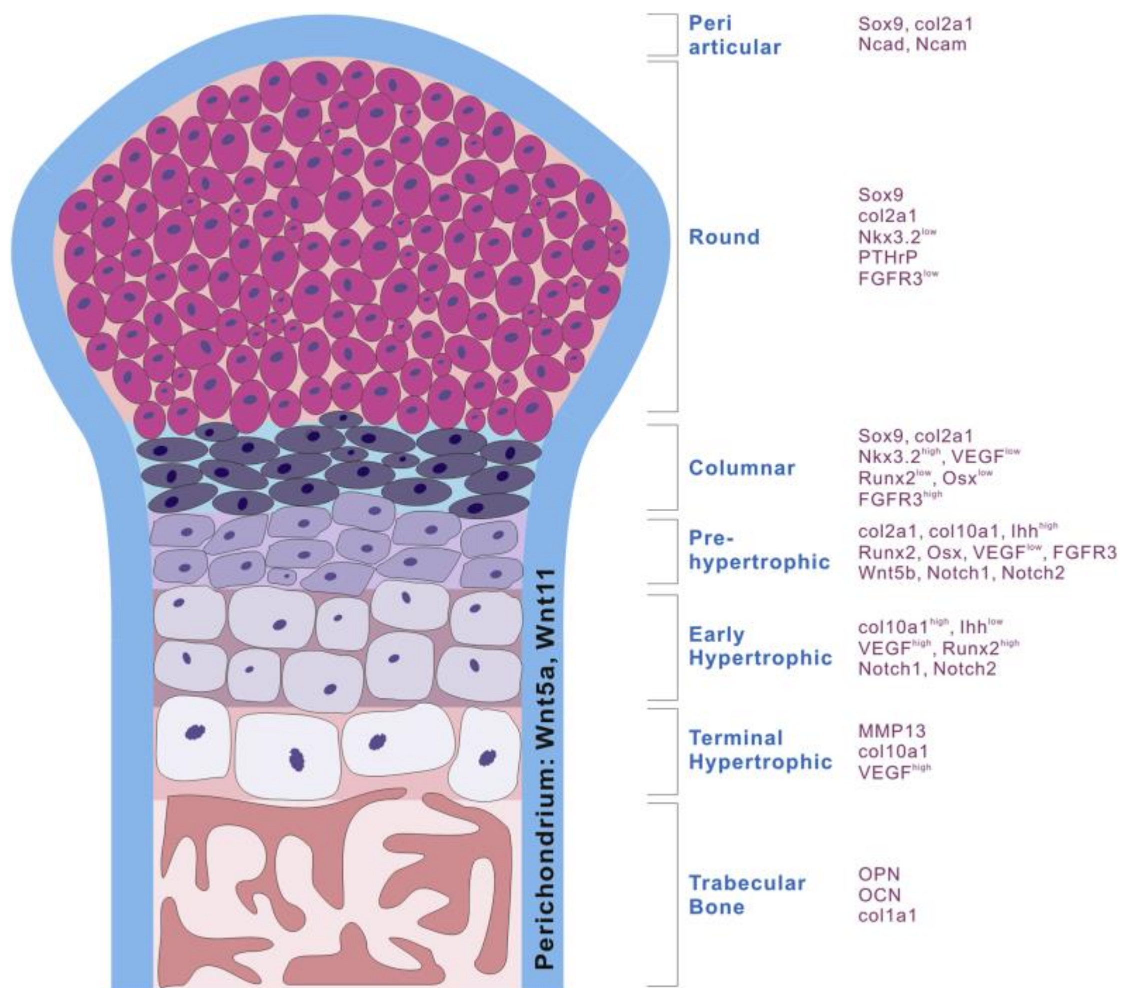


Figure 3. Maturational process of articular chondrocytes across the growth plate during endochondral ossification and differential expression of molecular markers that regulate terminal differentiation [51].

Risk factors associated with OA

Due to its multifactorial nature, predisposition to develop OA is associated with genetic, biological, and biomechanical risk factors both systemic and local. Depending on their nature, these factors can also be divided in modifiable and non-modifiable (as shown in Fig.4).

The most important association with OA is certainly age [52] and this could explain why initially this pathology was described as a “wear and tear” thus correlating the onset of the disease to mechanical degradation of cartilage and corresponding loss of regenerative capability of the joint tissue.

Prevalence data and clinical evidence clearly show that women are more often and more severely affected than men by OA. An association between female gender and symptomatic OA of the knee, hip and hand has also been proved [53-55]. In addition to that, the onset usually follows menopause [56] thus suggesting a potential connection with oestrogens, but, up to date, their role remains unclear. Cohort studies focusing on risk factors associated with OA onset are an important source of data, but are also impaired by difficulties in the follow-up of such vast study populations, therefore results based on patients who have completed the study should be taken into particular account [7]. Local factors such as muscular and bone strength and joint morphology could also be responsible of gender disparities [3] but, once again, lack of evidence prevents certainty in this regard.

Up to 40% of knee OA and 60% of hip OA are influenced by genetic predisposition [57]. Genome-wide studies have identified several loci associated with higher predisposition to OA but, to date, research has failed in identifying a single accountable gene for the majority of the genetic risk [6]. Several studies agree in identifying GDF-5 (growth/differentiation factor 5) mutations in association with knee OA [58-60]. This protein is a member of the bone morphogenetic protein family and is involved both in joint formation and musculoskeletal phenotypes of the hip and tendons [61, 62]. Despite current lack of information, further genome-wide studies are believed to be fundamental to uncover potential genetic association with OA development, which may be of great importance not only to detect high-risk individuals but also to identify specific genes or pathways that could be targeted for therapy.

Along with ageing population, the increase in prevalence of obesity in recent years has proven not only to represent an important morbidity and mortality cause but also a contributing factor to the onset of OA. The correlation between obesity and the development of OA is so relevant that recently a new subtype of OA, named metabolic OA, has been defined when pathological features of OA are present in association with metabolic syndrome [63, 64]. Obesity could act both mechanically, due to increased load on the joints due to high body weight, and systemically in association with inflammatory and metabolic factors [7]. Data obtained by a meta-analysis demonstrated a direct correlation between the increase on BMI (body-mass index) and risk of OA especially of peripheral joints such as the knee [65]. Interestingly, the most extended population study carried out in OA patients, the Framingham Study, has related weight loss to a decrease by 50% of risk of developing OA [66] as also confirmed by a more recent study [67]. These findings taken together speculate that almost 30% of knee OA could be prevented by weight loss and BMI <30 [6].

Another systemic factor that contributes to the etiopathogenesis of OA could be the diet and specifically nutritional intake of vitamins, such as vitamin C, K, and D, necessary for cartilage and

bone metabolism throughout early life and development. Deficiency of these nutrients has been linked to increased predisposition to OA, although no evidence has been found regarding a reduced risk in case of high intake [68, 69].

Local factors also contribute to OA development. Occupational or physical activities that require repetitive movements borne by the joints are associated with higher risk of OA [70, 71]. A specific correlation has been found in professional athletes although it is unclear if injuries could also play a role in this specific sub-population [72]. Post-traumatic OA is a is sub-type of osteoarthritis that follows joint related-injuries and particularly, in regard of OA, knee injuries that involve the rupture of the anterior cruciate ligament and the meniscus which combined lead to an increase up to 40% of prevalence of OA after 10-15 years [3, 73]. Other minor factors such as *quadriceps femoris* and knee extensor strength [74], leg length inequality (LLI ≥2 cm) [75], tibial and femoral morphology and joint alignment could contribute to an increased susceptibility of the joint to develop OA considering that joint biomechanics depend on anatomical and functional features. In spite of these association these risk factors should be considered of minor impact since in the majority of cases do not lead to development of OA [1]. In addition to that, some of the above-described features, specifically joint alignment and LLI, can be easily modified with surgery. Therefore, to date, among the modifiable factors taken into account, intervention is supported by data only in presence of obesity or in prevention of injuries [3].

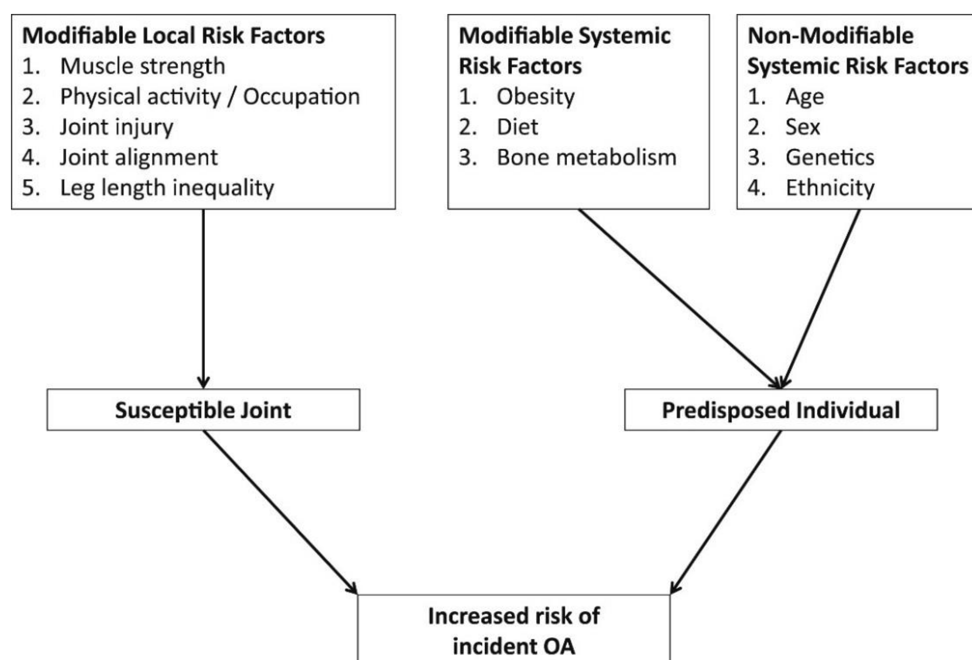


Figure 4. Risk factors associated with OA can be divided into modifiable (local and systemic) and unmodifiable [3].

Osteoarthritic modifications in articular cartilage

OA is now universally considered a whole joint disease, led by a disruption of homeostatic mechanisms that result in progressive degeneration of articular cartilage and loss of the essential mechanical properties that allow its function. Although the ECM is the primary target of cartilage degradation, the changes in articular chondrocytes, responsible for the anabolic and catabolic balance of the ECM, are predominant in instigating the onset of the disease. Chondrocytes meet a series of changes due to aging that lead to impaired metabolism that reflects, but is also exacerbated, in changes on the ECM and the other structures that form the joint. The process of OA can also be seen as a recapitulation of the processes normally occurring during endochondral ossification. The maturational course of chondrocytes, in adult cartilage, does not enter the hypertrophic stage, therefore stopping ahead in the already mentioned resting state. During OA, for reasons not yet completely known, the differentiation of chondrocytes restarts and leads to morphological and functional changes required for the progression of OA to the end-stage. The stages of endochondral ossification mimed in OA start with matrix remodelling arising from hypertrophy and terminal differentiation of chondrocytes, followed by progressive calcification of cartilage clinically associated with stiffness. Then, progressive ossification follows with the appearance of osteophytes; finally, vascularization and innervation link to the appearance of pain in patients. Several aspects have to be considered when discussing the etiopathogenesis of OA. The first one regards the homeostatic modifications occurring in the matrix. On one hand, ECM is no longer compact and therefore becomes sensitive to the attack of enzymatic and mechanic stressors released in the matrix from the surrounding tissues, such as the synovia, while chondrocytes cannot sustain repairing this damage [76]. On the other hand, the impaired function of articular chondrocytes implies a direct action in the degradation of the ECM. This process that identifies chondrocytes as both cause and consequence of ECM degradation was defined as “chondrocytic chondrolysis” and it represents the point of no return in OA progression [76]. The effectors of this degradation are a group of matrix-degrading proteases that cleave COLII molecules thus compromising the resistance of ECM and subsequently the joint bearing to mechanic loads. An essential family of proteins involved in ECM degradation are metalloproteases (MMP). Among these, metalloprotease 13 (MMP-13), also known as collagenase III, is the main culprit for COLII cleavage. MMP-13 is released in its pre-enzymatic form and later activated by two other MMPs: the stromelysin 1, or MMP-3, which is the most expressed in OA cartilage, and stromelysin 2, or MMP-10 [77]. Other MMPs involved in matrix degradation are the collagenases 1 and 2, also known as MMP-1 and MMP-8, the gelatinases MMP-2 and MMP-10, which further degrade COLII

after MMP-13, and finally matrilysin MMP-7 and the macrophage metalloprotease MMP-12 [78, 79]. At first, it was believed that MMPs were also responsible for aggrecan degradation, but a study by Sandy et al. conducted in bovine cartilage, demonstrated that the cleavage site of aggrecans also occur in the intraglobular domain (IGD) of the core protein but at a different site compared to the one used by MMPs [80]. It was therefore concluded that a different enzyme, named "aggrecanase", was responsible for the complete loss of function of the ECM structure due to the release of GAG from the complex. [81]. Later studies led to the discovery of aggrecanase 1 and 2 [80, 82], now identified as ADAMTS-4 and ADAMTS-5, respectively. More recently ADAMTS-1 was also found to have aggrecanase activity in articular cartilage [83]. The ADAMTS (a disintegrin and a metalloproteinase domain with thrombospondin motifs) proteins along with the ADAMs (proteins with a disintegrin and metalloproteinase) belong to the metallopeptidase M12 family [84] and functionally are zinc-dependent metalloproteases [76]. These enzymes are overexpressed in OA, due to the metabolic changes in the microenvironment, and their combined activity is not counteracted properly by the anabolic activity of chondrocytes especially in the SZ of cartilage, more sensitive to degradation. Another factor that favours ECM degradation is the progressive accumulation of the so-called advanced glycation end-products (AGEs), due to normal aging mechanisms occurring in articular chondrocytes. These molecules are the result of non-enzymatic glycation reaction between lysine and arginine residues of proteins and reducing sugars such as glucose, fructose and ribose [85]. The accumulation of AGEs and the expression of their receptors (RAGEs) can cause an increase in cross-linking phenomena with collagen. In the long run, these abnormal complexes can impact the biomechanical properties of cartilage leading to joint stiffness, a typical symptom of OA [86]. Another interesting parallelism with endochondral ossification is that the expression of molecular markers, that regulate this process in the embryo, are expressed by chondrocytes and actively sustain the changes in cell metabolism in OA. One pivotal factor, in this regard, is RUNX-2 that, as we have mentioned before, allows hypertrophy and differentiation in chondrocytes during endochondral ossification. RUNX-2 is not expressed in normal cartilage, but its expression along with that of some of its targets increases in early stages of OA [87]. Known targets of RUNX-2 include MMP-13, ADAMTS-4 and -5 [88]. As previously explained, these proteases are the main responsible of ECM degradation and normally in articular cartilage their action is inhibited by the presence of Tissue Inhibitors of Metalloproteases (TIMPs), the endogenous inhibitors of MMPs, ADAMTSs and ADAMs proteases [89]. Expression of several members of this family of inhibitors, especially TIMP-3 and TIMP-4, was found to be significantly decreased in OA [77] therefore suggesting a possible explanation for the uncontrolled activity of proteases. RUNX-2 expression is also increased by the presence of IHH and Hypoxia inducible

factor 2 alpha (HIF2α) [90, 91]. HIF2α is not expressed in healthy cartilage but its expression increases in OA, and it was found to sustain chondrocytes hypertrophy and vascularization of articular cartilage by activating MMP-13, NF-κb and VEGF [92]. In addition to that, collagen expression varies in OA where collagen type X, typically present during chondrogenesis, is predominantly expressed instead of COLII [27]. Evidence suggests, more and more, that a low-grade inflammation is pivotal for maintenance and progression of OA. A specific pattern of expression of inflammation mediators, defined by Loeser and colleagues senescence-associated secretory phenotype (SASP) [93], can be found in articular cartilage due to aging and could be relevant in the pathogenesis of OA. The most important inflammatory molecules expressed in articular cartilage include interleukin-1β (IL-1β), which is responsible for induction of other cytokines, such as IL-17, IL-6 and the tumor necrosis factor alpha (TNFα). The release of these local mediators of inflammation activates a cascade of interconnected mechanisms that results in further increase the inflammatory state and cartilage degradation [94]. A direct consequence of the release of cytokines in the microenvironment is swelling, another common clinical feature of OA, probably due to the inflammation of the synovium and effusion [64]. The reasons behind synovitis, i.e. inflammation of the synovia, are largely unknown [95], but a plausible hypothesis is that, once degraded, fragments of cartilage come in contact with the synovium thus activating a protective response by releasing inflammatory mediators in the synovial fluid. However, cytokines can induce a positive feedback and sustain further increase of inflammatory molecules and, simultaneously, activate pro-angiogenic factors in the synovia, thus perpetuating a vicious cycle of cartilage degradation [64]. Johnson and colleagues have proposed a model for OA development based on nine molecular features linked with aging chondrocytes [3]. Among these, evidence corroborates that one plays a particularly relevant role in the pathogenesis of OA: oxidative stress. This process is strictly related to the increased production of reactive oxygen species (ROS) in OA. This increased production could be due to mitochondrial dysfunction in chondrocytes, which leads to a reduced anti-oxidant capacity of the cell, as results from decreased levels of superoxide dismutase 2 (SOD2), thus leading to oxidative damage [96]. ROS activity modulates the expression of other catabolic pathways such as MAPK, MMP-13 and RAGE activation [20].

Figure 5 summarizes the processes, addressed above, involved in the onset and progression of OA.

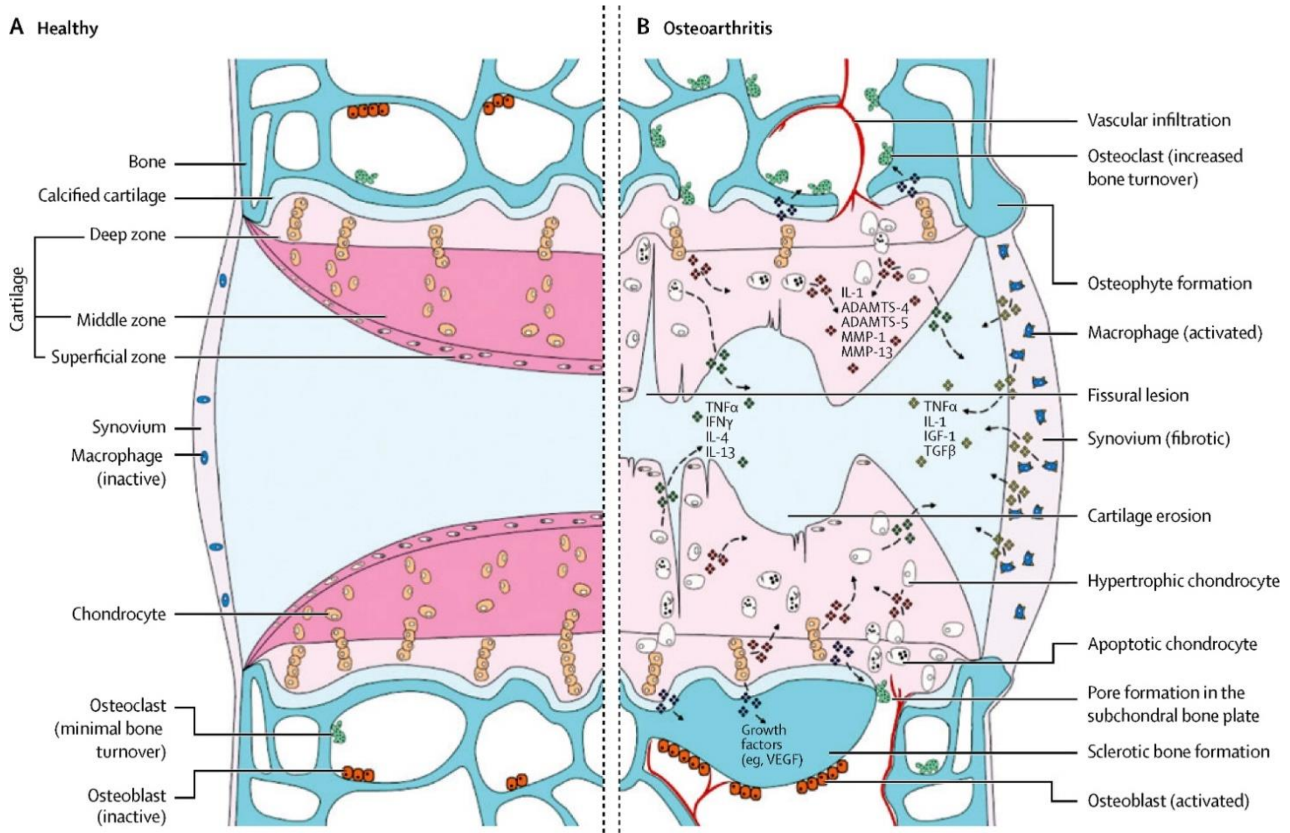


Figure 5. Modifications occurring in OA compared to healthy articular cartilage: degeneration of the extracellular matrix sustained by local inflammation disrupts chondrocytes homeostasis leading to terminal differentiation, vascularization and innervation of cartilage in end-stage OA [1].

Figure 5 suggests the importance of changes happening not only in chondrocytes and ECM, but also in the tidemark and calcified zone. As previously described, this is the interface between articular cartilage and the subchondral bone which is also impacted by the progression of OA, according with the new vision of OA as a "whole joint disease". In particular, attention is growing around the role played by the subchondral bone in relation with pain in OA. In fact, severe stages of the disease feature a progressive calcification of cartilage, due to terminal differentiation, that leads to an increased thickness of the subchondral bone. At this stage, a second front of perivascular ossification forms allowing the advancement of the tidemark and calcified zone. Consequently, flexibility and density of the underlying trabecular bone decrease leading to the osteophytes and worsening of pain perception [97]. Macroscopic modification can also appear in the tidemark and subchondral plate areas due to fragmentation of cartilage, compromising the compartmentalization between the different tissues forming the joint. A direct consequence of this disruption in tissues organization is the free traffic of fluids and molecules that further reinforce the inflammation-dependent damages. Fissures between the tissues are then filled by mesenchymal tissue and fibrocartilage [98]. In addition to that, ossification is accompanied by angiogenesis. Healthy cartilage is avascular, but this changes in late stage OA due to the disruption of the tidemark, which

allows the circulation of pro-angiogenic factors, particularly VEGF [99]. Formation of vessels in OA occurs through vascular channels that extend from the subchondral bone to the non-calcified layers, slowly triggering ossification around blood vessels [99, 100]. Finally, another factor contributing to end-stage OA is the expression of the nerve growth factor (NGF), which allows sprouting of sensory and sympathetic nerves, normally absent in articular cartilage, through the tidemark and calcified zone [101]. Cartilage innervation and vascularization are considered to play a primary role in the appearance of pain in patients, thus linking these phenomena to the clinical diagnosis and progression to end-stage OA.

Therapy and management of osteoarthritis

In spite of the increasing prevalence of OA, the lack of knowledge regarding its aetiology is probably the main reason for absence, to date, of disease modifying therapies (DMTs). Current treatment of OA mainly aims to reduction of inflammation and pain management, but drugs used for these purposes show therapeutic limitations and severe side effects. Therapeutic guidelines for OA currently follow a precise order consisting of behavioural intervention followed by prescription of analgesics and anti-inflammatory drugs, then minimally invasive medical procedures (intra-articular injections) and finally total knee replacement arthroplasty for end-stage patients [102]. Each one of these steps poses intrinsic issues. First, usage of analgesics with relatively light side effects, such as paracetamol, is limited for treatment of mild to moderate symptomatic OA, therefore this class of drugs is soon substituted in successive stages of the disease. In fact, more severe conditions, require the use of nonsteroidal anti-inflammatory drugs (NSAIDs) [103], currently the most common therapy for management of rheumatoid pathologies like OA, exerting their function through the inhibition of COX (cyclooxygenase) enzymes [104]. Although the majority of patients treated with NSAIDs report improvements in mobility and pain relief, prolonged use of these drugs leads to severe side effects predominantly borne by the gastrointestinal apparat due to the non-specific action of NSAIDs [105]. In fact, most NSAIDs inhibit both isoforms of COX enzymes: COX-2, the inducible form that increases in presence of inflammatory stimuli, and COX-1, the constitutively active isoform necessary for maintain homeostasis of tissues such as the gastrointestinal one [106]. In spite of this, both the European League Against rheumatism (EULAR) and the Osteoarthritis Research Society International (OARSI), in absence of alternative therapies, continue to recommend NSAIDs for OA patients with a cost-effective limitation for those who show severe side effects [107]. Opioid analgesics are another category of drugs used for pain management in OA, although the high risk of dependence given by this compound limits their use to patients who fail to respond to standard therapies [108].

An estimation reports that by 2030 the number of total knee replacements surgeries will jump up to 3.5 million yearly [109]. Surgery is, to date, the only available solution for end-stage disease and it is efficient in reducing pain and ameliorating joint functionality. In spite of this, total knee arthroplasty is not an optimal solution for patients who show onset of OA relatively early in age due to the limited lifespan of the prosthesis. Currently, research in the field is focused in developing more biocompatible materials that can integrate with the tissue thus slowing down the deterioration, but, although progresses have been made, the lifespan of prosthesis is up to 15-20 years [110]. This limit implicates multiple surgeries for patients with early onset of OA. Altogether, considering the risk-benefit ratio, current guidelines for OA therapy suggest arthroplasty as an option for those patients whose disease progression has severely impaired ambulation.

Current research is focusing on finding novel targets for the therapy of OA and possibly reliable biomarkers of OA. Biomarkers are indicators of normal or pathological processes or pharmacological effects in response to a therapeutic intervention, that can be objectively evaluated as defined by the Biomarkers Definitions Working Group [111]. Many possible targets as well as types of therapeutic alternatives have been proposed (Fig. 6).

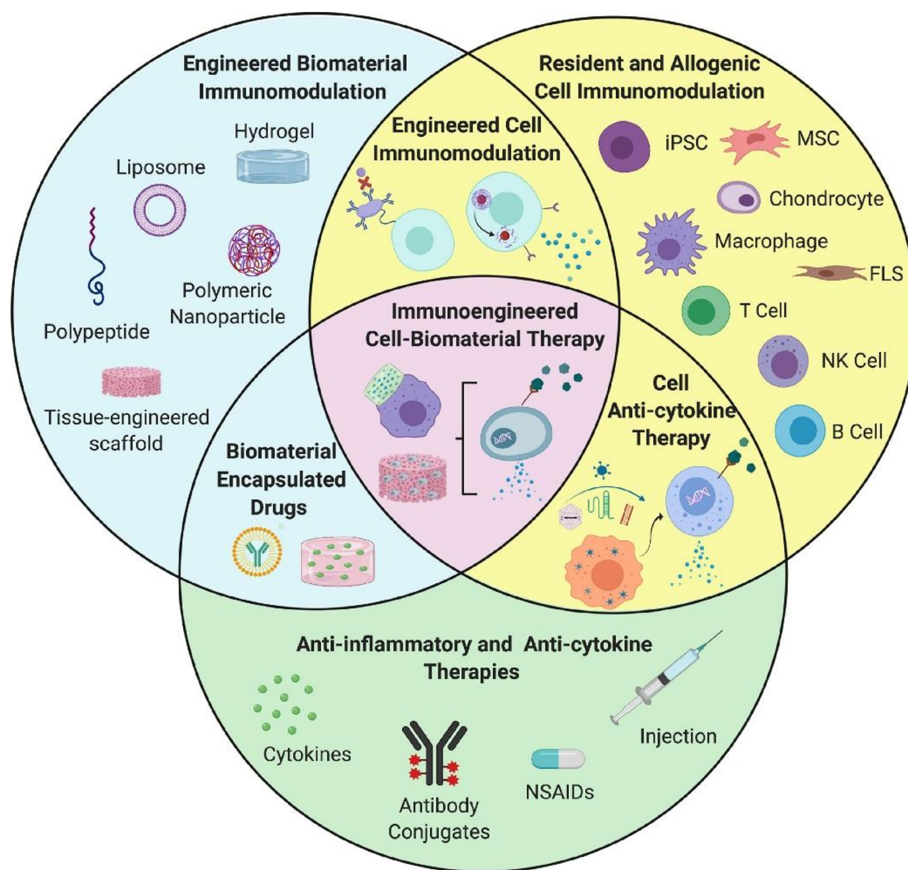


Figure 6. Currently available therapeutic options and future developments in OA therapy and management. The combination of multiple approaches along with identification of new therapeutic target seem to be the winning strategy [112].

For what concerns therapeutic strategies, at the present, immunoengineering poses the best foundation for future musculoskeletal diseases therapies [112]. Both cell- (mesenchymal stem cells, macrophages and induced pluripotent stem cells) and biomaterial-based (tissue-engineered scaffolds, polymeric nanoparticle) options have been explored in the past, but ever-new discoveries in the field suggest that a combination of these strategies with genetic engineering tools, such as CRISPR-Cas9 could be the best solution for fine tuning of mechanisms involved in OA [112].
The building of novel therapies for OA does not focus only on the best option for delivery but also on finding the best molecular target. For instance, inhibition of inflammatory-related pathways, like the Inducible Nitric Oxide Synthase (iNOS), could be a promising strategy considering the pivotal role of inflammation in the progression of OA previously described [105]. Another main feature of OA that could be targeted for therapy is the remodelling and degradation of ECM. In this regard, anti-MMP therapies could prove efficient in arresting this degenerative process [113]. More recently, Notch signalling pathway has aroused interest for the potential involvement of this family of membrane receptors in the molecular mechanisms driving OA changes in articular cartilage.

Notch signalling pathway

Notch is an evolutionary conserved signalling pathway in metazoans involved in the regulation of cellular processes from embryogenesis to adult life. Notch regulates cell fate using cell-cell interaction implementing cellular mechanisms, such as differentiation, proliferation and apoptosis to guide organogenesis [114]. The discovery of the first Notch mutant dates back to 1914 when John S. Dexter noticed the presence of *notches* in the wings of *Drosophila melanogaster* [115]. Shortly after, Thomas Hunt Morgan identified the mutant alleles correlating the abnormal phenotype previously observed in fly wings to a sex-linked mutation [116]. But it was only in the 1980s that Spryros Artavanis-Tsakonas and Michael Young managed to clone, independently, the Notch receptor [117]. These results sparkled a revolution and Notch became a focus of studies in a large number of fields that led to the discovery of the plethora of cellular mechanisms in which Notch is involved. In-depth studies in *Drosophila* have also elucidated the three main processes regulated by Notch during embryogenesis: lateral inhibition, lineage decision (or asymmetric assignation), and boundary formation. These mechanisms rely on space- and time-limited expression of Notch signalling that regulate cell fate and differentiation between sister cells derived from a common precursor. This fine regulation is kept through adult life where Notch plays a pivotal role in regulation of fundamental cellular mechanisms, maintenance of stem cells and general homeostasis of tissues [118]. Deregulation or abnormal expression of NOTCH signalling are linked to genetic and pathological conditions like cancer.

While in *Drosophila* there is only one, Notch signalling pathway in humans involves four genes that encode for as many receptors, namely NOTCH-1, NOTCH-2, NOTCH-3 and NOTCH-4. These receptors interact with five ligands: two homologs of Serrate ligands in *D. melanogaster* known in human as Jagged-1 (JAG-1) and Jagged-2 (JAG-2), and three Delta-like ligands (Dll-1, Dll-3, Dll-4). Table 1 shows chromosome mapping and general information for human Notch genes (Ensembl, Uniprot https://www.uniprot.org).

Notch receptors and ligands structure is also conserved and common features can be identified, as shown in detail in Fig. 7. Notch ligands belong to the Delta/Serrate/Lag2 DSL family of proteins [119]. The most important feature in the structure of these ligands is the region in the extracellular domain containing epithelial growth factor like repeats (EGF-like repeats), a series of repeated motifs that is essential for interaction with Notch receptors which seem to be regulated through another domain known as DSL. The main differences between jagged and delta-like ligands consists in the significantly smaller number of EGF-like repeats in the latter. Furthermore, Jagged

contains a cysteine-rich region that Dll completely lacks [120]. This region along with DSL is crucial for the interaction with the receptors [121].

Gene	**Localization**	**Exons**	**Transcript lenght**	**Number of aa residues**	**Protein weight (KDa)**
NOTCH-1	9q34.3	34	9371 bp (3 splicing variants)	2555 aa	272 KDa
NOTCH-2	1p12	34	11389 bp (8 splicing variants)	2471 aa	265 KDa
NOTCH-3	19p13.12	33	8666 bp (6 splicing variants)	2321 aa	243 KDa
NOTCH-4	6p21.32	30	6745 bp (5 splicing variants)	2003 aa	209 KDa

Table 1. Notch genes genome mapping and receptors structure.

Notch receptors are single passage type 1 transmembrane receptors, coded by a single precursor that becomes a non-covalently linked heterodimer. Notch receptors include an extracellular domain (NECD), a transmembrane domain (TMD), and a notch intracellular domain (NICD) which is the active subunit of the receptor [122]. The structure of the NECD is the most variable among the four receptors, although small differences can also be found in the NICD.

NECD is the largest part of the receptor and for the most it consists of EGF-like repeats, once again, essential for the interaction with the ligands. NOTCH-1 and -2 have each 36 EGF-like repeats, while NOTCH-3 and NOTCH-4 have, respectively, 34 and 29 repeats. The main consequence of this difference appears to be the different affinity with the ligands [121, 123]. Several studies have tried to elucidate the mechanism of interaction between Notch receptors and ligands and different solutions have been proposed [121, 124]. To date, a certain interaction structure has been determined only in regards to Notch-1 receptor, identifying the region encompassing EGF-like repeats 11-13 as the link point for interaction with ligands in human. In spite of this, the exact structure of the Notch receptor-ligand complex remains unsolved [125]. De Celis and Bray reported that inhibitory *cis* interactions are instead mediated by EGF-like repeats 24-29 [126].

Next to the EGF-like repeats, we find the negative regulatory region (NRR) that consists of three cysteine-rich Lin12-Notch repeats (LNR), and is pivotal in preventing Notch signalling activation in absence of ligand [127]. The NRR also plays an essential role in the activation of the signal, as described in detail later. The correct function of this domain is pivotal for normal signalling of Notch, and a demonstration of this comes from the discovery of point mutation in the NRR cause T-

Cell Lymphoblastic Leukemia or T-ALL in human [128]. Finally the last domain in the NECD is the heterodimerization domain (HD), a hydrophobic region, which allows the non-covalent but stable interaction between the N- and C- terminals halves of HD [127].
The single TMD is the smallest domain of the receptor and its function is determined by a "stop translocation" signal consisting in 3 /4 Arg/Lys residues [127].
In the NICD, the RBPjκ association module (RAM) domain is indispensable for the interaction with the DNA after the translocation to the nucleus. This domain is connected by a linker protein containing the nuclear localizing sequence (NLS) to seven Ankyrin (ANK) repeats that form the so called ANK domain, necessary for Notch transcriptional activation. An additional domain the transactivation domain (TAD) is present in Notch-1, 2 and 3 but absent in Notch-4; the functional implications regarding TAD are not yet clear. Finally, at the C-terminus we can find the PEST region a domain whose name derives from its conserved sequence of proline (P), glutamic acid (E), serine (S), and threonine (T) motifs [127]. The PEST domain is fundamental for the regulation of NICD stability and the nuclear transmission of the signal. The relevance of this repeated sequence is clear considering that most of the mutations identified in connection with an oncogenic role of Notch in cancer are found in the PEST domain [129].

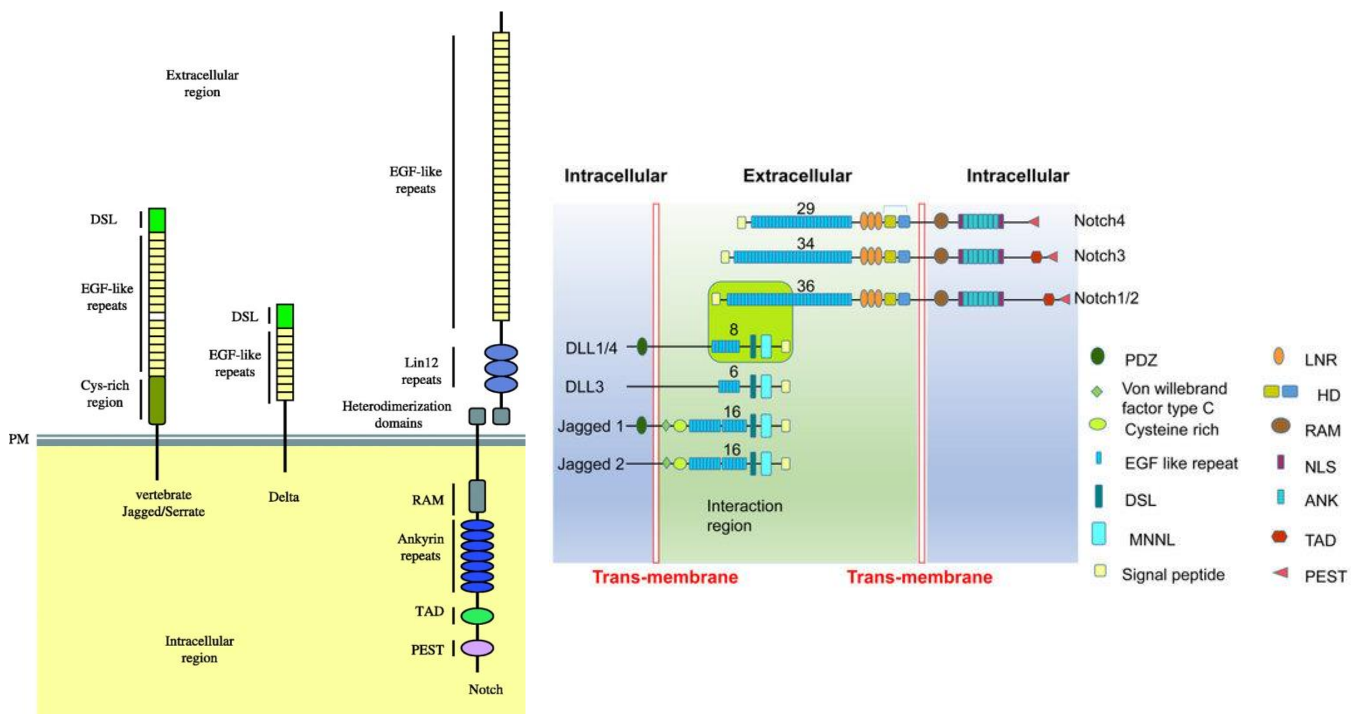


Figure 7. Detailed structure of Notch receptors and ligands. Preserved domains exert specific influence the affinity and interaction between receptors and ligands therefore influencing the signalling pathway activation [119, 130].

Canonical activation of Notch

Notch signalling is juxtacrine, meaning that the contact between a cell expressing the ligands and a cell expressing the receptor is necessary for canonical activation of the pathway. *In cis* interactions have also been reported and seem to induce the inhibition of Notch signalling [131]. Another important feature of Notch is its dual nature of transmembrane receptor and transcription factor. The canonical activation of Notch receptors depends not only on the interaction with the ligands but also on a series of proteolytic events culminating in the release of NICD in the cytoplasm (Fig. 8). The first of these enzymatic processes, commonly defined site 1 (or S1), occurs in the Golgi where a furin-like convertase cleaves the HD in Notch precursor, during the secretion process that precedes the exposure through the membrane [132]. Therefore, this first cleavage allows the formation of Notch receptor heterodimer conferring its mature structure. Once exposed at the cell membrane interface, Notch can interact with its ligands in adjacent cells. The interaction, as previously described, occurs in a specific site at the level of the EGF-like repeats. The affinity of ligand binding depends on several factors including interaction between the EGF-like repeats and calcium ions [133, 134] and the interaction with accessory molecules specifically glycosyltransferases that through their activity, i.e. addition of fucose to serine or threonine residues, can stabilize and/or induce preferential binding to Jagged or Dll ligands [135, 136]. In particular, in human two families of glycotransferases play this role. The first is the O-fucosyltransferase1, Pofut1, which is not required for activation but regulates the proper folding of Notch receptors and optimizes the binding with the ligands [137]. Further modification of the O-fucosylated sites occurs due to the action of Fringe, which in humans has three homologues: *Radical*, *Manic*, and *Lunatic Fringe*. In *Drosophila* the only fringe protein acts by inhibiting the interaction between Notch and serrate ligands thus favouring delta-like interactions [138]. In human, the function of these proteins has only partially been elucidated, but evidence suggests that each Fringe protein might interact differently with each receptor thus originating multiple effects on the ligand-receptor interaction [119].

After all these complicated factors have combined to allow formation of a Notch receptor-ligand complex, the second cleavage is induced at site 2 (S2) in the NRR domain [139, 140]. Some ambiguity still remains regarding the enzyme responsible for this proteolytic cut since two different metalloproteases, in human, seem to be involved in the process. The first is metalloprotease ADAM-10 (homolog of Kuzbanian), whose function was proven to be essential in all phyla [127], and the second is ADAM-17 or TACE (Tumor necrosis factor alpha converting enzyme), which is sufficient for Notch activation *in vitro* [141]. To date, it is unclear whether the synergic activity of

these ADAMs is necessary for S2 cleavage in human or if their activity is alternative. The release of the NECD creates an intermediate form defined as Notch extracellular truncation (NEXT), which is the substrate for the S3 cleavage operated by γ-secretase. This complex of enzymes includes five different proteases: presenilin-1 and-2 (PSEN1, PSEN2), nicastrin, presenilin enhancer 2 (PEN2), and anterior pharynx-defective 1 (APH1) [142]. Their activity, usually linked with accumulation of mutant amyloid β plaques in Alzheimer's disease, is essential for many cellular processes, including NICD release in the cytoplasm and thus Notch signalling activation. The mechanism of action of γ-secretase in S3 cleavage is still object of discussion among expert in the field. Evidence suggests that γ-secretases operate at the level of the cell membrane [143] cutting through the TDM at a specific site, starting at Val1744, in the NEXT fragment leading to the formation of a unique stable NICD (also known as V-NICD) [144]. This represents the active form of Notch receptor and can translocate to the nucleus and activate the transcription to modulate target genes. Anyway, later studies, conducted after the discovery of γ-secretase role in Notch activation, have identified other variants in which the cleavage is performed at residues different from Val1744 [145]. Although their existence is indisputable, this different isoforms of NICD are significantly less stable than V-NICD and seem to lead to a weaker activation of the signalling before being degraded by the proteasome [145, 146]. A possible explanation for this comes from the discovery of an alternative proteolytic activity of γ-secretase exerted in endocytic vesicles rather than the cell surface [147, 148]. Therefore, these results would suggest that different compartments are responsible for alternative γ-secretase-dependent cleavage of NICD thus producing multiple active isoforms. In addition to that, a fourth cleavage, namely S4, has been identified and, likewise S3, it is regulated by γ-secretase activity. S4 releases the Nβ peptide, a small fragment in NICD structure named after the Aβ peptide from the Amyloid Precursor Protein (APP), another substrate of γ-secretase commonly linked to the onset of Alzheimer's disease [149]. The function of this proteolytic process has not been clarified yet.

After S3 cleavage allows for its release, NICD translocates to the nucleus. RAM domain is essential for this task since it regulates the binding between NICD, which cannot bind DNA on its own, and RPBJϰ. This transcription factor also known as CSL or CBF1 (C-promoter binding factor 1) normally binds the DNA target genes region, in absence of NICD, forming an inhibitory complex with co-repressors such as the silencing mediator of retinoid and thyroid receptors (SMRT) [150]. The co-repressor complex is also associated with histone deacetylases to keep transcription blocked. When NICD reaches the nucleus, it binds RPBJϰ replacing the co-repressor complex and forming a ternary complex with the co-factor Mastermind (Mam) or mastermind-like (MAML). This complex

recruits histone acetyltransferases, chromatin remodelling factors and further transcription factors among which the most important is p300 [119, 151, 152].
The transcriptional activation of NICD has downstream effects on several target genes, some of which have, in turn, activity as transcription factors. The effect on further downstream targets mostly depends on cellular context. The primary targets of NICD are HES/HEY genes of the bHLH family (basic Helix-Loop-Helix), a large group of transcription factors mainly known for their involvement on organogenesis and tissue formation. Their name is due to the preserved helix-loop-helix structure, which allows the recognition between target sequences on DNA and basic domain of these proteins. These proteins also show hydrophobic residues that allow formation of dimers. Seven Hes transcription factors have so far been discovered in human (Hes1-7). Their activity, along with Notch, seems to depend on cellular context, but interestingly Hes can operate a negative feedback loop on its promotor therefore self-regulating its expression [153]. More and more targets of Notch are being found thanks to increased interest around this pathway. A known target, which seems to interact directly with RPBJκ in several cell types, is Cyclin D1, a pivotal regulator of cell cycle during activation of G1 phase. Other pathways interlink in its regulation, for instance Wnt pathway [154, 155]. Although no evidence of Notch-cyclin D1 regulation has been found in articular cartilage, to date, other proteins involved in OA, in particular MMP13, act as primary Notch targets. In addition to that, an important feature of Notch that must be taken into account to understand the importance of this signalling pathway in cell fate and differentiation is the crosslink with other regulatory pathways fundamental for cell homeostasis. Most evidence of interaction between Notch and other pathways comes from studies on embryogenesis, organogenesis and cancer. Among the vast number of interactions, it is particularly noteworthy the one with VEGF ligands and receptors in the “tip” and “stalk” process, essential for regulation of angiogenesis [156].
Notch signalling requires a transient activation, necessary to regulate the developmental processes regulated by this pathway. Therefore, Notch activation is oscillatory and finely regulated by feedback mechanisms. The first level of control starts form the PEST domain, which is promptly phosphorylated by CDK8 kinase and targeted for proteasomal degradation, exerted by E3 ubiquitin ligase (Fbw7/Sel10), during the transcriptional activation process [157].
Although the canonical activation is the preferred mechanism of activation of Notch signalling pathway, a non-canonical activation has also been described. In this alternative process the release of NICD can be ligand independent since triggering can come directly from cross-link with other pathways. Another main difference from canonical signalling regards the possibility of RBPJκ-independent transcription activation; in this case downstream targets are directly modulated without Hes/Hey genes intermediation.

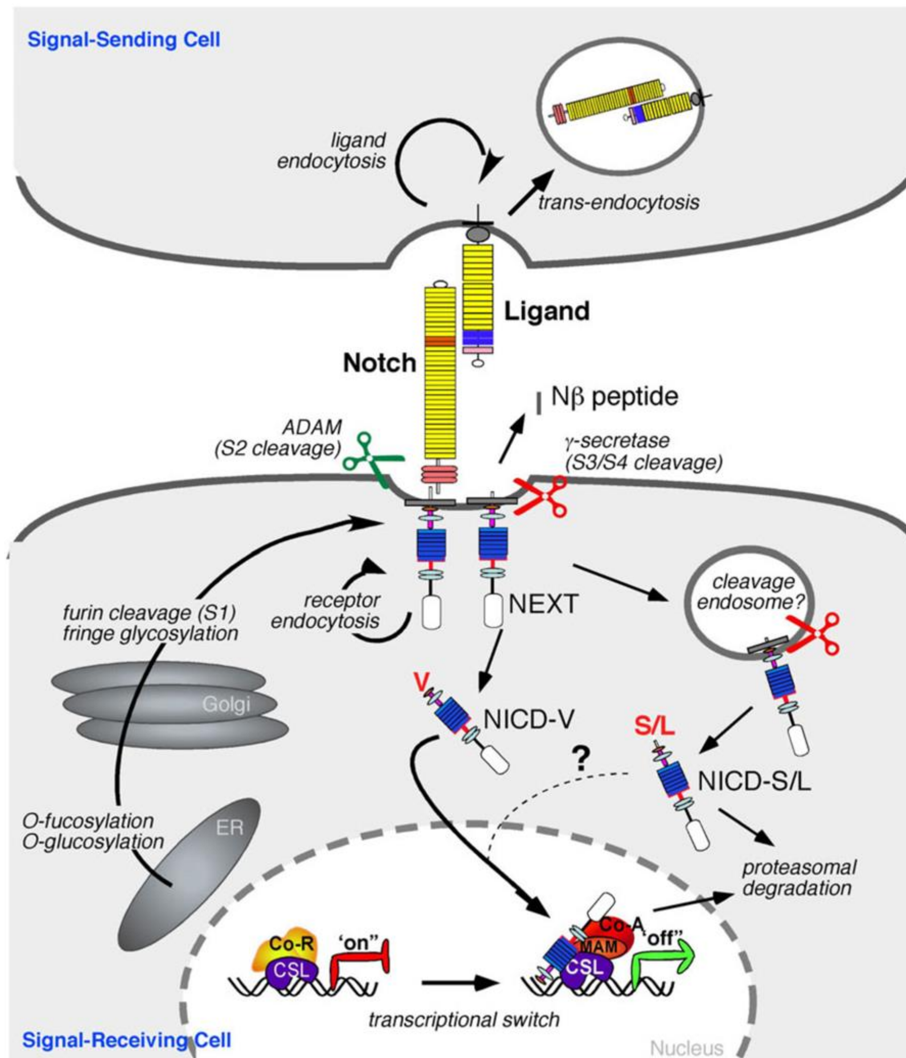


Figure 8. Notch signalling activation [127].

Notch in articular cartilage and osteoarthritis

Previously, we described in detail the involvement of Notch signaling pathway in chondrogenesis and endochondral ossification where the expression of NOTCH-1 is time- and space-restricted. In endochondral ossification, NOTCH-1 is essential in the initial stage of chondrocytes differentiation, for the commitment of mesenchymal progenitors, but is also switched on later, in the stages of prehypertrophic and early hypertrophic chondrocytes to promote differentiation progression [51]. Notch signaling is pivotal for the maintenance of homeostasis in most adult tissues, including articular cartilage. The expression of NOTCH receptors and ligands in healthy articular cartilage has been reported some years ago [158], as well as changes due to OA [43]. Only NOTCH-1 and NOTCH -2 were found expressed in human cartilage, but major changes in OA are related to NOTCH-1, whose increased expression in OA cartilage, particularly in the so-called "clusters"

suggests its involvement in the abnormal cell activation and differentiation process of OA chondrocytes. These clusters indeed represent an attempt to regenerate cartilage following the initial cartilage damage.

Jagged-1, one of the pivotal trigger of NOTCH-1 activation and a NF-κB target gene [159], resulted up-regulated at early (2 weeks) stages of OA induction in surgically induced OA (destabilization of the medial meniscus, DMM, the method of choice for surgically induced OA, closely mimicking the human pathology [15], as shown in a matched comparison of 9 microarrays carried out in similar conditions [160]. This suggests an early and pivotal involvement of NOTCH pathway in OA development.

OA onset and progression are sustained by changes in ECM remodeling [161]. Increased ECM remodeling may derive from increased MMP expression, increased activation and/or decreased inhibition by the Tissue Inhibitors of matrix metalloproteinases (TIMPs). Few studies have investigated the relationship of NOTCH signaling with MMPs in articular chondrocytes, and mainly using murine chondrocytes.

Notch signaling in chondrocytes has been previously linked to increased MMP expression, particularly of MMP-13, which represents the pivotal collagenase in cartilage degradation [161]. The first study suggested a direct connection between MMP-13 and NOTCH: employing NOTCH signaling inhibition (by DAPT, an inhibitor of γ-secretase) on murine chondrocytes, a significant reduction of MMP-13 mRNA and protein was found [162], particularly in cells at later passages while MMP-2 resulted unaffected. The NOTCH-MMP-13 connection was confirmed with several other findings derived from both *in vivo* and *in vitro* studies. In a mouse model with selective inhibition of NOTCH signaling (via SOX-9-Cre; $Rbpj^{fl/fl}$) a significant decrease of markers related to endochondral ossification (MMP-13) and angiogenesis (VEGFA) was observed, that paralleled decrease of HES-1, a main target gene of NOTCH-1. A similar pattern of regulation was observed in an inducible and articular cartilage specific murine model (Col2a1-Cre; $Rbpj^{fl/fl}$) where the inactivation of Rbpj (the transcriptional co-activator of the Notch signaling) was executed at skeletal maturity. This mouse was resistant to OA development as induced by mean of surgical induction of joint instability, and this resistance was phenocopied with intraarticular delivery of DAPT [163]. More recently, it was shown that inducible and articular specific HES-1 ablation in mice (in a Col2a1-Cre^{ERT}; $Rbpj^{fl/fl}$ background) prevented OA progression, and inhibited the expression of key matrix degrading enzymes in OA, i.e. MMP-13 and ADAMTS-5 [164].

Therefore, NOTCH signaling inhibition may represent one of the constraints that prevent the progression of articular chondrocytes toward hypertrophy and differentiation.

Nutraceuticals

The term "nutraceutical" was invented in 1989 by Dr. Stephen DeFelice, also founder of the Foundation for Innovation in Medicine (http://www.fimdefelice.org/), established in 1976. Although a precise definition of nutraceutical does not exist, we can define it as a food or a component of food with functional and beneficial physiological effects that could therefore be used as dietary supplement for its therapeutic properties. Although plant- and animal-derived compounds have been used for centuries in medicine for their properties, only recently the molecular mechanisms underlying the anti-inflammatory, antioxidant, anti-tumorigenic, etc. effects of food-derived compound have started to be elucidated. As a matter of fact, in the past two decades, research in this field has greatly extended, uncovering the great potential of these compounds as therapeutic agents. The first research field in which nutraceuticals have found application was oncology but research around bioactive compounds is now widespread in all fields.

In the context of OA, several nutraceutical compounds have been tested especially for their potential effect in pain management, although results from clinical trials are still lacking [165]. More data on the mechanisms of action of nutraceuticals emerge from pre-clinical and *in vitro* studies. Among botanical sources, avocado and soybean unsaponifiables (ASU), consisting of combined formulation of oils extracts of soy and avocado in relation of two to one thirds, contain minimal concentrations of phytosterols considered responsible for their bioactivity in chondrocytes [166]. They have shown both chondroprotective effects *in vitro* exerted through the enhancement of the transforming growth factor-β (TGF-β) [167] and ameliorated pain in human trials [168], even when compared with NSAID [169]. The anti-inflammatory effect of ASU could instead be due to inhibition of the expression of cytokines (IL-1β, TNFα, IL-6) and COX-2 by modulating NF-κB [170].

Another group of nutraceuticals known for their anti-inflammatory properties are boswellic acids. Derived from Boswellia resins commonly known as frankincense, they have been used for centuries in traditional medicine. 3-O-acetyl-11-keto-beta-boswellic acid (AKBA) is considered the active agent exerting its effect through the inhibition of pro-inflammatory and pro-oxidant factors [165]. The mechanisms by which boswellic acids exert their effects are mostly unknown but Akhtar and colleagues have speculated a modulation of major signalling pathways involved in the pathogenesis of OA, such as iNOS, MMP-3, MMP-13, and NO production [171].

Omega-3 fatty acids or long chain polyunsaturated fatty acids (LC-n-3 PUFAs) consists of alpha-linolenic (ALA,18:3 ω-3), eicosapentaenoic (EPA,20:5 ω-3), and docosahexaenoic (DHA, 22:6 ω-3) acids contained in fish oil. Thanks to their lipidic nature, they easily penetrate cell membrane

contributing to permeability and fluidity of plasma [166]. Considered as "conditionally essential" dietary components, EPA and DHA can be synthetized by elongases, at very low rate in human, from ALA precursor while this last PUFA is considered an essential fatty acid. Assumption of PUFAs is important for fetal development but also recommended in presence of degenerative diseases associated with inflammation [172]. These compounds are also particularly effective in OA due to the modulation exerted on the expression of OA markers of the ADAMTS and MMP families [173].

The largest family of nutraceuticals currently investigated in OA is that of polyphenols. These compounds are exclusively synthetized by plants and can be found in fruits, vegetables, green tea, and whole grains [174]. Polyphenols can be generically described as a large and heterogeneous class of compounds with chemical features of phenolic substances and anti-oxidant properties; polyphenols include at least two phenol groups and one or more hydroxyl substituents in their structure [174, 175]. Usually they are divided into two functional groups: flavonoids and non-flavonoids, with further sub-classification depending on the molecular structure [176]. Flavonols and flavones contain a carbonyl group in C4 position, where an oxygen atom is also located in flavonols. Depending on the position of attachment of the B ring (C2 or C3), they can be further classified as isoflavones. Flavonols differ for the presence of a hydroxyl group in C3. The most studied compounds belonging to this class are usually found in the aglycone form [177]. Other sub-groups include: flavanones, anthocyanidins, anthocyanins- and flavanols [174]. Nonflavonoids basic structure contains just one aromatic ring. The main class is represented by phenolic acids derivative by benzoic and cinnamic acids. These compounds are hard to find in their free form and are most commonly found conjugated to other polyphenols [178].

Curcumin, the active agent of *Curcuma longa* found in turmeric, is one of the most studied food-derived compounds for the therapeutic effects shown in a multiplicity of chronic conditions and cancer. Its role in OA has also been vastly investigated both *in vitro* and *in vivo* pre-clinical models, but also in clinical trials on patients with OA, showing chondroprotective, antioxidant and anti-inflammatory activity in chondrocytes. Curcumin can inhibit TNFα and MMP-13 expression by blocking the translocation to the nucleus of p65, the active subunit of NF-κB [179]. Further regulation of this pivotal pathway in the pathogenesis of OA, derives from upstream inhibition of toll-like receptor 4 (TLR4), involved in sustaining the inflammatory response in OA [180]. Curcumin may interfere with other essential cellular homeostatic mechanisms, such as autophagy, through the attenuation of AkT/mTOR pathway [181] and can reduce endoplasmic reticulum (RE) stress induced by oxidative stress in chondrocytes by modulating sirtuin 1 (SIRT-1) [182]. In spite of the indisputable beneficial effects in OA, curcumin, as many other nutraceuticals, has a main

drawback in scarce systemic bioavailability (below 1%) when transferred *in vivo* [180], and this issue could be further exacerbated in case of dietary supplementation in patients. Several studies have focused on alternative methods for administration and formulation of curcumin, from topical to intra-articular injection and creation of nanoparticle- or liposome-based complexes [110, 180, 183] in order to increase bioavailability of this compound. Exploitation of synergic activity with other nutraceuticals has also been tempted [183, 184]. Finally, although unconventional, promoting the accumulation of curcumin at the gastrointestinal mucosal interface through repetitive oral administration could be an effective strategy to increase the systemic absorption [185].

Green-tea polyphenols, a sub-group belonging to this vast family of compounds, are mostly flavonols known as cathechins and include epicatechin, epigallocatechin, epicathechin-3gallate, and epigallocathechin-3-gallate (EGCG) [166]. The last one is the most studied green-tea derivate, known for its antioxidant and anti-inflammatory activities. EGCG can inhibit gene expression of inflammation markers (iNOS and COX-2) in human chondrocytes [186]. MMP-13 and NF-κB are also modulated by this bioactive agent [186, 187]. ECGC has an important chondroprotective activity against ECM remodelling by modulating MMPs involved in this degenerative process as well as members of the ADAMTS family disintegrines [188]. Interestingly EGCG was also found to act as epigenome modulator especially by regulating the microRNA miR-9 [166], whose role in the pathogenesis of OA was previously demonstrated [189, 190].

Another class of polyphenols that is drawing increasing attention for the potential therapeutic role in OA is represented by olive-derived compounds. Olive oil is a basic component of the Mediterranean diet and its bioactive compounds oleocanthal (OC), oleuropein (OE), tyrosol, and hydroxytyrosol (HT) show interesting anti-inflammatory properties in many inflammatory or degenerative conditions. In an animal model of OA, dietary supplementation of extra virgin olive oil, in combination with moderate physical exercise, was associated with prevention of cartilage damage, suggesting an important potential role of this aliment in the prevention and management of OA [191] . In terms of concentration, the main polyphenols in olives are OE, its metabolite 2-(3,4-dihydroxyphenyl)-ethanol,known simply as HT, and α-tocopherol [192]. With a concentration up to 14% in the fruit, OE is the most present phenolic compound in olive, even though in the so called “green phase” of maturation of the fruit is associated with progressive decrease of OE concentration [193]. OE was discovered by Bourquelot and Vintilesco (1908) and is responsible for the bitter aroma of olive-oil. OE is a hydrophilic phenolic antioxidant and is oxidized to its catechol quinone during redox cycling. It has been used since the 19th century for malaria-induced fevers [192]. Its role as scavenger of free radicals and nitrogen species is also well-known [194, 195]. Several studies have also demonstrated antitumor properties of this compound [196, 197].

A possible mechanism for the metabolism of OE and its metabolites has been proposed and is shown in Fig. 9 [192]. After ingestion, OE undergoes acid hydrolysis in the stomach leading to the production of several metabolites whose concentration and distribution is highly related to pH conditions. Hydrolysis in acidic condition leads to the formation of aglycone that immediately splits into two dialdehyds. These highly instable intermediates are then converted in a lipophilic stable metabolite known as transposed secoiridoid, which can release HT along with methanol under prolonged and extreme acidic condition [192]. If OE instead reaches the small intestine unmodified, an event plausibly promoted by dietary supplementation in capsules, it may undergo a different fate. Due to its hydrophilic nature, OE cannot diffuse through cellular membrane and a few data available assert that probably just a small fraction of OE can reach systemic circulation [192]. Furthermore, in the intestine, OE can be cleaved by two lipases with consequent release of HT and another intermediate, methyl oleoside, which produces oleoside and methanol as final products [198]. Therefore, HT is formed through hydrolysis of OE catechol, occurring naturally during olive maturation, and it is undoubtedly the most important metabolite for the potential use as nutraceutical. Similarly to its precursor, HT shows antioxidant, and anti-inflammatory properties but an anti-atherogenic and anti-thrombotic activity has also been demonstrated *in vitro* [199, 200].

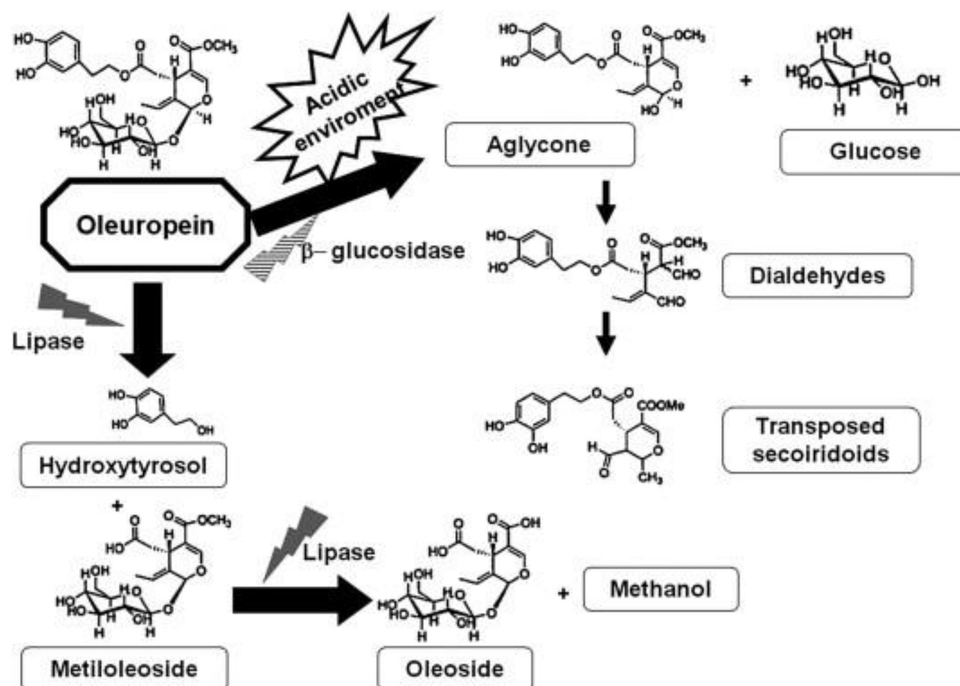


Figure 9. Metabolism of oleuropein and its main metabolite hydroxytyrosol [192].

Recent evidence have demonstrated the antitumor effect of these compounds, which, thanks to their roles as cytostatic and antiangiogenic agents and absence of side effects, could prove to be effective bioactive food-derived candidates for cancer therapy [192], once further investigations in patients

are conducted. In the context of OA, several studies reported benefits in supplementation with nutraceuticals, some of which are summarized in Fig. 10. In an animal model of OA, OE, has shown anti-osteoarthritic effects [201]. OE also modulated NF-κB and MAPK signalling pathways in human primary chondrocytes, therefore inhibiting downstream effectors such as iNOS, COX-2, MMPs and ADAMTS-5, involved in OA progression [202]. A comparative study focused on the differential absorption and metabolism of OE and HT conducted in rats has shown that the precursor form is probably more suitable for dietary supplementation *in vivo* due to the higher bioavailability [203]. Despite these promising premises, not much is known about the mechanisms of action in OA directly mediated by this compound, since most of the data regarding beneficial roles of olive-derived polyphenols have so far focused on HT. Facchini and colleagues reported an inhibition of key OA markers (iNOS, COX-2, MMP-13, RUNX-2, and VEGF) exerted by HT [204], which would suggest an important role in arresting chondrocytes progression through terminal differentiation and therefore OA progression. Furthermore, HT-mediated chondroprotective activity is also accomplished via stimulation of autophagy, an essential process in healthy chondrocytes impaired in OA, by modulating SIRT-1 [205]. A recent study focusing on the putative role of HT as epigenetic modulator has also linked this molecule to the regulation of demethylation of miR9 [189], which was, as already mentioned, linked to OA. HT is currently the most investigated olive-derived compound and its bioactive properties on cellular mechanisms along with positive results from studies on its safety profile [206] make it an exceptional candidate for food supplementation in degenerative conditions like OA.

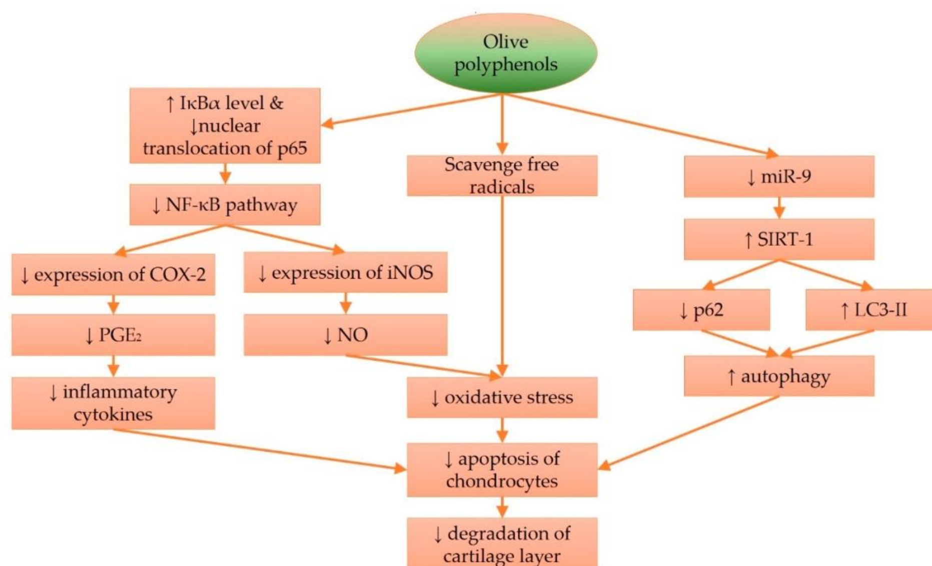


Figure 10. Effects of olive-derived polyphenols in OA [207].

Material and methods

Cell isolation and culture

For our experimental set up, we used two *in vitro* models: primary chondrocytes derived from patients with OA undergoing total knee replacement surgery (arthroplasty) and C28/I2, an immortalized human chondrocytes line. For primary chondrocytes both monolayer and 3-D culture models were set up.

After approval from the Ethical Committee, patients undergoing total knee replacement arthroplasty were recruited at Rizzoli Orthopaedic Institute. Fragments of articular cartilage (femoral condyle and tibial plate) were transferred to the Immunorheumatology and Tissue Repair Laboratory for chondrocytes isolation and experimental testing as explicated in the informed consent signed by recruited patients ahead of donation. For privacy protection all personal data of patients not necessary for our experimental purpose, such as sex and age of the donor, were anonymized. A total number of 20 patients took part into the different phases of this study. Candidates with a BMI ≥ 35, were excluded from the study. Cartilage kept in physiologic solution was immediately dissected after delivery, broken into small pieces and weighted. Then it was transferred in Petri dish and incubated in High Glucose Dulbecco's Modified Eagle medium DMEM supplemented with 10% fetal bovine serum (FBS, ECS5000L, Euroclone) and 100 IU/mL Penicillin and 0.1 µg/mL Streptomycin (P4333, Sigma), overnight at 37°C to optimize cell yield. Subsequently cartilage was washed twice with PBS, we then proceeded with enzymatic digestion. Enzymes were weighted and dissolved in DMED without FBS then sterilized by filtration with 0.22 µm filters (Sarstedt).

The first digestion was done with Pronase 0.5% (P-5147, Sigma) 5 mg/mL for 1h at 37°C in DMEM without FBS. We considered a volume of 10 mL of Pronase solution for 1 g of cartilage.

A second digestion was then carried out using Collagenase II 0.2% (C6885, Sigma) 2 mg/mL in DMEM without FBS in thermostatic bath at 37°C in magnetic agitation (~ 200 g) until complete digestion. The progression of the reaction is checked through the turbidity of the solution. To avoid cell death the digestion was interrupted in any case after a maximum of 2 h.

The cellular suspension was filtered with a 100 µm filter and then with a 70 µm filter (Sarstedt) and enzymatic digestion was blocked by adding an equal volume of DMEM added with 10 % FBS. Chondrocytes were centrifuged at 1000 g for 10' counted with Neubauer/Burker chamber and seeded at a density of 25,000 cells/cm^2 in T150 flasks (Sarstedt). Cells were then cultured to

confluence; culture medium was changed twice a week. For all experiments cells at passage P0-P1 were used.

C28/I2 cells were cultured on Petri dish (Sarstedt) and used between passage 7 and 40 for experiments. Monolayer cultures were employed to evaluate the effect of the treatments on gene and protein expression through real time RT-PCR and western blot analysis or tested for ROS production.

Cell treatment

Primary chondrocytes were seeded at high confluence (~200,000 cells/cm^2) in 12 well plates. After 48 h from seeding, cells were starved for 24 h. C28/I2 chondrocytes were seeded and maintained in DMEM with 10 % FBS. Then, both cellular models were pre-treated for 16 h with nutraceuticals 100 µM Oleuropein (OE, 92167, Sigma) and 100 µM Hydroxytyrosol (HT, 70604, Cayman chemical, MI, USA) before stimulation with 10 µg/mL lipopolysaccharide (LSP, L-2654, Sigma) for 6 h for real time RT-PCR analysis, 6 h or 24 h for Western Blot analysis or 2-4-6-24-48-120 h for ROS production evaluation. Pellets were then collected and dry-frozen at -80° C until use.

RNA interference mediated NOTCH-1 gene silencing

Primary chondrocytes at confluence were detached by means of 0.5% Trypsin (EuroClone), centrifuged at 1000 g for 8' and resuspended in DMEM with 10% FBS in absence of antibiotics. Cells were counted and seeded at a density of ~ 25,000 cells/cm^2 in 6 wells multiwell plates. Chondrocytes are seeded at a 70% confluence increase silencing efficiency allowing since aggregates could prevent liposomes penetration. Cells were cultured at 37°C for 24h. Following, 5 µL of Lipofectamine® RNAiMAX (Invitrogen) was added to 245 µL of Optimem® (Invitrogen) culture medium for well. Lipofectamine is a lipidic molecule able to bind nucleic acids via electrostatic interactions allowing their passage through the cell membrane bilayer. siRNA CTRL (NC) or siRNA NOTCH-1 (N1) (ON-TARGET Plus Non-targeting Pool; ON-TARGET Plus NOTCH-1 siRNA Human, Dharmacon) were added to the solutions with a final concentration of 25 nM. On target siRNA used for NOTCH-1 silencing consisted of a pool of siRNAs, whose specific sequence are protected by manufacturer's copyright, built to bind multiple target to improve silencing efficiency. Solutions containing Lipofectamine and CTRL-NOTCH-1 siRNAs were mixed in equal volume (250 µL each, final volume 500 µL) and incubated at room temperature (R.T.) for 25' to allow liposomes formation. 500 µL of culture medium were removed from each well and an equal volume of Lipofectamine + NC or Lipofectamine + N1 were added. Primary chondrocytes were incubated at 37°C. After 6 h, culture medium was changed to prevent toxic effects of

Lipofectamine with DMEM + 10% FBS and antibiotics. Cells were collected 48 h after gene silencing and used to assess the efficiency of silencing in Western Blot and real time RT-PCR. In addition to that, part of the transfected cells was employed for setting up 3-D constructs (micromasses) for gene expression analysis in real time RT-PCR.

RNA interference mediated SIRT-1 gene silencing

For transient silencing of sirtuin-1 (SIRT-1) in C28/I2 we replicated the protocol described above. This time we used 25 nM ON-TARGETplus Human Sirt1 siRNA (siSIRT1, Dharmacon) or ON-TARGETplus non-targeting pool (NC, Dharmacon) dissolved in Lipofectamine® RNAiMAX Reagent and Optimem® Medium (Invitrogen). The cells were incubated for 48 h before LPS treatment and subsequently collected at 6 h post stimulation for real time RT-PCR analysis and 24 h post LPS for evaluation of protein expression by Western Blot.

3-D cultures (micromasses) set up

3-D cultures, in particular micromasses, were set up starting from monolayer cells previously silenced for NOTCH-1. Chondrocytes were collected 48 h after gene silencing with Trypsin (ECB3052D, Euroclone) and centrifuged at 1000 g for 8' then resuspended in DMEM + 100 IU/mL Penicillin and 0.1µg/mL Streptomycin (P4333, Sigma) and supplemented with 50 µg/mL Ascorbic Acid (A-5960, SIGMA), to allow differentiation. Cells were then counted and resuspended at a density of 250,000 cells/mL. 1 mL of solution was then seeded in conic tubes (Microtube, Sarstedt, Ref. 72.632.005) and centrifuged at 1000 g for 10' at 4°C. Tubes were incubated at 37°C, the top of the tube was left partially open to allow CO_2 exchange. The micromasses were allowed to mature across 3 weeks, culture medium was changed every other day with 500 µL DMEM 10% FBS added with 50 µg/mL L-Ascorbic Acid. In culture, micromasses will start to assume the characteristic shape around day 2-3 after seeding. Complete maturation characterized by spherical morphology and presence of ECM components is reached at 1 week (1w) after seeding. At selected time points (1w, 2w and 3w) parallel samples were dry frozen at -80°C for subsequent Western Blot or real time RT-PCR analysis. Supernatants were also collected for MMPs measurement and kept at -80°C till the moment of the analysis. For each experimental condition at least 4-6 replicate were established.

Proliferation assay (PicoGreen)

For proliferation evaluation by means of PicoGreen (Thermofisher), NC and N1 cells were collected 48 h post transfection and seeded in 96 plate multiwell at an approximate density of 3000 cells/cm^2. For a homogeneous seeding a multichannel micropipette was used (final volume of 200 µL/well). Plates were incubated at 37°C and collected for analysis at established time points: Day 0 (considered has the day after seeding to avoid cell loss) then day 3-5-7-10-12. At time of collection culture medium was removed and plates were conserved dry frozen at -20° C up to analysis. Plates were then defrosted at R.T., then 100 µL of Cell Lysis Buffer (C-7027, Thermofisher) and 100 µL of TE Working Solution containing PicoGreen dsDNA quantification reagent probe (P11496, Thermofisher) were added. For both solutions a dilution of 1:200 from stock solution was used. Lysis buffer allows cell membrane rupture but leaves unaltered the nuclei and DNA. After a 5' incubation fluorescence intensity of PicoGreen probe was measured by means of Plate Fluorimeter (Spectramax Gemini Plate Fluorimeter). Setting for measurement was 480 nm for excitation and 540 nm for emission (cut off 515 nm). Data elaboration was executed on five replicates for each experimental condition, normalization was done on mean value from Day 0 [208]. Analysis of mean values ± SD was expressed as comparison between NC and N1 samples.

RNA extraction and real time RT-PCR

Cellular pellet or 3-D cultures were kept at -80°C until extraction, then resuspended in Trizol Reagent (Ambion). 100 µL of Chloroform (Sigma) were added, samples were briefly agitated to allow the formation of the aqueous and organic phases. After a 15' centrifugation at 12,000 g (4°C), the aqueous phase was collected and added with 500 µL of Isopropanol (Sigma) and incubated overnight at -20°C to optimize RNA yield. A second centrifugation at 12,000 g (4°C) for 10' followed; supernatant was removed and RNA was washes with 75% ethanol for 5' at 7,500 g (4°C). Dried pellets were resuspended in RNase Free water and quantified with Nanoveu. Total RNA (10 ng) was reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Invitrogen) following manufacturer's protocol. Real time RT-PCR analysis were performed using QuantiTect SYBR Green PCR Kit (TaKaRa) and according to the following standard protocol: Taqman DNA Polymerase (TaKaRA) (45 cycles; denaturation 95°, amplification annealing temperature variable according to primers design, as indicated in Table 1). mRNA quantification for primary chondrocytes samples was calculated for each target gene and normalized using GAPDH as reference gene. Results were expressed according to the formula $2^{-\Delta Ct}$ and expressed as a percentage of the reference gene or expressed as number of molecules per 100,000 GAPDH molecules to

reduce inter-patient variability in gene expression. Primers specificity was established by evaluation of melting curves.

Gene	***Forward Primer***	***Reverse Primer***	***Amplicon Size (annealing T)***
GAPDH	TGGTATCGTGGAAGGACTCA	GCAGGGATGAGTTCTGGA	*123bp (56°C)*
NOTCH-1	CCTGAAGAACGGGGCTAACA	GATGTCCCGGTTGGCAAAGT	*127bp (60°C)*
HES-1	AAGAAAGATAGCTCGCGGCA	TACTTCCCCAGCACACTTGG	*134bp (60°C)*
ADAMTS-5	GCACTTCAGCCACCATCAC	AGGCGAGCACAGACATCC	*187bp (58°C)*
MMP-13	TCACGATGGCATTGCT	GCCGGTGTAGGTGTAGA	*277bp (60°C)*
RUNX-2	GGAATGCCTCTGCTGTTATG	AGACGGTTATGGTCAAGGTG	*105bp (58°C)*
NFKB1	CAGGAGACGTGAAGATGCTG	AGTTGAGAATGAAGGTGGATGA	*109bp (60°C)*
IKKα	GCACAGAGATGGTGAAAATCATTG	CAACTTGCTCAAATGACCAAACAG	*86bp (60°C)*
IL-6	TAGTGAGGAACAAGCCAGAG	GCGCAGAATGAGATGAGTTG	*184bp (60°C)*
IL-8	CCAAACCTTTCCACCC	ACTTCTCCACAACCCT	*153bp (60°C)*
VEGFA	TGATGATTCTGCCCTCCTC	GCCTTGCCTTGCTGCTC	*82bp (58°C)*

Table 1. Target genes primers sequences.

Western Blot

Pellet of monolayer was resuspended in Ripa Buffer (Thermoscientific) added with Protease/Phosphatase inhibitors and EDTA 100x, according to manufacturer's instruction, then sonicated for 90" and centrifuged for 10' at 11,000 g. For micromasses the same procedure was used with the addition of mechanical rupture of the 3-D constructs by means of a pestle in Ripa Buffer. Supernatant was collected and total proteins were quantified by means of Bradford Assay with Coomassie Brilliant Blue G-250 (AppliChem) dye. Samples were diluted with Ripa Buffer to have equal concentration then added with denaturant loading buffer and boiled 5'. SDS-PAGE was performed using a BioRad apparat (Biorad) at 150 V on 10-12% Acrylamide gels loaded with 20-40 μg of denaturated proteins. Page Ruler™ Plus Prestained Protein Ladder (26619, Thermofisher) was added as molecular weight marker. Blotting was then employed to transfer proteins on nitrocellulose membrane (10600007, GE Healthcare Life Science) subsequently incubated for 1 h at R.T. in agitation in Tris-Buffered Saline with 0.05% Tween 20 (TBS-T) + 5% milk for saturation. Membranes were then incubated overnight at 4°C in agitation with the following primary antibodies diluted in TBS-T: NOTCH1 (polyclonal rabbit anti-human NOTCH-1, used at 0.1 μg/mL, Santa

Cruz Biotechnology sc-6014R, 1:1000, 5% BSA), Cleaved NOTCH-1 Val1744 (monoclonal rabbit anti-human cleaved NOTCH-1, D3B8, Cell Signaling Technology, 1:1000, 5% BSA), RUNX-2 (polyclonal goat anti-human RUNX-2, used at 1:20,000, 5% BSA), PHOSPHO-SAPK/JNK (Thr183/Tyr185) (polyclonal rabbit anti-human p-JNK, 92515, Cell Signalling Technology, 1:1000, 5% BSA), SIRT-1 (monoclonal mouse anti-human SIRT-1 B-7, sc-74465, Santa Cruz Biotechnology, used at 1:400, 5% milk).
After three repeated washing in TSB-T, membranes were incubated 1 h at R.T. in TSB-T + 3% milk added with either anti-mouse, anti-rabbit or anti-goat secondary antibody (1:2000) (Horseradish peroxidase-conjugated anti-mouse IgG, 70765, Cell Signaling Technology; or Horseradish peroxidase-conjugated anti-rabbit IgG, 70745, Cell Signaling Technology; Horseradish peroxidase-conjugated anti-goat IgG, Jackson). Western Blot bands were detected using ECL Select (GE Healthcare) with a ChemiDoc MP system (BioRad). β-actin (A4316, Sigma) or GAPDH (mouse monoclonal, clone, used at 0.8 μg/mL, MAB374, Sigma) served as loading control and for normalization of band intensity quantification done with the data-analysis software Image Lab (Version 4.1.).

MMPs quantitative assessment

The Bio-Plex Pro™ Human MMP Panel, 9-Plex (171AM001M, BioRad), is a highly sensitive multiplex bead-based sandwich immunoassay kit that allows to simultaneously evaluate multiple targets. Supernatants of micromasses at 3 time point (1w-2w-3w) derived from 3 control siRNA (NC) or NOTCH-1 siRNA (N1) micromasses were used to test the role of NOTCH-1 on the expression of major matrix metalloproteinases. The assay was performed in 96 multiwell plates. A standard curve was made to normalize the data. 50 μL of supernatant of NC and N1 micromasses were used. All buffers were reconstituted in DMEM+10% FBS supplemented with 50 μg/mL Ascorbic Acid, according to manufacturer's instructions. Magnetic beads were diluted to 1x concentration from 20x stock solution.
Magnetic beads are covalently attached to target antibodies and added to the supernatant samples separated. Following the protocol, samples were washed twice with Bio-Plex Buffer (BioRad) to remove unattached beads. Supernatant samples were added and incubated for 1 h at R.T. away from light, to allow interaction between the magnetic beads and the samples. Then, a biotinylated antibody 1X diluted from 20X Bio-Plex detection stock (BioRad) was added to form the sandwich complex. A 30' incubation away from light, followed. Samples were washed twice with Bio-Plex Buffer (BioRad) and incubated 10' with 1x fluorescent reporter consisting of a streptavidin-picoeritrin (SA-PE) conjugate was added to the solution to allow detection. Samples were resuspendend in assay Buffer (BioRad) and we proceeded with detection. Different targets were

divided through magnetic separation. In particular, we analyzed the three collagenases (collagenase1/MMP-1, collagenase 2/MMP-8 and collagenase 3/MMP-13), the two stromelysins (stromelysin1/MMP-3 and stromelysin2/2MMP-10), the matrylisin/MMP-7 and the macrophage metalloelastase MMP-12.

Viability assay in 3-D constructs

Loss of maturational arrest leads to hypertrophy and terminal differentiation in chondrocytes which comes along with cell death. This process can be replicated *in vitro* with 3w micromasses [208]. Therefore, cell viability was assessed in NC and N1 micromasses at 3w maturation by means of CellTiterGlo^{TM} (Promega) viability assay, a product specifically built for evaluation of cell death in 3-D cultures. Viability is measured by luminescence intensity released by a thermostable luciferase that binds ATP. The luminescence signal, defined as "glow type" is directly proportional to ATP in the sample. A lysis buffer, provided by manufacturer, was added to the cells to allow extraction of ATP from viable cells. Luminescence was measured with NANO M+ plate reader (Tecan). Measurements for N1 samples were normalized on relative control (NC). N1 increased viability of 3 weeks samples of 1.82 ± 2.29 (mean ± standard deviation, p=0.04, n=3) fold.

Alizarin-RED staining

Calcium and apatite crystals deposits were evaluated by means of Alizarin-RED S (AR-S) staining [209]. These dye gives an orange-red to crystals easily identifiable in micromasses. A solution of 2% Alizarin-RED (A5533, SIGMA) diluted in water at 4.2 pH was used. Staining was conducted on NC-N1 at 1-2-3-week maturation included in Optimum Cutting temperature (OCT) and conserved at -20°C and subsequently cut by means of cryostat in slices (5 µm) and laid on silanated slides. Micromasses slices were then fixed with 100 µl 4% PFA for 30' at R.T.; samples were then washed in PBS 1x for 5' then immerged in water. A drop of AR-S was put on each slice (using a syringe with 0.45 µm filter) and incubated at R.T. for 5'. Then, samples were washed in water to remove the excess of dye then dehydrated sequentially in 70%, 80%, 100% A and 100% B EtOH solutions for 2' each. A 5' incubation in 2 solutions of 100% xylene followed. Finally, slices were mounted with Entellan (Sigma); images were acquired by means of Eclipse 901 Nikon microscope using 10x magnification.

Toluidine Blue staining

Proteoglycans and GAG deposits in micromasses were evaluated by means of Toluidin-Blue staining. This dye has a higher affinity to sulphur present in macromolecules in articular cartilage and maintains a high staining efficiency even if the matrix is depleted from these components [210], as it occurs in 3-D cultures of primary chondrocytes. NC and N1 micromasses were prepared following the procedure previously described for AR-S staining with the only variation of the dye used. The sections were stained with freshly filtered 1% toluidine blue in 0.5% sodium borate, pH 4.2. Then, the protocol was the same as the one of Alizarin Red.

Immunoistochemical and immunofluorescent staining

NC and N1 micromasses were included in OCT compound then cut in 5 µm thick slices. Following, samples were fixed in 4% PFA for 30' at R.T., then washed 5' in IHC-TBS. During all subsequent incubations slides were kept in humid chamber and washings done in rotatory agitation.
First, an antigen unmasking was performed using 0.02 U/mL Chondroitinase ABC (C3667, Sigma) diluted in 50 mM pH 8 HCl Tris buffer (20' at 37°C) then washed in 1x TBS 5'. Blocking of non-specific binding followed (5% BSA, 5% donkey serum and 0.1% Triton X-100 in TBS for 30'). Afterwards, N1 samples were incubated 2h at R.T. with primary antibodies diluted in TBS, 3% donkey serum + 2% BSA + 0.1% Triton X-100 with a final concentration of 500 µg/mL. Samples were treated either with anti-human Runx-2 (R&D MAB2006, rat monoclonal, 1:100) or anti-HES-1 (Invitrogen PA5-28802, rabbit policlonal, 1:200). After 2 washing with TBS 5', the Supersensitive ICH Detection System (Biogenex) was added to detect mouse, rat or rabbit primary antibodies exploiting the avidin-biotin amplification system. To localize the alkaline phosphatase enzymes at antigen sites finally we used FAST RED substrate (5 mL Naftol phosphate in Tris Buffer, provided by manufacturer). The progression of the reaction was monitored by means of microscope observation and blocked after 30'. Micromass slices were then mounted with Aquamount; pictures were acquired by means of Eclipse 901 Nikon microscope using 20x magnification.
For immunofluorescence detections of NOTCH-1 (Novus Biologicals, NBP1-78292, 1:200) and HES-1 (Invitrogen, PA5-28802, 1:200), NC and N1 chondrocytes were seeded onto chamber slides (8 well-chamber slides, at a density of 10,000 cells per cm^2), and fixed in 4% PFA as previously described. After the incubation with primary antibodies, samples were rinsed in TBS, and the signal was revealed by a 15µg/ml donkey anti-rabbit Alexa Fluor 555 secondary antibody conjugate (Novex) in TBS with 3% BSA 0.1% Tween and incubated 30' at R.T. together with 1µg/ml

Hoechst 33342 (Sigma) for nuclear counterstaining. At the end, the samples were mounted with the addition of anti-fading (1% 1.4 Diazobicyclo (2.2.2) Octane (Sigma) in 90% glycerol, in 0.1 M pH 8.0 Tris-HCl), sealed with nail-polish and stored refrigerated and away from light for subsequent analysis.

DCFDA Cellular ROS Assay

C28/I2 chondrocytes were seeded at a density of ~15,000 cells/cm^2 in 96-well with clear flat bottom (Sarstedt). After 24h they were pre-treated with OE and HT 100 µM for 16 h. Afterwards, DCFDA (2',7'-dichlorofluorescein) was added, following manufacturer's instruction for Cellular ROS detection assay kit (ab13851, Abcam). Cells were washed once with 100 µL 1X Buffer and then exposed to 25 µM DCFDA in 1X Buffer for 30' at 37°C repaired from light. The solution was removed and stained cells were washed again with 100 µL 1X Buffer. Then, cells were treated with LPS 10 µg/mL. DCFDA signal was detected at 2-4-6-24-48-120 h, at Ex/Em = 485/535 nm with Infinite NANO M+ plate reader (Tecan). Tert-Butyl Hydrogen Peroxide (TBHP) 55 µM (Abcam) and hydrogen peroxide 100 µM were used as positive control. Each experimental condition was tested in triplicates or quadruplicates; the experiment was repeated five times.

Statistical analysis

Data are represented as mean ± standard deviation (SD) and compared by mean of Student's T test using the GraphPad Prism 5.0 software (GRAPHPAD SOFTWARE, La Jolla, CA, USA). Differences were considered significant when $p < 0.05$ with *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

Aim of the study

Current lack of disease modifying therapies in OA requires urgent identification of novel therapeutic targets and clarification of the molecular mechanisms involved in the onset and progression of this degenerative disease.

Our study aims to investigate the role of NOTCH-1 signalling pathway in OA and the mechanisms by which it exerts its action.

Furthermore, our goal is to identify downstream effectors of NOTCH-1 signalling already known to be involved in the etiopathogenesis of OA. By elucidating this interaction, we hope to better understand the processes that control the progression of the disease.

Finally, we aim to investigate the potential therapeutic role of two olive-derived polyphenols, OE and HT, in OA. In particular, we mean to test the chondroprotective properties of these compounds and their effects on the modulation of OA markers.

Results

Validation of NOTCH-1 silencing

The efficiency of NOTCH-1 transient silencing by means of siRNA transfection was determined by Western Blot and real time RT-PCR (Fig. 1). At the protein level, assessment of NOTCH-1 active domain, NICD1 (about 110 KDa), was evaluated 48 h after transfection showing a reduction by 58 % ± 20 (mean ± SD) compared to the siRNA negative control samples. The analysis was conducted on a set of five samples of primary chondrocytes derived from patients with OA.

At the same time point, mRNA levels for NOTCH-1 were assessed by real time RT-PCR, in an equal number of samples, pointing at a percentage decrease of expression of 82 ± 14 (mean ±SD) compared to the control.

The high variability of NOTCH-1 gene expression among the samples of primary cultures evaluated reflects the uneven pathophysiological condition of the population from which the cells derive. To overcome this issue, variance normalization for the comparison is represented by using a logarithmic (Log_{10}) scale. Noticeably, the silencing was more efficient at the mRNA level compared to the active protein one. This difference may be justified by the longer time required by the cell to get rid of highly expressed proteins, such as NOTCH-1, after transfection compared to

the transcript. However, the levels of both protein and mRNA for NOTCH-1 resulted significantly reduced after silencing compared to the control.

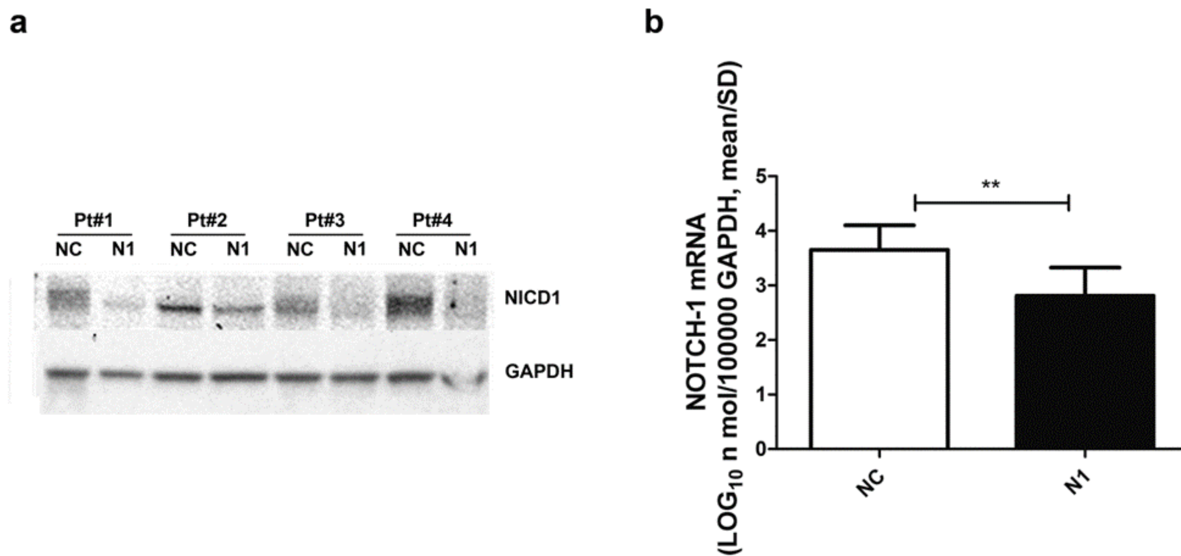


Figure 1. Efficiency of NOTCH-1 silencing: a) evaluation of NOTCH-1 KD in 4 different samples by means of Western Blot; the band shown corresponds to NICD1 (110 KDa), the active domain of NOTCH-1, at 48 h post transfection. Reduction of protein expression was quantified and GAPDH was used as loading control. b) NOTCH-1 KD expression assessed by real time RT-PCR. (mean ±SD, n=5). NC, control and N1, NOTCH1 silenced chondrocytes.

NOTCH-1 silencing interferes with chondrocytes proliferation

Considering that chondrocyte maturation is usually associated with proliferation and the role exerted by NOTCH-1 in the regulation of this process, we investigated if there was any variation in cell proliferation after silencing. Cell proliferation by quantitative DNA analysis was evaluated for 12 days starting from 48 h after transfection. Figure 2a shows DNA staining of NOTCH1 KD (N1) cells compared to control (NC) cells. The graph reports the cumulative data of three experiments, each performed in quintuplicate. N1 cells appeared to proliferate at a significant lower rate than NC cells. Presumably, this effect is a direct consequence of NOTCH-1 ablation and could be the result of its involvement in DNA replication, as suggested by fluorescence signal showing its increased expression in dividing cells (Fig. 2b). Supporting this observation, NOTCH-1 primary target HES-1 was found to be localized particularly in the nuclei.

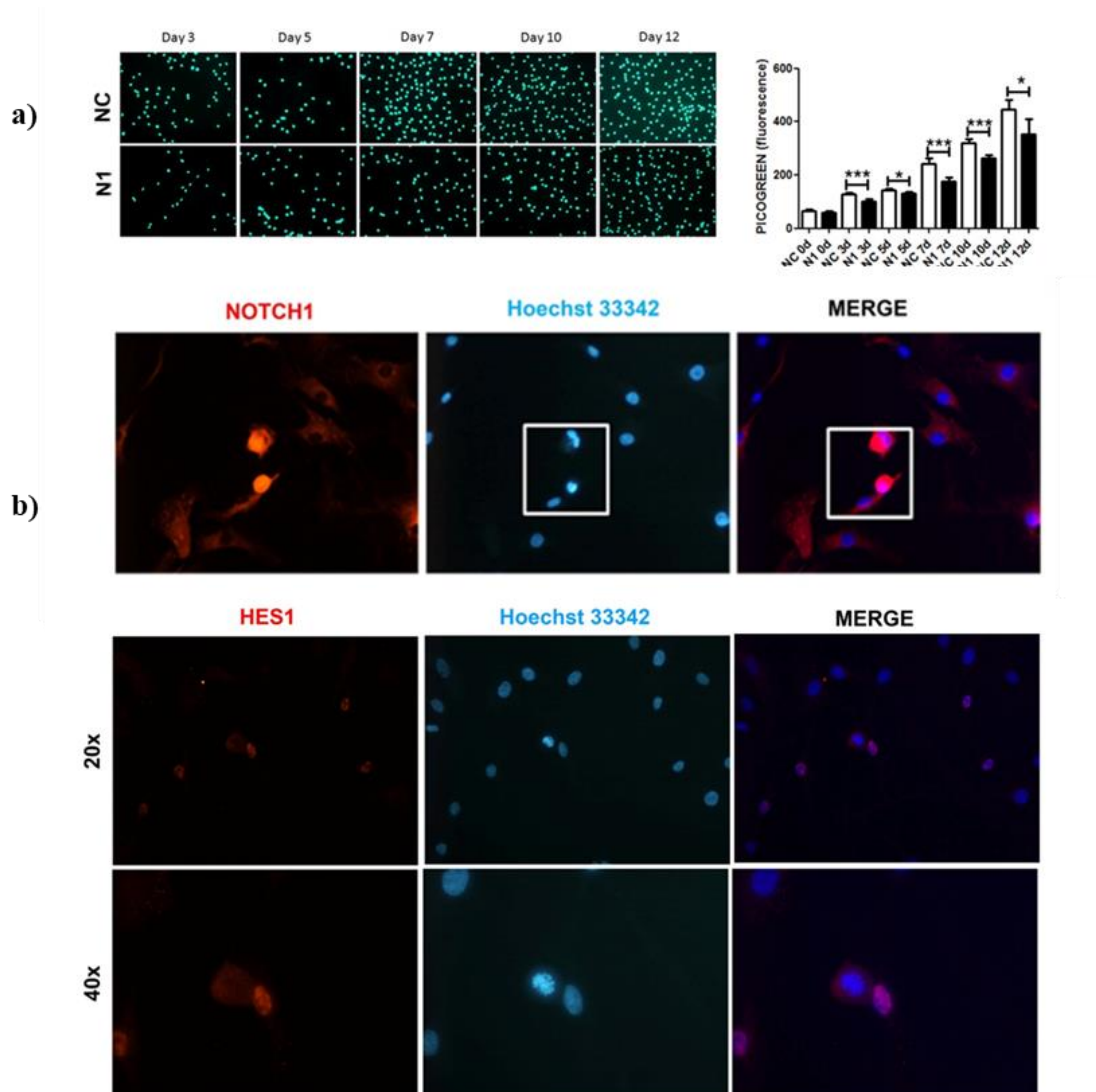


Figure 2. Effects of NOTCH-1 KD on chondrocyte proliferation. Primary chondrocytes were seeded at 48 h post transfection (Day 0) and then analysed at assigned time points (Day 3,5,7,10,12). a) DNA staining by means of PicoGreen of NC (upper row) compared to N1 silenced (lower row) cells; the column graph shows the mean ± SD of the fluorescence intensity obtained by scanning the bottom of the quintuplicate wells (n=3); b) Top: Immunofluorescence of NOTCH-1 in dividing cells; Bottom: immunofluorescence of HES-1 co-localizing with Hoechst staining of nucleus.

NOTCH-1 knock down delays primary chondrocytes differentiation in 3-D cultures

The role of NOTCH-1 silencing was investigated in depth in regard to maturation of chondrocytes towards terminal differentiation. To better assess the effects of NOTCH-1 in these pivotal mechanisms of OA pathogenesis, we used 3-D cultures, also known as micromasses. This *in vitro* model of OA allows to replicate differentiation process in a similar fashion to what happens in

physiological conditions in cartilage during endochondral ossification. 3-D cultures were set up from monolayer primary chondrocytes cultures silenced for NOTCH-1 or from control cells. To avoid bias connected to inter-patient variability, NOTCH-1 dependent variations in expression of differentiation markers were analysed in micromasses at 1-week maturation (Fig. 3).

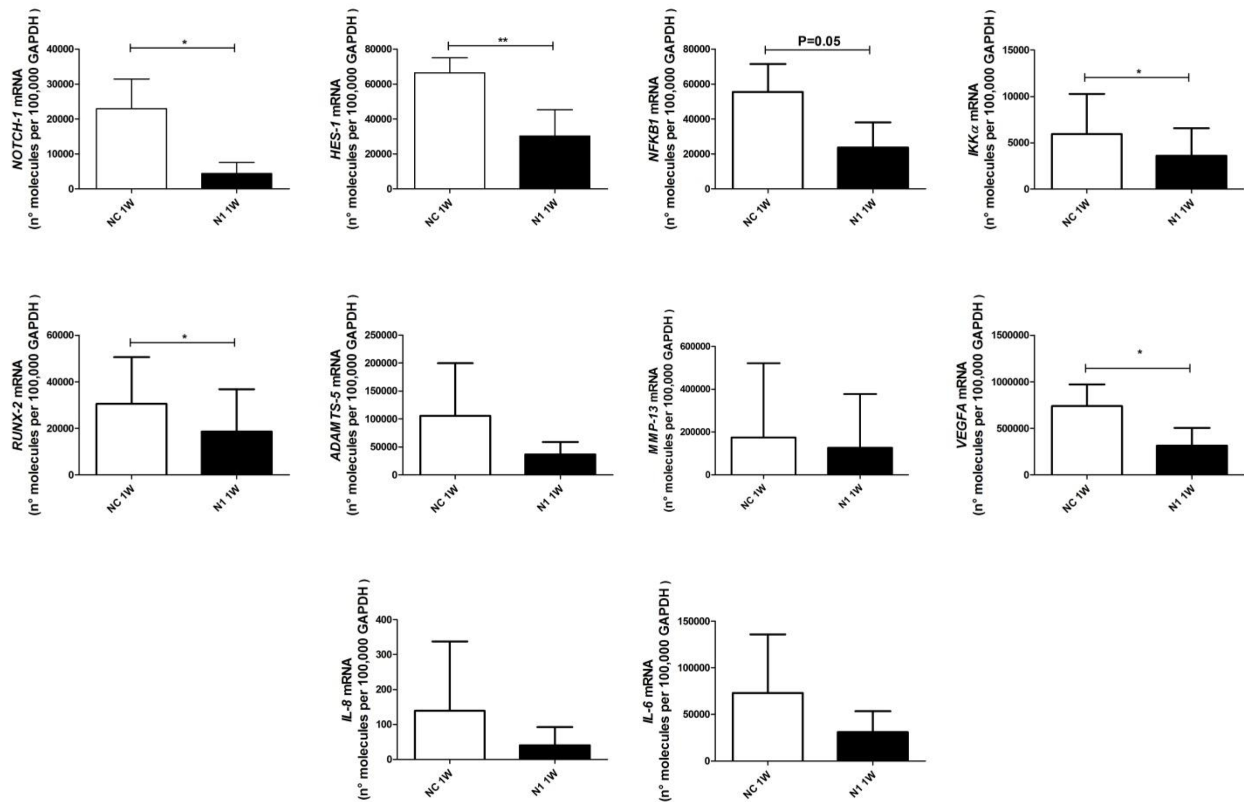


Figure 3. Effects of NOTCH-1 silencing on the transcript expression of OA markers. Total RNA was extracted from 3-D cultures of NC and N1 samples at 1 week maturation. mRNA levels were calculated for each target gene and normalized using the reference housekeeping gene GAPDH according to the formula $2^{-\Delta Ct}$ and expressed as number of molecules per 100,000 GAPDH molecules.

First, to demonstrate that modulation of target genes was strictly correlated to NOTCH-1 KD, we assessed NOTCH-1 gene expression levels. Data show a significant decrease of NOTCH-1 expression, with knockdown level pointing at a percentage reduction of 73 ± 30 (mean ± SD, p= 0.0134, n=5), in N1 transfected samples compared to control, therefore proving a persistence of siRNA mediated gene silencing at this time point. Then, we proceed by assessing the effects of the KD on NOTCH signalling intermediates and potential targets. As expected, we found a significant decrease of NOTCH-1 primary target HES-1 in silenced micromasses compared to control. We then proceed to investigate potential targets of NOTCH-1 involved in OA. Interestingly, we found a statistically significant reduction of NFKB1, i.e, p105, and its upstream activating kinase IKKα/CHUK, which, in addition to being critically involved in OA, is also known for its interaction with NOTCH pathway. Furthermore, we investigated modulations in gene expression of differentiation markers linked to progressive differentiation of chondrocytes from resting to

hypertrophic and finally terminally differentiate state, as described previously. Our results show a significant decrease of RUNX-2 expression in silenced micromasses compared to NC. RUNX-2 is a transcription factor pivotal in the pathogenesis of OA, since it sustains differentiation of chondrocytes towards hypertrophy. Furthermore, the expression of ADAMTS-5 and MMP-13, the two proteases mostly responsible for ECM remodelling and degradation, decreased significantly after silencing of NOTCH-1. Lastly, we observed a significant decrease in inflammatory genes, particularly IL-6 and IL-8, involved in the maintenance of low-grade inflammation typical of late stage of OA. Another marker of OA progression and more specifically of cartilage vascularization, VEGF, resulted reduced in silenced samples compared to controls.

As these results pointed to an involvement of NOTCH-1 silencing in delaying terminal differentiation of chondrocytes, possibly through a reduction of RUNX-2 mRNA expression, we assessed the effects of NOTCH-1 silencing on RUNX-2 at the protein level. Western Blot for RUNX-2, normalized on β-actin, carried out on micromasses at 1 week maturation clearly shows a significant decrease ($p < 0.05$, n=3) in N1 samples compared to control (Fig. 4).

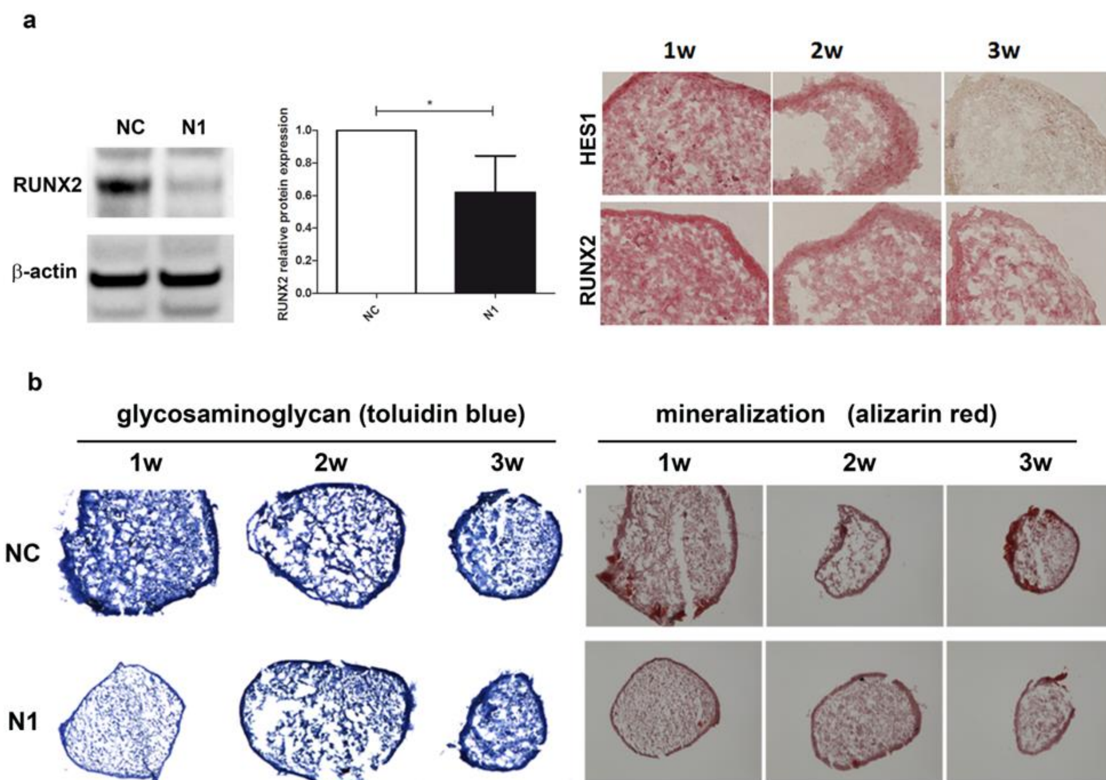


Figure 4. Effects of NOTCH-1 silencing on chondrocytes differentiation in micromasses. a) NOTCH1 silencing impacts RUNX2 expression: left, a representative Western Blot indicates that RUNX2 protein is significantly reduced in NOTCH1 silenced 3-D cultures at 1w, as confirmed in the cumulative right graph (n=5, p<0.05); right, immunohistochemistry imaging of the correlated reduced expression of RUNX2 and HES1 in micromass maturation across 1-3 weeks. b) NOTCH1 silencing impacts ECM remodelling. Left, Toluidin Blue staining of showed delayed GAG deposition across week 1-3 weeks in N1 KD samples compared to control. Right: calcium deposition, evidenced by Alizarin Red staining on 3-D constructs, showed a marked decrease at all time points in N1 KD micromasses compared to control.

Furthermore, expression of RUNX-2 in chondrocytes, investigated through immunoistochemical staining of micromass slices, shows a progressive decrease of RUNX-2 expression across 3 weeks of maturation. This reduction goes along with the almost ablated expression of NOTCH-1 primary target, HES-1, in the same conditions.

In addition to that, and probably as a consequence of RUNX-2 reduced expression, we observed a delayed maturation of the ECM as established by a marked decrease in glycosaminoglycan (GAG) deposition in N1 1w micromasses compared to control (Fig. 4). Furthermore, calcium crystal deposition, a sign of calcification often observed in end-stage OA progression, was investigated by means of Alizarin red staining, showing a progressive reduction across the 3 weeks of maturation.

NOTCH-1 transient silencing ameliorates matrix remodelling in micromasses

Since the data obtained from gene expression of pivotal OA markers of differentiation and disease progression pointed to an effect of NOTCH-1 silencing in slowing down terminal differentiation of chondrocytes, we expected to observe a similar trend in ECM remodelling. To assess this hypothesis, we investigated the release of catabolic enzymes in 3-D cultures. The analysis was carried out using the supernatant of micromasses across the three weeks period of maturation. Chondrocytes derived from samples of four different patients were isolated and used to set up 3-D cultures post NOTCH-1 transfection. For each patient, triplicates obtained from different micromasses were tested in parallel by means of Bioplex assay. This allowed the investigation of the release of MMPs repertoire, the most prominent enzymes involved in ECM remodelling in OA. Results show a clear effect of NOTCH-1 KD on downstream effectors of ECM remodelling. In particular, a reduction of MMPs release was significant in the early phases of maturation, i.e, 1 week, as shown in Fig 5. Quantification of major collagenases MMP-1 and MMP-13 showed a statistically significant decrease in KD samples (N1) compared to NC. In addition, the levels of the two stromelysines MMP-3 and MMP-10 were significantly reduced in N1 samples. In particular MMP-10 release was almost completely ablated at 1 week. This result is particularly relevant, since MMP-10 is pivotal in the activation of collagenases such as MMP-13, directly involved in degradation of the matrix. MMP-2 and MMP-7 were also tested among the set of MMPs but statistical analysis did not show a significant decrease compared to control (data not shown).

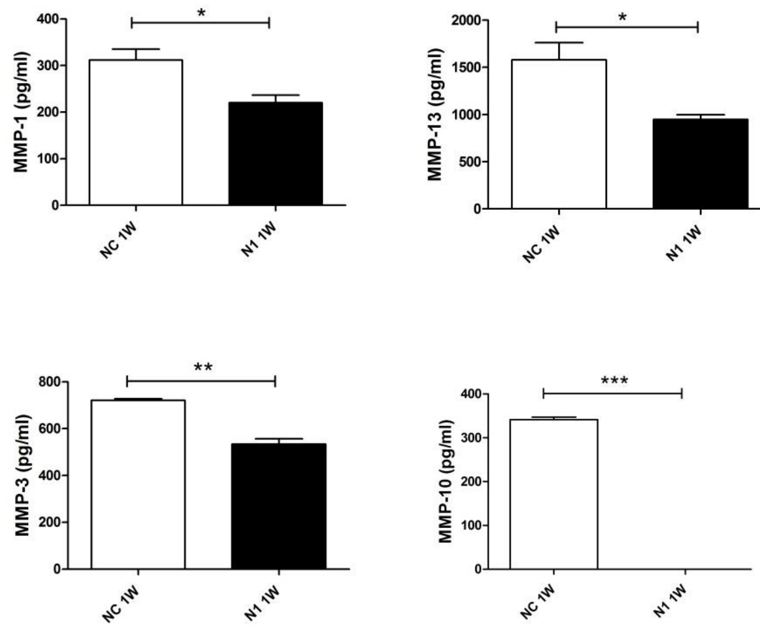


Figure 5 NOTCH-1 silencing affects ECM degradation in micromasses. Graphs show the quantification of the repertoire of MMPs analysed by means of BioPlex assay of a representative patient out of four analysed. Data are relative to supernatants of triplicate 3-D cultures and are expressed as mean ± SD. Negative control samples (NC) at 1 week maturation are compared to NOTCH-1 (N1) silenced ones.

Chondrocytes viability increases after NOTCH-1 KD

Chondrocytes 3-D cultures recapitulate the progression from hypertrophy to terminal differentiation leading to cell death in OA. Attenuation of cellular loss can be obtained by targeting specific differentiation effectors in OA [208]. Therefore, we investigated whether NOTCH-1 transient silencing had any effect in this regard. Since cellular death in OA begins at later phases of disease progression, we analysed micromasses after 3 weeks of maturation in order to better assess long term effects of NOTCH-1 KD. Interestingly, cell viability, measured by means of Cell Titer GLO 3-D assay, was found to be significantly increased in NOTCH-1 deficient cells compared to control (Fig. 6). These results are consistent with the previous data showing a possible delay in terminal differentiation of primary chondrocytes induced by NOTCH-1 KD.

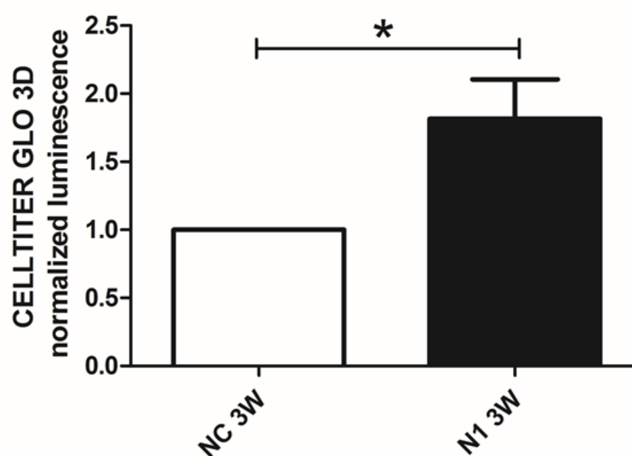


Figure 6. Effects of NOTCH-1 on chondrocytes viability in 3-D constructs. Cell viability was assessed in micromasses at 3 week maturation by means of the Cell Titer GLO 3D cell viability assay. N1 samples exhibited a statistically significant increased viability compared to control. Data represent N1 luminescence normalized as fold change compared to NC values and are expressed as mean±SD (n=3). Statistical analysis was performed by Student's t test for paired samples.

Lipopolysaccharide stimulates inflammation markers in C28/I2 and primary chondrocytes

After establishing a clear involvement of NOTCH-1 in the OA-related micromass changes, we decided to further investigate its role by establishing an *in vitro* model that replicates the microenvironment present in articular cartilage in OA and particularly the inflammatory state universally considered to sustain the progression to final stages of the disease. To do so we exposed primary chondrocytes to high doses of lipopolysaccharide (LPS).

To overcome the limitation given by limited source of material derived from patients with OA, we exposed an immortalized chondrocytes cell line, C28/I2, to the same stimulus. In both cellular models, LPS showed to be a reliable inflammatory stimulus as demonstrated by increased expression of inflammation markers iNOS and COX-2 (Fig. 7). Due to the large variability in gene expression of these markers encountered in patient-derived chondrocytes, we represented the results normalized on GAPDH molecules to obtain more comparable data. In C28/I2 this adjustment was not necessary given the more stable response of this cell line to the stimulus. Taken together our results suggest that the two *in vitro* models are comparable in our experimental setting.

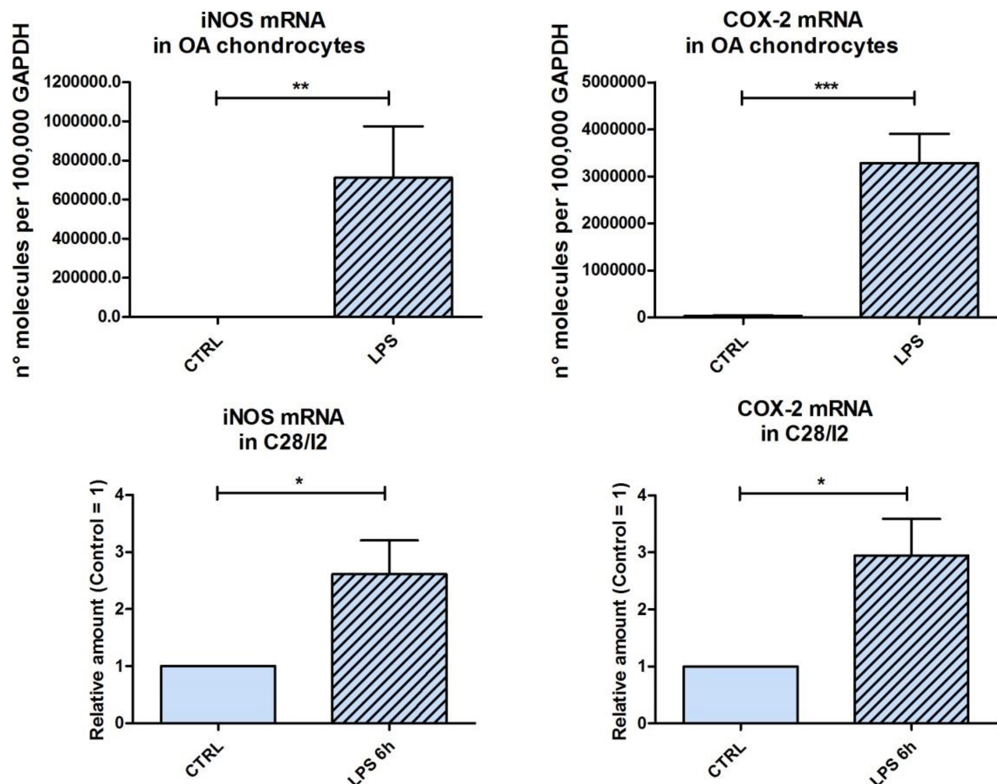


Figure 7. Effects of lipopolysaccharide (LPS) on inflammatory response. mRNA expression of inflammation markers in primary chondrocytes and C28/I2 chondrocytes significantly increased after 6 h LPS stimulation. Quantification of expression levels was calculated for each target gene and normalized using the reference housekeeping gene GAPDH according to the formula $2^{-\Delta Ct}$ and expressed as number of molecules per 100,000 GAPDH molecules in OA chondrocytes to flatten inter-patient variability. In C28/I2 cells, relative control was posed equal to 1 since less variability occurred.

Olive-derived nutraceuticals are efficient in limiting the effects of LPS treatment

We moved on to investigate whether the effects of LPS could be prevented by exposing cells to nutraceuticals. These food-derived compounds are more and more studied in the field of OA due to their potential therapeutic effect. To establish which compounds are more effective in our experimental setting, we started by treating primary chondrocytes with a set of seven different nutraceuticals. These compounds were chosen based on results from literature and previous research conducted by our group. Five of these nutraceuticals are classified as polyphenols (EGCG, OE, HT, OC and curcumin), whereas two, EPA and DHA, are PUFAs. Our results showed that among the compounds tested only three, all olive-derived, showed a consistent effect in preventing LPS-induced inflammation, as clearly highlighted by a statistically relevant decrease of inflammation markers iNOS and COX-2. After this preliminary evaluation, we decided to focus on two olive-derived compounds, HT and its precursor OE. OC was excluded because, in spite of its

undoubted efficacy, it proved to be highly toxic on chondrocytes at very low concentrations compared to other nutraceuticals.

OE and HT show antioxidant properties against LPS-induced ROS production

Among the known properties of polyphenols, such as OE and HT, the anti-oxidative effect is the most studied and has been proven in several models. On these premises, we moved on to test if these effects were also present in our experimental conditions. As in previous experiments LPS showed a pro-inflammatory activity, we expected that this stimulus sustained an increase in ROS production as well. Oxidative stress was investigated by means of 2'-7'-dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that allows a sensitive quantification of ROS within the cells. A time-course of the level of ROS after LPS is shown in Fig. 8a. LPS treatment provoked a progressive and significant increase in ROS production starting from 2h up to 120 h. The increment in ROS production given by LPS was compared with two positive controls, hydrogen peroxide and its stabilised form tert-butyl hydrogen peroxide (TBHP), resulting in values higher, but still comparable to LPS (data not shown). Our data show that the longer the incubation with LPS, the larger is the increase in ROS production. Anyway, variability of samples at 120 h post stimulation was very high, therefore we considered the previous time point (48 h) for statistical analysis, demonstrating a significant increase in ROS production induced by LPS in C28/I2 chondrocytes (Fig. 8b). Then, we moved on to investigate the antioxidant properties of HT and OE. C28/I2 were pre-treated with nutraceuticals and then stimulated with LPS for the previously established time points before ROS assay. As expected, both OE and HT showed a relevant capacity in preventing LPS-induced ROS increase. Again, the most significant anti-oxidant effect resulted 48 h after stimulation. These results confirm that OE and HT exert an anti-oxidant action and a consequent protective effect versus oxidative stress in our experimental model.

a)

b)

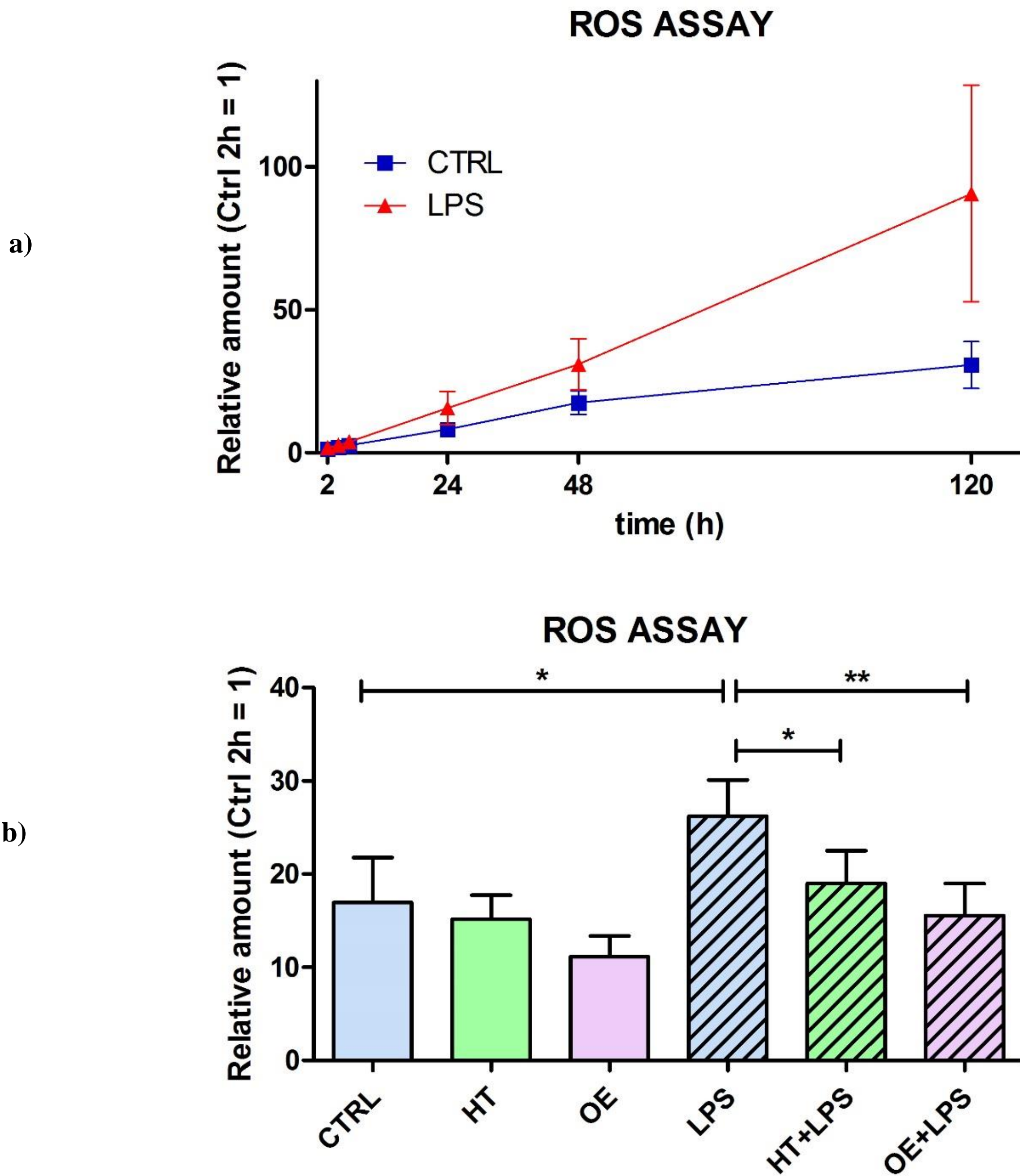


Figure 8. Antioxidant effect of OE and HT in C28/I2 chondrocytes. a) LPS treatment increase ROS production progressively from time 2 h to 120 h compared to control. ROS were assessed by fluorescence intensity of DCFDA. b) pre-treatment of C28/I2 chondrocytes with olive-derived nutraceuticals OE and HT showed a statistically significant prevention of LPS-induced oxidative stress at 48 h. Data represent results from multiple analysis (n =5) of quadruplicate samples for each experimental condition expressed as mean ± SD and normalized with respect to 2h control. Statistical analysis was performed by Student's t test for paired samples.

OE and HT exert chondroprotective effects against markers of inflammation

Given the encouraging results obtained in C28/I2 chondrocytes, we moved on to test whether OE and HT exert a chondroprotective effect in primary chondrocytes and particularly if their activity could replicate that of siRNA in modulating NOTCH-1 and its targets.

First, having previously proven the efficacy of LPS in activating the expression of inflammatory markers such as iNOS and COX-2, we started our investigation by testing if the combination with nutraceuticals prevented their increase. As shown in Fig. 9, the expression of iNOS and COX-2 after OE or HT alone is comparable to the appropriate control and both olive-derived nutraceuticals were effective in acting against LPS-related increase of inflammatory markers.

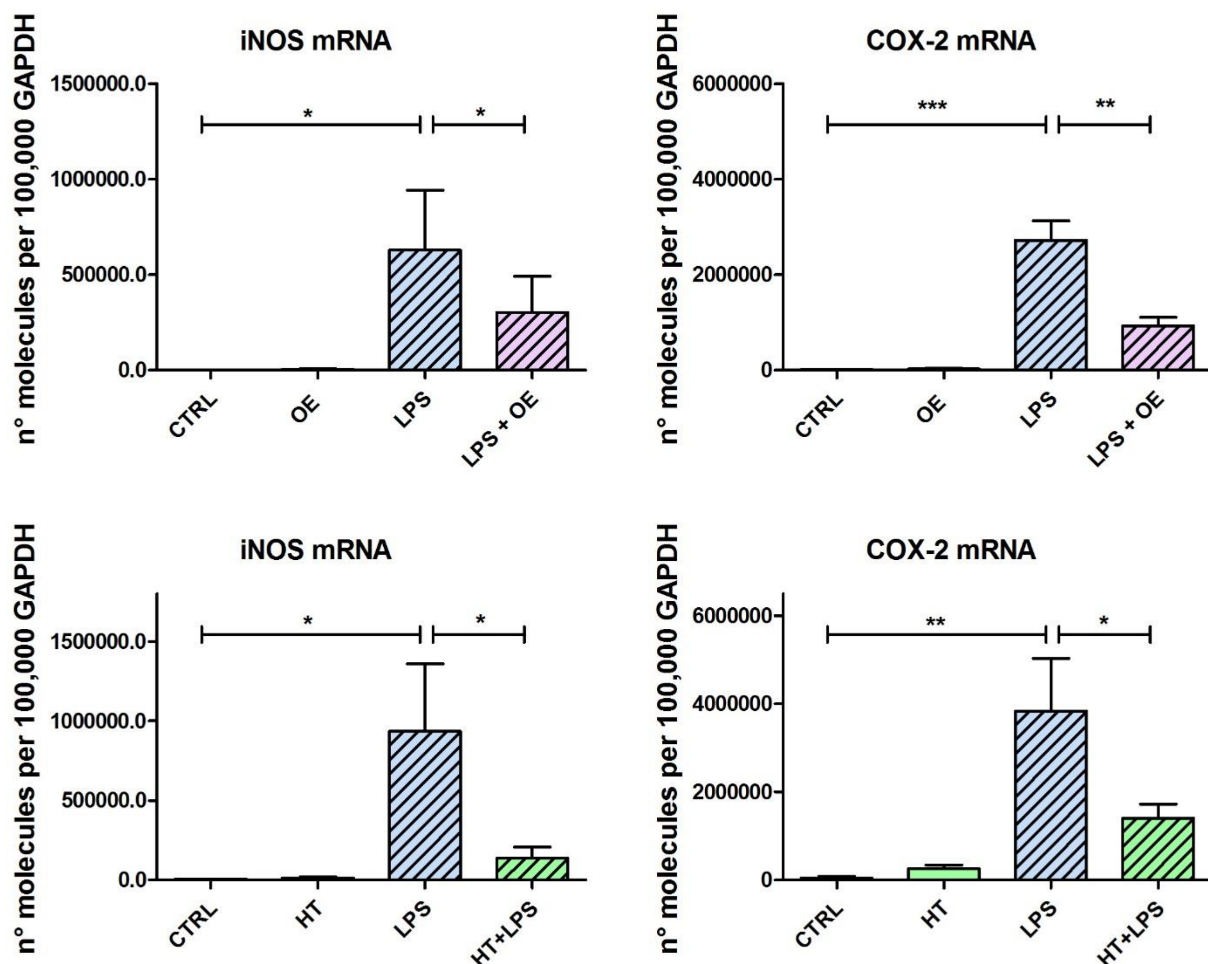


Figure 9. OE and HT affect LPS-induced inflammatory response. Gene expression of inflammation markers previously observed to be increased following LPS treatment are significantly reduced by nutraceuticals in human primary chondrocytes compared to appropriate control. Quantification of mRNA levels for each gene was normalized using the reference housekeeping gene GAPDH according to the formula $2^{-\Delta Ct}$ and expressed as number of molecules per 100,000 GAPDH molecules. Results are expressed as mean ± SD (n=5). Cells derived from the same patients were used for testing separately both nutraceuticals.

OE and HT reduce LPS-induced expression of NOTCH-1

After proving the potential beneficial effect of OE and HT in chondrocytes, we circled back to our target of interest NOTCH-1 to investigate a possible involvement of this signalling pathway in the mechanisms that lead to LPS-induced inflammation. Figure 10 shows a significant increase in NOTCH-1 expression by LPS in both C28/I2 and primary chondrocytes cultures.

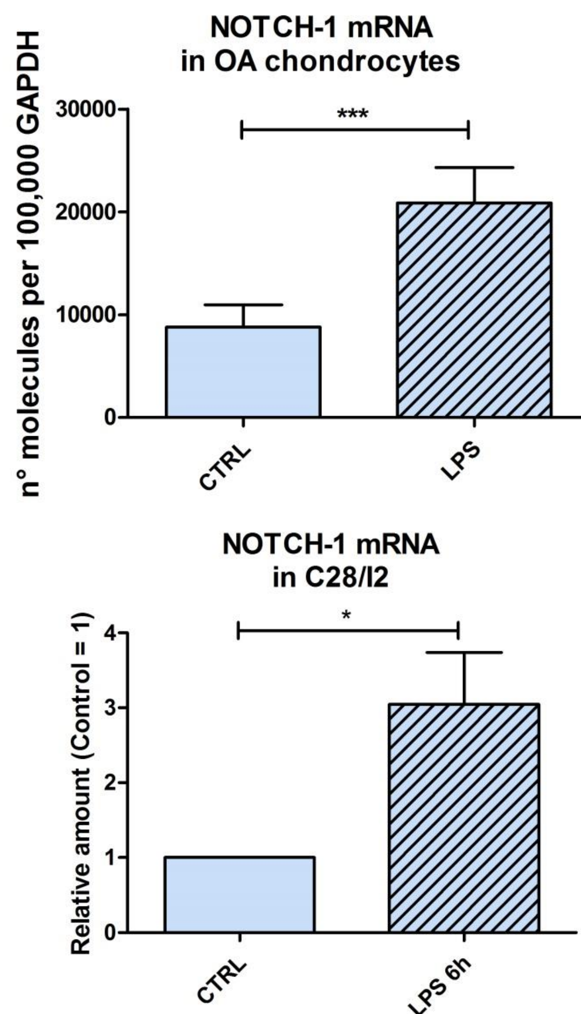


Figure 10. LPS induces NOTCH-1 pathway. Transcript expression of NOTCH-1 was investigated in C28/I2 and primary chondrocytes form patients with OA. In both models stimulation with LPS induced a significant increase of NOTCH-1 expression. LPS is expressed compared to control normalized using the reference housekeeping gene GAPDH according to the formula $2^{-\Delta Ct}$ *and expressed as number of molecules per 100,000 GAPDH molecules in OA chondrocytes to flatten inter-patient variability. In C28/I2 relative control was posed equal to 1 since less variability occurred (mean ± SD, n=5 for both models).*

Afterwards, we replicated the protocol used for assessing ROS production and pre-treated primary chondrocytes isolated from OA patients with OE and HT. Once again, we found that our

compounds of choice were able to decrease the transcript levels of NOTCH-1 significantly (Fig. 11).

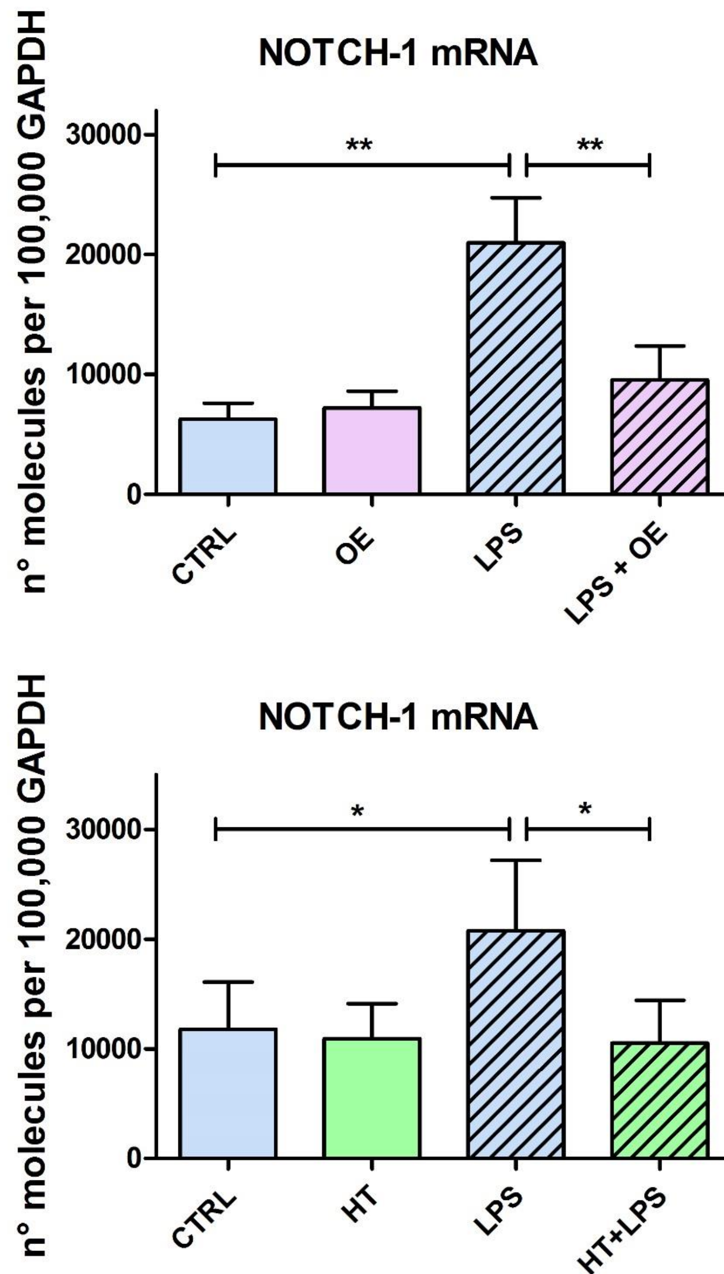
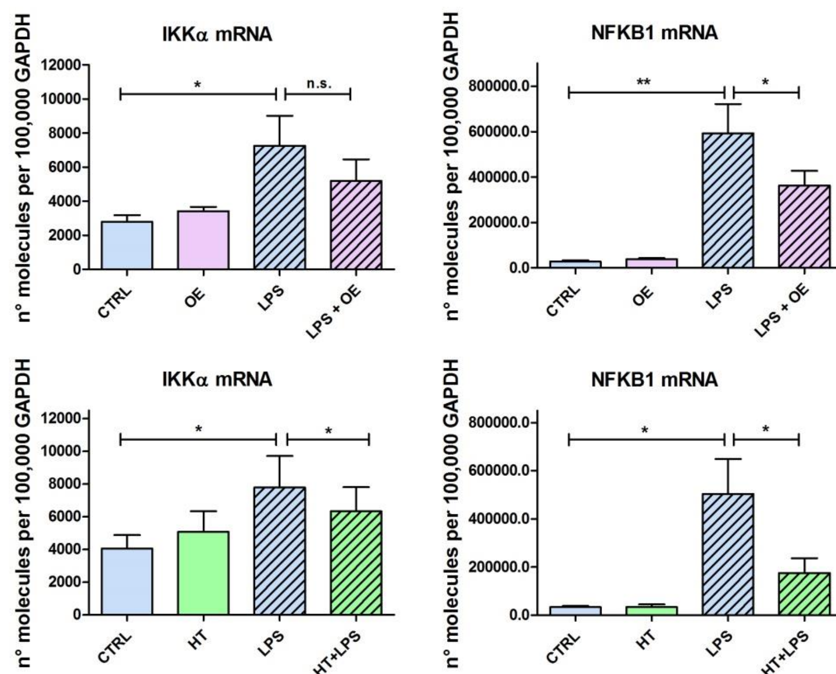


Figure 11. Effects of nutraceuticals on NOTCH-1 expression. OE (upper raw) and HT (lower raw) pre-treatment significantly reduced mRNA expression of NOTCH-1 induced by LPS. Analysis of multiple samples (n=5) is compared to control and normalised as in previous real time RT-PCR experiments.

Olive-derived nutraceuticals modulate OA-related and inflammation markers

To further elucidate the mechanism(s) by which NOTCH-1 may be involved in the effects of LPS, we have investigated whether other genes that we previously proved to be downstream effectors of Notch signalling were modulated in our experimental model. As shown in Fig. 12, LPS is effective in inducing an increase in the expression of NFKB1 and its upstream effector IKKα, two pivotal signalling pathways involved in the progression and sustainment of low-grade inflammation in OA. In addition to that, two mediators of inflammation, interleukin -6 and -8 (IL-6, IL-8), usually released in the ECM at late stages of disease, were significantly more expressed after LPS treatment compared to control. Taken together these results support the previously identified relation between these genes and NOTCH-1, which could be mediated by NOTCH signalling and other molecular mechanisms induced by LPS. Furthermore, for all the target genes analysed, data clearly demonstrate a strong chondroprotective effect exerted by OE and HT against LPS. This modulation suggests a potential beneficial role of OE and HT in reducing the inflammatory state that follows progression of OA.

a)



b)

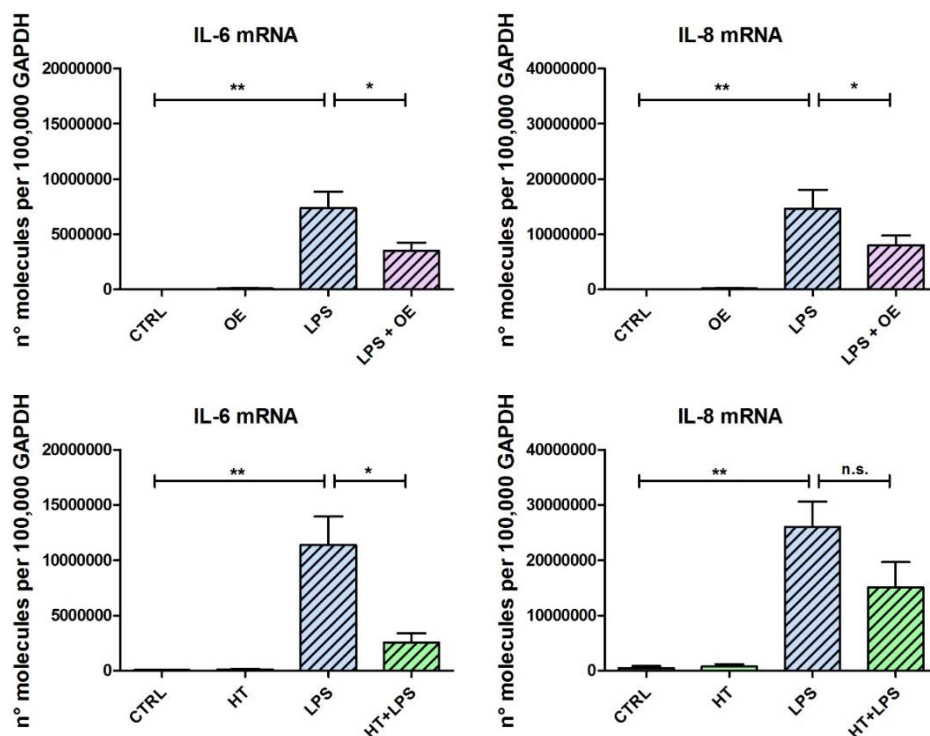


Figure 12 a-b. Effects of nutraceuticals on OA markers. LPS treatment increases markers of osteoarthritis in primary chondrocytes and combination with OE and HT efficiently prevents this effect. Data from cells treated with LPS or nutraceuticals + LPS combination were compared to appropriate control and obtained from the same set of samples (n=5); results are means ± SD, normalized using the reference housekeeping gene GAPDH according to the formula $2^{-\Delta Ct}$ *and expressed as number of molecules per 100,000 GAPDH molecules in OA chondrocytes to flatten inter-patient variability.*

Finally, to assess if these olive-derived compounds have a chondroprotective effect against ECM remodelling and degradation, we investigated the mRNA modulation of OA differentiation markers that sustain these processes. Contrarily to what observed in 3-D culture of chondrocytes, we did not observe modulation of neither RUNX-2, a pivotal differentiation marker in pre- and hypertrophic chondrocytes, nor VEGF, a primary effector of vascularization in OA articular cartilage (data not shown). Presumably, since RUNX-2 and VEGF underlie processes occurring in later stages of chondrocyte maturation and not reproducible in monolayer cultures, these discrepancies depend on the different *in vitro* model used for the experiments.

Nevertheless, we were able to observe a relevant modulation of MMP-13 expression, the major protease involved in ECM degradation and remodelling, which is reduced in presence of nutraceuticals in spite of LPS stimulation (Fig. 13). Altogether, these results support what demonstrated previously regarding the strict interaction between MMP-13 and its upstream mediator NOTCH-1 in chondrocytes.

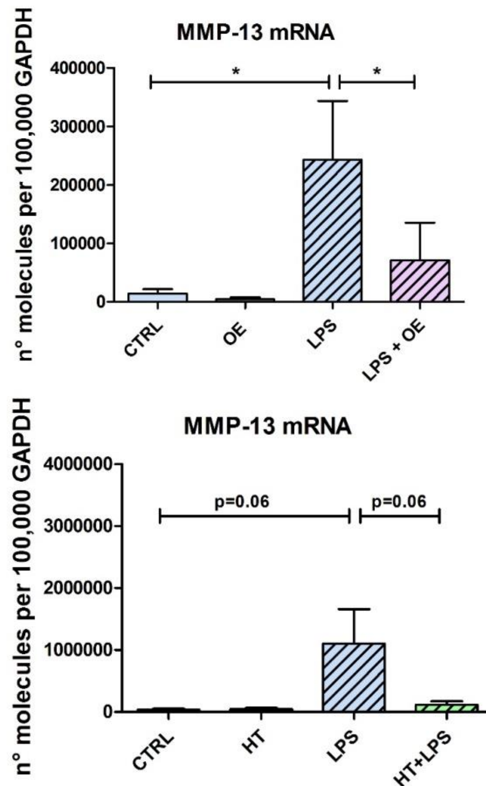


Figure 13. Nutraceuticals effects on ECM remodelling and degradation. LPS-induced mRNA expression of the NOTCH-1 target MMP-13 is significantly decreased by OE and HT. As in previous real time RT-PCR data, analysis was conducted on a set of 5 primary chondrocytes samples derived from patients with OA (n=5) and expressed as mean ± SD. Samples were normalized using the reference housekeeping gene GAPDH according to the formula $2^{-\Delta Ct}$ and expressed as number of molecules per 100,000 GAPDH molecules in OA chondrocytes to flatten inter-patient variability. For samples treated with OE data were not statistically significant (p=0.06).

Further sustaining the role of NOTCH-1 in MMP-13 activation, we tested its modulation after the inhibition of NICD release, by using DAPT (10 μM) in C28/I2 cells. This compound acts by blocking S3 cleavage operated by γ-secretase complex, therefore preventing the translocation of the active domain to the nucleus. Interestingly preliminary data show that DAPT treatment efficiently blocked NOTCH-1 activation as well as the expression of MMP-13 after LPS stimulation (as shown in Fig. 14).

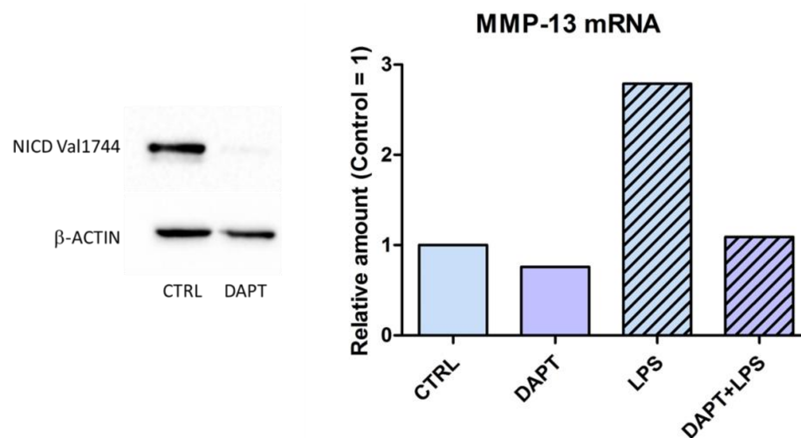


Figure 14. Effects of NOTCH-1inhibition on MMP-13 expression. DAPT blockage of NICD release, demonstrated by Western Blot decrease of NICD band (110 KDa) led to a decreased expression of NOTCH-1 target MMP-13 observed in real time RT-PCR.

Crosstalk between NOTCH-1 and other pathways regulates its activation and downstream effects. For instance, NOTCH-1 could be activated by upstream factors that interact with this signalling pathway due to LPS-elicited mechanisms. An early effector of these interactions could be c-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family [211]. The early activation of this pathway by phosphorylation of JNK, reported after LPS stimulation, usually requires a shorter incubation time compared to other protein targets. Therefore, we checked the amount of phosphorylated (active) protein in primary chondrocytes treated with LPS for 6 h after pre-treatment with nutraceuticals. As shown in Fig. 15, phosphorylated JNK increased in presence of LPS compared to the relative control; OE and HT seem to prevent this effect by re-establishing the levels of phosphorylated protein comparable to the control. This result, although to be confirmed by further experiments, would suggest a correlation between JNK and NOTCH-1 signalling.

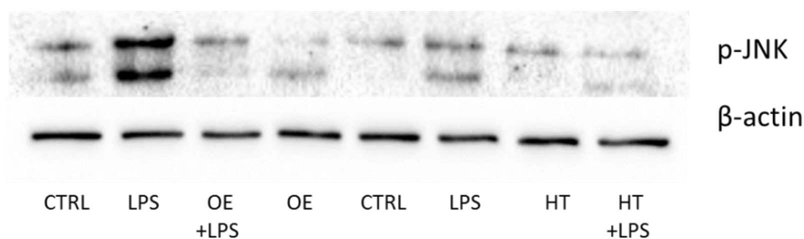


Figure 15. Up-regulation of JNK by LPS. Stimulation with LPS (6 h) increased the levels of phosphorylated JNK in primary chondrocytes samples.

Another key protein of pivotal importance for chondrocyte homeostasis is SIRT-1 [205, 212-214]. This enzyme was found able to modulate NOTCH-1 pathway in other cell types [215, 216]. In order to assess if this correlation exists in our experimental model, we induced transient silencing of SIRT-1 in C28/I2 chondrocytes. After verifying the efficiency of silencing by Western Blot, we investigated the modulation of NOTCH-1 and its possible target MMP-13. Preliminary data showed

an increased expression of both genes in SIRT-1-depleted cells (Fig. 16), thus suggesting a crosstalk between these pathways, that could be relevant in OA.

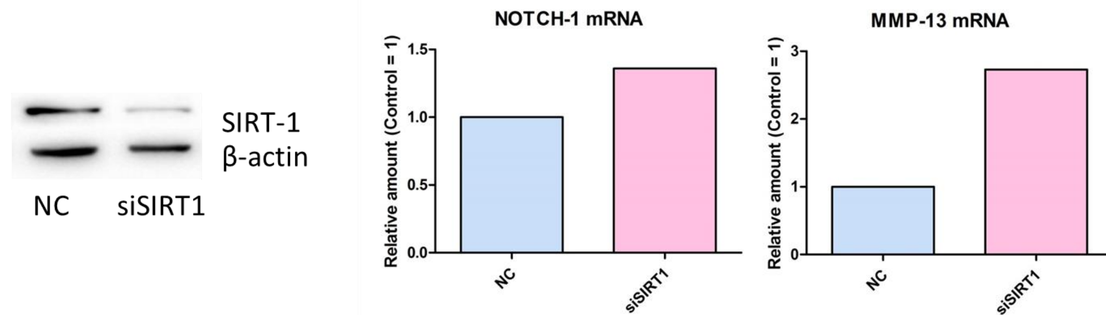


Figure 16. Effect of SIRT-1 silencing on NOTCH-1 and MMP-13 expression. SIRT-1 knock down, shown by Western Blot, increased the expression of NOTCH-1 and its target MMP-13 suggesting a potential crosslink between the two pathways, with SIRT1 upstream (preliminary data).

Discussion

The aging of population is strictly connected with the increasing of age-related diseases, such as OA. Lack of therapies due to the limited knowledge of the underlying pathological mechanisms further aggravates the burden of this disease.

The first aim of our study was to elucidate the role of a newly identified target of OA, i.e. NOTCH-1. This signalling pathway, normally involved in the regulation of endochondral ossification during embryogenesis [44], is also necessary for the homeostasis of cartilage in the adult [158], but it was found to be overexpressed in OA [217]. Recently, NOTCH-1 importance has been described in the temporomandibular OA, a subtype of the pathology that affects only this joint [218].

Our study investigated NOTCH-1 role in primary chondrocytes cultured in micromasses to better replicate the physio-pathological conditions occurring in cartilage. Our results showed that the silencing of NOTCH-1 not only was efficient but it prevented the expression of its primary target HES-1. Furthermore, transient knock down of NOTCH-1 decreased several hallmarks of cartilage degeneration. In particular, we proved that NOTCH-1 sustains proliferation of chondrocytes, which is reduced by its down-regulation. Proliferation is usually linked to the re-activation of the maturational process of chondrocytes in OA. This process replicates the events occurring during endochondral ossification and ultimately leads to cell death. In keeping with this observation, our data also showed a significant increase in cell viability in 3-D models post NOTCH-1 silencing and at the same time we found a reduction in RUNX-2 expression, related to terminal differentiation, and in the deposition of calcium crystals, representative of cartilage calcification.

Moreover, the expression of many genes linked to the pathogenesis of OA, such as RUNX-2 and VEGFA, was significantly decreased. Also, a relevant modulation of inflammation-related genes,

such as NFKB1 and its regulator IKKα, was observed. Matrix degradation, a pivotal process that sustains OA progression, was also investigated. We evaluated the release of the main metalloproteases (MMPs) involved in this mechanism, in particular MMP-1, MMP-3, MMP-10 and MMP-13, and we found that they were significantly decreased by NOTCH-1 silencing. Interestingly, MMP-13 was previously reported as target of NOTCH-1 signalling [162, 163].
Taken together, our results suggest a key role of NOTCH-1 pathway in sustaining the differentiation of articular chondrocytes toward terminal differentiation. Furthermore, the regulation of MMPs, and especially of MMP-13, could support this hypothesis. In fact, NOTCH-1 silencing prevented all these phenomena.
The second aim of our study was to evaluate the effects of two olive-derived compounds, OE and HT, against OA modifications in chondrocytes. To accomplish this, we established an experimental model of OA, by exposing chondrocytes to high concentrations of LPS, thus replicating the inflammatory state that sustains the progression of the disease. Our results proved that OE and HT significantly prevented LPS-mediated increase of ROS production in C28/I2 cells after prolonged exposition to the inflammatory stimulus. This antioxidant effect of olive-derived polyphenols, although previously reported in OA chondrocytes, was proven for the first time in relation to LPS treatment. Furthermore, it was sustained by data obtained regarding the modulation of generic inflammatory markers, such as iNOS and COX-2, but also of other, such as NFKB1, IKKα, IL-6 and IL-8, more stringently linked to OA. The chondroprotective effect of OE and HT was also exerted on NOTCH-1 expression, which was found to be increased by LPS treatment but efficiently prevented by pre-treatment with nutraceuticals in primary chondrocytes. Previously Gualillo and colleagues [219, 220] have demonstrated a chondroprotective action of oleocanthal, another phenolic compound derived from olive, in a similar experimental setting, but to our knowledge this is the first study that describes these effects for HT and OE in primary chondrocytes and C28/I2 cells. In addition to that, our study also proved the modulation exerted by these compounds on NOTCH-1 expression and its putative target MMP-13 in the context of OA. Moreover, we investigated the direct link between LPS stimulation and NOTCH-1 effects by blocking the activation of the pathway via DAPT. Our preliminary data would suggest a decreased expression of MMP-13 as a direct consequence of NOTCH-1 inability to complete the S3 cleavage.
In addition to that, we investigated the crosslinking with other pathways that could exert a regulatory activity on NOTCH-1 signalling. An immediate upstream regulation could be due to the LPS-induced increase of JNK activation. This effect has been previously investigated [211], and our preliminary data seem to support this hypothesis. Furthermore, we noticed that OE and HT decreased the phosphorylation of JNK, thus suggesting a further chondroprotective mechanism.

Finally, we observed an increase of NOTCH-1 and, interestingly, even of its target MMP-13 as a consequence of SIRT-1 downregulation in C28/I2 chondrocytes. A connection between NOTCH-1 pathway and this pivotal degrading enzyme was previously established in different cellular models [215, 216], hinting that a similar interaction could occur in our experimental model. Although more experiments need to be performed in order to confirm these last results, the data so far collected suggest an even more complex role of NOTCH-1 in the mechanisms underlying the onset and progression of OA.

Conclusions

Our study supports a key role for NOTCH-1 in the pathogenesis of OA, thus suggesting this signalling pathway as a potential target for therapy. Furthermore, we showed that nutraceuticals, and particularly olive-derived polyphenols, have remarkable chondroprotective effects in the OA-related context also by modulating NOTCH-1 pathway. These data strongly support the urgency to investigate *in vivo* this chondroprotective action, in order to consider these compounds as a therapeutic option for OA management.

References

1. Glyn-Jones, S., et al., *Osteoarthritis.* Lancet, 2015. **386**(9991): p. 376-87.
2. Hunter, D.J. and D.T. Felson, *Osteoarthritis.* BMJ, 2006. **332**(7542): p. 639-42.
3. Johnson, V.L. and D.J. Hunter, *The epidemiology of osteoarthritis.* Best Pract Res Clin Rheumatol, 2014. **28**(1): p. 5-15.
4. Peat, G., et al., *Clinical classification criteria for knee osteoarthritis: performance in the general population and primary care.* Ann Rheum Dis, 2006. **65**(10): p. 1363-7.
5. Kellgren, J.H. and J.S. Lawrence, *Radiological assessment of osteo-arthrosis.* Ann Rheum Dis, 1957. **16**(4): p. 494-502.
6. Suri, P., D.C. Morgenroth, and D.J. Hunter, *Epidemiology of Osteoarthritis and Associated Comorbidities.* Pm&R, 2012. **4**: p. S10-S19.
7. Neogi, T. and Y. Zhang, *Epidemiology of osteoarthritis.* Rheum Dis Clin North Am, 2013. **39**(1): p. 1-19.
8. Altman, R.D., et al., *Atlas of individual radiographic features in osteoarthritis.* Osteoarthritis Cartilage, 1995. **3 Suppl A**: p. 3-70.
9. Litwic, A., et al., *Epidemiology and burden of osteoarthritis.* Br Med Bull, 2013. **105**: p. 185-99.
10. Cross, M., et al., *The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study.* Ann Rheum Dis, 2014. **73**(7): p. 1323-30.
11. Vos, T., et al., *Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 2012. **380**(9859): p. 2163-96.
12. Nuesch, E., et al., *All cause and disease specific mortality in patients with knee or hip osteoarthritis: population based cohort study.* BMJ, 2011. **342**: p. d1165.
13. Palazzo, C., et al., *Risk factors and burden of osteoarthritis.* Ann Phys Rehabil Med, 2016. **59**(3): p. 134-138.
14. Flandry, F. and G. Hommel, *Normal anatomy and biomechanics of the knee.* Sports Med Arthrosc Rev, 2011. **19**(2): p. 82-92.
15. Glasson, S.S., T.J. Blanchet, and E.A. Morris, *The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse.* Osteoarthritis Cartilage, 2007. **15**(9): p. 1061-9.
16. Brody, L.T., *Knee osteoarthritis: Clinical connections to articular cartilage structure and function.* Phys Ther Sport, 2015. **16**(4): p. 301-16.
17. Bobick, B.E., et al., *Regulation of the chondrogenic phenotype in culture.* Birth Defects Res C Embryo Today, 2009. **87**(4): p. 351-71.
18. Schiller, A., *Pathology of osteoarthritis.* Osteoarthritic disorders, 1995(American Academy of Orthopaedic Surgeons): p. pp. 95-102.
19. S. Bhatia, N.G., N. Verma, *Overview of articular cartilage.* Biologic joint reconstruction, 2009: p. pp. 5-12.
20. Goldring, M.B. and K.B. Marcu, *Cartilage homeostasis in health and rheumatic diseases.* Arthritis Res Ther, 2009. **11**(3): p. 224.
21. Goldring, M.B., K. Tsuchimochi, and K. Ijiri, *The control of chondrogenesis.* J Cell Biochem, 2006. **97**(1): p. 33-44.
22. Walker, J., *Cartilage of human joints and related structures.* Athletic injuries and rehabilitation, 1996: p. pp. 120-151.
23. Ströbel, S., et al., *Anabolic and catabolic responses of human articular chondrocytes to varying oxygen percentages.* Arthritis Research & Therapy, 2010. **12**(2): p. R34.
24. Mobasheri, A., et al., *Hypoxia inducible factor-1 and facilitative glucose transporters GLUT1 and GLUT3: putative molecular components of the oxygen and glucose sensing apparatus in articular chondrocytes.* Histol Histopathol, 2005. **20**(4): p. 1327-38.
25. Schipani, E., et al., *Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival.* Genes Dev, 2001. **15**(21): p. 2865-76.

26. Mackie, E.J., et al., *Endochondral ossification: how cartilage is converted into bone in the developing skeleton.* Int J Biochem Cell Biol, 2008. **40**(1): p. 46-62.
27. Houard, X., M.B. Goldring, and F. Berenbaum, *Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis.* Curr Rheumatol Rep, 2013. **15**(11): p. 375.
28. Culav, E.M., C.H. Clark, and M.J. Merrilees, *Connective tissues: matrix composition and its relevance to physical therapy.* Phys Ther, 1999. **79**(3): p. 308-19.
29. Buckwalter, J.A., H.J. Mankin, and A.J. Grodzinsky, *Articular cartilage and osteoarthritis.* Instr Course Lect, 2005. **54**: p. 465-80.
30. Long, F. and D.M. Ornitz, *Development of the endochondral skeleton.* Cold Spring Harb Perspect Biol, 2013. **5**(1): p. a008334.
31. Fell, H.B., *The histogenesis of cartilage and bone in the long bones of the embryonic fowl.* Journal of Morphology, 1925. **40**(3): p. 417-459.
32. O'Rahilly, R. and E. Gardner, *The timing and sequence of events in the development of the limbs in the human embryo.* Anat Embryol (Berl), 1975. **148**(1): p. 1-23.
33. Olsen, B.R., A.M. Reginato, and W. Wang, *Bone development.* Annu Rev Cell Dev Biol, 2000. **16**: p. 191-220.
34. Tickle, C., *Patterning systems--from one end of the limb to the other.* Dev Cell, 2003. **4**(4): p. 449-58.
35. Barna, M. and L. Niswander, *Visualization of Cartilage Formation: Insight into Cellular Properties of Skeletal Progenitors and Chondrodysplasia Syndromes.* Developmental Cell, 2007. **12**(6): p. 931-941.
36. DeLise, A.M., L. Fischer, and R.S. Tuan, *Cellular interactions and signaling in cartilage development.* Osteoarthritis Cartilage, 2000. **8**(5): p. 309-34.
37. Ng, L.J., et al., *SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse.* Dev Biol, 1997. **183**(1): p. 108-21.
38. Minina, E., et al., *Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation.* Dev Cell, 2002. **3**(3): p. 439-49.
39. Enomoto, H., et al., *Cbfa1 is a positive regulatory factor in chondrocyte maturation.* J Biol Chem, 2000. **275**(12): p. 8695-702.
40. Ma, B., et al., *WNT Signaling and Cartilage: Of Mice and Men.* Calcified Tissue International, 2012. **92**(5): p. 399-411.
41. Guidotti, S., et al., *Enhanced Osteoblastogenesis of Adipose-Derived Stem Cells on Spermine Delivery via β-Catenin Activation.* Stem Cells and Development, 2013. **22**(10): p. 1588-1601.
42. Haller, R., et al., *Notch1 signaling regulates chondrogenic lineage determination through Sox9 activation.* Cell Death Differ, 2012. **19**(3): p. 461-9.
43. Grogan, S.P., et al., *Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis.* Arthritis Res Ther, 2009. **11**(3): p. R85.
44. Watanabe, N., et al., *Suppression of differentiation and proliferation of early chondrogenic cells by Notch.* Journal of Bone and Mineral Metabolism, 2003. **21**(6): p. 344-352.
45. Hayes, A.J., et al., *The distribution of Notch receptors and their ligands during articular cartilage development.* Journal of Anatomy, 2003. **202**(6): p. 495-502.
46. Inada, M., et al., *Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification.* Proceedings of the National Academy of Sciences, 2004. **101**(49): p. 17192-17197.
47. Inada, M., et al., *Maturational disturbance of chondrocytes inCbfa1-deficient mice.* Developmental Dynamics, 1999. **214**(4): p. 279-290.
48. Ferguson, C.M., et al., *Common Molecular Pathways in Skeletal Morphogenesis and Repair.* Annals of the New York Academy of Sciences, 1998. **857**(1 MORPHOGENESIS): p. 33-42.
49. Gerber, H.-P., et al., *VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation.* Nature Medicine, 1999. **5**(6): p. 623-628.
50. Vu, T.H., et al., *MMP-9/Gelatinase B Is a Key Regulator of Growth Plate Angiogenesis and Apoptosis of Hypertrophic Chondrocytes.* Cell, 1998. **93**(3): p. 411-422.
51. Green, J.D., et al., *Multifaceted signaling regulators of chondrogenesis: Implications in cartilage regeneration and tissue engineering.* Genes Dis, 2015. **2**(4): p. 307-327.
52. Felson, D.T., *Osteoarthritis: New Insights. Part 1: The Disease and Its Risk Factors.* Annals of Internal Medicine, 2000. **133**(8): p. 635.

53. Jordan, J.M., et al., *Prevalence of knee symptoms and radiographic and symptomatic knee osteoarthritis in African Americans and Caucasians: the Johnston County Osteoarthritis Project.* J Rheumatol, 2007. **34**(1): p. 172-80.
54. Jordan, J.M., et al., *Prevalence of hip symptoms and radiographic and symptomatic hip osteoarthritis in African Americans and Caucasians: the Johnston County Osteoarthritis Project.* J Rheumatol, 2009. **36**(4): p. 809-15.
55. Haugen, I.K., et al., *Prevalence, incidence and progression of hand osteoarthritis in the general population: the Framingham Osteoarthritis Study.* Ann Rheum Dis, 2011. **70**(9): p. 1581-6.
56. Srikanth, V.K., et al., *A meta-analysis of sex differences prevalence, incidence and severity of osteoarthritis.* Osteoarthritis and Cartilage, 2005. **13**(9): p. 769-781.
57. Valdes, A.M. and T.D. Spector, *The contribution of genes to osteoarthritis.* Rheum Dis Clin North Am, 2008. **34**(3): p. 581-603.
58. Dai, J., et al., *Association of a single nucleotide polymorphism in growth differentiate factor 5 with congenital dysplasia of the hip: a case-control study.* Arthritis Res Ther, 2008. **10**(5): p. R126.
59. Rouault, K., et al., *Evidence of association between GDF5 polymorphisms and congenital dislocation of the hip in a Caucasian population.* Osteoarthritis Cartilage, 2010. **18**(9): p. 1144-9.
60. Williams, F.M., et al., *GDF5 single-nucleotide polymorphism rs143383 is associated with lumbar disc degeneration in Northern European women.* Arthritis Rheum, 2011. **63**(3): p. 708-12.
61. Loughlin, J., *Osteoarthritis year 2010 in review: genetics.* Osteoarthritis Cartilage, 2011. **19**(4): p. 342-5.
62. Posthumus, M., et al., *Components of the transforming growth factor-beta family and the pathogenesis of human Achilles tendon pathology--a genetic association study.* Rheumatology (Oxford), 2010. **49**(11): p. 2090-7.
63. Kluzek, S., J.L. Newton, and N.K. Arden, *Is osteoarthritis a metabolic disorder?* British Medical Bulletin, 2015. **115**(1): p. 111-121.
64. Berenbaum, F., F. Eymard, and X. Houard, *Osteoarthritis, inflammation and obesity.* Current Opinion in Rheumatology, 2013. **25**(1): p. 114-118.
65. Jiang, L., et al., *Body mass index and susceptibility to knee osteoarthritis: A systematic review and meta-analysis.* Joint Bone Spine, 2012. **79**(3): p. 291-297.
66. Felson, D.T., *Obesity and osteoarthritis of the knee.* Bull Rheum Dis, 1992. **41**(2): p. 6-7.
67. Christensen, R., et al., *Effect of weight reduction in obese patients diagnosed with knee osteoarthritis: a systematic review and meta-analysis.* Annals of the Rheumatic Diseases, 2006. **66**(4): p. 433-439.
68. McAlindon, T.E., et al., *Do antioxidant micronutrients protect against the development and progression of knee osteoarthritis?* Arthritis & Rheumatism, 1996. **39**(4): p. 648-656.
69. Neogi, T., et al., *Low vitamin K status is associated with osteoarthritis in the hand and knee.* Arthritis & Rheumatism, 2006. **54**(4): p. 1255-1261.
70. Messier, S.P., et al., *The Intensive Diet and Exercise for Arthritis (IDEA) trial: design and rationale.* BMC Musculoskeletal Disorders, 2009. **10**(1).
71. Hansen, P., M. English, and S.E. Willick, *Does Running Cause Osteoarthritis in the Hip or Knee?* Pm&R, 2012. **4**: p. S117-S121.
72. Hunter, D.J. and F. Eckstein, *Exercise and osteoarthritis.* Journal of Anatomy, 2009. **214**(2): p. 197-207.
73. Slauterbeck, J.R., et al., *Geographic Mapping of Meniscus and Cartilage Lesions Associated with Anterior Cruciate Ligament Injuries.* The Journal of Bone and Joint Surgery-American Volume, 2009. **91**(9): p. 2094-2103.
74. Slemenda, C., et al., *Reduced quadriceps strength relative to body weight: a risk factor for knee osteoarthritis in women?* Arthritis Rheum, 1998. **41**(11): p. 1951-9.
75. Golightly, Y.M., et al., *Symptoms of the knee and hip in individuals with and without limb length inequality.* Osteoarthritis Cartilage, 2009. **17**(5): p. 596-600.
76. Aigner, T., et al., *Mechanisms of Disease: role of chondrocytes in the pathogenesis of osteoarthritis—structure, chaos and senescence.* Nature Clinical Practice Rheumatology, 2007. **3**(7): p. 391-399.
77. Troeberg, L. and H. Nagase, *Proteases involved in cartilage matrix degradation in osteoarthritis.* Biochim Biophys Acta, 2012. **1824**(1): p. 133-45.

78. Nagase, H. and M. Kashiwagi, *Aggrecanases and cartilage matrix degradation.* Arthritis Research & Therapy, 2003. **5**(2): p. 94.
79. Nagase, H., *Metalloproteases.* Current Protocols in Protein Science, 2001. **24**(1).
80. Sandy, J.D., et al., *Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain.* Journal of Biological Chemistry, 1991. **266**(14): p. 8683-8685.
81. Sandy, J.D., et al., *The structure of aggrecan fragments in human synovial fluid. Evidence for the involvement in osteoarthritis of a novel proteinase which cleaves the Glu 373-Ala 374 bond of the interglobular domain.* Journal of Clinical Investigation, 1992. **89**(5): p. 1512-1516.
82. Lark, M.W., et al., *Cell-mediated Catabolism of Aggrecan.* Journal of Biological Chemistry, 1995. **270**(6): p. 2550-2556.
83. Hurskainen, T.L., et al., *ADAM-TS5, ADAM-TS6, and ADAM-TS7, Novel Members of a New Family of Zinc Metalloproteases.* Journal of Biological Chemistry, 1999. **274**(36): p. 25555-25563.
84. Abbaszade, I., et al., *Cloning and Characterization of ADAMTS11 , an Aggrecanase from the ADAMTS Family.* Journal of Biological Chemistry, 1999. **274**(33): p. 23443-23450.
85. Verzijl, N., et al., *AGEing and osteoarthritis: a different perspective.* Current Opinion in Rheumatology, 2003. **15**(5): p. 616-622.
86. Chen, A.C., et al., *Induction of advanced glycation end products and alterations of the tensile properties of articular cartilage.* Arthritis & Rheumatism, 2002. **46**(12): p. 3212-3217.
87. Wang, X., et al., *Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage.* Osteoarthritis and Cartilage, 2004. **12**(12): p. 963-973.
88. Tetsunaga, T., et al., *Regulation of mechanical stress-induced MMP-13 and ADAMTS-5 expression by RUNX-2 transcriptional factor in SW1353 chondrocyte-like cells.* Osteoarthritis and Cartilage, 2011. **19**(2): p. 222-232.
89. Brew, K. and H. Nagase, *The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2010. **1803**(1): p. 55-71.
90. Saito, T. and H. Kawaguchi, *HIF-2α as a possible therapeutic target of osteoarthritis.* Osteoarthritis and Cartilage, 2010. **18**(12): p. 1552-1556.
91. Lin, A.C., et al., *Modulating hedgehog signaling can attenuate the severity of osteoarthritis.* Nature Medicine, 2009. **15**(12): p. 1421-1425.
92. Saito, T., et al., *Transcriptional regulation of endochondral ossification by HIF-2α during skeletal growth and osteoarthritis development.* Nature Medicine, 2010. **16**(6): p. 678-686.
93. Loeser, R.F., *Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix.* Osteoarthritis and Cartilage, 2009. **17**(8): p. 971-979.
94. Loeser, R.F., J.A. Collins, and B.O. Diekman, *Ageing and the pathogenesis of osteoarthritis.* Nature Reviews Rheumatology, 2016. **12**(7): p. 412-420.
95. Sellam, J. and F. Berenbaum, *The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis.* Nat Rev Rheumatol, 2010. **6**(11): p. 625-35.
96. Wang, Y., et al., *Mitochondrial Biogenesis Is Impaired in Osteoarthritis Chondrocytes but Reversible via Peroxisome Proliferator-Activated Receptor γ Coactivator 1α.* Arthritis & Rheumatology, 2015. **67**(8): p. 2141-2153.
97. Suri, S. and D.A. Walsh, *Osteochondral alterations in osteoarthritis.* Bone, 2012. **51**(2): p. 204-11.
98. Hayes, D.W., Jr., R.L. Brower, and K.J. John, *Articular cartilage. Anatomy, injury, and repair.* Clin Podiatr Med Surg, 2001. **18**(1): p. 35-53.
99. Fenwick, S.A., P.J. Gregg, and P. Rooney, *Osteoarthritic cartilage loses its ability to remain avascular.* Osteoarthritis and Cartilage, 1999. **7**(5): p. 441-452.
100. Walsh, D.A., et al., *Angiogenesis in the synovium and at the osteochondral junction in osteoarthritis.* Osteoarthritis and Cartilage, 2007. **15**(7): p. 743-751.
101. Suri, S., et al., *Neurovascular invasion at the osteochondral junction and in osteophytes in osteoarthritis.* Annals of the Rheumatic Diseases, 2007. **66**(11): p. 1423-1428.
102. Goldring, M.B. and F. Berenbaum, *Emerging targets in osteoarthritis therapy.* Current Opinion in Pharmacology, 2015. **22**: p. 51-63.
103. Flood, J., *The role of acetaminophen in the treatment of osteoarthritis.* Am J Manag Care, 2010. **16 Suppl Management**: p. S48-54.
104. Vane, J.R. and R.M. Botting, *Mechanism of action of aspirin-like drugs.* Semin Arthritis Rheum, 1997. **26**(6 Suppl 1): p. 2-10.

105. Mobasheri, A., *The future of osteoarthritis therapeutics: targeted pharmacological therapy.* Curr Rheumatol Rep, 2013. **15**(10): p. 364.
106. Lipsky, P.E., *Role of cyclooxygenase-1 and -2 in health and disease.* Am J Orthop (Belle Mead NJ), 1999. **28**(3 Suppl): p. 8-12.
107. Zhang, W., et al., *OARSI recommendations for the management of hip and knee osteoarthritis, part I: critical appraisal of existing treatment guidelines and systematic review of current research evidence.* Osteoarthritis Cartilage, 2007. **15**(9): p. 981-1000.
108. Zhang, W., et al., *OARSI recommendations for the management of hip and knee osteoarthritis, Part II: OARSI evidence-based, expert consensus guidelines.* Osteoarthritis and Cartilage, 2008. **16**(2): p. 137-162.
109. Kurtz, S., et al., *Projections of Primary and Revision Hip and Knee Arthroplasty in the United States from 2005 to 2030.* The Journal of Bone & Joint Surgery, 2007. **89**(4): p. 780-785.
110. Zhang, W., et al., *Current research on pharmacologic and regenerative therapies for osteoarthritis.* Bone Research, 2016. **4**(1).
111. *Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework.* Clinical Pharmacology & Therapeutics, 2001. **69**(3): p. 89-95.
112. Klimak, M., et al., *Immunoengineering the next generation of arthritis therapies.* Acta Biomaterialia, 2021.
113. Clutterbuck, A.L., et al., *Targeting matrix metalloproteinases in inflammatory conditions.* Curr Drug Targets, 2009. **10**(12): p. 1245-54.
114. Artavanis-Tsakonas, S., *Notch Signaling: Cell Fate Control and Signal Integration in Development.* Science, 1999. **284**(5415): p. 770-776.
115. Dexter, J.S., *The Analysis of a Case of Continuous Variation in Drosophila by a Study of Its Linkage Relations.* Am Nat. , 1914. **48**(576): p. 712–758.
116. Morgan, T.H., *A Demonstration of Genes Modifying the Character "Notch.".* In: Contributions to the Genetics of Drosophila melanogaster, 1919. **278**: p. 345-88.
117. Artavanis-Tsakonas, S., M.A. Muskavitch, and B. Yedvobnick, *Molecular cloning of Notch, a locus affecting neurogenesis in Drosophila melanogaster.* Proceedings of the National Academy of Sciences, 1983. **80**(7): p. 1977-1981.
118. Bray, S.J., *Notch signalling: a simple pathway becomes complex.* Nature Reviews Molecular Cell Biology, 2006. **7**(9): p. 678-689.
119. Fiúza, U.-M. and A.M. Arias, *Cell and molecular biology of Notch.* Journal of Endocrinology, 2007. **194**(3): p. 459-474.
120. Lissemore, J.L. and W.T. Starmer, *Phylogenetic analysis of vertebrate and invertebrate Delta/Serrate/LAG-2 (DSL) proteins.* Mol Phylogenet Evol, 1999. **11**(2): p. 308-19.
121. Rebay, I., et al., *Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor.* Cell, 1991. **67**(4): p. 687-99.
122. Blaumueller, C.M., et al., *Intracellular Cleavage of Notch Leads to a Heterodimeric Receptor on the Plasma Membrane.* Cell, 1997. **90**(2): p. 281-291.
123. Haines, N. and K.D. Irvine, *Glycosylation regulates Notch signalling.* Nature Reviews Molecular Cell Biology, 2003. **4**(10): p. 786-797.
124. Brennan, K., et al., *Wingless modulates the effects of dominant negative notch molecules in the developing wing of Drosophila.* Dev Biol, 1999. **216**(1): p. 210-29.
125. Hambleton, S., et al., *Structural and functional properties of the human notch-1 ligand binding region.* Structure, 2004. **12**(12): p. 2173-83.
126. de Celis, J.F. and S.J. Bray, *The Abruptex domain of Notch regulates negative interactions between Notch, its ligands and Fringe.* Development, 2000. **127**(6): p. 1291-302.
127. Kopan, R. and M.X. Ilagan, *The canonical Notch signaling pathway: unfolding the activation mechanism.* Cell, 2009. **137**(2): p. 216-33.
128. Weng, A.P., et al., *Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia.* Science, 2004. **306**(5694): p. 269-71.
129. Katoh, M. and M. Katoh, *Precision medicine for human cancers with Notch signaling dysregulation (Review).* International Journal of Molecular Medicine, 2019.
130. Meng, L., et al., *DLL4+ dendritic cells: Key regulators of Notch Signaling in effector T cell responses.* Pharmacological Research, 2016. **113**: p. 449-457.

131. Klein, T., K. Brennan, and A.M. Arias, *An intrinsic dominant negative activity of serrate that is modulated during wing development in Drosophila.* Dev Biol, 1997. **189**(1): p. 123-34.
132. Logeat, F., et al., *The Notch1 receptor is cleaved constitutively by a furin-like convertase.* Proc Natl Acad Sci U S A, 1998. **95**(14): p. 8108-12.
133. Raya, A., et al., *Notch activity acts as a sensor for extracellular calcium during vertebrate left-right determination.* Nature, 2004. **427**(6970): p. 121-8.
134. Cordle, J., et al., *Localization of the delta-like-1-binding site in human Notch-1 and its modulation by calcium affinity.* J Biol Chem, 2008. **283**(17): p. 11785-93.
135. Sakamoto, K., et al., *Intracellular Cell-Autonomous Association of Notch and Its Ligands: A Novel Mechanism of Notch Signal Modification.* Developmental Biology, 2002. **241**(2): p. 313-326.
136. Okajima, T. and K.D. Irvine, *Regulation of Notch Signaling by O-Linked Fucose.* Cell, 2002. **111**(6): p. 893-904.
137. Stahl, M., et al., *Roles of Pofut1 and O-fucose in mammalian Notch signaling.* J Biol Chem, 2008. **283**(20): p. 13638-51.
138. Panin, V.M., et al., *Fringe modulates Notch-ligand interactions.* Nature, 1997. **387**(6636): p. 908-12.
139. Kopan, R., et al., *Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain.* Proc Natl Acad Sci U S A, 1996. **93**(4): p. 1683-8.
140. Greenwald, I., *Structure/function studies of lin-12/Notch proteins.* Curr Opin Genet Dev, 1994. **4**(4): p. 556-62.
141. Brou, C., et al., *A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE.* Mol Cell, 2000. **5**(2): p. 207-16.
142. Shah, S., et al., *Nicastrin Functions as a γ-Secretase-Substrate Receptor.* Cell, 2005. **122**(3): p. 435-447.
143. Kaether, C., et al., *Amyloid Precursor Protein and Notch Intracellular Domains are Generated after Transport of their Precursors to the Cell Surface.* Traffic, 2006. **7**(4): p. 408-415.
144. Schroeter, E.H., J.A. Kisslinger, and R. Kopan, *Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain.* Nature, 1998. **393**(6683): p. 382-6.
145. Tagami, S., et al., *Regulation of Notch signaling by dynamic changes in the precision of S3 cleavage of Notch-1.* Mol Cell Biol, 2008. **28**(1): p. 165-76.
146. van Tetering, G. and M. Vooijs, *Proteolytic Cleavage of Notch: “HIT and RUN”.* Current Molecular Medicine, 2011. **11**(4): p. 255-269.
147. Ray, W.J., et al., *Cell Surface Presenilin-1 Participates in the γ-Secretase-like Proteolysis of Notch.* Journal of Biological Chemistry, 1999. **274**(51): p. 36801-36807.
148. Pasternak, S.H., et al., *Presenilin-1, Nicastrin, Amyloid Precursor Protein, and γ-Secretase Activity Are Co-localized in the Lysosomal Membrane.* Journal of Biological Chemistry, 2003. **278**(29): p. 26687-26694.
149. Okochi, M., et al., *Presenilins mediate a dual intramembranous gamma-secretase cleavage of Notch-1.* EMBO J, 2002. **21**(20): p. 5408-16.
150. Kao, H.Y., et al., *A histone deacetylase corepressor complex regulates the Notch signal transduction pathway.* Genes Dev, 1998. **12**(15): p. 2269-77.
151. Wallberg, A.E., et al., *p300 and PCAF Act Cooperatively To Mediate Transcriptional Activation from Chromatin Templates by Notch Intracellular Domains In Vitro.* Molecular and Cellular Biology, 2002. **22**(22): p. 7812-7819.
152. Kurooka, H. and T. Honjo, *Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5.* J Biol Chem, 2000. **275**(22): p. 17211-20.
153. Iso, T., L. Kedes, and Y. Hamamori, *HES and HERP families: Multiple effectors of the notch signaling pathway.* Journal of Cellular Physiology, 2003. **194**(3): p. 237-255.
154. Yang, K., M. Hitomi, and D.W. Stacey, *Variations in cyclin D1 levels through the cell cycle determine the proliferative fate of a cell.* Cell Division, 2006. **1**(1): p. 32.
155. Ronchini, C. and A.J. Capobianco, *Induction of Cyclin D1 Transcription and CDK2 Activity by Notch: Implication for Cell Cycle Disruption in Transformation by Notch
(ic).* Molecular and Cellular Biology, 2001. **21**(17): p. 5925-5934.
156. Welti, J., et al., *Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer.* Journal of Clinical Investigation, 2013. **123**(8): p. 3190-3200.
157. Fryer, C.J., J.B. White, and K.A. Jones, *Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover.* Mol Cell, 2004. **16**(4): p. 509-20.

158. Ustunel, I., et al., *The immunohistochemical localization of notch receptors and ligands in human articular cartilage, chondroprogenitor culture and ultrastructural characteristics of these progenitor cells.* Acta Histochem, 2008. **110**(5): p. 397-407.
159. Johnston, D.A., B. Dong, and C.C. Hughes, *TNF induction of jagged-1 in endothelial cells is NFkappaB-dependent.* Gene, 2009. **435**(1-2): p. 36-44.
160. Gardiner, M.D., et al., *Transcriptional analysis of micro-dissected articular cartilage in post-traumatic murine osteoarthritis.* Osteoarthritis Cartilage, 2015. **23**(4): p. 616-28.
161. Goldring, M.B., et al., *Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis.* Eur Cell Mater, 2011. **21**: p. 202-20.
162. Blaise, R., et al., *Involvement of the Notch pathway in the regulation of matrix metalloproteinase 13 and the dedifferentiation of articular chondrocytes in murine cartilage.* Arthritis Rheum, 2009. **60**(2): p. 428-39.
163. Hosaka, Y., et al., *Notch signaling in chondrocytes modulates endochondral ossification and osteoarthritis development.* Proc Natl Acad Sci U S A, 2013. **110**(5): p. 1875-80.
164. Sugita, S., et al., *Transcription factor Hes1 modulates osteoarthritis development in cooperation with calcium/calmodulin-dependent protein kinase 2.* Proc Natl Acad Sci U S A, 2015. **112**(10): p. 3080-5.
165. Wang, A., et al., *Nutraceuticals and osteoarthritis pain.* Pharmacology & Therapeutics, 2018. **187**: p. 167-179.
166. D'Adamo, S., et al., *Nutraceutical Activity in Osteoarthritis Biology: A Focus on the Nutrigenomic Role.* Cells, 2020. **9**(5): p. 1232.
167. Boumediene, K., et al., *Avocado/soya unsaponifiables enhance the expression of transforming growth factor ?1 and ?2 in cultured articular chondrocytes.* Arthritis & Rheumatism, 1999. **42**(1): p. 148-156.
168. Christiansen, B.A., et al., *Management of Osteoarthritis with Avocado/Soybean Unsaponifiables.* Cartilage, 2014. **6**(1): p. 30-44.
169. Thierry Appelboom, J.S., *Symptoms modifying effect of avocado/soybean unsaponifiables (ASU) in knee osteoarthritis.* Scandinavian Journal of Rheumatology, 2009. **30**(4): p. 242-247.
170. Ownby, S.L., et al., *Expression of pro-inflammatory mediators is inhibited by an avocado/soybean unsaponifiables and epigallocatechin gallate combination.* Journal of Inflammation, 2014. **11**(1): p. 8.
171. Akhtar, N., M.J.S. Miller, and T.M. Haqqi, *Effect of a Herbal-Leucine mix on the IL-1β-induced cartilage degradation and inflammatory gene expression in human chondrocytes.* BMC Complementary and Alternative Medicine, 2011. **11**(1).
172. Swanson, D., R. Block, and S.A. Mousa, *Omega-3 Fatty Acids EPA and DHA: Health Benefits Throughout Life.* Advances in Nutrition, 2012. **3**(1): p. 1-7.
173. Zainal, Z., et al., *Relative efficacies of omega-3 polyunsaturated fatty acids in reducing expression of key proteins in a model system for studying osteoarthritis.* Osteoarthritis and Cartilage, 2009. **17**(7): p. 896-905.
174. Singla, R.K., et al., *Natural Polyphenols: Chemical Classification, Definition of Classes, Subcategories, and Structures.* J AOAC Int, 2019. **102**(5): p. 1397-1400.
175. Han, X., T. Shen, and H. Lou, *Dietary Polyphenols and Their Biological Significance.* International Journal of Molecular Sciences, 2007. **8**(9): p. 950-988.
176. Cardona, F., et al., *Benefits of polyphenols on gut microbiota and implications in human health.* J Nutr Biochem, 2013. **24**(8): p. 1415-22.
177. Oliveira, L.d.L.d., M.V.d. Carvalho, and L. Melo, *Health promoting and sensory properties of phenolic compounds in food.* Revista Ceres, 2014. **61**(suppl): p. 764-779.
178. Chang, J., J. Reiner, and J. Xie, *Progress on the Chemistry of Dibenzocyclooctadiene Lignans.* Chemical Reviews, 2005. **105**(12): p. 4581-4609.
179. Wang, J., et al., *Regulation of type II collagen, matrix metalloproteinase-13 and cell proliferation by interleukin-1β is mediated by curcumin via inhibition of NF-κB signaling in rat chondrocytes.* Molecular Medicine Reports, 2017. **16**(2): p. 1837-1845.
180. Yan, D., et al., *Involvement of TLR4 in the protective effect of intra-articular administration of curcumin on rat experimental osteoarthritis.* Acta Cirurgica Brasileira, 2019. **34**(6).

181. Zhang, G., et al., *Curcumin improves age-related and surgically induced osteoarthritis by promoting autophagy in mice.* Bioscience Reports, 2018. **38**(4).
182. Feng, K., et al., *Curcumin Inhibits the PERK-eIF2α-CHOP Pathway through Promoting SIRT1 Expression in Oxidative Stress-induced Rat Chondrocytes and Ameliorates Osteoarthritis Progression in a Rat Model.* Oxidative Medicine and Cellular Longevity, 2019. **2019**: p. 1-17.
183. D'Ascola, A., et al., *Exploiting Curcumin Synergy With Natural Products Using Quantitative Analysis of Dose–Effect Relationships in an Experimental In Vitro Model of Osteoarthritis.* Frontiers in Pharmacology, 2019. **10**.
184. Kim, L. and J.Y. Kim, *Chondroprotective effect of curcumin and lecithin complex in human chondrocytes stimulated by IL-1β via an anti-inflammatory mechanism.* Food Science and Biotechnology, 2018. **28**(2): p. 547-553.
185. Vareed, S.K., et al., *Pharmacokinetics of Curcumin Conjugate Metabolites in Healthy Human Subjects.* Cancer Epidemiology Biomarkers & Prevention, 2008. **17**(6): p. 1411-1417.
186. Singh, R., et al., *Epigallocatechin-3-gallate inhibits interleukin-1?-induced expression of nitric oxide synthase and production of nitric oxide in human chondrocytes: Suppression of nuclear factor ?B activation by degradation of the inhibitor of nuclear factor ?B.* Arthritis & Rheumatism, 2002. **46**(8): p. 2079-2086.
187. Rasheed, Z., et al., *Green tea polyphenol epigallocatechin-3-gallate inhibits advanced glycation end product-induced expression of tumor necrosis factor-α and matrix metalloproteinase-13 in human chondrocytes.* Arthritis Research & Therapy, 2009. **11**(3): p. R71.
188. Leong, D.J., et al., *Green tea polyphenol treatment is chondroprotective, anti-inflammatory and palliative in a mouse posttraumatic osteoarthritis model.* Arthritis Research & Therapy, 2014. **16**(6).
189. D'Adamo, S., et al., *Effect of oxidative stress and 3-hydroxytyrosol on DNA methylation levels of miR-9 promoters.* Journal of Cellular and Molecular Medicine, 2019. **23**(11): p. 7885-7889.
190. Jones, S.W., et al., *The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-α and MMP13.* Osteoarthritis and Cartilage, 2009. **17**(4): p. 464-472.
191. Musumeci, G., et al., *Extra-virgin olive oil diet and mild physical activity prevent cartilage degeneration in an osteoarthritis model: an in vivo and in vitro study on lubricin expression.* The Journal of Nutritional Biochemistry, 2013. **24**(12): p. 2064-2075.
192. Carrera-González, M.P., et al., *Protective role of oleuropein and its metabolite hydroxytyrosol on cancer.* Trends in Food Science & Technology, 2013. **31**(2): p. 92-99.
193. Amiot, M.J., A. Fleuriet, and J.J. Macheix, *Importance and evolution of phenolic compounds in olive during growth and maturation.* Journal of Agricultural and Food Chemistry, 2002. **34**(5): p. 823-826.
194. Manna, C., et al., *Protective effect of the phenolic fraction from virgin olive oils against oxidative stress in human cells.* J Agric Food Chem, 2002. **50**(22): p. 6521-6.
195. de la Puerta, R.o., et al., *Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission.* Life Sciences, 2001. **69**(10): p. 1213-1222.
196. Valko, M., et al., *Role of oxygen radicals in DNA damage and cancer incidence.* Molecular and Cellular Biochemistry, 2004. **266**(1/2): p. 37-56.
197. Hamdi, H.K. and R. Castellon, *Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor.* Biochemical and Biophysical Research Communications, 2005. **334**(3): p. 769-778.
198. Furneri, P.M., et al., *In vitro antimycoplasmal activity of oleuropein.* International Journal of Antimicrobial Agents, 2002. **20**(4): p. 293-296.
199. Stefanon, B. and M. Colitti, *Original Research: Hydroxytyrosol, an ingredient of olive oil, reduces triglyceride accumulation and promotes lipolysis in human primary visceral adipocytes during differentiation.* Experimental Biology and Medicine, 2016. **241**(16): p. 1796-1802.
200. Bertelli, M., et al., *Hydroxytyrosol: A natural compound with promising pharmacological activities.* Journal of Biotechnology, 2020. **309**: p. 29-33.
201. Horcajada, M.N., et al., *Oleuropein or rutin consumption decreases the spontaneous development of osteoarthritis in the Hartley guinea pig.* Osteoarthritis and Cartilage, 2015. **23**(1): p. 94-102.
202. Feng, Z., et al., *Oleuropein inhibits the IL-1β-induced expression of inflammatory mediators by suppressing the activation of NF-κB and MAPKs in human osteoarthritis chondrocytes.* Food Funct., 2017. **8**(10): p. 3737-3744.

203. López de las Hazas, M.-C., et al., *Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids.* Journal of Functional Foods, 2016. **22**: p. 52-63.
204. Facchini, A., et al., *Hydroxytyrosol Prevents Increase of Osteoarthritis Markers in Human Chondrocytes Treated with Hydrogen Peroxide or Growth-Related Oncogene α.* PLoS ONE, 2014. **9**(10): p. e109724.
205. Cetrullo, S., et al., *Hydroxytyrosol prevents chondrocyte death under oxidative stress by inducing autophagy through sirtuin 1-dependent and -independent mechanisms.* Biochimica et Biophysica Acta (BBA) - General Subjects, 2016. **1860**(6): p. 1181-1191.
206. Hu, T., et al., *Hydroxytyrosol and Its Potential Therapeutic Effects.* Journal of Agricultural and Food Chemistry, 2014. **62**(7): p. 1449-1455.
207. Chin, K.-Y. and K.-L. Pang, *Therapeutic Effects of Olive and Its Derivatives on Osteoarthritis: From Bench to Bedside.* Nutrients, 2017. **9**(10): p. 1060.
208. Olivotto, E., et al., *Differential requirements for IKKalpha and IKKbeta in the differentiation of primary human osteoarthritic chondrocytes.* Arthritis Rheum, 2008. **58**(1): p. 227-39.
209. Schumacher, H.R., et al., *Intra-articular apatite crystal deposition.* Annals of the Rheumatic Diseases, 1983. **42**(Supplement 1): p. 54-59.
210. Camplejohn, K.L. and S.A. Allard, *Limitations of safranin 'O' staining in proteoglycan-depleted cartilage demonstrated with monoclonal antibodies.* Histochemistry, 1988. **89**(2): p. 185-188.
211. Tsao, P.N., et al., *Lipopolysaccharide-induced Notch signaling activation through JNK-dependent pathway regulates inflammatory response.* J Biomed Sci, 2011. **18**: p. 56.
212. Dvir-Ginzberg, M., et al., *Regulation of Cartilage-specific Gene Expression in Human Chondrocytes by SirT1 and Nicotinamide Phosphoribosyltransferase.* Journal of Biological Chemistry, 2008. **283**(52): p. 36300-36310.
213. Dvir-Ginzberg, M., *Towards elucidating the role of SirT1 in osteoarthritis.* Frontiers in Bioscience, 2013. **18**(1).
214. Gabay, O., et al., *Sirtuin 1 enzymatic activity is required for cartilage homeostasis in vivo in a mouse model.* Arthritis & Rheumatism, 2013. **65**(1): p. 159-166.
215. Marcel, N., et al., *The lysine deacetylase Sirtuin 1 modulates the localization and function of the Notch1 receptor in regulatory T cells.* Science Signaling, 2017. **10**(473): p. eaah4679.
216. Bai, X., et al., *Acetylation-Dependent Regulation of Notch Signaling in Macrophages by SIRT1 Affects Sepsis Development.* Frontiers in Immunology, 2018. **9**.
217. Lin, N.-Y., et al., *Inhibition of Notch1 promotes hedgehog signalling in a HES1-dependent manner in chondrocytes and exacerbates experimental osteoarthritis.* Annals of the Rheumatic Diseases, 2016. **75**(11): p. 2037-2044.
218. Ruscitto, A., et al., *Notch Regulates Fibrocartilage Stem Cell Fate and Is Upregulated in Inflammatory TMJ Arthritis.* Journal of Dental Research, 2020. **99**(10): p. 1174-1181.
219. Scotece, M., et al., *Oleocanthal Inhibits Catabolic and Inflammatory Mediators in LPS-Activated Human Primary Osteoarthritis (OA) Chondrocytes Through MAPKs/NF-κB Pathways.* Cellular Physiology and Biochemistry, 2018. **49**(6): p. 2414-2426.
220. Iacono, A., et al., *Effect of oleocanthal and its derivatives on inflammatory response induced by lipopolysaccharide in a murine chondrocyte cell line.* Arthritis & Rheumatism, 2010. **62**(6): p. 1675-1682.