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PATOGENESI DEL DANNO DELLA CARTILAGINE ARTICOLARE IN CORSO DI OSTEOARTRITE NEL CAVALLO. STUDIO *IN VITRO* DI UN BIOMATERIALE UTILIZZATO IN INGEGNERIA TISSUTALE PER LA RIPARAZIONE CARTILAGINEA.

PATHOGENESIS OF ARTICULAR CARTILAGE DAMAGE IN EQUINE OSTEOARTHRITIS. *IN VITRO* STUDY OF A BIOMATERIAL USED IN TISSUE ENGINEERING FOR ARTICULAR CARTILAGE REPAIR.

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Riassunto

L'osteoartite è una patologia infiammatoria a carattere degenerativo di cruciale importanza clinica nell'uomo e negli animali. Nella specie equina, l'osteoartite nell'insieme di degenerazione della cartilagine articolare, alterazione dell'osso subcondrale e sinovite, costituisce una debilitante causa di zoppie e performances carenti, responsabili del prematuro allontanamento del cavallo dalla carriera sportiva. L'osteoartite equina colpisce più frequentemente le articolazioni degli arti, in animali atleti e/o anziani o giovani animali geneticamente predisposti.

Accanto alla terapia tradizionale, essenzialmente sintomatica (tuttora prevalente nel contesto equino), recenti progressi volti all'indagine della patogenesi dell'osteoartite nell'uomo hanno evidenziato l'importanza di complessi meccanismi molecolari nell'instaurarsi e nella progressione della patologia. Sfruttare questi meccanismi molecolari come target di specifici farmaci può contribuire a modificare significativamente il decorso dell'osteoartrite.

Inoltre la medicina rigenerativa ambisce a una *restitutio ad integrum* della cartilagine degenerata attraverso varie tecniche. In particolare, l'ingegneria tissutale è un campo in grande sviluppo nel trattamento delle patologie ortopediche e mira a creare un tessuto ingegnerizzato, combinando cellule da biopsia a specifici supporti costituiti da biomateriali. Una cartilagine articolare artificiale, idealmente il più simile possibile a quella nativa, può essere impiantata in un ricevente in sostituzione della cartilagine danneggiata.

Il chitosano rappresenta uno dei più interessanti biomateriali di origine naturale per disponibilità in natura, plasticità negli impieghi e biocompatibilità. In questo lavoro si è esaminato l'aspetto istologico, sfruttando varie colorazioni, di una cartilagine ingegnerizzata mantenuta *in vitro*, ottenuta da scaffold in chitosano e chitosano + acido ialuronico, seminati con condrociti in coltura da articolazioni del nodello di equidi regolarmente macellati. I risultati nella colonizzazione degli scaffold, nella morfologia cellulare e nella secrezione di componenti caratteristiche della matrice extracellulare sono incoraggianti, benché nuove ricerche siano necessarie per realizzare una fedele imitazione della cartilagine nativa.

General anatomical features of articular cartilage

Introduction

Proximal and distal ends of diarthrodial joints are covered by articular cartilage (AC).

Mature articular cartilage is a specialized connective tissue (hyaline cartilage) in which few cells (2-5% of tissue volume), the chondrocytes, are embedded in a dense and hydrated extracellular matrix (ECM), which they themselves synthesize (Huber, Trattnig, & Lintner, 2000).

ECM is primarily composed of water (65-75% wet weight) and macromolecules, including collagens (mainly type II collagen) (15-20% wet weight), proteoglycans (10-15% wet weight), and non-collagenous proteins (Poole A. R., 2001) (Huber, Trattnig, & Lintner, 2000).

AC has an apparently smooth surface, with a translucent appearance and variable thickness in the various joints or, in the same joint, in the various articular areas depending on the different pressure load.

Indeed, specific structural organization of articular cartilage matrix endows this tissue with peculiar mechanical properties such as tensile strength and elasticity, which enable it to absorb (resistance to compressive forces) and distribute loads; together with the synovial fluid, AC allows frictionless movement of the surfaces of articulating joint components (Huber, Trattnig, & Lintner, 2000).

AC is aneural, alymphatic, and avascular (Huber, Trattnig, & Lintner, 2000), therefore the supply of nutrients and oxygen and the elimination of the catabolites, from and through the synovial fluid, occur by diffusion through the matrix and are determined by the consequent compression/decompression cycles to joint movement.

Chondrocytes are therefore adapted to these hypoxic conditions (Milner, Fairfax, Browning, Wilkins, & Gibson, 2006), which have also shown to trigger essential positive signals for the chondrocyte phenotype (Semenza, 2001) (Semenza, 2012) (Smith, Robbins, & Ratcliffe, 2008). Adaptation to this avascular environment is mediated by hypoxia-inducible factors HIF-1 and HIF-2 (Semenza, 2001).

AC formation starts developing in utero, along with other joint tissues. All joint tissues differentiate from the mesoderm, which originates mesenchyme and therefore most connective tissues. With regard to AC development, after mesenchymal cells condensation, chondrocyte differentiation and ECM synthesis, namely “chondrogenesis” (Umeda, et al., 2015) is committed by the expression of several factors including Sox9 that has a key role.

AC thickness seems to be mainly determined during the process of embryonic synovial joint development (through extracellular signal-regulated kinases Erk signalling, upregulated by expression of proteins such as Lin28a and similarly Kras) (Kobayashi & Kozlova, 2018). AC reaches its final size and maturity in early adulthood when chondrocytes switch from a cartilage-building activity to a cartilage-maintenance activity (Correa & Lietman, 2016).

Molecular composition of articular cartilage

AC components and their roles are portrayed in the following table (table 1).

Components	**Functions**
Chondrocytes (2-5%)	Synthesis of matrix macromolecules, degradative enzymes (MMPs) and their inhibitors (TIMPs). Synthesis of cytokines (IL-1, IL-6, IL-8, TNF-α) and growth factors (TGF-β, IGF-1). Proliferative and differentiation activity in repair processes.
Water (65%)	Fluid motion; hydrostatic lubrication.
Collagen (15%) - Collagen II (95% collagen) - Minor collagens (5% collagen) • Collagen VI • Collagen IX • Collagen XI • Collagen X	Fibre network containing and limiting expansion of PGs aggregates; resistance to tension and traction. In the ECM connects collagen II and PGs with cell surface. Interfibrillar connection and interaction with other molecules. Regulates collagen II fibrillogenesis. In the calcified zone of mature cartilage and in hypertrophic zone in growing cartilage; involved in calcification.
HA (0,5-1%)	Binds aggrecans. Constitutive molecule in synovial fluid.
Proteoglycans PGs (10-12%) - GAGs: • Condroitin-6-sulphate • Condroitin-4-sulphate • Keratan-sulphate - Aggrecans - Others: • Decorin	Confer polyanionic character to aggrecan. Determine swelling pressure, deformability and elasticity of the ECM. Regulates collagen II fibrillogenesis.

• Biglycan • Fibromodulin • Lumican • Perlecan	Binds collagen VI and TGF-β. Binds collagen I and II; binds TGF-β. In pericellular zone, interacts with CII and regulates fibrillogenesis.
Non-collagenous proteins (3%) - Core protein - Binding protein - COMP - CMP - Tenascin - Fibronectin - Anchorin CII - Thrombospondin - Chondroadherin - Asporin - Chondrocalcin - CD44 and integrins	 Binds GAGs in aggrecan. Stabilize binding between aggrecan and HA. Marker of ECM degradation. Role in chondrogenesis; binds aggrecan. Role in chondrogenesis. Cellular adhesion to the ECM; interacts with GAGs and collagen. Cellular adhesion to the ECM; binds collagen. Adhesion; binds Calcium and hydroxyapatite. It binds CII in territorial zone and interacts with cartilage. Involved in cartilage calcification. Membrane protein receptor.

Table 1: Main componentsof AC and their functions.

Structure of articular cartilage

The structure of AC consists of four layers, starting from the articular surface (see figure 1, panel A and B): the superficial, transitional, deep, and calcified cartilage zones. The zonal organization is of functional importance, and the size of the zones varies among joints and between various species. Also, chondrocytes show different size and shape and a biosynthetic activity varying in quality and quality according to their position in the different layers of cartilage (Huber, Trattnig, & Lintner, 2000). The chondrocytes are flattened and elongated, of fibroblastic appearance in the superficial layer, ovoid in shape, single or sometimes paired in the middle layer, larger in size and paired in doublets or triplets in the deep layer, while in the proximity of the calcified layer they can arrange

themselves in short columns or groups of 2-4 cells. Hypertrophic and metabolically active chondrocytes are present within the calcified layer.

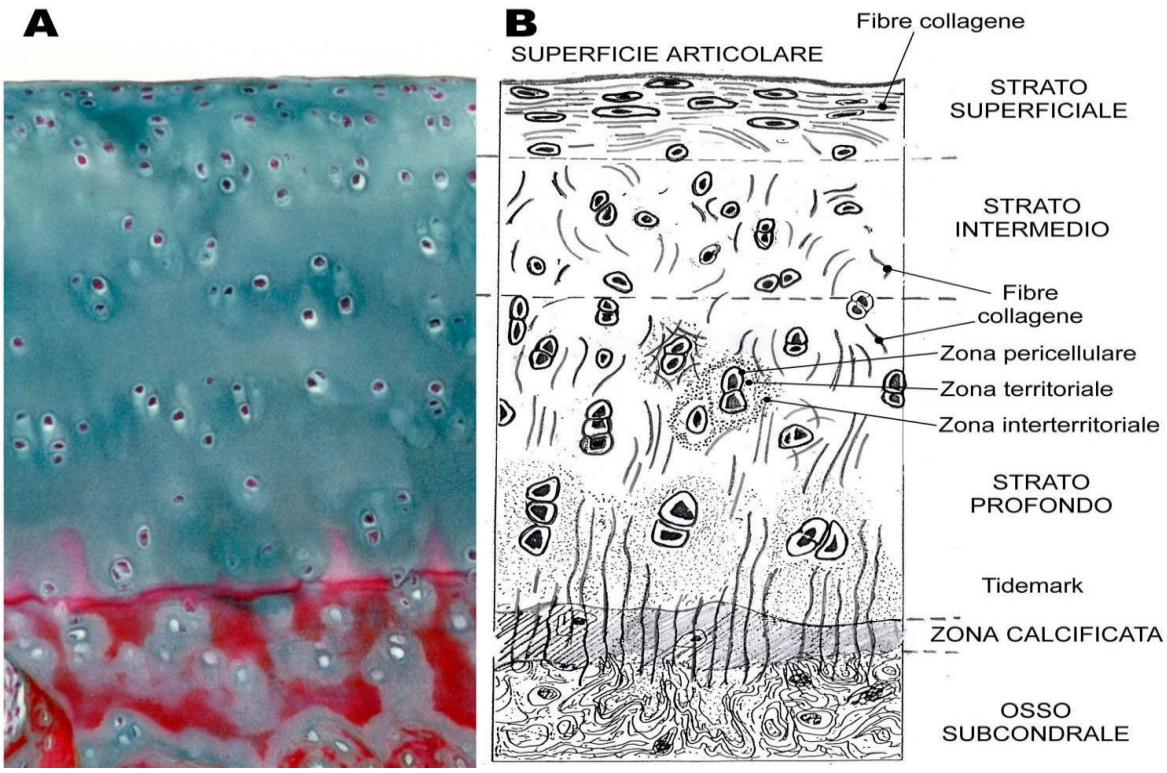


Figure 1: Schematic of AC. Panel A: Safranin O stained AC histologic sample. Panel B: schematic drawing representing AC zonal organisation. Proceeding from the articular surface (to the top, indicated as "superficie articolare", these zones are distinguished (Fig. 22 and 27): 1) Surface layer or superficial zone, 2) Intermediate or transitional layer, 3) Deep or radial layer, 4) Calcified layer in which the matrix is partially mineralized. The radial layer is separated from the calcified cartilage by a line called "tidemark" which represents the ossification front resulting from the epiphyseal osteocartilaginous growth and whose progression is blocked at the level of mature cartilage. Below the calcified cartilage is the subchondral bone. Reproduced by permission of Prof. Paolo Borghetti.

The superficial zone (tangential zone) is the thinnest zone of articular cartilage. Composed by flattened chondrocytes (see figure 1, panel B and figure 2, panel A), it has the lowest proteoglycan content, and densely packed layers of uniform collagen fibres that are thin in diameter (Weiss, Rosenberg, & Helfet, 1968). It also consists of two layers distinguished by different collagens organization: the first one, known as the *lamina splendens*, is characterised by fine fibrils covering the articular surface in the form of thickly packed bundles of collagen fibres arranged parallel to each other to give the tissue its mechanical properties (Minns & Steven, 1977); the second one consists of collagen fibres arranged perpendicularly to the articular surface (Huber, Trattnig, & Lintner, 2000).

Three-dimensional surface analysis showed that, compared with the international standards for surface preparations used by machinists for metal surfaces, the articular cartilage surface could be described as being smooth (Bloebaum & Radley, 1995) (Huber, Trattnig, & Lintner, 2000).

The transitional zone (intermediate or middle zone) is composed of rounded chondrocytes surrounded by extracellular matrix (see figure 2, panel A). The collagen fibres are arranged randomly, and the proteoglycan content is increased (see figure 2, panel B) (Huber, Trattnig, & Lintner, 2000).

In the deepest zone (radial or radiate zone), the chondrocytes are mainly organized in columns, and the cell volume is at its lowest (see figure 2, panel A) (Huber, Trattnig, & Lintner, 2000). This part of the matrix is most remote from the chondrocytes. The proteoglycan content is high and the concentration of water is the lowest (see figure 2, panel C).

The collagen fibres in the middle and deep zones are generally oriented toward the articular surface in large bundles approximately 55 μm across (Minns & Steven, 1977) and are randomly arranged (see figure 2, panel B) (Huber, Trattnig, & Lintner, 2000).

A wavy, irregular line known as the tide mark separates the deep zone from the calcified zone; ultrastructurally, it is formed by a band of fibrils that may serve as a tethering mechanism for the collagen fibrils in the non-calcified zone to prevent them from being sheared of anchorage to the calcified zone (Huber, Trattnig, & Lintner, 2000); small gaps in the tide mark may provide channels for the passage of nutrients (Redler, Mow, Zimny, & Mansell, 1975).

The calcified zone is characterized by rounded chondrocytes located in uncalcified lacunae and by the absence of proteoglycans (see figure 2, panel A and C) (Huber, Trattnig, & Lintner, 2000); collagen fibres are arranged perpendicular to the articular surface and are anchored in a calcified matrix (see figure 2, panel B) (Huber, Trattnig, & Lintner, 2000).

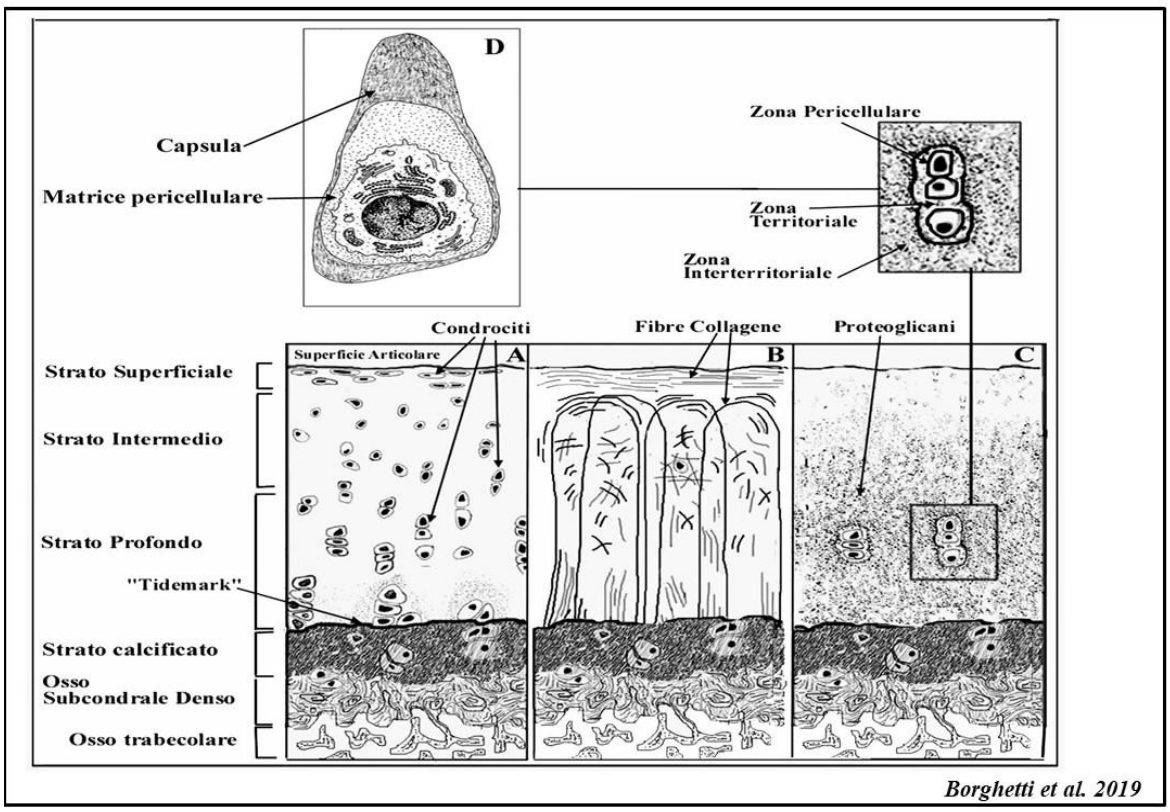


Figure 2: Schematic of chondrocytes (panel A), collagen fibres (panel B) and proteoglycans (panel C) organisation within the different layers of AC. In panel A, chondrocytes are flattened in the superficial zone, grow rounder in the intermediate zone, tend to be grouped in columns and assume a hypertrophic appearance in the deep zone and especially in the calcified zone. In panel B, it can be noticed that collagen fibres assume a different spatial orientation (mainly flat and tangential in the superficial zone, crossed and random in the intermediate zone, perpendicular to the articular surface in the deep and calcified zone). In panel C, proteoglycans appear more concentrated in the intermediate and deep zones, while they are present at a very low content in the superficial and the calcified zones. On top left, panel D represents a chondron, i.e. the chondrocyte with its pericellular matrix. Reproduced by permission of Prof. Paolo Borghetti.

Chondrocytes

Chondrocytes are highly specialised, highly differentiated cells originating from mesenchymal stem cells, amounting to 1-2% of tissue volume (Huber, Trattnig, & Lintner, 2000) (Fox, Bedi, & Rodeo, 2009). Due to their high specialisation, they indeed have limited potential for replication and therefore limited intrinsic healing capacity, their survivability depending on optimal chemical and mechanical environment
(Fox, Bedi, & Rodeo, 2009).

They stabilize a specialized microenvironment producing, developing, maintaining and repairing the ECM and driving the organization of collagens, proteoglycans, and non-collagenous proteins into a highly ordered structure (Fox, Bedi, & Rodeo, 2009) (Huber, Trattnig, & Lintner, 2000). Chondrocytes are eventually trapped and retained within the matrix (Fox, Bedi, & Rodeo, 2009). The chondrocyte number, shape, organization and metabolic activity and consequently the cartilage molecular structure differ into the various

layers of the AC; for instance, in the superficial zone vs. the deep zone chondrocytes appear flatter, smaller, at a greater density (Huber, Trattnig, & Lintner, 2000).

Chondrocytes receive mechanical, electrical and physicochemical signals transmitted by the ECM and respond by regulating their metabolic activity, balancing between anabolism and catabolism, and by pursuing a continual internal remodelling as the cells replace matrix macromolecules lost through degradation (Huber, Trattnig, & Lintner, 2000).

The cartilage extracellular matrix

The ECM is primarily composed of tissue fluid and macromolecules, including collagens, proteoglycans, and non-collagenous proteins, with differences in content and distribution depending on the joint, the site in the joint, and increasing age (Meachim, 1971) (Gurr, Mohr, & Pallasch, 1985).

AC can be considered a slow-metabolism tissue with very low matrix turnover: in healthy cartilage, the aggrecan core protein of cartilage proteoglycan has a half-life of 3-24 years, while the collagen network has a half-life of more than 100 years (Goldring & Marcu, 2009).

The matrix has been subdivided into the pericellular, territorial, and interterritorial regions around each chondrocyte (Huber, Trattnig, & Lintner, 2000); these regions are defined by structural differences and a specific distribution of proteoglycans, link protein, and hyaluronic acid (or hyaluronan) (Poole, Pidoux , Reiner, & Rosenberg, 1982), which function synergistically to produce an integrated, hydroelastic suspension system capable of resisting compression. (Poole, Flint, & Beaumont, 1984)

The chondrocyte and its pericellular microenvironment together represent the *chondron* (see above in figure 2, panel D) (Poole C. A., 1997): this thin, pericellular matrix provides hydrodynamic protection for the chondrocyte during physiological loading and that plays a metabolic role in pericellular retention (Huber, Trattnig, & Lintner, 2000); each single chondrocyte is linked to a high surrounding concentration of sulphated proteoglycans, as well as hyaluronan, biglycans (Miosge, Flachsbart, Goetz, & et al., 1994), and a range of matrix glycoproteins; the so-structured transparent pericellular glycocalyx is enclosed by a fibrillar pericellular capsule (Poole C. A., 1997).

The territorial region is characterized by large collagen fibres in radial bundles and proteoglycans rich in chondroitin sulphate (Huber, Trattnig, & Lintner, 2000) (Meachim & Stockwell, 1979). The interterritorial region is characterized by the largest collagen fibres

(Huber, Trattnig, & Lintner, 2000) and proteoglycans rich in keratan sulphate (Meachim & Stockwell, 1979).

ECM components

The complex ECM organisation and components are portrayed below in figure 3 and described in detail in the following chapters.

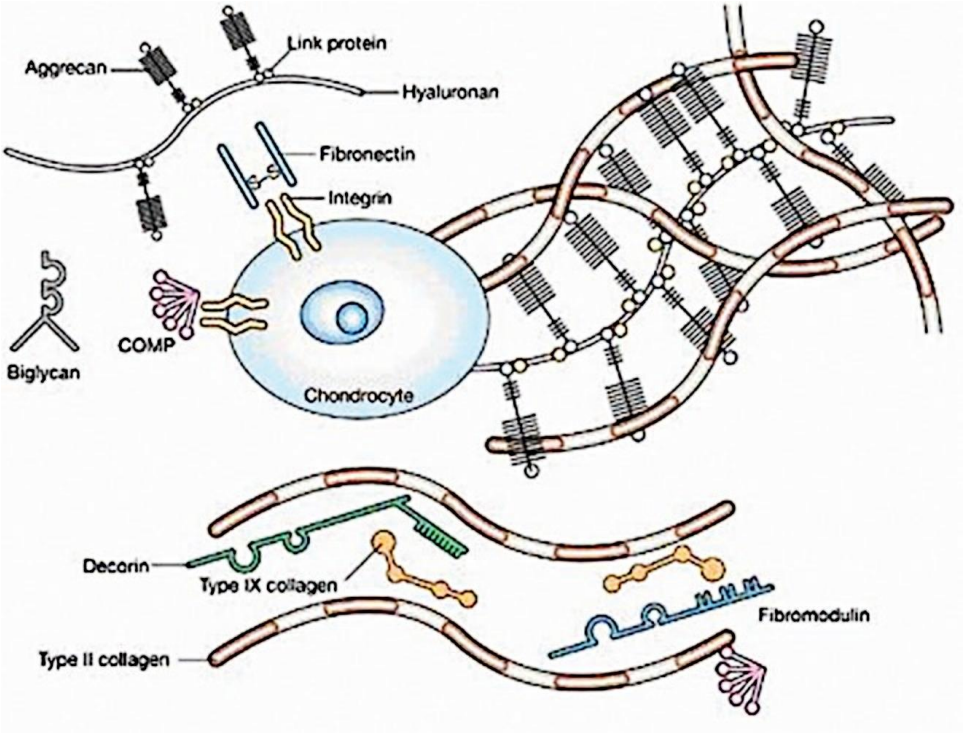


Figure 3: ECM of articular cartilage. Two major load-bearing macromolecules are present in articular cartilage: collagens (mainly, type II) and proteoglycans (notably, aggrecan). Smaller classes of molecules, such as noncollagenous proteins and smaller proteoglycans, are present in smaller amounts. The interaction between the highly negatively charged cartilage proteoglycans and type II collagen provides the compressive and tensile strength of the tissue. Reprinted by Fox, Bedi, & Rodeo, 2009 with permission from Chen, Rousche, & Tuan, 2006.

Water

Water makes up to 80% of articular cartilage wet weight, thus establishing as the most abundant component. Water content is divided into the intracellular compartment in minimal quantity and the extracellular one for the vast majority: the latter involve about 30% of total water associated with the intrafibrillar space within the collagen and approximately 70% of total water contained in the pore space of the matrix (Fox, Bedi, & Rodeo, 2009) (Maroudas, Wachtel, Grushko, Katz, & Weinberg, 1991) (Torzilli, 1985).

Some inorganic ions, such as sodium, calcium, chloride, and potassium, are dissolved in water (Fox, Bedi, & Rodeo, 2009).

Water within the ECM can flow: as interfibrillar water appears as a gel, it may be moved through the ECM by pressure gradient or compression (Mow, Kuei, Lai, & Armstrong,

1980) (Mow, Ratcliffe, & Poole, 1992). Also, frictional resistance against this flow through the matrix is very high (Fox, Bedi, & Rodeo, 2009); this and the subsequent water pressurization are the key mechanisms that allow articular cartilage to withstand loads (Fox, Bedi, & Rodeo, 2009).

Water content decreases from superficial to deep zone (Buckwalter & Mankin, 1997).

Collagens

Collagen is the most abundant structural macromolecule in ECM and represents its endoskeleton or extracellular framework (see above in figure 3) (Huber, Trattnig, & Lintner, 2000). A region consisting of 3 polypeptide chains (α-chains), wound into a triple helix, characterizes all collagen types; this triple helix structure of the polypeptide chains, rich in glycine and proline and stabilized by hydrogen bonds, endows articular cartilage with unique shear and tensile properties (see below in figure 4) (Maroudas, 1979).

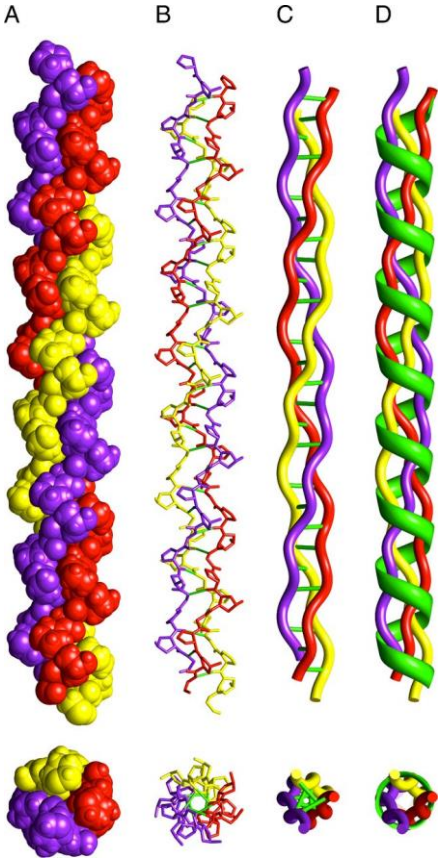


Figure 4:Different representation of collagen triple helix. A represents the space filling diagram. B is a stick diagram with hydrogens removed for clarity (hydrogen bounds are represented as green sticks). C shows the ladder of hydrogen bonds. D presents the left-handed superhelix in green, which describes mathematically the collagen triple helix as a continuous helix of repeating units across the three individual strands. Reproduced from Bella, 2016.

Collagen type II is about 90% of total collagen in hyaline cartilage; collagen types VI, IX and XI, all which help in collagen II fibrillar network stabilization (main cross-linking

occurs between collagen II and IX and XI; see above in figure 3), represent the remaining 10% (Fox, Bedi, & Rodeo, 2009) (Huber, Trattnig, & Lintner, 2000).

Proteoglycans

Proteoglycans are molecules consisting in one or more glycosaminoglycan chains covalently attached to a protein core (Huber, Trattnig, & Lintner, 2000), accounting for 10% to 15% of the wet weight (Fox, Bedi, & Rodeo, 2009). Glycosaminoglycan chains are composed by more than 100 monosaccharides; they extend out from the protein core, remaining separated from one another because of charge repulsion. Articular cartilage contains a variety of proteoglycans, essential for normal function, including aggrecan, decorin, biglycan, and fibromodulin (Fox, Bedi, & Rodeo, 2009).

Aggrecan is the largest and most abundant among ECM proteoglycans (Fox, Bedi, & Rodeo, 2009). Aggrecan (see in figure 5) is made of a protein core, which binds keratan sulphate and chondroitin sulphate glycosaminoglycan chains (their content varies depending on load-bearing necessity in different joints) (Huber, Trattnig, & Lintner, 2000). It provides a hydrated gel structure thank to its interaction with hyaluronan and link proteins that endows the cartilage with load-bearing properties (exemplified above in figure 3) (Kiani, Chen, Wu, Yee, & Yang, 2002).

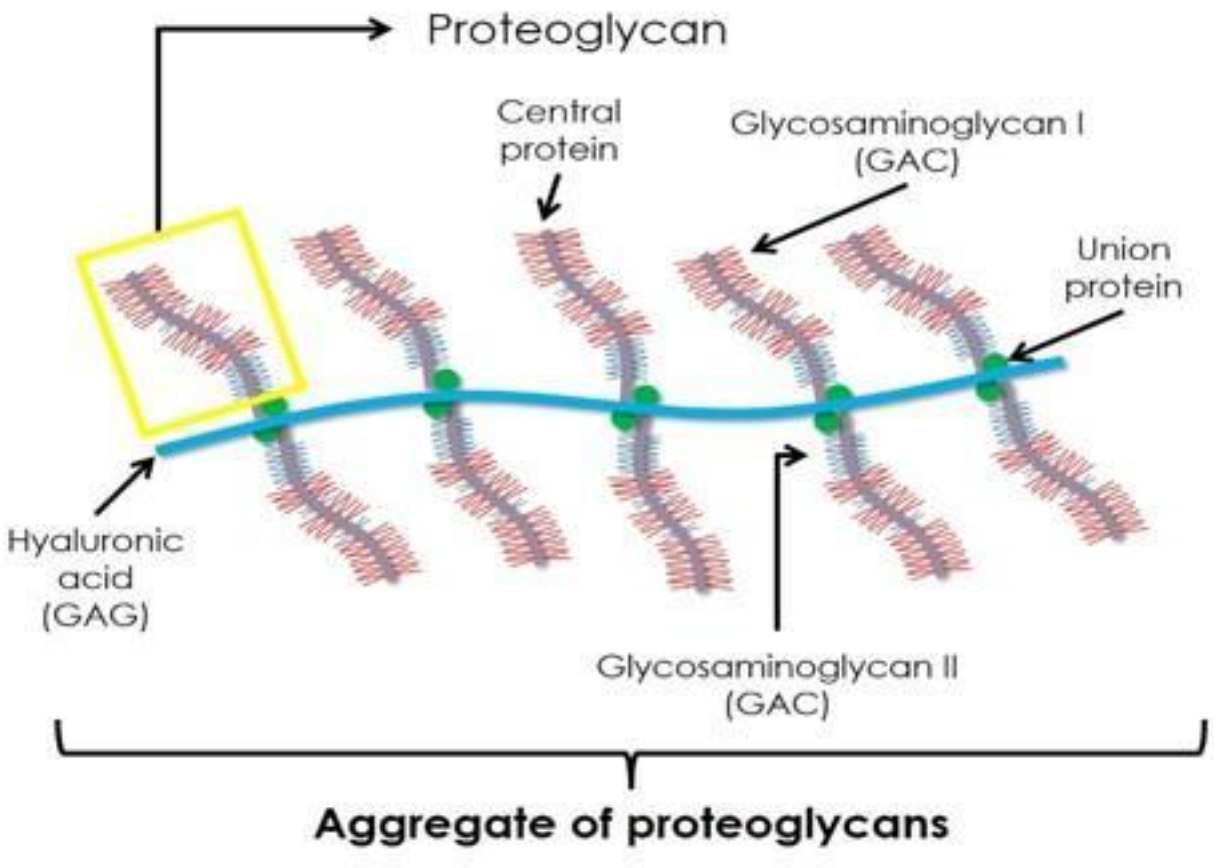


Figure 5: Schematics of aggrecan complex structure. HA is the spine of the whole proteoglycans aggregate, binding several core, or central, proteins via union proteins. These core proteins bind keratan sulphate and chondroitin sulphate glycosaminoglycan chains and together with them form the proteoglycan, above marked with a yellow rectangle. Reproduced from https://www.europages.it/LipoProteoglicani/INDERMAL/cpid-5589475.html.

Hyaluronic acid (HA) or hyaluronan is abundantly synthetized by chondrocytes and presents partly bound to ECM structural proteins and partly free binding (Huber, Trattnig, & Lintner, 2000): in the first form, it binds and retains collagen fibrillar network, aggrecan and link proteins (Poole, Pidoux, Reiner, & Rosenberg, 1982) (Parkkinen, et al., 1996). HA has osmotic properties, which are critical to its ability to resist compressive loads.

Small proteoglycans or non-aggregating proteoglycans, such as decorin, biglycan, and fibromodulin, make up approximately 3% of the total proteoglycan mass. United by ability to interact with collagen, they bind to matrix macromolecules and help with matrix stabilization (Huber, Trattnig, & Lintner, 2000) (Fox, Bedi, & Rodeo, 2009).

Non-collagenous proteins and glycoproteins

A number of non-collagenous proteins and glycoproteins are found within articular cartilage, their specific function though has not been fully characterized; some likely have a role in organization and maintenance of the macromolecular structure of the ECM (Fox, Bedi, & Rodeo, 2009).

For instance, anchorin CII appears to help anchor chondrocytes to the collagen fibrils. (Mollenhauer, Bee, Lizarbe, & von der Mark, 1984). Other proteins, including tenascin and fibronectin, influence interactions between the chondrocytes and the matrix. They may play roles in response to arthritis and osteoarthritis (Huber, Trattnig, & Lintner, 2000) (Saltner, 1993) (Nishida, Inoue, & Murakami, 1995).

Cartilage matrix protein binds to chondrocytes and is seen to increase in osteoarthritis (Huber, Trattnig, & Lintner, 2000). Chondrocytes may express the cartilage matrix protein in response to arthritic stimuli (Saxne & Heinegard, 1992) (Okimura, et al., 1997).

Relationship between chondrocytes and extracellular matrix

The chondrocyte has a central role in the biology and physiology of AC. Chondrocytes are responsible for the metabolic homeostasis of the matrix on which mechanical functions of the tissue depend. In turn, due to its structural characteristics, the matrix ensures a physico-chemical homeostasis suitable for normal cell nutrition, metabolism and synthetic activity.

Chondrocytes show the following functions related to their biosynthetic activity (Huber, Trattnig, & Lintner, 2000) (Syggelos, Altras, Smirlaki, & Skandalis, 2013):

1. Synthesis of matrix macromolecules and degradative enzymes (metalloproteases or MMPs) and metalloproteases inhibitors (TIMPs), involved in the balance of cartilage metabolism and ECM production;
2. Synthesis of several cytokines (IL-1, TNF-α, IL-6, IL-8), growth factors (TGF-β, IGF-1), of prostaglandin E_2 (PGE2), proteases, plasminogen activator (PA);
3. Release of free radicals of O2 and NO;
4. Proliferative activity and changes in differentiation during reparative processes.

Under physiological conditions, the matrix is slowly but continuously degraded and replaced by new matrix; the replacement of proteoglycans is rapid, in terms of days or weeks, while the turnover of fibrillar collagen is much slower and occurs in months or even years. Fragments derived from degradation, together with the action of mechanical loading, are signals capable of regulating the activity of the chondrocyte.

With the aging of the tissue, the number of chondrocytes and their proliferative capacity in response to growth factors is reduced while the metabolic equilibrium moves towards degradation with an increase in MMPs and a reduction in their inhibitors.

The balance between production and degradation of the cartilaginous matrix is regulated by several factors (mechanical forces, inflammatory signals, cytokines, aging, etc.), some

favouring the anabolic and cell proliferative action, others with catabolic and degradative activity (see in figure 6).

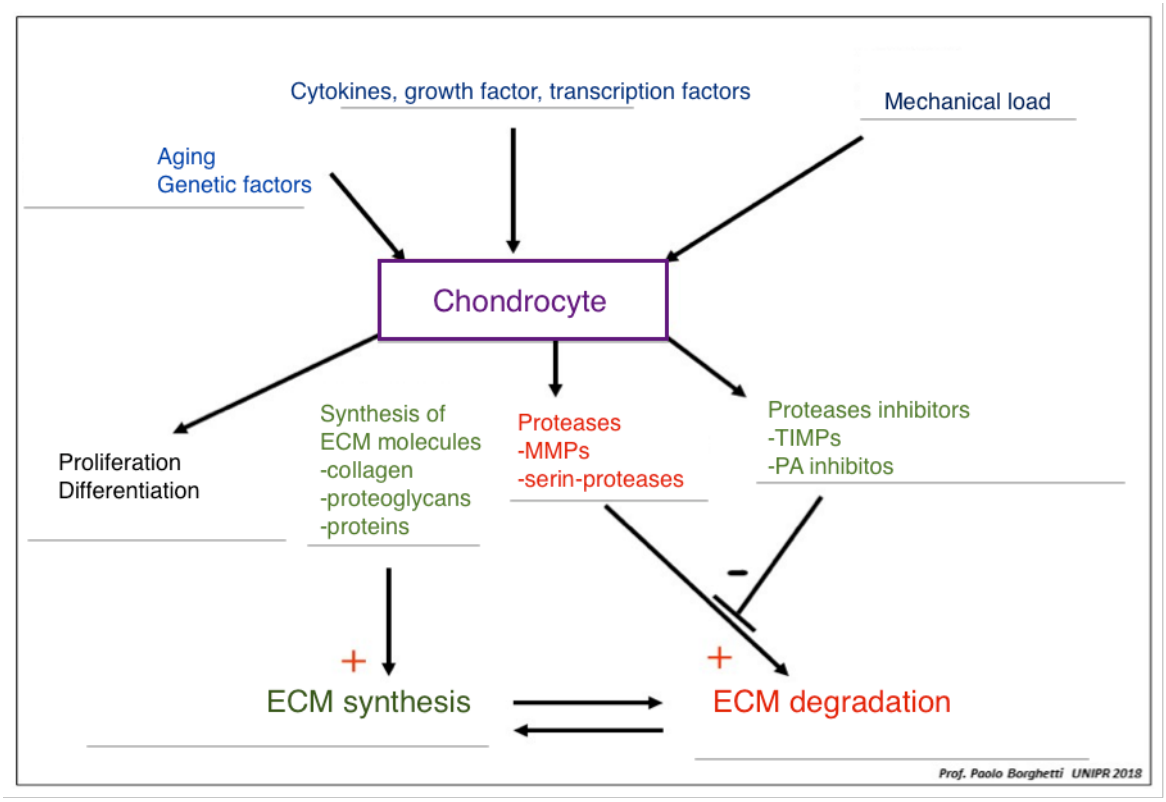


Figure 6: Main factors acting on chondrocyte behaviour and its responses towards a balance between ECM synthesis and degradation. By permission of Prof. Paolo Borghetti.

The chondrocytes regulate the synthesis and degradation of the cartilaginous matrix also through mechanisms that depend on the interactions of the chondrocytes, through integrins, with ECM molecules such as collagen, proteoglycans and non-collagenous proteins (fibronectin, laminin, vitronectin, osteopontin etc.) and which regulate chondrocyte homeostasis through mechanisms involving nitric oxide and PGE2.

Osteoarthritis in horses

Definition and clinical relevance

Equine osteoarthritis (OA) is a disease of huge clinical relevance, given that it might be related to up to 60% of lameness in the horse (United States Department of Agriculture, 2000) (Caron & Genovese, 2003).

OA, also known as degenerative joint disease (Zhang, Ouyang, Dass, & Xu, 2016), can be defined as a group of disorders characterised by a common end stage: progressive deterioration of the articular cartilage accompanied by changes in the subchondral bone and soft tissues of the joint (McIlwraith, 2005), with involvement of ligaments, capsule, synovial membrane and peri-articular tissues.

The primary lesions are characterized by articular cartilage degeneration with fibrillation, fissures, ulceration, and full thickness loss of cartilage tissue and bone surface exposure (Brandt, Dieppe, & Radin, 2008);

Clinically the horse shows pain, deformity, loss of motion, decreased function, lameness in the involved joint(s) (Schlueter & Orth, 2004). Structural origin of equine OA might be disease in synovial membrane, fibrous joint capsule, subchondral bone, ligaments, articular cartilage or a combination of the above (McIlwraith, 2005).

OA primarily develops in equine athletes and elderly animals. In the first category it is often seen at a very young age, even before 36 months (Garcia, Melo, Ferreira, Toscano, & Cruz, 2009), mainly due to premature start of horse training during early musculoskeletal system development or due to excessive and/or prolonged mechanical loads on immature articular cartilage, with peri-articular tissues inadequately developed to support intense loads (Garcia, Melo, Ferreira, Toscano, & Cruz, 2009) (Schlueter & Orth, 2004)

In older animals degeneration of articular cartilage is often seen related to ageing and prolonged exposure to mechanical stressors (Schlueter & Orth, 2004).

The aetiology of equine OA recognizes a variety of causes, such as (Schlueter & Orth, 2004):

1. Trauma, which creating direct insult on joint structures is believed to be the major etiopathogenetic factor;
2. Immobilization, leading to atrophy of articular cartilage;
3. Joint conformation and of legs, predisposing to an improper joint loading and therefore of abnormal forces placed on joint surfaces (Thompson & Herring, 1994);

4. Age, as a point of convergence of lifelong detrimental effects and perhaps of a different biochemical structure in articular cartilage.

Most mechanisms are attributed to two general patterns, namely “abnormal” loading on “normal” cartilage or “normal” loading on “abnormal” cartilage (Goldring & Goldring, 2007).

Frequently affected joints are: carpus, fetlock, proximal interphalangeal joint and distal intertarsal/tarsometatarsal joint (Schlueter & Orth, 2004). Joints characterised by close fitting articular surfaces can quickly develop linear erosions and wear lines in association with osteochondral fragmentation (McIlwraith, Frisbie, & Kawcak, 2012).

It is also seen that most lamenesses and injuries involve the forelimbs, since they carry 60-65% of the horse’s weight and are subjected to higher load rates than the hind limbs (Back, Schamhardt, Hartman, & Barneveld, 1995) (McDuffee, Stover, & Coleman, 2000) (Stashak, 2002).

For instance, racehorses tend to present with spontaneous OA in the metacarpophalangeal joint, followed by carpal joint (McIlwraith, Frisbie, & Kawcak, 2012). In the carpus of a racehorse, considerable damage may be inflicted directly to the articular cartilage in the regions of concussion by cyclic mechanical stress (as exemplified by fractures and chondral lesions not associated with fracture), and there is often primary damage to the subchondral bone that often leads to secondary damage to the articular cartilage, from either loss of bone support or release of cytokines.

Subchondral sclerosis, acute synovitis and capsulitis might as well contribute to the degenerative process in articular cartilage through decreased shock absorption, release of enzymes, inflammatory mediators and cytokines (main patterns are summarized in figure 7) (McIlwraith, Frisbie, & Kawcak, 2012) (McIlwraith, 1996) (McIlwraith & Vachon, 1988).

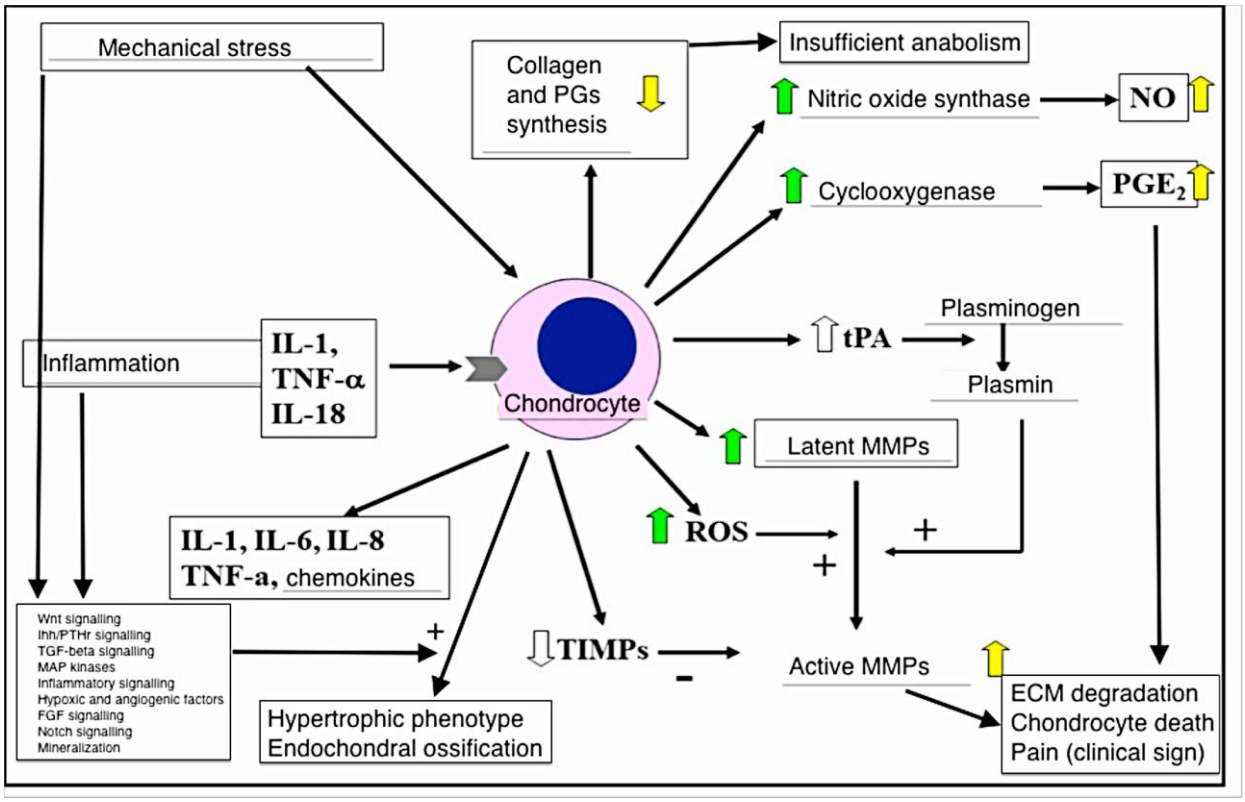


Figure 7: Schematic representing main molecular pathways triggering the chondrocyte to change phenotype, produce degragative substances, while decreasing ECM molecules and protective factors synthesis. Adapted by permission of Prof. Paolo Borghetti.

Patogenesis and gross lesions

Microscopically, OA cartilage is characterized by loss of ECM molecules, namely collagen and proteoglycans (Brocklehurst, et al., 1984) (Venn & Maroudas, 1977), thus perturbing the cartilage matrix structure and impairing its biomechanical properties (Pearle, Warren, & Rodeo, 2005). Chondrocytes near the superficial layer form clusters, whereas in the deep and calcified layers, they undergo apoptotic death (Sandell & Aigner, 2001) (Khan, Williams, & Archer, 2009) (Del Carlo & Loeser, 2008) Chondrocyte proliferation is somewhat activated, but cannot compensate the predominant catabolic activity (Sandell & Aigner, 2001).

During OA progression, osteoarthritic chondrocytes produce matrix-degrading enzymes including matrix metalloproteinase 13 (MMP13), which degrades collagen and Adamts-5 (A disintegrin, a metalloproteinase with thrombospondin motifs-5) that targets aggrecan (Dreier, 2010) (van den Berg, 2011). The synthesis of degradative enzymes further exacerbates the breakdown of articular cartilage.

The biomechanical and biochemical changes would together disrupt cartilage homeostasis and contribute to the pathogenesis of OA, which leads to joint space narrowing, painful cartilage destruction, and loss of function.

Numerous studies suggest that chondrocyte death is a key player in cartilage degeneration in OA (Hashimoto, Ochs, Komiya, & Lotz, 1998) (Blanco, Guitian, Vazquez-Martul, de Toro, & Galdo, 1998) (Sharif, Whitehouse, Sharman, Perry, & Adams). Chondrocyte death by apoptosis (Hashimoto, Ochs, Komiya, & Lotz, 1998) (Blanco, Guitian, Vazquez-Martul, de Toro, & Galdo, 1998), necrosis, chondroptosis, or combination of these processes has been implicated in the pathogenesis of OA (Zamli & Sharif, 2011). Apoptosis has been positively correlated with the severity of cartilage destruction and matrix depletion in human osteoarthritic tissue specimens (Musumeci, Aiello, Szychlinska, di Rosa, Castrogiovanni, & Mobasheri, 2015).

In addition to cartilage degeneration, OA usually affects all structures in the synovial joint. Aberrant hypertrophy and calcification are reported in several OA cases, which is similar to the terminal differentiation process during endochondral ossification. (Dreier, 2010) (Ea, et al., 2011) (Fuerst, et al., 2009). Osseous outgrowths called osteophytes often form at the joint margins (van der Kraan & van den Berg, 2007) (Felson, et al., 2005). Subchondral bone sclerosis (Hayami, Pickarski, Zhuo, Wesolowski, Rodan, & Duong, 2006), meniscal tear and extrusion (Dixon, Jacoby, Berry, & Hamilton, 1988), and synovial membrane inflammation (synovitis) (Benito, Veale, FitzGerald, van den Berg, & Bresnihan, 2005) may also occur due to the mechanical changes in OA cartilage, and make OA disease more debilitating.

Typical changes in joint tissues during OA, as exemplified below in figure 8, involve synovitis, cracking of AC, subchondral bone sclerosis. Anatomopathological lesions may appear as in figure 9.

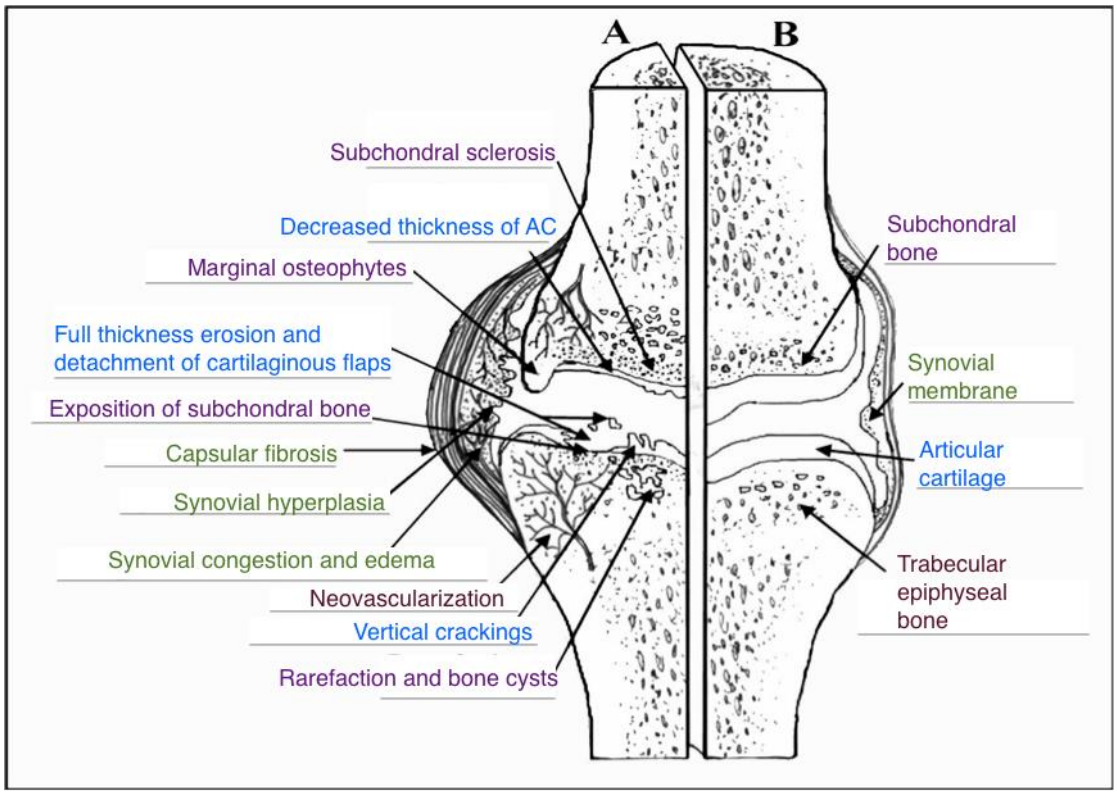


Figure 8: Schematic of gross lesions in OA (panel A), compared with a healthy joint (panel B). Blue: changes in AC (decreased thickness, erosion, flap detachment, cracking). Purple/red: changes in subchondral bone/epiphyseal bone (sclerosis, osteophyte formation, exposition, rarefaction and bone cysts, neovascularization). Green: changes in synovium (fibrosis, hyperplasia, congestion and edema). By permission of Prof. Paolo Borghetti.

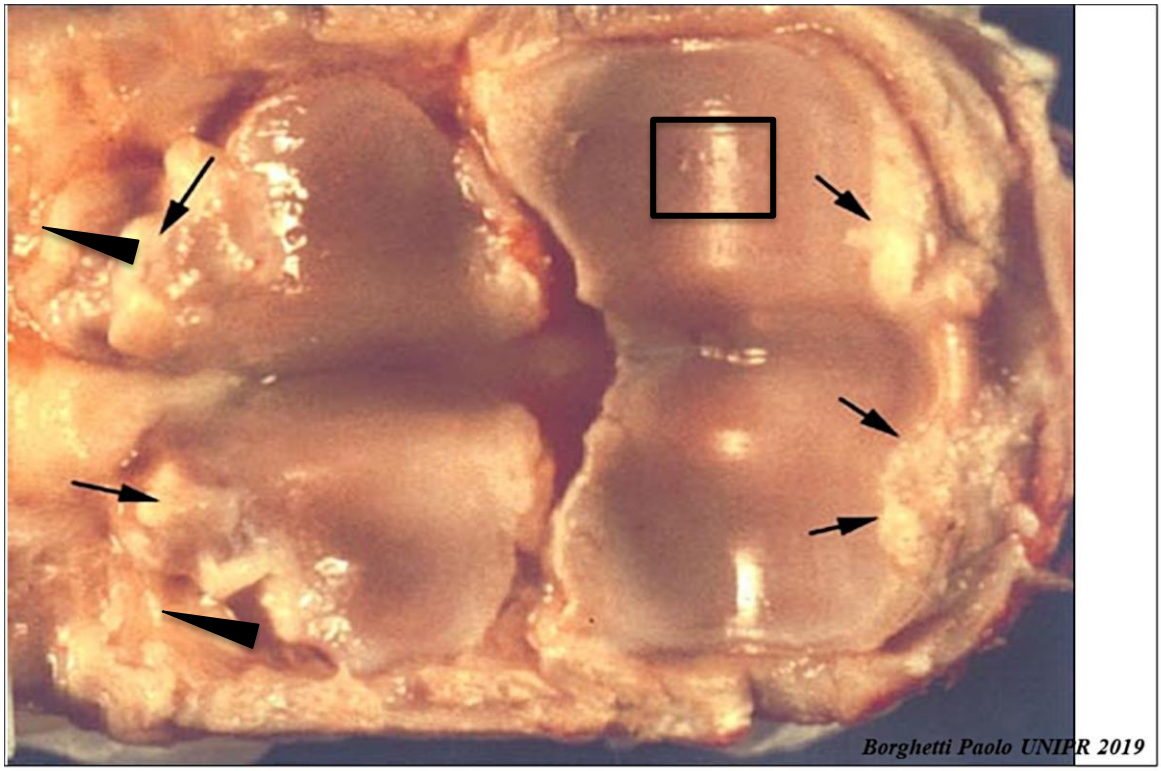


Figure 9: Macroscopic lesions in equine OA: extended erosion of AC (outlined by rectangle) with osteophyte formation (arrows) in the cranial margin of first phalanx and in sesamoid bones; subacute-chronic synovitis and capsular fibrosis (arrowhead). By courtesy of Prof. Paolo Borghetti.

The following table 2 represents in detail changes undergone by joint tissues during OA.

Joint tissues	**Tissue changes**
Cartilage	Fibrillation, degeneration and fragmentation
	Hypocellularity
	Empty and/or debris in lacuna
	Chondrocyte clustering/death
	Proteoglycan loss
	Collagen II loss
	Cartilage ossification
Subchondral bone	Sclerosis
	Marginal osteophyte formation
	Increase in BMD
	Increase in bone turnover
	Cyst formation
	Joint space narrowing
	Alteration of subchondral trabecular architecture
Synovium	Capsular fibrosis
	Synovial hyperplasia
	Synovitis
	Production of MMPs and ROS
Synovial fluid	Increase in MMPs
	Increase in IL-1β
	Increase in TNF-α
	Increase in ROS
	Increase in PGE
	Increase in aggrecan fragments
	Decreased viscosity

Table 2:Summary of pathological features of OA. Adapted from Zamli & Sharif, 2011

OA and molecular pathways

Anabolic/catabolic balance in OA pathogenesis

Several mediators are involved in the subtle anabolic/catabolic balance in cartilage homeostasis. Many studies have investigated the role of these molecules and main characteristics are summarized in the following table 3 (Mueller & Tuan, 2011) (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010) (Musumeci, Aiello, Szychlinska, di Rosa, Castrogiovanni, & Mobasheri, 2015).

Name	Expression in OA and role in AC homeostasis	Characteristics and actions
Cytokines		
➢ Interleukin 1 beta or IL-1β (Mueller & Tuan, 2011)	Increased Detrimental	• Found in synovial fluid • Expressed by synoviocytes and chondrocytes • Increases level of matrix-degrading enzymes and proteoglycans loss • Reduces synthesis of ECM proteins by chondrocytes • Its receptor IL-1R1 in increasingly expressed in OA • Induces synergistic cytokines: IL-6, which augments the catabolic effects of IL-1β and TNF-α; IL-17 is increased as well • Up-regulates BMP-2, anabolic growth factor, though inhibited if also NO is present • At low doses stimulates BMP-7 expression, whereas at high doses suppresses BMP-7 expression; BMP-7 counteracts IL-1β-induced MMP-13 expression
➢ Tumor necrosis factor alfa or TNF-α (Mueller & Tuan, 2011)	Increased Detrimental	• Synergic action together with IL-1β • Suppresses ECM synthesis (proteoglycan, collagen) • Stimulates expression of matrix-degrading enzymes (such as metalloproteinases or MMPs) • Synergy with and induction of other cytokines, like IL-6 and IL-17 • Chondrocytes stimulated by these pro-inflammatory cytokines increase expression of prostaglandin E2 (PGE2) and bone morphogenetic protein-2 (BMP-2) in attempt to compensate loss of ECM with increased

		collagen II expression
Mediators ➢ Nitric Oxide or NO (Mueller & Tuan, 2011)	Increased Detrimental	• Inflammatory catabolic mediator • Increased through overexpression of inducible nitric oxide synthase (iNOS) in OA chondrocytes • Induced by mechanical factors and pro-inflammatory cytokines • Inhibits proteoglycan and collagen synthesis • Mediates MMPs induction via IL-1β • Chondrocyte apoptosis inducer • Positive regulator of nuclear factor (NF)- κB pathway, which mediates transcription of pro-inflammatory genes • Perpetrates catabolic processes that lead to tissue degeneration
➢ Prostaglandins (Mueller & Tuan, 2011)	Increased Beneficial/ detrimental	• Main mediator of inflammation and pain in OA • Produced by enzymes cyclo-oxigenase-2 (COX-2) and prostaglandin E synthase, both induced by IL-1β • IL-1β and TNF-α stimulate expression of PGE2, which stimulates expression of collagen II in a positive feedback regulation • Drugs blocking COX-2 expression reduce pain but do not help with slowing down cartilage loss
Growth factors ➢ Transforming growth factor beta or TGF- β (Mueller & Tuan, 2011) (Musumeci, Aiello, Szychlinska, di Rosa, Castrogiovanni, & Mobasheri, 2015)	Increased Mostly beneficial	• Family of dimeric proteins involved in multiple biologic signalling activities, cellular proliferation and differentiation • In OA chondrocytes promotes expression of collagen II and aggrecan, while downregulating matrix degrading enzymes • Loss of efficacy (reduction) upon aging • Counteracts IL-1β-induced suppression of proteoglycan biosynthesis • In some conditions, may shift molecular pathways towards chondrocytes terminal differentiation and apoptosis
➢ Bone morphogenetic proteins or BMPs (Mueller & Tuan, 2011)	Increased Mostly beneficial	• Members of TGF-β superfamily, involved in many processes as maintenance of articular cartilage • BMP-2 is up-regulated in OA chondrocytes by IL-1β and TNF-α, while BMP-7 is down-regulated; regulation is also performed by BMP

		antagonist, like gremlin or follistatin • Increase expression of ECM genes like collagen II • BMP-7 has positive effect on ECM synthesis and is the strongest in contrasting IL-1β-mediated degradation • BMP-4 has anabolic effects on chondrocytes • Dual anabolic/catabolic role: BMP signalling stimulates both matrix synthesis and terminal chondrocyte differentiation, which results in increased MMP-13 production, which increases matrix degradation
➢ Insulin-like growth factor-1 or IGF-1 (Mueller & Tuan, 2011)	Increased Beneficial, but effectiveness is strongly regulated	• Crucial role in cartilage homeostasis by stimulating ECM production and inhibiting matrix degradation • Expression induced by growth hormone (GH); • Highly expressed, with its receptor, in OA chondrocytes, though receptors are highly occupied by ineffective IGF binding proteins, highly expressed in OA • Involved in response against inflammatory signalling from IL-1β, NO • Regulates IL-1β signalling by up-regulation of the IL-1 decoy receptor IL-1R2 that binds IL-1β, inhibiting downstream signal transduction, and by inhibition of IL-1β-induced MMP-13 expression
➢ Fibroblast growth factors or FGFs (Mueller & Tuan, 2011)	Increased Dubious (FGF-2) / beneficial (FGF-18)	• Mostly studied are FGF-2 and FGF-18 • FGF-2 increased in OA, mainly acts through FGF receptor 1; FGF-18 is increased in OA, mainly acts through FGF receptor 3 • FGF-2 may have an anabolic role for cartilage repair, though mainly in terms of cell proliferation rather than appropriate ECM synthesis, with potential loss of chondrocytic phenotype (high expression of collagen I): repaired cartilage would resemble fibrous cartilage • FGF-2 counteracts anabolic growth factor such as IGF-1 and BMP-7 and may stimulate expression of MMPs • FGF-18 has clear anabolic effects, increasing cell proliferation as well as ECM synthesis whilst reducing cartilage degeneration

Enzymes		
➢ Metalloproteinases or MMPs (Mueller & Tuan, 2011)	Increased Detrimental	• Zinc-dependent endopeptidase enzymes involved in ECM degradation • Low expression may contribute to healthy ECM remodelling, while in OA there is overexpression • Proinflammatory cytokines trigger a pathway (NF-κB and AP-1) resulting in up-regulation of MMPs; expression is down-regulated by tissue inhibitors of metalloproteinases (TIMPs) • MMP-13 or collagenase-2 is up-regulated by many catabolic pathways in OA • MMP-13 is highly active in cleaving collagen II; its inhibition protects against cartilage erosion • MMP-13 also degrades aggrecan
➢ Aggrecanases (Mueller & Tuan, 2011)	Increased Detrimental	• A disintegrin and metalloproteinase with thrombospondin motifs ADAMTS-4 (or aggrecanase-1) and ADAMTS-5 (or aggrecanase-2) are principally responsible for aggrecan degeneration in degenerative cartilage diseases • ADAMT-4 and ADAMT-5 expression is stimulated by TNF-α and IL-1 • TIMP-3 is a potent inhibitor of ADAMT-4 and ADAMT-5 activity
➢ Heme oxigenase-1 or HO-1 (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010)	Decreased by inflammation Beneficial	• Induced as a protective response against oxidative stress in many cell types • Expression is down-regulated by proinflammatory cytokines such as IL-1β, IL-17 and TNF-α, but up-regulated by the anti-inflammatory cytokine IL-10 • Causes diminished proteoglycan release with increased synthesis and expression of aggrecan and type II collagen
Transcriptional regulation		
➢ *Sox9* (Mueller & Tuan, 2011)	Increased/ decreased Beneficial	• Anabolic growth factors such as IGF-1, TGF-β, and BMP-2, act at least in part by induction of Sox9, a transcription factor • Key regulator of mesenchymal chondrogenesis during embryonic development: it enhances the expression of cartilage-specific genes, such as collagen type II and aggrecan • Used to characterize chondrogenic and

		chondrocytic phenotype
➢ *NF-κB* and *AP-1* (Mueller & Tuan, 2011)	Increased Detrimental	• NF- κB has a central role in catabolic processes and its activation in an inflammatory environment negatively affects progenitor cells recruitment for cartilage repair • NF- κB mediates transcription of pro-inflammatory genes, such as IL-1β, TNF-α, iNOS, MMPs, and others • NF- κB expression is activated by IL-1β and TNF-α • NF- κB supresses Sox9 expression, thus down-regulating ECM proteins • NF- κB is involved in FGF-2-mediated anabolic processes • AP-1 is a transcription factor activated by growth factors and cytokines such as TGF-β, FGF-2, TNF-α • AP-1 regulates expression of most MMPs • Curcumin, a phytochemical, can inhibit IL-1-induced NF- κB activation in chondrocytes
Others		
➢ Small leucine-rich repeat proteoglycans or SLRPs (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010)	Increased Detrimental	• Family of proteins, components of the extracellular matrix that may provide a number of targets for OA treatment • SLRP asporin, a component of cartilage and bone, inhibits the anabolic effects of TGFβ1 and is involved in the pathogenesis of OA
➢ Syndecans (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010)	Unknown Beneficial	• Heparan sulfate proteoglycans, expressed on the surface of adherent cells that interact with growth factors, cytokines, proteinases, adhesion receptors and extracellular matrix components, through their heparan sulfate chains • Modulate homeostatic processes and tissue injury • Control aggrecanases expression
➢ Discoidin domain receptor 2 or DDR2 (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, &	Increased Detrimental	• Collagen receptor, whose expression is increased in articular chondrocytes of mice that develop OA • Overexpression of DDR2 is present in OA and causes collagen II-dependent induction of MMPs and proinflammatory cytokines

Guillén, 2010)		• Involved in the MAP kinases pathway
➢ Proteinase-activated receptor 2 or PAR-2 (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010)	Increased Detrimental	• Its activation participates in inflammatory reactions • Significantly up-regulated in OA chondrocytes by proinflammatory cytokines • Its activation induces MMP-1, MMP-13 and cyclo-oxygenase-2 as well as phosphorylation of ERK1/2 and p38 (MAP kinases pathway)
➢ Oxidative stress (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010) (Musumeci, Aiello, Szychlinska, di Rosa, Castrogiovanni, & Mobasheri, 2015)	Increased markers Detrimental	• NO and reactive oxygen species (ROS) are present in OA cartilage and play a role in chondrocyte insensitivity to anabolic actions of IGF-1 • Oxidative stress results in mitochondrial DNA damage, mitochondrial dysfunction, apoptosis, necrosis and senescence of chondrocytes • In OA cartilage important oxidative defense genes are expressed, including genes for superoxide dismutase (SOD) 2, SOD 3, and glutathion peroxidase 3 • Free radical scavengers have been suggested as potential therapeutic agents for the protection of articular cartilage against progression of OA • In human OA cartilage, IL-1β, TNF-α and oxidative stress induce the expression of hypoxia-inducible factor 1a (HIF-1a) in chondrocytes

Table 3: Main mediators involved in OA pathogenesis.

Mechanisms of cell death in OA

Numerous studies suggest that chondrocyte death is a key player in cartilage degeneration in OA (Hashimoto, Ochs, Komiya, & Lotz, 1998) (Blanco, Guitian, Vazquez-Martul, de Toro, & Galdo, 1998) (Sharif, Whitehouse, Sharman, Perry, & Adams). Chondrocyte death by apoptosis (Hashimoto, Ochs, Komiya, & Lotz, 1998) (Blanco, Guitian, Vazquez-Martul, de Toro, & Galdo, 1998), necrosis, chondroptosis (a variant of apoptosis), or combination of these processes has been implicated in the pathogenesis of OA (Zamli & Sharif, 2011).

Apoptosis plays an important role in normal physiological processes (e.g. endochondral ossification and cell turnover) as well as in pathology (e.g. autoimmunity and cancer). It is induced through two main, alternative pathways: death receptor-mediated (or extrinsic) and mitochondria-dependent (or intrinsic), both lead to the different cascades of executor

caspases (Musumeci, Loreto, Carnazza, & Martinez, 2011) (Huppertz , Frank, & Kaufmann, 1999) (Zamli & Sharif, 2011) (Musumeci, Loreto, Carnazza, Strehin , & Elisseeff, 2011) In the death receptor pathway, the death receptors such as tumor necrosis factor (TNF) or Fas receptors are activated by specific death ligands, TNF-α and Fas ligand respectively. On the other hand, the mitochondrial pathway it is initiated by stimuli that change mitochondrial membrane permeability toward pro-apoptotic proteins (Zamli & Sharif, 2011).

On the contrary, necrosis is a pathological form of cell death, non-programmed, caspase- and energy-independent (Zamli & Sharif, 2011). Cells die by necrosis when there is tissue damage as a result of exposure to highly toxic substances or extreme physiological conditions; unlike apoptosis, necrosis is accompanied by inflammatory reaction. In addition, necrotic cells also show other morphological changes, such as formation of cytoplasmic vacuoles, swelling of mitochondria and other organelles, which eventually lead to total cell lysis (Zamli & Sharif, 2011).

Both apoptotic and non-apoptotic forms of cell death have been reported in OA cartilage (Zamli & Sharif, 2011). Chondrocyte death in osteoarthritic cartilage is supported by the presence of large numbers of empty lacunae, hypocellularity and is correlated with mechanical injury, increased production of reactive oxygen species (ROS), disruption of ECM integrity and loss of production of growth factor by the cells (Zamli & Sharif, 2011). Thus, although the concept of increased cell death in OA is generally accepted, the precise mechanism of cell death is yet to be established (Zamli & Sharif, 2011).

Apoptosis has been positively correlated with the severity of cartilage destruction and matrix depletion in human osteoarthritic tissue specimens (Musumeci, Aiello, Szychlinska, di Rosa, Castrogiovanni, & Mobasheri, 2015). Freshly isolated chondrocytes from human OA cartilage exhibited morphological evidence of apoptosis, clear cytoplasmic, cell-surface blebs, altered nuclear shape, apoptotic bodies and a parallel loss of nuclear volume. Chondrocytes from normal donors did not show any cytoplasmic features of apoptotic cell death. These findings suggest that the OA chondrocytes demonstrate differences in predisposition towards apoptosis (Musumeci, Loreto, Carnazza, & Martinez, 2011) (Musumeci, Loreto, Carnazza, Strehin , & Elisseeff, 2011).

Apoptosis as a cause of OA

Strength of correlation between chondrocyte apoptosis and cartilage degeneration in human OA is suggested by the finding that, in macroscopically normal cartilage of elder donors, unusually high numbers of apoptotic chondrocytes can be seen (Hashimoto, Ochs, Komiya, & Lotz, 1998).

There may be a number of possible mechanisms involved in chondrocyte apoptosis-mediated cartilage damage and development of OA. First, with age chondrocytes may undergo phenotypic changes, making them more vulnerable to pro-apoptotic and other catabolic stimuli and also less responsive to anti-apoptotic and anabolic factors (Zamli & Sharif, 2011). As a result, small but increased numbers of chondrocytes die by apoptosis, leading to hypocellular cartilage. Hypocellularity causes that chondrocytes are no longer able to maintain the vast ECM and therefore there is a net degradation and loss of cartilage in these joints, resulting in OA (Zamli & Sharif, 2011). More direct damage to cartilage may be caused by apoptotic bodies, the end product of apoptosis. Cartilage is avascular, there are no phagocytic cells in cartilage, therefore apoptotic bodies in cartilage are not cleared quickly and accumulation of these bodies in pericellular or interterritorial matrices, especially in advanced OA, lead to further cartilage matrix damage (Hashimoto, et al., 1998). Apoptotic bodies may also produce alkaline phosphatase and induce precipitation of calcium, which results in abnormal calcification in the subchondral bone, and subsequent cartilage degradation (Hashimoto, Ochs, Komiya, & Lotz, 1998).

Apoptosis as a consequence of OA

The concept that chondrocyte apoptosis could be secondary to cartilage degradation is supported by the fact that cell-matrix interaction is vital for chondrocyte survivability. The phenomenon of "anchorage dependence" states that cells need to attach to ECM or to each other for survival, and therefore when the ECM is damaged by either mechanical load or inadequate synthesis and/or expression of extracellular matrix molecules, chondrocytes may undergo apoptosis and exacerbate existing cartilage matrix breakdown (Zamli & Sharif, 2011). In addition, it appears that alterations in pericellular matrix properties and cell-matrix interactions, secondary to genetic, epigenetic, metabolic, or biomechanical stimuli, could in fact serve as initiating or progressive factors for OA, suggesting that many of the characteristics and influences of OA are present and possibly initiated in the pericellular matrix (Guilak, Nims, Dicks, Wu, & Meulenbelt, 2018).

Chondrocyte survivability can be mediated by integrins, a/b-heterodimeric receptors that connect ECM components such as collagen, laminin and fibronectin to various intracellular cytoskeletal proteins (Zamli & Sharif, 2011); loss of this adhesion may trigger chondrocytes to endure apoptosis.

Extent of chondrocyte apoptosis is positively correlated with expression of fibronectin, key ECM molecule involved in communication between chondrocytes and surrounding matrix, and whose up-regulation is associated with the severity of articular cartilage damage (Zamli & Sharif, 2011). Both expression of fibronectin and chondrocyte apoptosis are early events and could be involved in initiation of cartilage degradation in OA (Zamli & Sharif, 2011)

Abnormal mechanical loading is a major risk factor in OA development and vigorous cyclic loading of normal cartilage can cause collagen denaturation (Clements, Hollander, Sharif, & Adams, 2004), expel GAGs from articular cartilage (Summers, Merrill, Sharif, & Adams, 2008), and induce cell death (Clements, Bee, Crossingham, Adams, & Sharif, 2001), possibly by apoptosis (Hashimoto, et al., 2009).

Significant pathways involved into changing hypertrophic chondrocyte phenotype

A prominent feature of OA is cartilage degradation, which is attributed to an elevated production of proteolytic enzymes, such as matrix metalloprotease 13 (MMP13) and aggrecanases. These degrade important cartilage matrix components, such as type II collagen (COL2A1) and aggrecan (Ripmeester, Timur, Caron, & Welting, 2018).

In addition to this, other features in OA cartilage include expression of chondrocyte hypertrophic markers (such as type 10 collagen or COL10A1), neovascularization, and focal calcification: these features together resemble the endochondral ossification process, which occurs in the hypertrophic zone of the growth plate. Therefore it has been hypothesized that OA is a disease characterized by ectopic recapitulation of the endochondral ossification process. Post-developmental healthy articular cartilage homeostasis is thought to be “protected” against hypertrophic or catabolic changes by several pathways employing soluble mediators, including BMPs, TGF-β, and hedgehog signalling (Dreier, 2010) (Pitsillides & Beier, 2011). These pathways transcriptionally control the chondrocyte phenotype by tuning the activity and levels of major chondrocyte phenotype-determining downstream transcription factors, such as SOX9, RUNX2, and SMADs (van der Kraan & van der Berg, 2012).

Wnt signalling

The Wnt/β-catenin pathway involves the interactions of Wnt ligands with frizzled receptors and LRP-5 or -6 co-receptors (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010). Wnt signalling is involved in embryonic development of cartilage and bone and is considered a key regulator of joint remodelling. Nevertheless, the response of chondrocytes to a canonical Wnt stimulus is affected by alterations in extracellular matrix components, as in OA cartilage (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010).

The canonical Wnt/β-catenin pathway is clearly associated with chondrocyte hypertrophy; in addition, expression levels of Wnt signalling- and β-catenin-inducing factors, as well as downstream Wnt effectors, such as LEF1 and AXIN2, are directly or indirectly associated with both initiation and progression of OA (Ripmeester, Timur, Caron, & Welting, 2018).

Cartilage-specific SMURF2-mediated ubiquitination and proteasomal degradation of GSK-3β results in increased β-catenin signalling (Wu, et al., 2009); in this context, early hypertrophic changes in the articular cartilage of mice can be noticed and are subsequently followed by cartilage degeneration and osteophyte formation with aging, suggesting a direct relationship between early hyper-trophic changes followed by OA development (Wu, et al., 2009).

EZH2 is the catalytic unit of the polycomb repressive complex 2 (PRC2), responsible for transcriptional silencing of a multitude of genes involved in differentiation (Ripmeester, Timur, Caron, & Welting, 2018). EZH2 expression is higher in OA chondrocytes compared to healthy chondrocytes and overexpression of EZH2 in normal chondrocytes result in activation of β-catenin signaling, including higher mRNA expression of its downstream effectors, *AXIN2* and *LEF1*. In fact intra-articular injection with a pharmacological EZH2 inhibitor in a surgically induced mouse OA model results in reduced cartilage degradation (Ripmeester, Timur, Caron, & Welting, 2018).

The expression of Wnt and BMP antagonists: dickkopf 1 homolog (*DKK1*), frizzled-related protein (*FRZB*), and Gremlin 1 (*GREM1*) is reduced in OA cartilage compared to post-mortem healthy controls (Leijten, et al., 2013). These Wnt and BMP antagonists are able to inhibit hypertrophic chondrocyte differentiation even when added to chondrogenically differentiated mesenchymal stem cells (MSCs) (Ripmeester, Timur, Caron, & Welting, 2018).

Ihh/PTHrP signalling

Parathyroid hormone-related protein (PTHrP) is a protein member of the parathyroid hormone family; it acts as an endocrine, autocrine, paracrine and intracrine hormone. It regulates endochondral bone development by maintaining the endochondral growth plate at a constant width. It also has role in epithelial-mesenchymal interactions during the formation of the mammary glands and it is sometimes overexpressed in malignant paraneoplastic hypercalcemia.

Indian hedgehog (Ihh) is a protein involved in chondrocyte differentiation, proliferation and maturation especially during endochondral ossification; it regulates its effects by feedback control of PTHrP (Vortkamp, Lee, Lanske, Segre, Kronenberg, & Tabin, 1996).

PTHrP and Indian hedgehog signalling pathways generate a feedback loop which is involved in controlling the chondrocyte phenotype in the growth plate in skeletal development, as well as in determining the homeostasis that keeps articular cartilage healthy (Kronenberg, 2003). Given that PTHrP maintains the function of proliferating chondrocytes in the growth plate and inhibits chondrocyte differentiation towards hypertrophy, it seems that PTHrP may be protective against OA (Kronenberg, 2003). Several studies demonstrate direct evidence that inhibiting hypertrophic processes results in protection against OA (Ripmeester, Timur, Caron, & Welting, 2018).

PTHrP experimental treatment reduces GAG loss, increases expression of *Col2a1* (gene codifying for collagen type II alpha chain (orpha.net, 2019) levels and reduces *Col10a1* (gene codifying for collagen type I alpha chain (GeneCards, 2019) levels in the cartilage, which together reduces OA severity in a chemically induced mouse OA model (Ripmeester, Timur, Caron, & Welting, 2018).

Ihh expression is considered as a marker gene for hypertrophic chondrocytes in growth plates (Weisser, et al., 2002). Inhibition of the Ihh pathway indeed protects against OA development (Zhou, et al., 2014).

TGF-β Superfamily signalling

The transforming growth factor β (TGF-β) superfamily is a group of multifunctional cytokines that play critical roles in cartilage homeostasis and have well-established roles in endochondral ossification (Dangelo, Sarment, Billings, & Pacifici, 2001). In the classical TGF-β superfamily signalling pathway, superfamily ligands, such as TGF-β and BMP isoforms, bind to their respective cell surface receptors and, upon type I and -II receptor dimerization, activate a signalling cascade which includes the recruitment,

phosphorylation, and interaction of different SMAD proteins (Ripmeester, Timur, Caron, & Welting, 2018). Upon activation, SMAD complexes translocate to the nucleus to drive transcription of genes regulating a variety of biological responses (van der Kraan, Blaney Davidson, Blom, & van der Berg, 2009). TGF-β superfamily ligands can signal in chondrocytes *via* SMAD2/3 and also *via* SMAD1/5/8 (van der Kraan, 2017).

TGF-β is implicated in OA pathogenesis, since deregulation of TGF-β signalling is clearly associated with OA (van der Kraan, Blaney Davidson, Blom, & van der Berg, 2009) (van der Kraan, 2017). Moreover an increased expression of TGF-β superfamily members or their receptors and down-stream signalling molecules can be noticed in human OA chondrocytes, compared to non-OA chondrocytes (Ripmeester, Timur, Caron, & Welting, 2018).

MAP Kinases

The mitogen-activated protein kinases (MAP kinases or MAPKs) pathway consists of a sequence of intracellular signalling proteins, which transduce a signal from various cell receptors to the nucleus (Schaeffer & Weber, 1999). Activity of MAPKs is regulated in response to environmental stress and to cytokines and growth factors, such as members of the Wnt family (Bikkavilli & Malbon, 2009) or the TGF-β superfamily (Derynck & Zhang, 2003).

The three major MAPK pathways include p38, c-Jun N-terminal (JNK) kinase, and extra-cellular-regulated kinases (ERK) (Johnson & Lapadat, 2002). MAPKs are currently considered as signalling mediators involved in the endochondral ossification process, but they are also able to regulate the activity of multiple mediators of cartilage destruction (Ripmeester, Timur, Caron, & Welting, 2018) (Loeser, Erickson, & Long, 2008).

ERK pathway seems involved in destructive OA responses, while the p38 pathway is OA protective (Ripmeester, Timur, Caron, & Welting, 2018). Pharmacological ERK inhibition, together with hyaluronic acid treatment, results indeed in a synergistic chondro-protective effect, compared to hyaluronic acid treatment only (Ripmeester, Timur, Caron, & Welting, 2018). In contrast to pERK, p38 appears negatively associated with OA: p38 expression levels are higher in wild-type murine articular cartilage, compared to *Smad3* knockout mice displaying cartilage damage (Li, et al., 2010), and lower in human OA articular chondrocytes, compared to healthy human articular chondrocytes (Prasadam, van Gennip, Friis, Shi, Crawford, & Xiao, 2010). On the contrary, under certain conditions even p38

activation may result in hypertrophic differentiation of cultured chondrocytes (Ripmeester, Timur, Caron, & Welting, 2018).

To summarize, it can be said that pERK has pro-hypertrophic effects while p38 mostly has contra-hypertrophic effects (Ripmeester, Timur, Caron, & Welting, 2018) (Prasadam, van Gennip, Friis, Shi, Crawford, & Xiao, 2010). Taken together, specific MAP kinases have been demonstrated to regulate both hypertrophic and chondrogenic responses in the chondrocyte; inhibition of specific MAP kinases could potentially be a strategy to block OA progression *via* modulating the hypertrophic chondrocyte phenotype (Ripmeester, Timur, Caron, & Welting, 2018).

Inflammatory signalling

NF-kB activation determines the expression of a wide range of inflammatory and catabolic mediators in joint tissues (Alcaraz, Megìas, Garcìa-Arnandis, Clérigues, & Guillén, 2010). Not surprisingly, regarding the inflammatory nature of OA, the involvement of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway has been described as involved in the regulation of hypertrophic differentiation in OA (Marcu, Otero, Olivotto, Borzi, & Goldring, 2010). Many stimuli (such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens) activate NF-κB, mostly through IκB kinase-dependent (IKK-dependent) phosphorylation and subsequent degradation of NF-κB inhibitory IκB proteins. The liberated NF-κB dimer enters the nucleus, where it regulates transcription of diverse target genes (Marcu, Otero, Olivotto, Borzi, & Goldring, 2010).

IKKα and IKKβ knockdown in OA chondrocyte micromass cultures result in increased GAG content, COL2A1 expression, and reduced calcium deposits. IKKα and IKKβ also have differential activities, since IKKα knockdown, but not IKKβ knockdown, results in smaller OA hypertrophic chondrocytes (Olivotto, et al., 2008) (Olivotto, et al., 2013).

Besides NF-κB, ELR+ CXC chemokines (named this way after their structure, where the two N-terminal cysteines are separated by one amino acid, indicated with an “X”), characterized by their glutamic acid-leucine-arginine (or ELR+) motif, provide new links connecting OA to hypertrophic changes (Wenke, Niebler, Grassel, & Bosserhoff, 2011). AP-2ε is a transcription factor, which acts in hypertrophic cartilage differentiation; its expression is increased in OA chondrocytes (Wenke, Grassel, Moser, & Bosserhoff, 2009) and is associated, together with CXCL1 expression (also triggered by AP-2ε), to hypertrophic differentiation of MSCs (Wenke, Niebler, Grassel, & Bosserhoff, 2011).

Another chemokine involved in OA pathogenesis is CXCL6, whose expression is reduced in human and animal model OA cartilage (Sherwood, et al., 2015).

In addition to chemokines, different inflammatory cytokines have been implicated in OA pathophysiology, one of them being TNFα (Lai, et al., 2014). There exists a positive feedback loop between TNFα and ADAMTS7, since they stimulate expression of each other. Interestingly, mice overexpressing ADAMTS7 display OA-like phenotypes, characterized by reduced cartilage GAG content, osteophyte formation, thinner cartilage, upregulation of hypertrophic markers expression, such as Col10a1 and MMP13. Furthermore, in mice ADAMTS7 overexpression seems to accelerate OA development and is associated with several skeletal developmental abnormalities, including a reduced hypertrophic zone and reduced COL10A1 levels in the growth plate and lower bone mineral density. (Ripmeester, Timur, Caron, & Welting, 2018) (Lai, et al., 2014)

The inflammatory S100A11, a ligand for the receptor for advanced glycation end products (RAGE), is associated with chondrocyte hypertrophy, even though deletion of RAGE is not chondroprotective in an instability induced knee OA mouse model (Cecil, et al., 2009).

The alternative patterning receptor CD36, marker for growth plate chondrocyte hypertrophy, promotes OA *via* mediation of inflammatory and differentiation responses but induces as well cartilage repair when exposed to inflammatory stimuli, displaying both pro-hypertrophic and chondroprotective effects (Cecil, et al., 2009).

The semicarbazide-sensitive amine oxidase (SSAO) found in the hypertrophic chondrocytes of the growth plate is known to be involved in leukocyte extravasation from the blood to the inflammation site. Additionally, it may be associated with the differentiation of chondrocytes towards a hypertrophic phenotype (Ripmeester, Timur, Caron, & Welting, 2018).

OA is a disease associated with aging, which could be a result of tissue accumulation of $p16^{INK4a}$ positive cells. $p16^{INK4a}$ is a tumor suppressor protein, important in cell cycle regulation due to causing deceleration of cell's progression from G1 to S phase, also increasingly expressed under influence of reactive oxygen species, DNA damage, senescence. $p16^{INK4a}$ is suggested to support chronic inflammation, as $p16^{INK4a}$ positive cells exhibit a specific secretome called SASP (senescence-associated secretory phenotype) including pro-inflammatory cytokines. OA chondrocytes are characterized by an accumulation of $p16^{INK4a}$ (Ripmeester, Timur, Caron, & Welting, 2018).

Since inflammation and angiogenesis are closely correlated in the pathogenesis of OA, it can be hypothesized that there are common regulators controlling both processes simultaneously: for example, overexpression of the inflammation responsive transcription factor SAF-1 leads to severe cartilage degradation and OA and causes neo-vascularization in the perichondrium and synovium, suggesting a link between angiogenesis and inflammation; SAF-1 expression is related to other molecules in a complex OA-inducing network (Ripmeester, Timur, Caron, & Welting, 2018).

Another factor involved in inflammatory signalling and OA disease progression is type 1 sirtuin (SIRT-1). SIRT-1 indirectly inhibits NF-κB, thus modulating the inflammatory signalling pathway (Liu-Bryan, 2015). Furthermore, inhibition of SIRT-1 induces *COL10A1* expression. Since OA chondrocytes display decreased SIRT-1 expression compared to healthy controls, this may provide a route for chondrocytes to acquire an endochondral cellular phenotype (Fujita, et al., 2011). Besides SIRT-1, type 6 sirtuin (SIRT-6) also modulates inflammation: *SIRT-6* haplo-insufficiency in mice can enhance OA progression by stimulating the inflammatory response, as shown by increased expression of *Tnf*α and *IL-6* (Ailixiding, et al., 2015), and in addition might be correlated with an increase in osteophytes and synovial tissue, with increased infiltration of inflammatory cells, as well as increased MMP13 expression in the cartilage.

To conclude, cytokines and chemokines and their downstream intracellular pathways have been revealed to lead to hypertrophic as well as anti-hypertrophic changes in the chondrocyte. While inflammation-induced hypertrophic changes have been established as associated with processes involved in cartilage matrix degradation, inflammation-induced anti-hypertrophic changes have been described to lead to chondroprotective responses (Ripmeester, Timur, Caron, & Welting, 2018).

Hypoxic and angiogenic factors

The proliferative zone of the growth plate is an avascular hypoxic mesenchymal tissue (Maes, Carmeliet, & Schipani, 2012). Intriguingly, chondrocytes are competent at surviving and differentiating in this challenging environment; this survival and capability of differentiation could happen, at least in part, by virtue of the actions of hypoxia-inducible factors (HIFs), such as HIF-1α.

Moreover, the process of endochondral ossification in the growth plate is driven by vascularization and hypertrophic chondrocytes in the growth plate that secrete angiogenic stimuli, which actively support vascularization (Maes, Carmeliet, & Schipani, 2012). This

hypertrophy-associated angiogenic switch is a major driver of active growth plate cartilage remodeling toward bony tissue (Maes, Carmeliet, & Schipani, 2012). Therefore OA pathophysiology may involve hypoxic/angiogenic mediators, such as HIFs and VEGF. For instance, expression of HIF-2α, localized predominantly in the hypertrophic zone of mouse growth plates, seems to cause reduced proliferative and hypertrophic zone lengths, together with impaired bone length, indicating impaired endochondral ossification during limb development; as well as that, its expression is increased in surgically induced OA mouse models, while in the same model gene knockout produces significant resistance to cartilage degradation, osteophyte formation, and subchondral bone sclerosis (Saito, et al., 2010).

Dio2 is upregulated in OA cartilage (Nagase, et al., 2013) and is responsible for active thyroid hormone (T3) production. T3 in turn induces terminal chondrocyte differentiation with increased HIF-2α expression, as well as *Col10a1, ALPL, osteocalcin, RUNX2, MMP13,* and *ADAMTS5* expression (Nagase, et al., 2013) (Ripmeester, Timur, Caron, & Welting, 2018). The upregulation of *HIF-2*α expression after T3 treatment suggests a link between DIO2 levels and OA development *via* HIF-2α signalling and activating mutations in the *DIO2* allele result in a predisposition for OA development in human patients (Ripmeester, Timur, Caron, & Welting, 2018).

In addition to HIF-2α, VEGF is involved in OA development and inhibition of its expression results in less cartilage degradation in OA-induced mice, also accompanied by reduction in *Col10a1* levels (Zhang, Crawford, & Xiao, 2016a).

Another protein essential in vascularization during endochondral ossification is chondromodulin. Proliferative chondrocytes in the growth plate are resistant to vascular invasion because of the presence of angiogenic inhibitors, such as chondromodulin (ChM-1) (Ripmeester, Timur, Caron, & Welting, 2018). Chondromodulin overexpression protects against OA development in surgically induced rat OA models and against TNFα-induced chondrocyte hypertrophy (Zhang, Crawford, & Xiao, 2016a) (Zhang, Prasadam, Fang, Crawford, & Xiao, 2016b).

Vascularization is essential for the endochondral ossification process and can be associated with OA. Indeed, OA cartilage is invaded by blood vessels into the non-calcified articular cartilage, likely due to an increased production of pro-angiogenic factors (Ripmeester, Timur, Caron, & Welting, 2018). This could be attributed to the increase in subchondral bone porosity, which may result in disruption of the osteochondral junction, with subsequent invasion of blood vessels and further structural damage, leading to progression of OA (Ripmeester, Timur, Caron, & Welting, 2018).

In conclusion, these results reveal the importance of angiogenesis in enabling OA disease progression. The differential activation of hypoxic and angiogenic pathways observed in OA appear to be key factors in OA development.

FGF signalling

Fibroblast growth factors (FGFs) comprise a group of morphogens involved in wound healing, angiogenesis, in processes such as proliferation and differentiation of different cell types (Turner & Grose, 2010), and in endochondral ossification, since FGF23 and FGF receptor 1 are produced by growth plate hypertrophic chondrocytes (Raimann, Ertl, Helmreich, Sagmeister, Egerbacher, & Haeusler, 2013). Provided that OA chondrocytes also produce FGF family members, studies have investigated the involvement of FGF signalling in the chondrocyte phenotypic alterations observed in OA cartilage (Ripmeester, Timur, Caron, & Welting, 2018). Expression of FGFR1, FGF23, and its co-receptor KLOTHO is higher in OA chondrocytes compared to non-OA chondrocytes; exogenous addition of FGF23 to human OA primary chondrocytes results in hypertrophic changes, as evidenced by *Col10a1* and *VEGFA* induction *via* FGFR1 (Bianchi, et al., 2016). On the other hand, FGFR3 seems to be crucial for cartilage homeostasis and inhibition of a hypertrophic phenotype, since FGFR3 knockout mice display OA-like defects with an increased expression of hypertrophic markers Col10a1 and MMP13 (Zhou, et al., 2016).
Thus FGF signalling pathway is active in OA and also provided new insights in the contribution of this signalling pathway to OA disease initiation or progression.

Notch signalling

The notch-signalling pathway consists of five identified ligands (Jagged 1, Jagged 2, Dll1, Dll3, and Dll4) that can interact with four receptors (Notch 1–4). Notch signalling is involved in endochondral ossification and, therefore, studies have investigated its involvement in OA development as well (Ripmeester, Timur, Caron, & Welting, 2018).
Many studies provide evidence that the notch-signalling pathway is positively associated with hypertrophic changes in chondrocytes and its inhibition may be used as a therapeutic tool to block OA disease progression, reducing hypertrophic changes in the chondrocyte (Ripmeester, Timur, Caron, & Welting, 2018).

Mineralization

Mineralization is also an important consequence of chondrocyte hypertrophy in OA (Ripmeester, Timur, Caron, & Welting, 2018). The mineralization process is analogous to

the last phase of endochondral ossification as it is observed in the growth plate (Kronenberg, 2003). Here, hypertrophic chondrocytes secrete matrix vesicles containing high concentrations of phosphatases. Polarized budding and pinching-off from the plasma membranes form vesicles, containing the first mineral crystals, made by phosphatases hydrolysing inorganic pyrophosphate (PPi) to create inorganic phosphate (Pi). Pi ions in turn crystallize with calcium, resulting in crystals then released through the vesicles membranes. When these pre-formed hydroxyapatite crystals come in contact with the extracellular fluid containing Ca^{2+} and $(PO_4)^{3-}$ ions a process of continuous crystal formation takes place in the matrix (Anderson, 2003). This mineralized matrix is then vascularized, enabling the infiltration of osteoblasts and osteoclasts.

Several mediators can influence cartilage mineralization: AKT1, RAC1 stimulate respectively mineralization and chondrocyte hypertrophy, while MEPE and Gla-rich protein (GRP) act as matrix mineralization inhibitors, protective factors against OA and GRP particularly exerts anti-inflammatory effects in chondrocytes and synoviocytes (Ripmeester, Timur, Caron, & Welting, 2018).

Articular cartilage of many OA patients hosts calcium-containing crystals, which are present in the superficial and deep layers of the cartilage, as well as in synovial fluid and the meniscus (Nguyen, et al., 2013) (Ripmeester, Timur, Caron, & Welting, 2018). Treatment of murine chondrocytes with BCP results in an increase of IL-6 secretion, which in turn induces the expression of pro-mineralizing genes that control extracellular Pi and PPi levels, critical determinants of mineralization. Expression of these regulatory proteins is further increased in chondrocytes with calcium-containing crystals, forming a positive feedback loop (Ripmeester, Timur, Caron, & Welting, 2018) (Nguyen, et al., 2013).

Pharmacologic therapy of equine OA

With regard to pharmacologic therapy, all pharmaceutical compounds used to treat OA have been classified in two main categories from human medicine: disease modifying osteo-arthritic drug (DMOAD) and symptom-modifying osteo-arthritic drug (SMOAD).

NSAIDs

NSAIDs may be classified as SMOADs. They inhibit the expression of enzyme known as cyclooxygenase (COX), which is responsible for arachidonic acid oxidation into prostaglandin (PGs). COX may be divided into COX-1, constitutive, and COX-2, inducible in response to inflammation. Inflammation control is fundamental to help with pain

reduction and slowdown of OA progression, therefore ideal NSAIDs should be able to selectively inhibit COX-2. On the other hand, it seems NSAIDs are generally detrimental to proteoglycan synthesis (Goodrich & Nixon, 2006).

NSAIDs are relatively safe. Though relatively uncommon if NSAIDs are administered at a recommended dose, NSAIDs toxicity in equines may present as (Stewart) (Goodrich & Nixon, 2006):

- Gastric and sometimes oral ulcer, due to inhibition of the protective secretion of PGE2
- Nephrotoxity, due to momentary renal hypotension, with renal crest or papillary necrosis
- Colic, especially in right dorsal colon, and/or diarrhoea

Most used NSAIDs are phenylbutazone (PBZ), flunixin, ketoprofen, naproxen, carprofen. NSAIDs can be administered orally, IM (unless flunixin and carprofen that might cause myonecrosis) or IV one or more times per day. They produce a clinical amelioration with reduction in lameness (*via* analgesia), joint temperature, synovial fluid volume and synovial PGE2. Some NSAIDs, such as flunixin, ketoprofen, carprofen, are capable of accumulating at the inflammatory foci providing a long lasting effect compared to a short systemic half-life.

Intra-articular steroids

Glucocorticoids (GCs), classifiable as SMOADs, bind to cell receptors (GCRs), which go to the cell nucleus and modulate expression of target genes. GCs contrast inflammation by upregulating a protein that inhibits NF-κB, protein responsible for inflammation enhancement (Boumpas & Wilder, 2001). GCs also contrast PGs synthesis inhibiting phospholipase A_2 (PLA_2) via the steroid-inducible group of proteins called lipocortin (Di Rosa, 1985), preventing membrane phospholipids from mobilizing arachidonic acid (Goodrich & Nixon, 2006).

In spite of the apparent reduction of outward inflammation signs, high doses of GCs have several detrimental effects on articular cartilage: decrease in GAGs and their synthesis, decrease in proteoglycan synthesis, chondrocyte necrosis and hypocellularity. Cartilage degeneration may present together with local bone necrosis. Lower doses might on the contrary support GAGs and DNA synthesis. What is more, intra-articular administration is a risk factor for joint infection (Goodrich & Nixon, 2006).

Methylprednisolone acetate (MPA), betamethasone and triamcinolone acetonide are commonly used GCs. For instance, MPA minimizes transcription of harmful molecules, such as IL-1β, MMP13 (collagenase 3) and others that directly cause matrix degeneration, but might cause chondrocyte necrosis, inhibition of proteoglycan core protein and procollagen synthesis. Lower doses treatments are being studied in attempt to control inflammation while preserving normal joint environment (Goodrich & Nixon, 2006).

Hyaluronic acid

Hyaluronic acid (HA) is synthesized by synoviocytes and chondrocytes. In the synovial fluid, HA provides viscoelasticity to the joint fluid, lubrication to intra-articular soft tissues and modulates chemotactic cell response within synovial membrane. Exogeneously administered HA might restore synovial fluid properties and inhibit leukocytes chemotaxis; ideal molecular weight to achieve effectiveness in treatment is controversial (Goodrich & Nixon, 2006).

HA can be administered intra-articularly or IV. Its effects are mainly anti-inflammatory, with no better or worse modification on GAG content, synthetic rate, or morphologic scoring in articular cartilage; because of that, it should be considered a SMOAD.

Polysulphated glycosaminoglycans (PSGAGs)

Polysulphated glycosaminoglycans (PSGAGs) exhibit chondroprotecive properties in cartilage, being thus classified as DMOADs: they can alter OA progression sustaining chondrocyte metabolic activity and inhibiting the detrimental effects of cytokines and prostaglandins. PSGAGs deposit in the ECM where they inhibit degradative enzymes, such as lysosomal elastase, catepsin, lysosomal hyrolases, serine proteinases, neutral metalloproteinases, plasminogen activators, inducible nitric oxide. Moreover they inhibit chemotaxis and sustain biosynthesis of HA, GAG and collagen (Goodrich & Nixon, 2006). PSGAGs are GAGs, primarily chondroitin sulphate, of animal origin. They may be administered intra-articularly, with the main adverse effect of severe joint infection.

Oral glucosamine/chondroitin sulphate

These oral compounds, classified as DMOADs or biological response modifiers, are used as supplements for OA. Glucosamine (GS) and chondroitin sulphate (CS) are fundamental "building blocks" for ECM molecules (Goodrich & Nixon, 2006). Together their chondroprotective effect is synergistic, since glycosaminoglycan production is stimulated

by glucosamine and matrix degradation is inhibited by chondroitin sulphate (Das & Hammad, 2000) (Orth, Peters, & Hawkins, 2002).

In spite of this, oral absorption and bioavailability is doubted especially with chondroitin sulphate, a large molecule.

Cosequin (oral GS/CS), the most commonly used compound in human and dog clinical trials, has been given to horses with OA obtaining some improvements in lameness scores (Hanson, 1996).

OA and regenerative medicine

Regenerative medicine is the branch of medicine that develops methods to regrow, repair or replace damaged or diseased cells, organs or tissues. It includes the generation and use of therapeutic stem cells, tissue engineering and the production of artificial organs (nature.com, 2019).

In equine practice, regenerative medicine involves use of different techniques (exemplified in figure 10):

- Mesenchymal stem cells, with or without scaffolds;
- Gene therapy;
- Compounds that enhance the endogenous healing responses, named Biofactors, such as PRP, HA, autologous conditioned serum (interleukin receptor antagonist protein) (Abu-Seida, 2015);
- Tissue engineering approaches: autologous chondrocyte implantation, scaffold therapies, with or without cells (Koch, et al., 2009) (Luyten & Vanlauwe, 2012).

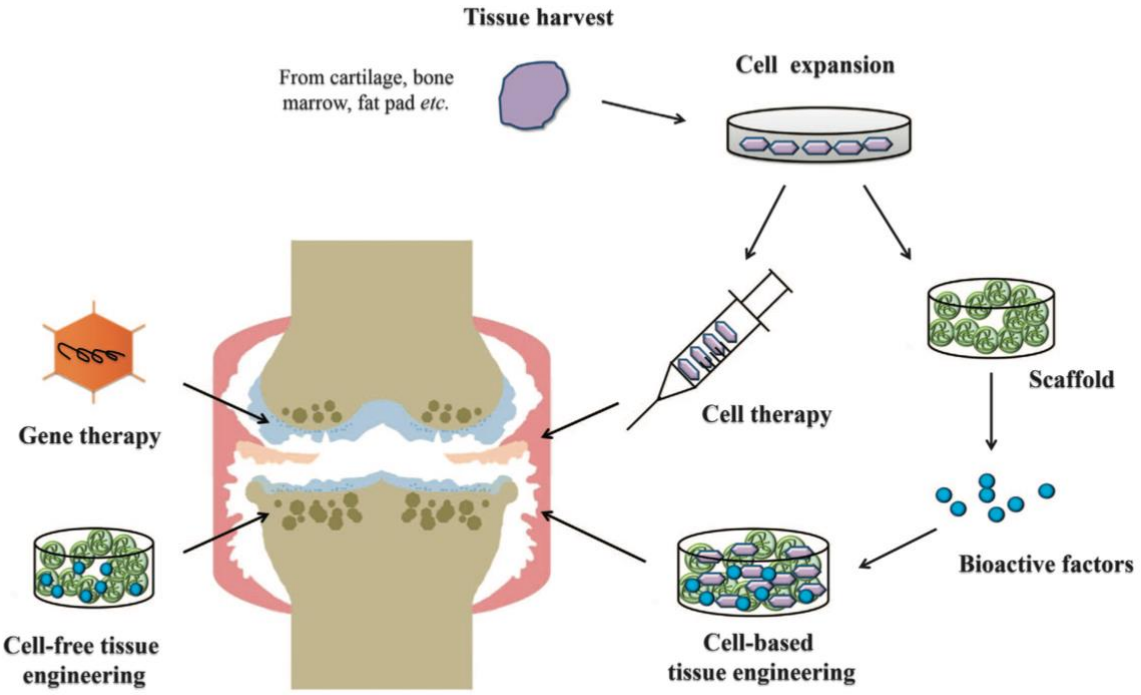


Figure 10: Current regenerative therapies for OA treatment. Joint represented is a human knee. From Zhang, Ouyang, Dass, & Xu, 2016.

These different techniques aim at either enhance intrinsic repair mechanisms, or introduce cells into the lesion, or resurface the articular cartilage through tissue engineering (Koch, et al., 2009) (Luyten & Vanlauwe, 2012).

Mesenchymal stem cells

Introduction

Cells used in AC tissue engineering are either derived from cartilage (which provides chondrocytes) or through chondrogenic differentiation of MSCs obtained from different origins and then directed towards selected phenotype with growth factors (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

With regard to tissue engineering, chondrocytes and MSCs can be harvested, cultured, differentiated (if needed), expanded in vitro and seeded in scaffolds (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

Committed MSCs and/or differentiated chondrocytes are desired to produce a typical hyaline cartilage ECM, characterized by collagen II.

There some obstacles for an efficient utilisation of these cells are:

- Loss of phenotype and/or altered differentiation status, often when a two-dimensional culture is used during cell expansion (Giannoni & Cancedda, 2006); e.g. production of collagen I instead of collagen II

- Aging of organism impacts negatively chondrocyte biosynthetic and mitotic activities, response to growth factors, aggrecans dimension and uniformity (Adkisson, Gillis, Davis, Maloney, & Hruska, 2001).

Promising stem cells for use in equine practice have been classified as multipotent mesenchymal stromal cells (MSC) (Dominici, et al., 2006) (Horwitz, et al., 2005).
MSCs can derive from different tissues, express specific surface markers and have tri-lineage differentiation potential towards osteoblasts, adipocytes, and chondroblasts (Koch, et al., 2009). The term mesenchymal stem cells should be reserved for cells that have shown *in vivo* long-term survival, with self-renewal capacity and tissue repopulation with multi-lineage differentiation (Horwitz, et al., 2005).
Mesenchymal stem cells are clonogenic in nature, meaning that multiple discrete cell populations are isolated from a single bone marrow sample (Koch, et al., 2009). Each of these cell populations has a common ancestral cell, the putative stem cell, and the different cell populations may vary with regard to reparative potential and lifespan (Kassem, et al., 2004).
The use of undifferentiated cells depends on the local tissue environment, the so-called "niche", an important condition to commit the stem cells into the desired phenotype and function; this though may be compromised, especially in diseased or injured tissues (Koch, et al., 2009).
A problem is that cell-based therapies rely on an intact stromal connective tissue in diseased or injured tissue; in fact, cell-based therapies may fail in case of widespread fibrosis and scar tissue, because of the lack of blood supply and/or of a microenvironment and biological signals suitable to provide the "niche" for attracting and supporting cell differentiation, proliferation, and function (Koch, et al., 2009).
Usually, injection-based stem cell therapy is attractive because of its minimal invasiveness, the relative ease of the procedure, the ability of incorporated scaffolds to conform to normal anatomic form, and the reduced cost, morbidity, and decreased recovery time when compared with that of transplantation by open surgeries (Elisseeff, 2004).
In cartilage tissue engineering and repairing models, the compressive load exerted on the injected cells and scaffolds has proven to be a major challenge (Elisseeff, 2004) (Caplan & Dennis, 2006).

Current stem cell therapies in the horse

In the horse, only the therapeutic use of adult MSCs derived from bone marrow has been reported (Guest, et al., 2008) (Smith, et al., 2003) (Pacini, et al., 2007) (Wilke, et al., 2007) (Crovace, et al., 2007) (Blatt, et al., 2005). The efficacy of these treatments is difficult to determine, since the use of control animals is rarely reported and often the stem cell treatment is combined with other biological factors, as described afterwards (Koch, et al. 2009).

The utilization of these cells has mainly been in treatment of lesions of the superficial digital flexor tendon (Koch, et al., 2009), while in equine cartilage repair mostly it has been restricted to *in vitro* studies (Koch, et al., 2009).

Gene-therapy in equine medicine

The logical basis for gene therapy is that a virus vector is able to invade the endogenous cell type of interest and alter its function by integrating an exogenous gene into its genome, thus enabling the cells to overexpress an existing gene or express a gene novel to that cell type (Koch, et al., 2009). Subsequently, the cells will transcribe the implanted gene material into mRNA and translate it into functional protein (Koch, et al., 2009). Gene therapy for various biofactors has mainly been investigated in vitro and experimentally in horses for the purpose of arresting osteoarthritis and/or improving joint cartilage healing.

Encouraging results have been obtained in equine arthritis models by over-expression of interleukin-1 receptor antagonist protein (IL-1Ra) and IGF-1 but with time-limited effect (Koch, et al., 2009). More hyaline-like cartilage was present in equine cartilage defects treated with chondrocytes transfected to over-express IGF-1 than in defects treated with native chondrocytes, even though the expression of IGF-1 decreased over time (Goodrich, et al., 2007).

Equine bone marrow-derived MSCs transfected by using recombinant and modified adenoviruses have shown greater permissiveness and sustained expression of transgenes than equine chondrocytes and synoviocytes (Ishihara, et al., 2006).

Morisset *et al.* (2007), in an equine model of induced chondral defects, have investigated *in vivo* gene transfer as follows: adenovirus vectors containing the genes coding for IL-1Ra and IGF-1 have been injected intra-articularly after a cartilage defect had been created; no macroscopic or histological differences have been noted between the treatment, control, and placebo groups after 16 weeks; however, biochemical assays revealed increased type II

collagen and proteoglycan content in the early repair tissue phase of the treatment group compared with that in the other groups.

Tissue repair by enhancing the endogenous healing response

Enhancement of intrinsic repair mechanisms includes stimulation of cell proliferation, differentiation, tissue formation and remodelling, and the recruitment of endogenous progenitor populations into the damaged tissue (Luyten & Vanlauwe, 2012).

If insufficient intrinsic repair leads to clinical symptoms and signs, with loss of function, extrinsic repair needs to be considered, i.e. tissue engineering approaches using cell populations and combinations products that can contribute mostly locally to the tissue repair processes (Luyten & Vanlauwe, 2012).

Biofactors are used indirectly in equine medicine, within stem cell-based therapies, as mentioned above; stem cells are often combined with platelet rich plasma, bone marrow supernatant, or autologous serum. No purified biofactors are licensed for use in the horse (Koch, et al., 2009).

Growth factors

The commitment maintenance of the chondrocyte phenotype is obtained through molecular signalling between several growth factors, such as transforming growth factor β1 or β3 (TGF-β1 or TGF-β3), insulin-like growth factor-1 (IGF-1), growth/differentiation factor-5 (GDF-5), and bone morphogenic proteins (BMPs; BMP-2, BMP-4, and BMP-7) (Chen, Liang, & Thouas, 2013).

Main properties of some key molecules are described as follows in table 4.

Factor	Actions
TGF-β1	- stimulates large-scale DNA and GAGs synthesis (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019) - strongly facilitates chondrogenesis in hydrogel cultured MSCs improving differentiation (Bosnakovski, Mizuno, Kim, Takagi, Okumura, & Fujinaga, 2006)
BMP-2	- stimulates chondrogenesis (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019)
BMP-4	- stimulates chondrogenesis (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019) - promotes MSCs differentiation into mature chondrocytes (Hatakeyama, Tuan, & Shum, 2004)
GDF-5	- increases production of prechondrogenic precursors and the transcription factor sox-9 (Giannoni & Cancedda, 2006) (Angel, Sgaglione, & Grande, 2006)
IGF-1	- favours selective, dose-dependent production of collagen II and PGs (aggregan, GAGs) (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019)

	(Fortier, Lust, Mohammed, & Nixon, 1999) - prevents release and degradation of PGs, expression of procollagen I, IIA (Fortier, Lust, Mohammed, & Nixon, 1999) - maintains rounded phenotype, prevents dedifferentiation (Fortier, Lust, Mohammed, & Nixon, 1999) - reinforces bonds between damaged cartilaginous tissue and subchondral bone, while increasing the proportion of chondrocytes (Nixon, et al., 2007)
Collagen II	- helps with chondrogenic differentiation and phenotype maintenance in hydrogel cultured MSCs (Bosnakovski, Mizuno, Kim, Takagi, Okumura, & Fujinaga, 2006)

Table 4: Main molecules involved in maintenance of the chondrocyte phenotype.

PRP injections

The use of intra-articular injections of platelet rich plasma (PRP) is another interesting "multidrug" treatment for joint surface defects and early and established OA (Luyten & Vanlauwe, 2012). PRP takes in essence advantage of the high concentrations of growth factors including platelet derived growth factors (PDGF), fibroblast growth factors (FGFs) and transforming growth factors (TGF-β) present in platelets (Tschon, et al., 2011). Thus a cocktail of growth factors is released and delivered locally, thereby potentially influencing several steps of the tissue healing processes, such as cell proliferation, chemotaxis, cell migration, cell differentiation and matrix synthesis (Luyten & Vanlauwe, 2012). Due to its complexity and lack of characterization, it cannot be considered as a targeted drug treatment (Luyten & Vanlauwe, 2012).

In vitro studies on the effect of platelet rich plasma (PRP) on equine ligament and tendon explants have demonstrated increased transcription (up-regulation) of anabolic genes and increased secretion of proteins, including cartilage oligomeric matrix protein (COMP), which is a protein believed to play an organizational role in tendon and ligament matrices (Schnabel, et al., 2007) (Smith, et al., 2006).

Bone marrow derivatives

Acellular bone marrow supernatant produced a better effect than PRP on protein and COMP secretion (Smith, et al., 2006); it was also shown to have an anabolic effect on tendon matrix products that was superior to that of platelet poor plasma, plasma, or blood (Schnabel, et al., 2008).

Other techniques currently under study in human medicine involve as well use of bone marrow cell concentrates and so-called superfactors (Marc, 2018).

Local application of bone marrow cell concentrates (BMC) harvested by bone puncture, often combined with a hyaluronic acid (HA)-based scaffold soaked in PRP or even

associated with the microfracture technique, has only been described in case reports or small case series on localized cartilage defects so far (Marc, 2018). On one hand, it is difficult to determine the specific effect of BMC in these multiple therapeutic combinations and, on the other hand, the second-look arthroscopy shows only dubious results with a neocartilage more fibro-cartilaginous than natural hyaline; this histological result is similar to those observed with chondrocyte grafts for the treatment of *osteochondritis dissecans* (Grässel & Andres, 2012).

Other biofactors

Other biofactors such as prostaglandin factor 2 (PGF2) or fibroblast growth factor 18 (FGF18), referred to as sprifermin, are currently being studied (Lohmander, 2014). Some promising results were obtained on cartilage thickness after cycles of injections of sprifermin (Roemer, et al., 2018). Moreover, a PRP/HA combination (CM-PRP-HA), obtained using a dedicated medical device called Cellular Matrix A-CP-HA kit, has the advantages of being a validated simple, inexpensive and clinically effective procedure in terms of pain, stiffness and joint function, while remaining non-invasive and safe (Abate, et al., 2015) (Marc, 2018).

Insulin-like growth factor-1 (IGF-1) has been shown to improve cellular and molecular healing parameters in an *in vivo* equine collagenase-induced tendonitis model (Dahlgren, et al., 2002).

Joint resurfacing by tissue engineering

Tissue engineering (TE) is an emerging multidisciplinary field in regenerative medicine that combines the principles and technologies from the life, material, and engineering sciences to develop functional substitutes for damaged tissues and organs and/or promote endogenous regeneration (Obregón, et al., 2017) (Furth & Atala, 2014). TE generally involves the use of a tissue scaffold for the formation of new viable tissue for a medical purpose (Wikipedia, 2019).

TE consists in cell collection from a donor (who might as well be the recipient of the engineered tissue), cell isolation, culture and expansion, seeding in devices made of certain biomaterials (in scaffolds, beads, etc.), further cultivation to allow neo-tissue formation, and final implantation of the engineered tissue (Melero-Martin & Al-Rubeai, 2007). Figure 11 shows TE steps with regard to AC; these steps will be reviewed in detail in this paper in the experimental section.

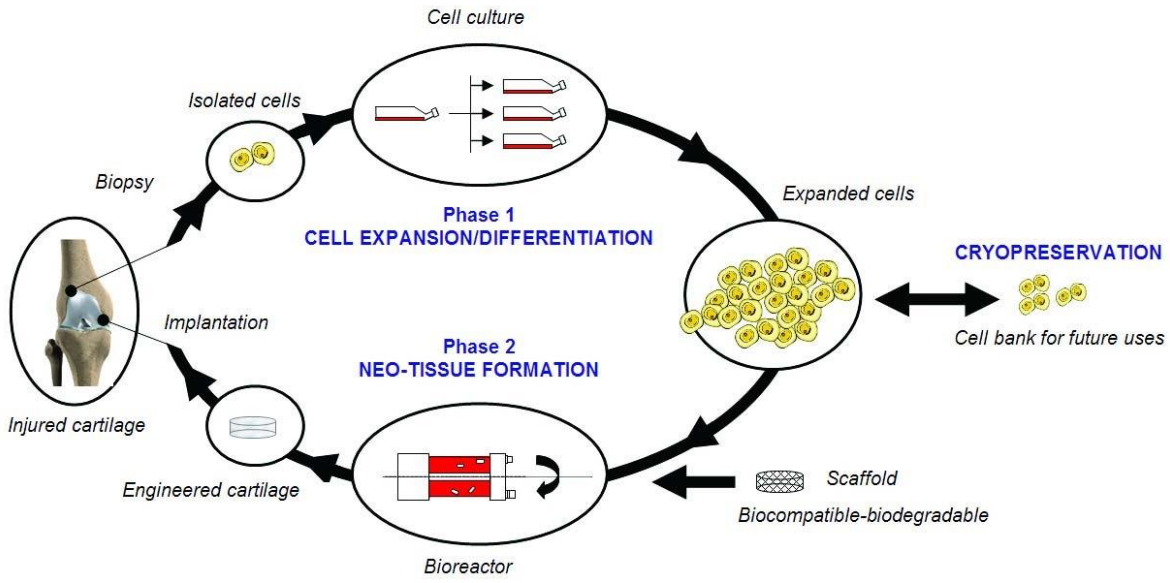


Figure 11: Steps in articular cartilage tissue engineering; from Melero-Martin & Al-Rubeai, 2007, adapted from reference Martin, Smith, & Al-Rubeai, 2005.

The mechanisms through which the cellular therapies and combination products contribute to tissue repair and interfere with disease processes are multiple and involve direct engraftment, proliferation and differentiation to tissue specific cell types, but also include paracrine actions such as the secretion of growth and differentiation factors that enhance local tissue repair (Luyten & Vanlauwe, 2012).

Indeed, cellular products typically function as multi-signal delivery systems and interact with the microenvironment (Caplan, 2010); in addition, it appears that adult stem cells display anti-inflammatory and immunomodulatory properties (Luyten & Vanlauwe, 2012).

Marrow stimulation techniques

Microfracture is the current main technique based to achieve a regenerative response from bone marrow stimulation (Pridie, 1959) (Johnson L. L., 2001) (Steadman, Miller, Karas, Schlegel, Briggs, & Hawkins, 2003).

Microfracture consists in debridement of the focal lesion on articular cartilage to stable borders, followed by complete removal of the calcified cartilage layer on top of the subchondral plate; then with a sharp awl the subchondral plate is perforated at the border and in the centre of the defect to a depth of several mm (in terms of defect filling, in a rabbit cartilage defect model, deep as 6 mm is better than shallow as 2 mm (Chen, et al., 2011)), every 3 to 4 mm. Bleeding from the underlying bone is allowed through these punctures, leading to the formation of a “superclot” containing marrow derived components, including stromal cells; this clot subsequently reorganizes into a new tissue

coverage of local defect in the joint surface (Luyten & Vanlauwe, 2012) (the procedure is shown in Figure 8, panel A).

The newly formed tissue consists of a mixture of fibrous and cartilaginous tissue, with the presence of some collagen type II and proteoglycans in the matrix as in articular cartilage (Saris, et al., 2008) (Minas, Gomoll, Rosenberger, Royce, & Bryant, 2009). Nevertheless this tissue tends over time to degenerate into fibrous tissue or sometimes to form so-called "intralesional osteophytes" through endochondral bone formation, with recurrence of symptoms (Minas, Gomoll, Rosenberger, Royce, & Bryant, 2009).

Autologous chondrocyte implantation ACI

First generation ACI

Brittberg et al. reviewed first attempt to this procedure (Brittberg, Lindahl, Nilsson, Ohlsson, Isaksson, & Peterson, 1994); Cell populations were prepared by enzymatic release from a biopsy of articular cartilage taken from an unloaded area in the symptomatic joint during arthroscopy (Figure 1, panel B). The chondrocytes were subsequently expanded in vitro, and re-implanted through arthrotomy in the joint surface defect under a periosteal flap, taken from the tibia from the same patient. This was then followed by a long rehabilitation to reach its optimal outcome at 18-24 months (Luyten & Vanlauwe, 2012).

Main drawback resides in the need for a periosteal cover of the defect, especially when is necessary to harvest large flaps and for the risk of tissue hypertrophy of the periosteal cover (Henderson, Tuy, & Oakes, 2004) (Luyten & Vanlauwe, 2012)

Some progress has been made since then with studies aiming to improve and standardize the preparation of the autologous chondrocytes, the development of other delivery systems for the chondrocytes and the replacement of the periosteal flap by a membrane of diverse composition, and a series of clinical studies (Luyten & Vanlauwe, 2012). Microfracture is clearly associated with a clinical benefit and a filling of the joint surface defect with repair tissue, but it is generally accepted that this repair is less durable, resulting in a consistent decline in clinical outcome over the long term. It is the aim of ACI to repair the cartilage defect with high quality tissue matching the characteristics of the neighbouring tissue, thereby resulting in improved long-term outcomes. (Luyten & Vanlauwe, 2012)

Second generation ACI

The so-called “second generation” ACI, cells cultured in/on a matrix (Figure 8, panel C) might have some distinct advantages over the “classical” cell suspensions implanted under a periosteal flap. Culturing the cells in 2D-plus (in multilayer on a membrane) or 3D conditions (distributed in a matrix) seems to favor the phenotypic stability of the chondrocytes (Benya & Shaffer, 1982) (von der, Gauss, von der, & Muller, 1977). Chondrocytes are cultured on/in a matrix that is biocompatible and functions as growth and delivery scaffold for implantation (Luyten & Vanlauwe, 2012). It is known that these procedures encounter fewer complications such as arthrofibrosis and the risk for infection. At this moment, several matrices are in use in a clinical setting, but it is unclear which is the ideal matrix at this time; they include natural and synthetic types (Luyten & Vanlauwe, 2012).

So far, the short-term and medium-term results of the first and second generations seem to be comparable (Luyten & Vanlauwe, 2012).

The “third-generation” joint surface repair techniques address some of the limitations of prior approaches. Some of these third-generation techniques use several novel approaches including those designed for a “1-stage” procedure; these include the use of allogeneic chondrocytes, chondro-inductive and chondro-conductive matrices, and techniques to mechanically condition the developing tissue in a bioreactor setup, aiming to improve the material properties of the cell-matrix implant in advance of surgical application (Hettrich, Crawford, & Rodeo, 2008).

The next wave of developments relate to the use of autologous and/or allogenic progenitor cell populations for the repair of joint surface defects in combination with new biomaterials, and the application of gene therapy (Kessler, Ackerman, Dines, & Grande, 2008). Adult bone marrow stromal cells, also called mesenchymal stem cells (MSC) are lead candidates for their use in the repair of cartilage defects and other skeletal tissues such as bone defects (Charbord, et al., 2011). Other tissue sources have been explored to prepare MSC populations including adipose tissue, synovium and periost (De Bari, Dell'accio, & Luyten, 2001) (De Bari, Dell'accio, Tylzanowski, & Luyten, 2001) (De Bari, et al., 2006). It is not known what the safety and durability is of an allogenic approach; prerequisites for successful outcomes may include a better understanding of the differentiation protocols towards the proper stable articular cartilage phenotype, and the immune tolerance at the short and long term of the allogenic cells (Luyten & Vanlauwe, 2012).

Acellular approaches

Many acellular approaches have been taken so far; few approaches appear worth mentioning such as autologous matrix induced chondrogenesis (AMIC). This technique was developed by Behrens et al. in an attempt to improve the microfracture procedure (Behrens, 2005) (Benthien & Behrens, 2010): one of the improvements was the containment of the blood clot in the defect by covering the microfracture treated lesion in the knee with a collagen type I/III membrane (Chondrogide, Geistlich Biomaterials, Wolhusen, Germany).

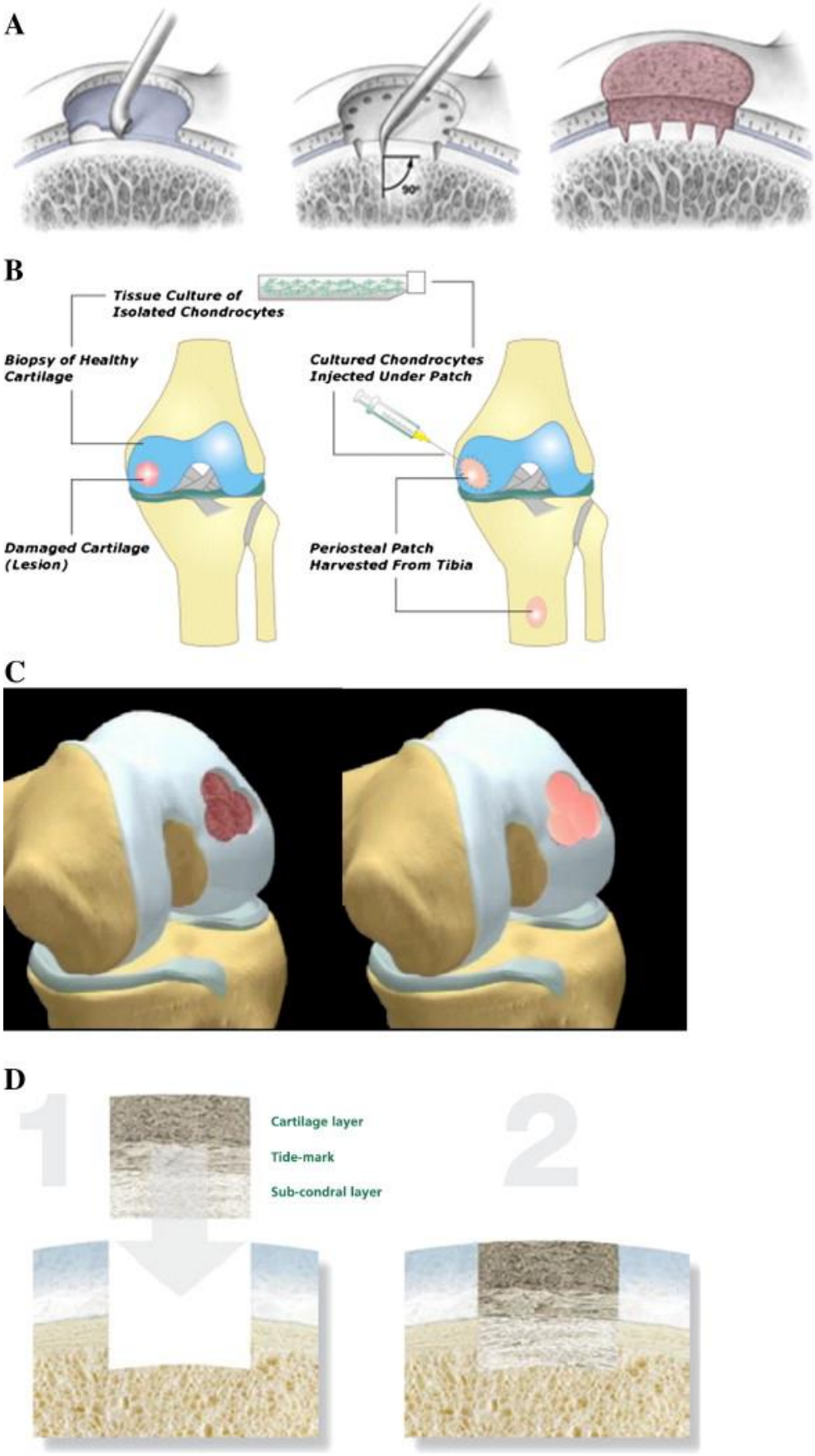


Figure 12: A. Microfracture, B. autologous chondrocyte implantation, C. second generation ACI (Hyalograft, Anika therapeutics, Bedford MA, USA), and D. acellular scaffold (Maioregen, Finceramica, Faenza, Italy) from Luyten & Vanlauwe, 2012.

Scaffolds in equine medicine

Scaffold use

Recently several new scaffolds were introduced into the market for the treatment of osteochondral defects (above in figure 12, panel D). All of them are applied in the same fashion by surgically (arthroscopic or open) delivering them into a created osteochondral defect. The depth of the lesion is dependent on the type of scaffold used. They are either composed of synthetic or natural materials (Luyten & Vanlauwe, 2012). Scaffolds can be injectable, non-injectable, simple, complex, biological, or synthetic in nature (Koch, Berg, & Betts, 2009).

An important paradigm to be considered when evaluating any scaffold-based therapy is the interaction between scaffold and tissue (Figure 13). The ideal scaffold has sufficient strength to protect cells from compression and shearing forces, while still having injury site anchoring potential and porosity to allow nutrient and differentiation factors to diffuse through it. The scaffold must also degrade at a rate that optimizes cellular growth and tissue regeneration.

Such ideal scaffolds have not yet been designed. The optimal time point for evaluation of a scaffold-based treatment is also critical, and the best determination of treatment success can probably be made only after the "scaffold-tissue transition phase" has passed, which depends on the scaffold, cells, and tissue in question (Koch, Berg, & Betts, 2009).

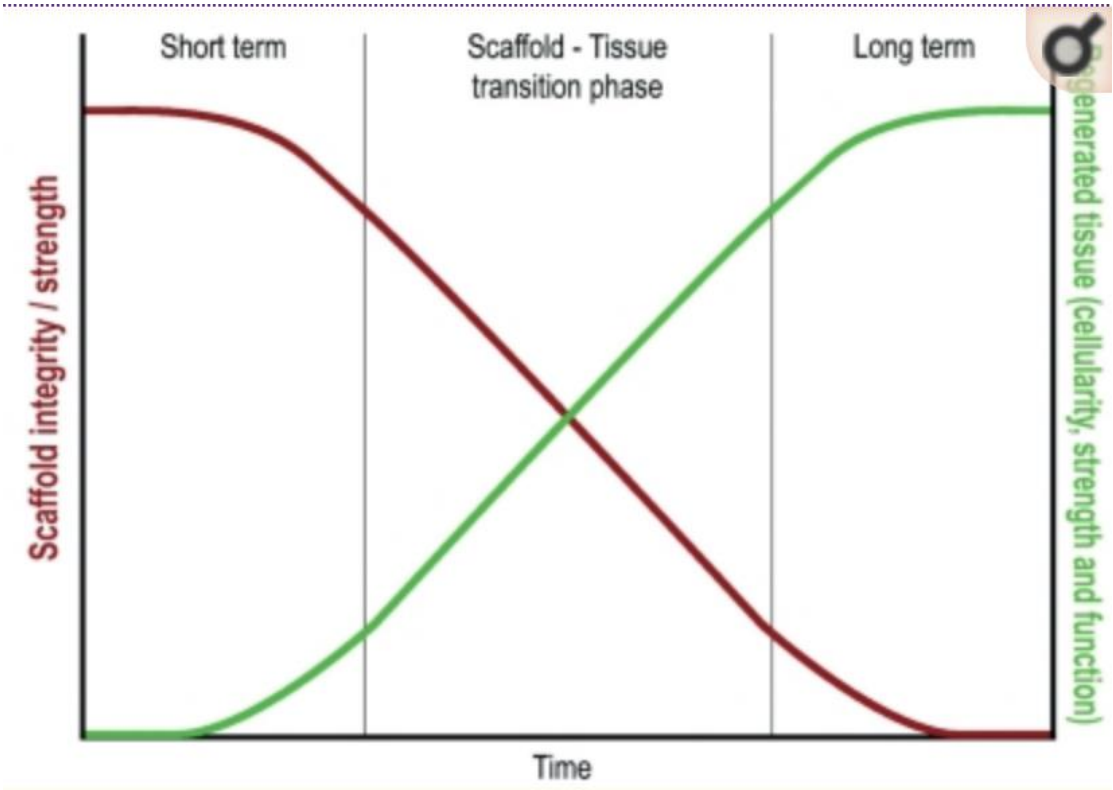


Figure 13: Ideally, any scaffold is degraded at a rate optimum for allowing complete tissue regeneration and ultimately replaced entirely by the regenerated tissue. Survival of the transplanted cells and successful tissue integration relies on diffusion of biological factors through the scaffold. The scaffold-tissue transition phase might be associated with decreased mechanical strength and function, leading to treatment failure if the scaffold degrades faster than the tissue can regenerate. On the other hand, a slowly degrading scaffold might impair and, potentially, prevent proper tissue healing. These concepts should be considered when evaluating scaffold-based studies and the time point chosen for evaluation of treatment success. Figure by Koch and Berg from Koch, Berg, & Betts, 2009.

Simple scaffold therapies

One biological and 1 synthetic extracellular matrix scaffold are commercially available for use in the horse at this time (Koch, Berg, & Betts, 2009).

Porcine urinary bladder matrix (UBM) is marketed as an acellular biological scaffold (Acell Vet; Acell, Columbia, Maryland, USA) for use in the horse. The product is available as powder, sheet, or granules; as a gel; and in disc form. Possible applications according to the manufacturer include, but are not limited to, tendon and ligament injuries, hoof and hoof wall injuries, corneal ulcers, dental extractions, full and partial thickness wounds, burns, and post surgical skin closures (Koch, Berg, & Betts, 2009).

Synthetic extracellular matrix scaffolds (EquitrX; Sentrex Animal Care, Salt Lake City, Utah, USA) have recently become available to the equine practitioner for the treatment of skin wounds. The scaffolds are hydrogel films made of chemically cross-linked glycosaminoglycans (Koch, Berg, & Betts, 2009).

Complex scaffold therapies

Replacing whole joints with artificial joints has benefited thousands of human patients and the longevity of these implants continues to increase with improved patient selection, improved surgical technique, and better implants (Eingartner, 2007) (Breusch, Lukoschek, Thomsen, Mau, Ewerbeck, & Aldinger, 2005) (Parsch, Jung, Thomsen, Ewerbeck, & Aldinger, 2007) (Eingartner, Heigele, Dieter, Winter, & Weise, 2003); development of techniques that can replace the diseased joint in a more biological way, allowing life-long treatment success, is being investigated (Koch, Berg, & Betts, 2009).

There are no reports on the use of engineered complex tissues in the horse

In sheep, engineered osteochondral-like plugs for use in mosaic arthroplasty have been investigated (Pilliar, Kandel, Grynpas, Zalzal, & Hurtig, 2007), using autologous chondrocytes, not stem cells, loaded on to a polycalciumphosphate scaffold; this biphasic construct was cultured in vitro prior to placement in induced defects in the stifle. Improved healing was noted histologically when compared with that in the sham operated contralateral joint. However, the compressive and shear strength of these constructs is below that of native osteochondral plug; compressive and shearing forces are higher in the horse than in the sheep, so compression- and shearing-induced injuries might be of significance if the technique were to be transferred to the horse in its current form. Nevertheless, mosaic arthroplasty, using autologous or allogenic osteochoindral plugs, is currently used in selected equine patients and continues to be investigated in experimental models (procedure shown in figure 14) (Bodo, Hangody, Modis, & Hurtig, 2004). The correct cells combined with the correct scaffold may improve the outcome of mosaic arthroplasty in the future (Koch, Berg, & Betts, 2009).

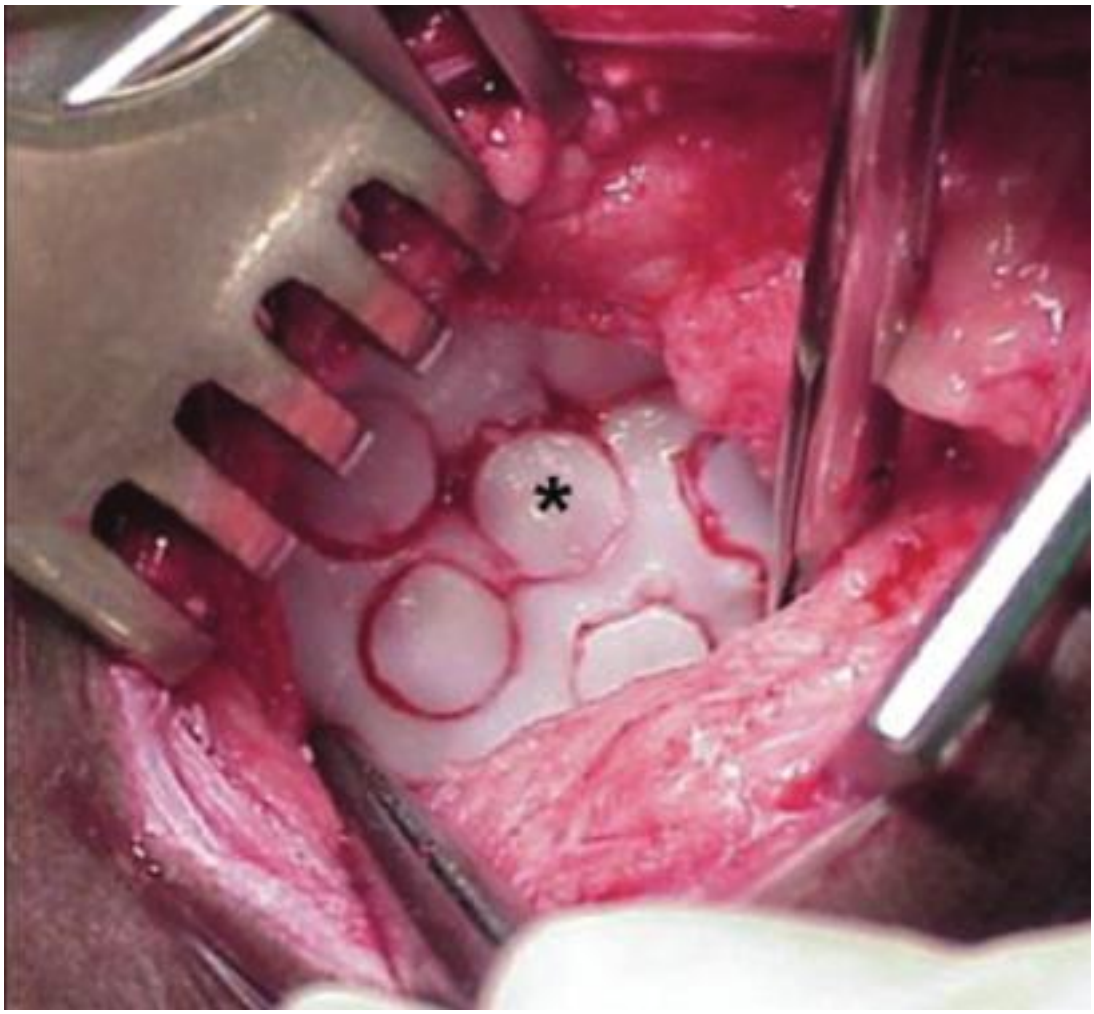


Figure 14: Autologous osteochondral grafts are currently used in mosaic arthroplasty for selected focal cartilage defects in the horse. Incongruency between graft and native cartilage, as well as reduced biomechanical properties of the graft compared with native cartilage and bone, are the main limitations of the technique today. In the future, tissue engineering and stem cell-based therapies may help to negate these limitations Image: Courtesy of Dr. Mark Hurtig, University of Guelph, from Koch, Berg, & Betts, 2009.

Tissue engineering approaches for OA: biomaterials and scaffolds

Mechanical properties

Proper mechanical stimulation is fundamental for cartilage development (Khan, Redman, Williams, Dowthwaite, Oldfield, & Archer, 2007) (Mikic, Isenstein, & Chabra, 2004), as well as for *in vitro* chondrogenesis and tissue regeneration (Salisbury Palomares, Gerstenfeld, Wigner, Lenburg, Einhorn, & Morgan, 2010) (Takahashi, et al., 1998); also, optimal neocartilage growth requires a specific combination between Young's modulus (stiffness or resistance to elastic deformation under load (Helmenstine, 2018)), lubricant coefficient and viscoelasticity.

Thus, mechanical properties such as compression, shear stress and hydrostatic pressure must be considered when designing any system for joint cartilage repair.

In vitro, when the chondrogenic phenotype is adequately maintained, i.e. by using an appropriate combination of scaffold and cells, can regenerate cartilage. However, when a scaffold is *in vivo*, the implanted material must permanently bond to the local cartilage tissue; interestingly, subjecting the scaffold to biomechanical loads before implantation generates an adequate phenotype (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019), and simulating a natural mechanical environment, chondrocytes are able to secrete growth factors and differentiate themselves into an appropriate route (Hu & Athanasiou, 2006).

The load-bearing nature of the joint environment in which the engineered scaffold/construct will be implanted must be considered when designing the mechanical properties of the scaffold. Design considerations with respect to mechanical strength will depend on whether the scaffold will be used *in vitro* or *in vivo* (Izadifar, Chen, & Kulyk, 2012). If the scaffold will be implanted into a joint *in vivo* shortly after fabrication, its mechanical characteristics should ideally match those of natural cartilage to support the loads encountered in the joint; on the other hand, if it is designed to initially promote engineered tissue growth *in vitro* before implantation *in vivo*, it may not require the same level of mechanical strength as natural cartilage, since it will largely act as a supportive environment to help formation of the tissue construct (Izadifar, Chen, & Kulyk, 2012). As an example of load-bearing capacity required, the force applied to a human knee cartilage,

during normal physiological loading, ranges from 1.9 to 7.2 times body weight, meaning about 0.84 to 3 MPa for a 70 kg person (Izadifar, Chen, & Kulyk, 2012).

Eventually the newly formed tissue or neocartilage-scaffold construct must achieve the required mechanical properties to replace the damaged tissue *in vivo* (Izadifar, Chen, & Kulyk, 2012).

Mechanical properties of TE scaffolds, for instance, are also influenced by the variation of architectural parameters, such as porosity, pore size and shape, fibre diameter and spacing (Izadifar, Chen, & Kulyk, 2012).

Hydrogels

Hydrogels are easily prepared and embedded with chondrocytes and enable them to retain their phenotype and morphology through impregnation. Hydrogels can be made of a wide variety of biomaterials, including natural materials, which may be carbohydrate-based (e.g., alginate, agarose, chitosan, hyaluronic acid (HA)), protein-based (e.g., fibrin glue, collagen type I and II, silk), or some combination of the two, and synthetic materials (such as poly(hydroxyethyl methacrylate), polyethylene glycol and its derivatives, or poly(vinyl alcohol) (PVA)) (Izadifar, Chen, & Kulyk, 2012). Exhibiting characteristics similar to soft tissues, hydrogels therefore provide a supportive matrix for chondrocyte activity and cartilage ECM secretion both *in vitro* and *in vivo*. High efficiency of cell encapsulation and uniform cell distribution within the hydrogel are advantages improving the quality of formed tissue. In spite of this, hydrogels have very weak mechanical properties, limiting their application for cartilage and making impossible any actual *in situ* placement.

In contrast to solid scaffolds, hydrogels do not provide much flexibility for creating structures with defined internal architectures. Some attempts to hybrid hydrogel scaffolds are being studied.

Scaffolds

The main rationale for biomimetic scaffolds is to provide an engineered tissue able to resemble structure, function, ECM of the articular cartilage (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

Therefore some aspects have to be carefully considered:

- Biomaterials
    - Nature

      Biomaterials should mimic a natural ECM, which provides diverse

chemical, physical, and biological signals for cell growth and function (Owen & Shoicet, 2010) (Shoichet, 2010).

- Biocompatibility

  Any implantable device and their degradation products should be innocuous to the host and low inflammatory in order to preserve integrity in adjacent tissue, health in the patient and achieve the best possible responses in the engineered tissue, (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019) (Ratner, 2011).

- Stability and duration

  An effective scaffold should withstand implantation, remain in place (Ratner, 2011) and ideally persist long enough so that, over time, it becomes fully replaced by neocartilage (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

- Architecture

  The scaffold should support adhesion, growth, proliferation, ECM production of chondrocytes or MSCs, allow free cell movement throughout its structure and present appropriate mechanical properties, similar to the ones of native hyaline cartilage.

  These characteristics are obtained through the selection of biomaterials, the scaffold design and fabrication (e.g., type of porosity) that enables cells preloading and subsequent ingrowth of new tissue (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

Types of Biomaterials

Biomaterials are substances engineered to take a form that, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure (Biomaterials, 2019).

They play a central role in current strategies of regenerative medicine and tissue engineering as suitable biophysical and biochemical environment that supports and directs cellular behaviour and function (Peppas & Langer, 1994) (Hubbel, 1995) (Langer & Tirrell, 2004). Biomaterials may facilitate restoration of structure and function of damaged or dysfunctional tissues, both in cell-based therapies, where carriers deliver transplanted cells or matrices induce morphogenesis in bioengineered tissues constructed ex vivo, and

in acellular therapies, when cell-free materials induce ingrowth and differentiation of cells from healthy residual tissues in situ (Lutolf & Hubbell, 2005).

Biomaterials should provide a provisional three-dimensional (3-D) support to interact biomolecularly with cells to control their function, guiding the spatially and temporally complex multicellular processes of tissue formation and regeneration (Lutolf & Hubbell, 2005).

Biomaterials can be either natural or synthetic. In general, materials from natural sources are advantageous for their inherent properties of biological recognition, such as presentation of receptor-binding ligands and susceptibility to proteolytic degradation and remodelling; on the contrary, natural materials may present complexities associated with purification, immunogenicity and pathogen transmission, while synthetic analogues could provide greater control over materials properties and tissue responses (Lutolf & Hubbell, 2005).

Key concepts about biomaterials are bioactivity and biocompatibility. Bioactivity indicates the property of exerting an effect on or response from a living organism (Medical Dictionary for the Health Professions and Nursing, 2012). Biocompatibility is the ability of a biomaterial to perform with an appropriate host response in a specific application (Williams, 1987), without eliciting any undesirable local or systemic effects (Williams, 2008).

An ideal biomaterial should be able to promote healing and regeneration of damaged structures, to potentially reside in the body for long periods with degrees of inflammation as low as possible (Ratner, 2011).

Tables 5 and 6 list principal natural and synthetic biomaterials and their advantages/disadvantages.

Material	**Advantages**	**Disadvantages**
Agarose	Allows cell differentiation; high glycosaminoglycan/DNA; reparative ability	Difficult migration of cells when polymerized at a high concentration; needs to be exposed to mechanical overload
Alginate	Allows interaction with cells	Not ideal mechanical properties
Chitosan	Unlimited resource; contains glycosaminoglycans and hyaluronan, similarly to native cartilage	Lacks fast gelling properties (cannot be applied *in situ*)
Collagen	Main component present in	Needs mechanical

	the ECM; good cell adhesion properties; achieved good clinical results with young patients	stimulation for improving loading capacity
Fibrin	Approved by the FDA; stimulates production of glycosaminoglycans; supports formation of the ECM	Success rate of 3 of 5 patients; results are dependent on cell seeding concentration
Hyaluronan	Glycosaminoglycan present in native cartilage; allows interaction with cells; improves expression of collagen type II	Needs growth factors for cell survival; decreases expression of collagen type I
Gellan gum	Water soluble; good rheological properties	Derived from microbial fermentation of *Sphingomonas paucimobilis*; poor mechanical strength

Table 5: Main natural biomaterials for AC repair. Adapted from Duarte Campos, Drescher, Rath, Tingart, & Fischer, 2012.

Material	**Advantages**	**Disadvantages**
Poly(ethylene glycol) or PEG	Allows interaction with chondrocytes; does not support angiogenesis (beneficial for chondrocytes)	Not ideal strength and compression modulus
Poly(N-isopropylacrylamide) or PNiPAAm	Copolymerization possible with AAC; gelling temperature around 37 °C; does not support angiogenesis; cells keep their phenotype	When polymerized, there is an output of water content; poor mechanical strength
Polylactic acid or PLA (semi-synthetic)	Able to maintain 3-D structure when implanted *in vivo*; expression of high levels of collagen types I and II	Needs growth factors for cell survival
Polyurethane	Ease of processing as injectable gel (*in situ*polymerization); good mechanical properties	Not completely biocompatible (mild host response)
Poly(vinyl alcohol) or PVA	Water soluble; excellent adhesion properties; allows interaction with cells	Not completely degradable (semidegradable); culture in bioreactor needed to increase compression modulus
Scaffold free	Production of an ECM rich in proteoglycans; derives sizable tissues	Poor mechanical strength

Table 6: Main synthetic biomaterials for AC repair. Adapted from Duarte Campos, Drescher, Rath, Tingart, & Fischer, 2012.

Cell-matrix interactions

A highly dynamic and complex network of biophysical and biochemical signals, transmitted from the outside of a cell by surface receptors and integrated by intracellular signalling pathways, converge to regulate gene expression and ultimately establish cell phenotype (see figure 15) (Lutolf & Hubbell, 2005).

The extracellular microenvironment surrounding cells is a highly hydrated network hosting three main effectors:

- Insoluble hydrated macromolecules (fibrillar proteins such as collagens, non-collagenous glycoproteins such as elastin, laminin or fibronectin, and hydrophilic proteoglycans with large glycosaminoglycan side chains) called physical signals in figure 15;
- Soluble macromolecules (growth factors, chemokines and cytokines);
- Proteins on the surfaces of neighbouring cells.

Thus, a cell differentiates, proliferates, migrates, synthetises ECM or degrades it under a coordinated and highly bidirectional response to the molecular interactions with these ECM effectors (Lutolf & Hubbell, 2005).

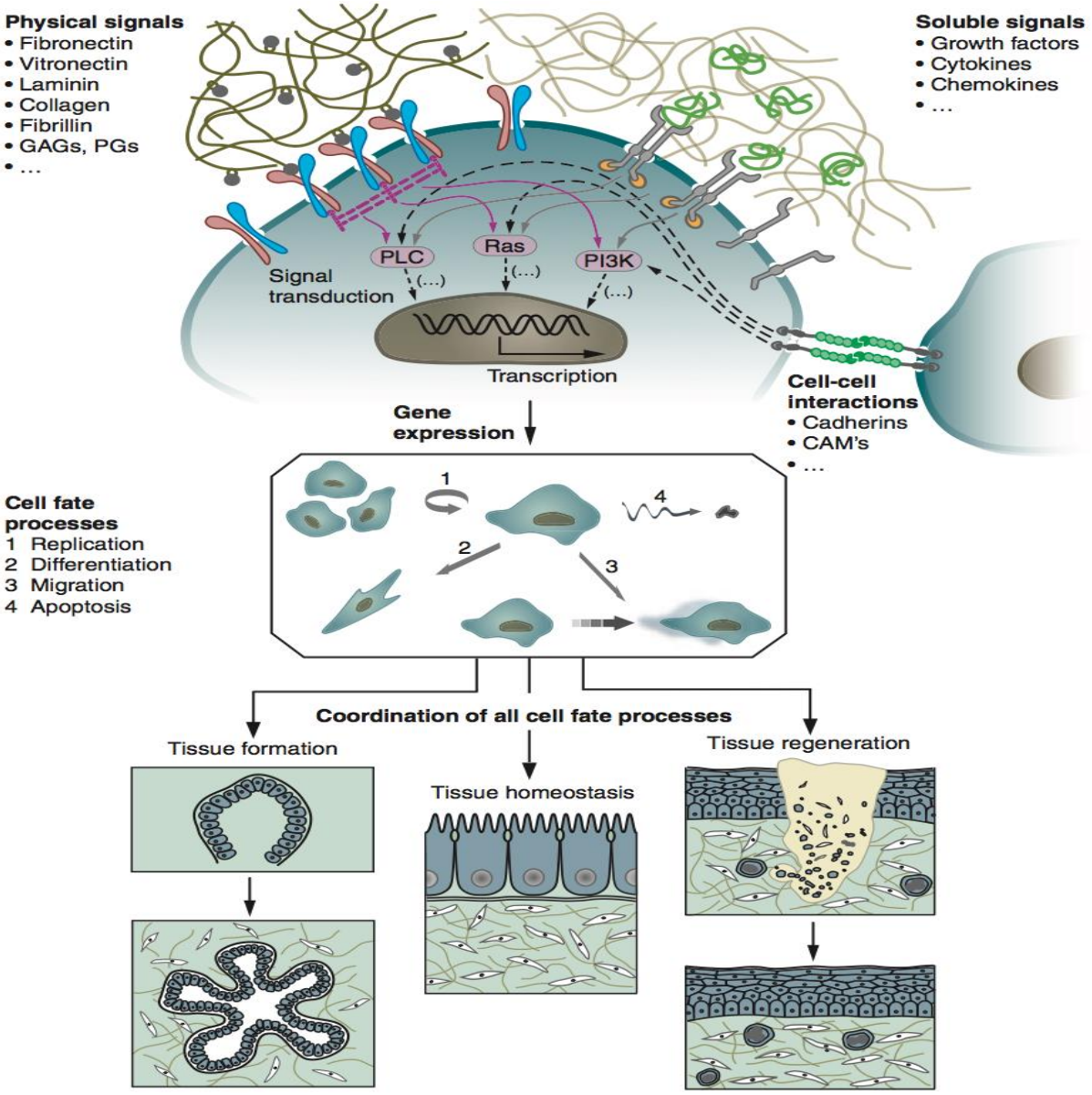


Figure 15: The behaviour of individual cells and the dynamic state of multicellular tissues is regulated by intricate reciprocal molecular interactions between cells and their surroundings. This extra- cellular microenvironment is a hydrated protein- and proteoglycan-based gel network comprising soluble and physically bound signals as well as signals arising from cell-cell interactions. Adapted from Bottaro, Liebmann-Vinson, & Heidaran, 2002 by Lutolf & Hubbell, 2005.

Natural biomaterials

- Hyaluronic acid

  HA is a fundamental component of cartilage ECM and has been utilised as a cartilage repair scaffold in cross-linked forms.

  Used by itself though, it has showed some drawbacks:

  - The repaired cartilage appeared different or thinner (Butnariu-Ephrat, Robinson, Mendes, Halperin, & Nevo, 1996)
  - HA oligosaccharides induce chondrocytic chondrolysis, including total loss

of stainable PG-rich matrix and activation of gelatinolytic activity (Knudson, Casey, Nishida, Eger, Kuetter, & Knudson, 2000)

On the contrary, combination with other materials showed better results (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

- Collagen

  Collagen I, component of bone tissue and fibrous cartilage, can be extracted from biological tissue with specific solutions (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019) and used in collagen-based scaffolds along with other biomaterials, such as elastin, chitosan or GAGs (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

  It presents some advantages:

  - Allows for contact between preloaded cells and endogenous cells located in joint tissues (Frenkel, Toolan, Menche, Pitman, & Pachence, 1997) (Grande, Halberstadt, Naughton, Schwartz, & Manji, 1997)
  - Cell-free (Sellers, et al., 2000) or loaded with chondrocytes (Frenkel, Toolan, Menche, Pitman, & Pachence, 1997), promotes articular-type cartilage repair similar to native tissue.
  - Combined with fibrin glue, supports cell survival and synthetic activity in culture, obtaining display of chondral properties *in vitro* (Deponti, et al., 2014)

- Alginate

  Alginate is a linear anionic natural polymer composed of repeating units of disaccharides, extracted from brown algae where it is abundant (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019). It is biocompatible and in solution exposes negative charges, which promote better and greater cell adhesion in 3-D scaffolds for cartilage (Yang, Chen, Liu, Kurokawa, & Gong, 2010) (Mai, 2017). Combined with chitosan, alginate provides a valid alternative to chitosan alone in terms of cell proliferation and chondrocyte expression (Li & Zhang, 2005).

- Chitosan

  Chitin is a cationic polysaccharide, a homopolymer of long chains of glucosamine units that also contain N-acetylglucosamine, bound through β(1➔4) glycosidic linkages (Jardine & Sayed, 2017) (Valappil Sajna, Devi

Gottumukkala, Sukumaran, & Pandey, 2015) (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019). Chitin is the second most abundant polysaccharide in nature, after cellulose, and it is mainly found in exoskeleton of arthropods (such as crustaceans, insects, arachnids) and in fungi (sources exemplified in figure 16) (Doucet & Retnakaran, 2012). Naturally, chitin is found in three crystalline polymorphic forms, characterized by different orientations of the microfibrils: α-chitin has antiparallel chains, β-chitin has parallel chains, and γ-chitin has the mixture of parallel and anti-parallel chains (Rudall & Kenchington, 1973) (Cabib, Bowers, Sburlati, & Silverman, 1988).

Chitosan is deacetylated chitin, produced by enzyme chitin deacetylase, obtained from fungi such as *Aspergillus nidulans* (Valappil Sajna, Devi Gottumukkala, Sukumaran, & Pandey, 2015). Degree of deacetylation ranges from 30% to 95% and chemically depends on the ratio of glucosamine units to N-acetyl-glucosamine units (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

Chitin and its derivatives have several applications in the fields of biotechnology, medicine and pharmacology, agriculture, cosmetics, and wastewater (Park & Kim, 2010) (Synowiecki & Al-Khateeb, 2003) (Felse & Panda, 1999) (Hajji, et al., 2014). From a biological stem point, they are endowed with properties such as non-antigenicity, biocompatibility, biodegradability and non-toxicity (Shahidi & Abuzaytoun, 2005) (Khor & Lim).

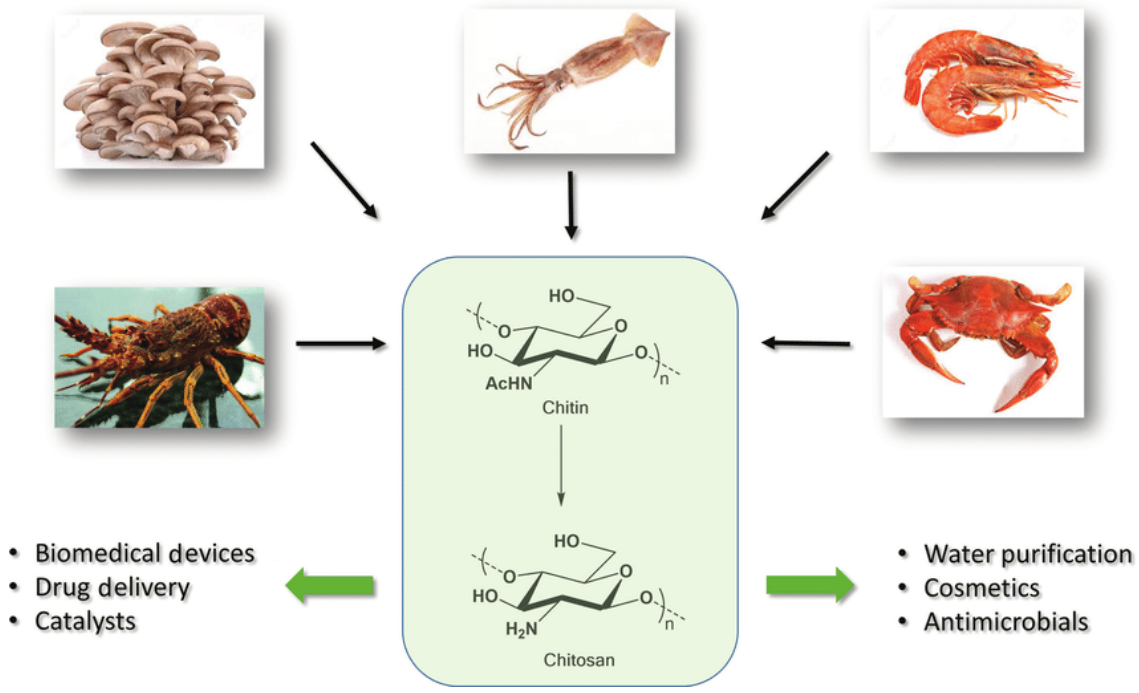


Figure 16: Chitin and chitosan sources and reported uses; from Jardine & Sayed, 2017.

The solubility of crystalline chitosan in aqueous solutions is pH dependent (insoluble above pH 7, more and more soluble downward (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

The PGs and GAGs in cartilage, which are anionic, contribute to electrostatic interactions with chitosan, which is cationic; this phenomenon enables retention and concentration of growth factors at the implant site (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019). Chitosan oligosaccharides are able to stimulate macrophages, both *in vitro* and *in vivo* (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

Chitosan can be used combined with other materials for bone and cartilage restoration and/or regeneration (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019) as well as, in the form of scaffolds, (Hu, Li, Wang, & Shen, 2004), alone or combined to facilitate cell seeding and enhance the mechanical properties of the implant (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

Chitosan-based scaffold, in a study by Jeon et al. (Jeon, Choi, Sung, Kim, Cho, & Chung, 2007), showed a longer durability resulting in greater maturation of the ECM network compared to a PLGA-based scaffold.

3-D printed chitosan and chitosan + HA scaffolds were used in the experiment described later in this paper.

Synthetic biomaterials

- Polylactic acid (PLA)

  MSCs seeded in polylactic acid-based scaffolds, treated with growth factors such as TGF-β1, BMP-2, undergo chondrocyte differentiation and high quality articular-like tissue can be developed; moreover, polylactic acid achieves secure positioning in the implantation site, prolongued durability and good integration with host tissue (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

- PGA, PLA, PLGA

  Polyglycolic acid (PGA), polylactic acid (PLA) and copolymer poly(lactic-co-glycolic acid) (PLGA) have been used in articular cartilage tissue engineering. Alone or mixed in different 3-D scaffolds arrangements, they showed firm hyaline cartilage development, mechanical properties resembling those of native tissue (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019); porosity, composition, architecture can also be manipulated to improve cell seeding, avoid scaffold delamination and overall better resemblance between engineered tissue and articular cartilage and subchondral bone (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

Architecture

The importance of 3-D structure

3-D matrices provide better model systems for physiologic situations particularly for cartilage regeneration (Friedl & Brèocker, 2000) (Cukierman, Pankov, Stevens, & Yamada, 2001) (Cukierman, Pankov, & Yamada, 2002) (Grinnell, 2003) (Abbott, 2003) (Schmeichel & Bissell, 2003). (Lutolf & Hubbell, 2005).

Natural ECM-derived biomaterials can be used as carriers for transplanted cells that are subsequently grafted into tissue defects (Bell, Ehrlich, Buttle, & Nakatsuji, 1981) (Yannas, Lee, Orgill, Skrabut, & Murphy, 1989), and also as cell infiltration matrices to induce regeneration and remodelling in vivo (Patino, Neiders, Andreana, Noble, & Cohen, 2002; Currie, Sharpe, & Martin, 2001). More pertinent for biomedical and materials engineering, naturally derived materials represent valuable models from which engineering principles to create artificial materials with similar biological function can be derived (Hubbell, 1999) (Griffith, 2002).

As natural cartilage tissue originally develops in a 3-D environment, there are distinct advantages of using 3-D *vs*. 2-D scaffold structures, including better maintenance of chondrocyte morphology and differentiation, and higher expression of genes regulating cell activities and ECM production (Izadifar, Chen, & Kulyk, 2012).

Different design parameters within all available architectures, including pore size and geometry, pore distribution, pore accessibility and tortuosity, and porosity, play significant roles in the morphology, composition, mechanical properties, and functionality of the engineered cartilage (Izadifar, Chen, & Kulyk, 2012).

Scaffold pore size is important in 3-D scaffolds, as macropores (>50 μm) promote cell migration and micropores promote cell–cell interaction and mass transport, which improve tissue formation especially *in vivo* (Izadifar, Chen, & Kulyk, 2012). Larger pore sizes (400 μm) may result in a significantly larger GAG content compared to constructs with 100 and 200 μm pore sizes, increasing the extension of ECM. Small pores might help with maintaining the chondrocyte phenotype, as chondrocytes are more likely to differentiate when the pore size is about 30 times the cell diameter (10–15 μm) (Izadifar, Chen, & Kulyk, 2012).

Gradient structures or composite scaffolds (micro to macropore structures), exhibiting a wide range of pore sizes, could better facilitate both chondrocyte activity and secretion of ECM (Izadifar, Chen, & Kulyk, 2012).

In addition to pore size, scaffold fibre size affects cell activity: large fibres (hundreds of μm) in fibrous scaffolds negatively influence cell function, because they present as a more 2-D attachment surface for individual cells; chondrocytes seeded on nanofiber meshes show indeed better chondrogenesis (Izadifar, Chen, & Kulyk, 2012).

Practically speaking, there is a compromise between porosity and mechanical properties of the scaffold, where the degree of stiffness tends to decrease as porosity increases.

Sponge scaffold designs generally have higher porosities than fibrous scaffolds, although the porosity of fibrous scaffolds can be precisely controlled in the fabrication process.

Pore interconnectivity can influence the ability of a scaffold to support chondrogenesis as well as the quality of formed tissue, even in scaffolds with similar porosities: interconnected structures with open pores are more capable of facilitating homogeneous cell seeding and better nutrient dispersion throughout the construct. Lack of sufficient pore interconnectivity can cause inhomogeneous chondrogenesis, with cartilage formation only evident on the peripheral boundaries of TE constructs (Izadifar, Chen, & Kulyk, 2012).

3-D printed scaffolds

3-D printing is known as an additive manufacturing method, building the required structure layer-by-layer, or even pixel-by-pixel. The printed structure is firstly modelled using computer-aided design software packages (see the process in figure 17) (Liu & Yan, 2018). The ideal role of 3-D printing in tissue engineering is to provide the suitable microenvironment for cells to induce cell proliferation and differentiation towards the functional tissue. Two main modes of 3-D printing using for tissue engineering currently: one is creating 3D cell-laden scaffolds that the cells are contained within the bioink, another is fabricating molds or scaffolds, which can be cultured with cells in-vitro after fabrication (Knowlton, Anand, Shah, & Tasoglu, 2018) (Kolesky, Truby, Gladman, Busbee, Homan, & Lewis, 2014).

Mechanical properties, such as elastic stiffness, fracture toughness, and relaxation rate, can be tailored according to the specific site in host tissue (Chaudhuri, et al., 2016) (Das, Gocheva, Hammink, Zouani, & Rowan, 2016).

Scaffolds must meet some specific requirements for their architecture and internal structure. It is crucial to have interconnected pore within the bulk scaffolds, to allow transferring of nutrients and oxygen for cell vascularization and proliferation.

Considering the trade-off between printing cost and biological performance, ideal pore size for scaffolds ranges from 200 to 500 with a porosity between 60 and 90% (Liu & Yan, 2018) (Thavornyutikarn, Chantarapanich, Sitthiseripratip, Thouas, & Chen, 2014).

Surface morphology of scaffolds, which affects cell adhesion, can be modified through plasma etching to improve its bioactivity, as well as reformed via other deposition methods (Liu & Yan, 2018).

The numerous 3-D printing techniques available are no further discussed in this essay.

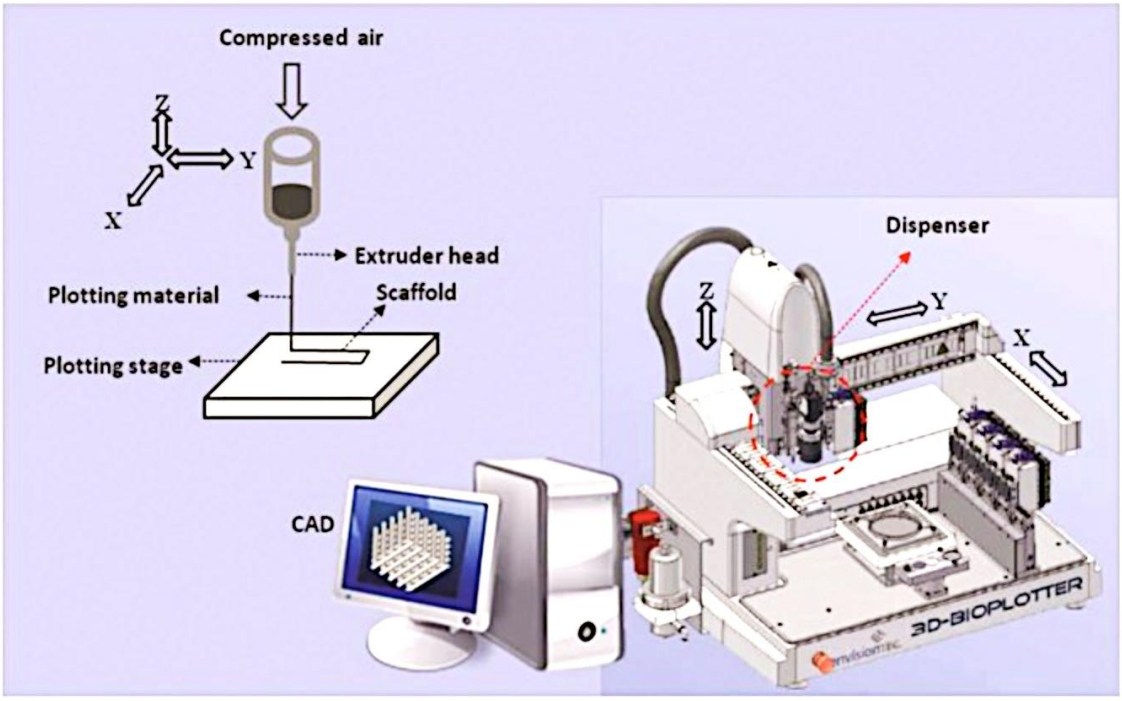


Figure 17: Schematic diagram of a 3-D plotter additive manufacturing fabrication technique. Adapted from Izadifar, Chen, & Kulyk, 2012 with courtesy of Envision TEC: BioPlotter V, 2012.

- Fibrous scaffolds

  Fibrous polymer scaffolds obtained through electrospinning (a fibre production technique capable of producing fibres up to some hundred nm in diameter) are laminar scaffolds, highly promising because of their robust support for damaged cartilage restoring, promotion of cartilaginous tissue growth in vitro and superior mechanical properties (compared with those of homogeneous material) (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

- Porous scaffolds

  Porous scaffolds are 3-D structured characterised by a certain set of cavities for cells to colonise and synthetize the ECM. In several studies hey have showed encouraging results in terms mechanical properties of the wet material, observing degradation based on four stages: quasi-stability, loss of force, loss of weight, fracture (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

- Porous multilayer scaffolds

  Multilayer grafts promote a higher growth of cells and may guide simultaneous regeneration of bone, cartilage and a calcified cartilage intermediate (Kang, Zeng, & Varghese, 2018).

  Porous multilayer scaffolds are advantageous because they require a smaller population of chondrocytes resemble natural cartilage in complexity, enable

mimicking of the mechanical properties of the natural tissue in each layer (see Figure 3), controlling orientation, morphology, and phenotype of seeded chondrocytes (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019). Indeed the calcified layer in cartilage could be filled with calcium phosphate (hydroxyapatite, b-tricalcium phosphate, octacalmium phosphate, etc.) to promote osteointegration and osteinduction of the bone tissue, which is close to subchondral bone (Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019).

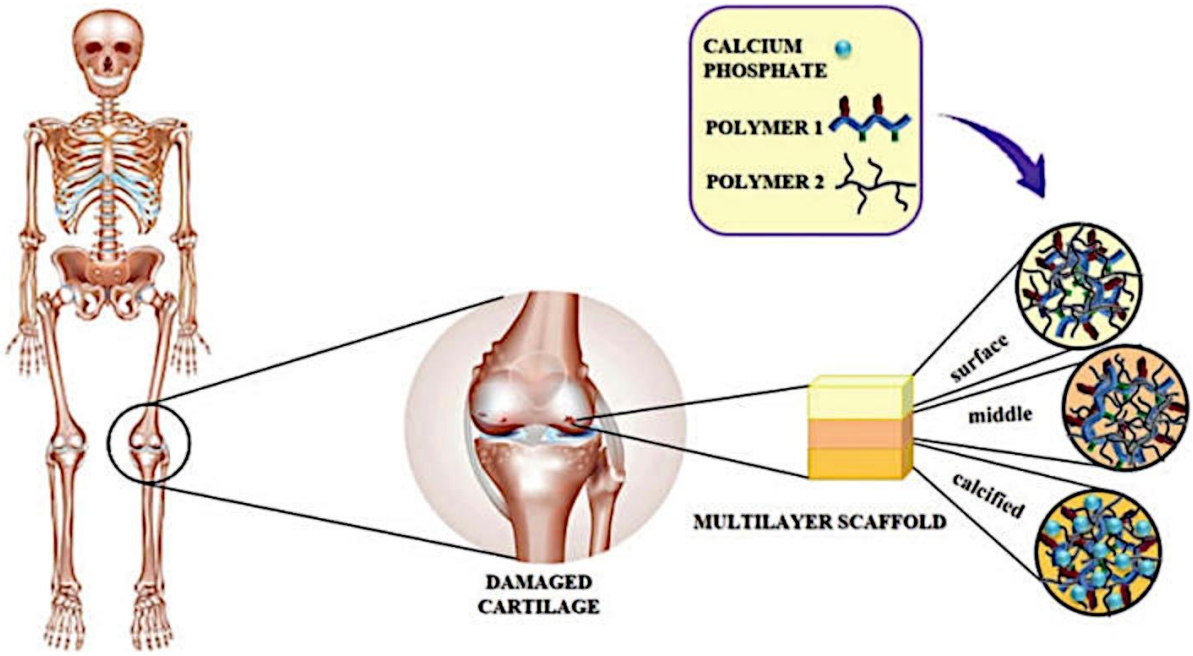


Figure 18: Multilayer scaffolds in human knee joint; the multi-layered layout allows mimicking of cartilage complexity, getting closer to the natural healthy tissue. From Campos, Almirall, Fuentes, Bloem, Kaijzel, & Cruz, 2019.

Experiment section

Materials and methods

Special thanks to Dr. Elena De Angelis (Veterinary sciences Department, Parma University) for the supervision and guidance during all the procedures involved in this experiment.

Animal tissue

- Chondrocytes were isolated from fetlock articular cartilage of regularly slaughtered horses (*Equus caballus*).
- Preparation was performed as follows:
    - Limbs were skinned, cut with a bone saw to remove the hoof and just above the fetlock joint if needed to handle the limb
    - In order to preserve sterility in the joint, limbs were sprayed with denatured alcohol 95%, flambé and brought under flow hood.

Explant

- To preserve sterility, this step was done under flow hood
- Using a scalpel 22, the fetlock joint was opened, evaluated for macroscopic lesions and therefore excluded, repeatedly washed with PBS with fungizone (amphotericin B) and penicillin/streptomycin 2X
- Using a scalpel 11, a full thickness grid with 2-3 mm squares was engraved in the articular cartilage
- The above squares were transferred in a Petri dish of 57 cm^2 (one for each joint used) with 20 ml PBS, then received 2-3 washings with PBS, lastly were added as medium 15ml of D-MEM 4,5 g/L glucose, 25 mM Hepes and fungizone/antibiotic, adding as well 10% FCS.
- Petri dishes were incubated at 37° C and 5% CO2 for 24-48 hours.

Cell isolation

- 2-3 PBS washings
- Using a sterile spoon, squares were transferred in 50 mL test tubes
- Protease 0,1% (0,1 g/100 mL) in D-MEM without FCS was added to the Petri dishes, then they were incubated for 1 hour at 37° C; this step aimed at optimising

digestion of protein nucleus and matrix proteoglycans.

- Protease was removed, the content placed in flasks with con 20 mL of collagenase 0,2% (0,2g/100mL) in D-MEM without FCS, then incubated in a shaking incubator for 2 hours at 37° C (110-120 rpm); this step enables breakdown of collagen network, releasing the chondrocytes.
- Content was filtered with nylon strainer 70 μM (VWR) in order to retain pieces that might not have been well digested, after that another filtration with nylon strainer 40 μM (VWR) was done to retain big cells aggregates.
- The cell suspension so obtained was placed in a 50 mL tubes, 5mL of medium D-MEM with FCS 10% were added to inactivate collagenases; followed centrifugation for 7 minutes at 2000 rpm; supernatant was removed, cells were re-suspended in 5 ml D-MEM with 10% FCS and again centrifuged for 7 minutes at 2000 rpm.
- Supernatant removed, cells were re-suspended in D-MEM with 10% FCS and counted using a Bürker chamber and trypan Blue to exclude dead cells.
- At this point isolated chondrocytes could either be seeded of frozen at -80° C and stored in liquid nitrogen at -196° C for long periods of time.

Culture systems

- 3-D printed chitosan and chitosan + HA scaffolds were produced by professor Bettini (Food and drug Sciences, Parma University).
- Chondrocytes were seeded in 0,25 cm^2 scaffolds and put in 96-well plates; target chondrocyte density was 800,000 cells/scaffold in 50 μL.
- Plates were incubated at 37° C for 3 hours.
- 0,2 mL of D-MEM with 10% FCS were added
- Cultures were incubated for 2 and 4 weeks at 37°C and 5% CO_2, medium was changed every 3-4 days for all duration of experiment.
- After 2 and 4 weeks, following analyses were performed:
    - Histology with different staining techniques:
        - Haematoxylin and eosin (HE)
        - Masson's trichrome (MT)
        - Alcian blue pH 2,5 (AB)
        - Collagen II immunohistochemistry (IHC)

Preparation for histology

- At 2 and 4 weeks cultured scaffolds received PBS washing and then fixation in paraformaldehyde (PFA) 4% in PBS for 1 hour at room temperature (RT).
- PFA was removed, followed washing for 3 times, for 5 minutes each, with spring water.
- In order to facilitate scaffold handling, a solution of agarose 2% in distilled water (DW) was added and left to gel.
- Scaffolds were put in embedding cassettes and processed as follows:
    - Dehydration, through increasing concentrations of alcohol
    - Clearing, through dipping in xylol.
    - Inclusion in paraffin; paraffin can penetrate thanks to previous clearing in xylol (paraffin is soluble in xylol) in dehydrated samples.
    - Solidification at RT, then on cooling plate.
    - Using a microtome, 5 μM sections were cut from samples, put on microscope slides and kept overnight in oven at 60° C to facilitate adhesion; after that, samples were ready for staining.

Staining

Haematoxylin and eosin stain (HE)

Probably the most widely used histologic staining, HE simply yet effectively portrays tissue components and architecture.
Haematoxylin dies nuclei in blue-purple and eosin dies the ECM and cytoplasm in red-pink, while other structured might stain with intermediate shades.
Procedure:

- Xylol for 30 minutes to remove paraffin
- Rehydration
    - Passages of 5 minutes each in decreasing concentrations of alcohol until DW
- Staining
    - Mayer's Haematoxylin for 5 minutes
    - Rinse with spring water for 10 minutes
    - Eosin for 1 minute
    - Quick rinse with spring water

- Dehydration
    - Alcohol at increasing concentrations
- Mounting
    - Clearing in xylol for few minutes
    - Coverslip positioning using a synthetic mounting medium Bio Mount (Bio-Optica)

Masson's trichrome stain

Stain of choice for connective tissue ECM; it stains nuclei in black, cytoplasm in red, collagen in blue.

In this experiment done using a kit (Bio-Optica) for MT with aniline blue:

- Reagent A: Weigert's iron haematoxylin
- Reagent B: Weigert's iron haematoxylin
- Reagent C: alcoholic picric acid solution
- Reagent D: Ponceau B solution
- Reagent E: phosphomolybdic acid solution
- Reagent F: Masson's aniline blue

Procedure:

- Xylol as in HE
- Rehydration as in HE
- Protocol from kit:
    - 6 drops of reagent A, then 6 drops of reagent B, left on for 10 minutes
    - No rinsing; dripping and addition of 10 drops of reagent C, left on for 4 minutes
    - Quick rinse (3-4 seconds) in DW (slides should remain yellowish); addition of 10 drops of reagent D, left on for 4 minutes
    - Rinse in DW; addition of 10 drops of reagent E, left on for 10 minutes
    - No rinsing; dripping and addition of 10 drops of reagent F, left on for 5 minutes
    - Rinse in DW
- Quick dehydration in increasing concentrations of alcohol (waiting about 1 minute in alcohol 100%)
- Mounting as in HE

Alcian blue stain

Alcian dyes bind polyanions of acid, sulphated mucopolysaccharides through saline bounds; pH 2,5 Alcian blue ionizes most acid mucins.
Alcian stains in light blue collagen, proteoglycans and agarose.
Nuclear fast red stains nuclei in bright pink-red.
Stains used:

- Alcian blue pH 2.5, Mowry's solution (Bio-Optica 05-M26003)
- Nuclear fast red 0,1% in 2,5 g of aluminium sulphate, brought to 100 mL with DW

Procedure:

- Rehydration as in HE
- Dipping in Alcian blue pH 2.5 for 30 minutes
- Rinse under spring water for 5 minutes
- Application of nuclear fast red solution for 3 minutes
- Rinse under spring water for 2 minutes
- Quick dehydration:
    - 30 seconds in 95% alcohol
    - 1 minute in 100% alcohol
    - 2 minutes in xylol I
    - 2 minutes in xylol II
- Fixation as in HE

Immunohistochemistry

Immunohistochemistry is an immunostaining technique involving selective antigen (proteins) identification in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vara & Miller, 2014).
There are two methods, direct and indirect: the direct method involves direct use of a single primary antibody (meant to bind tissue antigens), whilst the indirect method involves use of a primary (meant to bind tissue antigens) and a secondary (meant to bind the primary) antibody.

Among indirect methods, to enhance sensibility, signal amplification can be obtained, using chromogenic detection *via* different techniques (Immunohistochemistry (IHC) Detection, 2019). In this experiment, Avidin-Biotin Complex (ABC) method was used.

In the avidin-biotin complex (ABC) method, biotin conjugated secondary antibodies link tissue-bound primary antibodies with an avidin-biotin-peroxidase complex. The avidin molecule contains four binding sites for biotin. These binding sites enable complexes to form, where avidin molecules are linked together via the enzyme. A colourless substrate is then added and subsequently converted to a brown product by the peroxidase enzyme to mark the target antigen. The large complexes formed in the ABC method contain multiple copies of the reporter enzyme. Because of the high enzyme-to-antibody ratio, the ABC method increases sensitivity compared to direct conjugation of enzyme to the secondary antibody; principles are portrayed in Figure 1 (Immunohistochemistry (IHC) Detection, 2019).

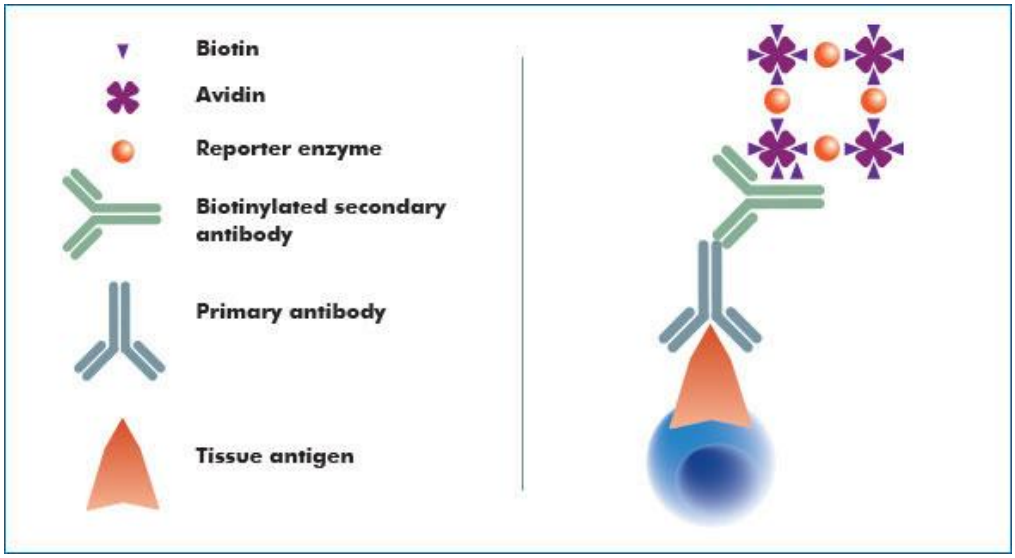


Figure 19: Principle of ABC method; from Immunohistochemistry (IHC) Detection, 2019.

IHC was directed towards collagen II, typical of hyaline cartilage, to assess synthesis. Haematoxylin stains nuclei in blue; due to interaction of various reagents, collagen should appear in brown-red after DAB reaction and ECM in light pink.

Procedure for collagen II IHC:

- Hydration as in HE
- ABC method:
    - Washing in PBS 1X pH 7,4 to stabilize samples
    - Samples covered in hyaluronidase at 2% in PBS for 30 minutes at 37° C
    - Re-washing in PBS

- Exogenous peroxidases were blocked putting H2O2 3% in DW for 12 minutes at RT
- Washing in PBS
- Samples were covered with PBS/BSA 1% for 30 minutes at RT to block specific antigens
- Addition of primary antibody anti-collagen II 1:100 (IgG, polyclonal orb156420 Biorbyt)
- Incubation ON at 4° C in wet environment
- Washing in PBS
- Addition of secondary antibody, biotin-conjugated, meant to bind primary antibody enabling visualisation; left of for 1 hour at RT
- Some washings in PBS
- For revelation was used Vector Kit (vector laboratories PK7800): Vector Kit contains avidin-biotin complex with peroxidase, conjugated with specific marker (in this case, peroxidase enzyme); this complex allows amplified formation of marked aggregates:
    - Colour development covering slides with DAB (3,3'-diaminobenzidine, it is a chromogen), which binds aggregates
    - Addition of H_2O_2 left on for 3 minutes; DAB in presence of peroxidase produces a brown-orange precipitate, insoluble in alcohol
    - Rinse with spring water

- Counterstaining with Mayer's Haematoxylin to highlight the nuclei
- Quick dehydration as in HE
- Mounting as in HE

Results

Slides were looked under light microscope and pictures were shot with Nikon Digital Sight system.

Scaffold structure

The scaffolds used in this experiment are 3-D, round, porous structures (see in figure 20 and 21). Above a basal chitosan layer, the printer always lays filaments horizontally. Parallel filaments are affixed in a second layer, then the printer changes direction, adding filaments perpendicularly to the first ones, and so on, until achieving five layers. The final product resembles the scaffold in figure 21.

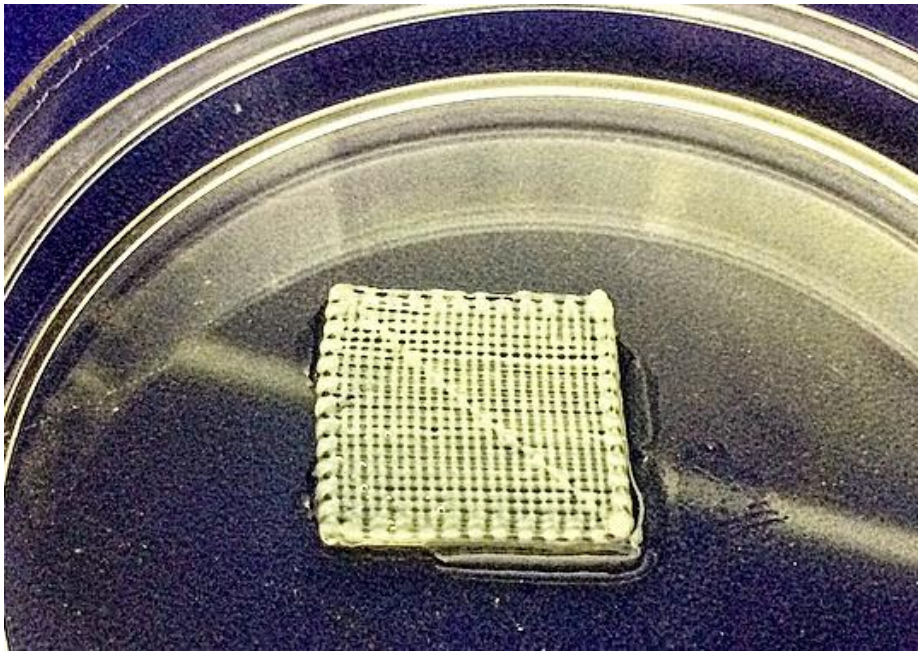


Figure 20: Square version of the scaffold used in this experiment. Scaffolds are printed in this square shape and then cut in a round shape to fit in the dwells for culture.

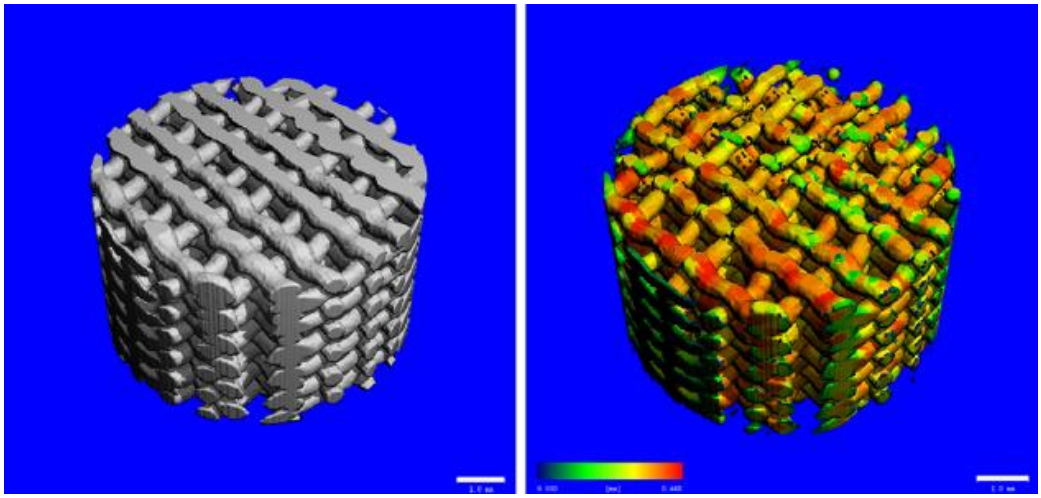


Figure 21: A 3-D printed scaffold similar to the one used in this experiment (the one portrayed above has more layers). Adapted from Yang, Yang, Ao, & Tang, 2016.

Scaffold architecture was based on data available in literature on AC TE, with a fixed porosity of 200 μm. Further data on filament dimension were not available. Main features are summarized below, in table 7.

Scaffold characteristic	**Description**
Concentration	6% weight/volume
Dimension (diameter)	15 mm
Inferior film	Chitosan 0,6 mm
Structure	5 alternate layers with 200 μm pores and film

Table 7: Chitosan and chitosan + HA scaffolds features.

Cells seeded in the scaffold tend to attach to the chitosan/chitosan + HA filaments, expected to fill the pores with ECM (figure 22).

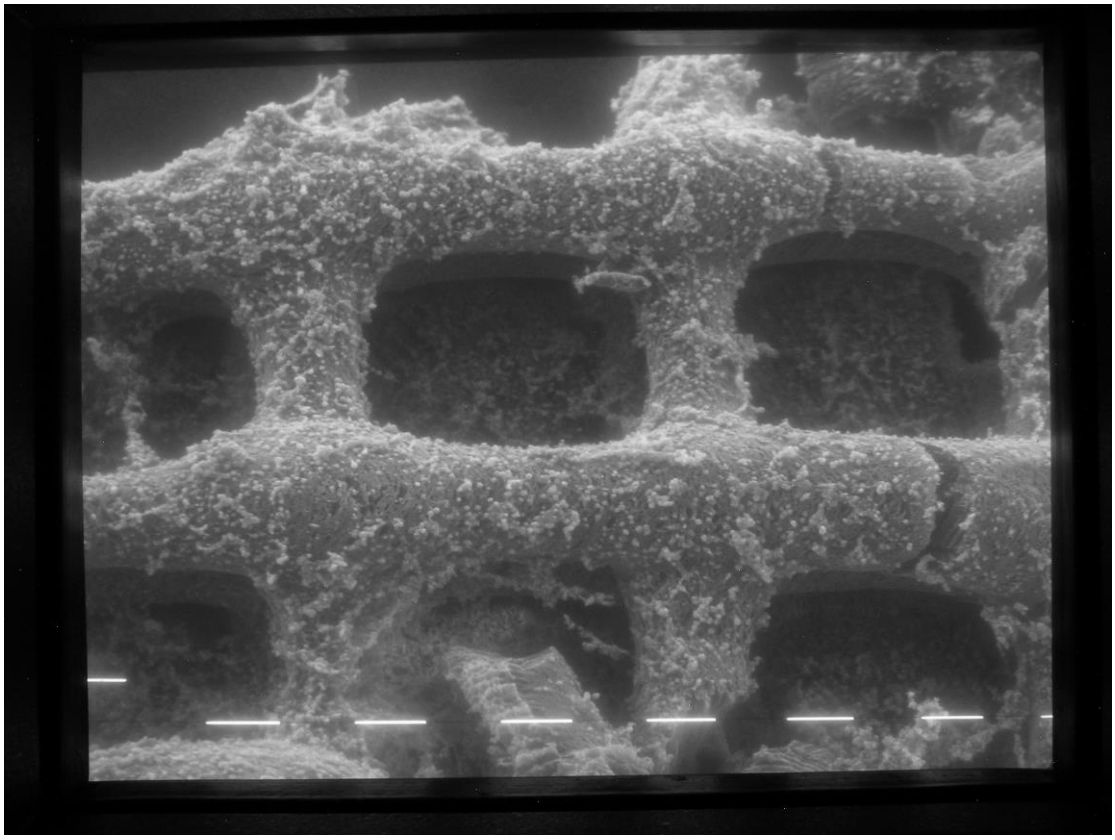


Figure 22: SEM image of scaffold seeded with chondrocytes at 2 weeks. Note the 3-D arrangement of the filaments. Cells tend to concentrate very close to the filaments. Deeply the basal chitosan layer, covered with cells, can be seen.

It should be taken into account that histology always presents virtually bi-dimensional sections. Therefore, the engineered tissue and scaffold 3-D architecture might seem apparently lost. Depending on the orientation of the scaffold under the microtome, fibres might be sectioned: often transversally, giving them a round appearance; rarely longitudinally, giving them a long, hair-like look; sometimes in an oblique way, giving them an oblong, ellipsoidal aspect. Thus most sections will present a couple of

transversally sectioned filaments, on top of each other, with perhaps some obliquely sectioned ones too (see figure 23).

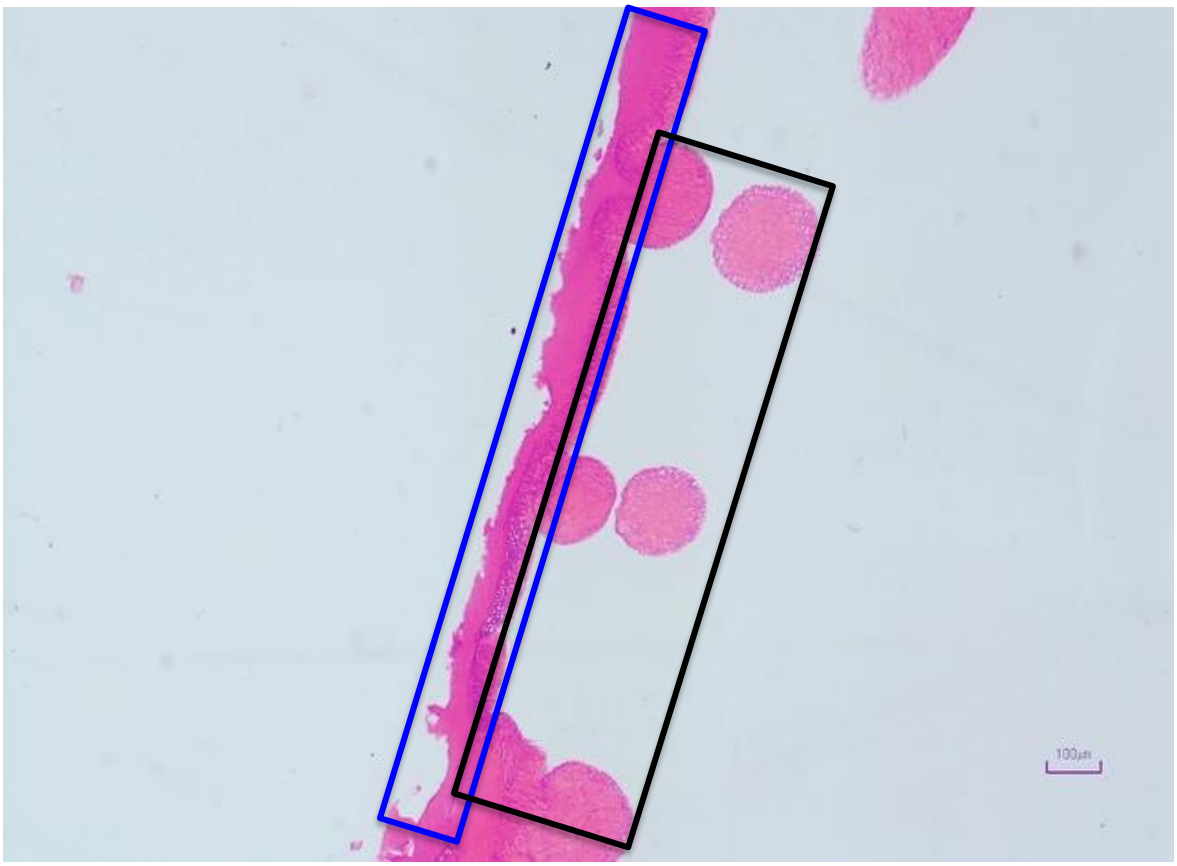


Figure 23: HE stained empty scaffold, 4X magnification. Note the basal chitosan layer (blue rectangle) and the transversally sectioned fibres (black rectangle); on top right there is a fragment that is obliquely sectioned.

Histology commentary

Haematoxylin and eosin stained samples at 2 weeks

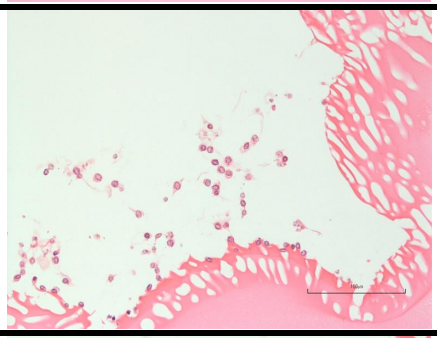
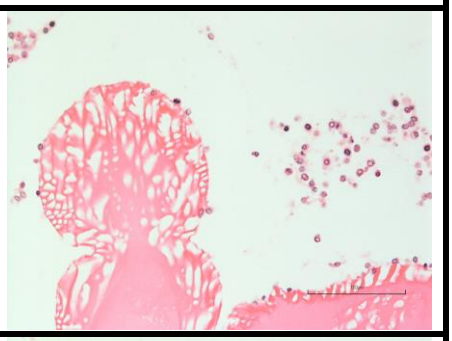
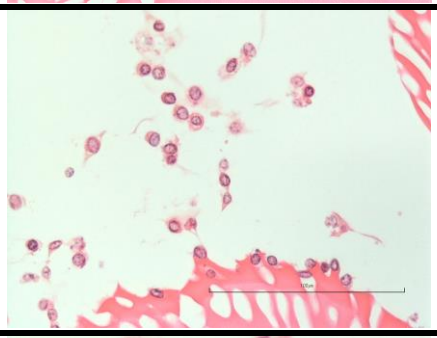
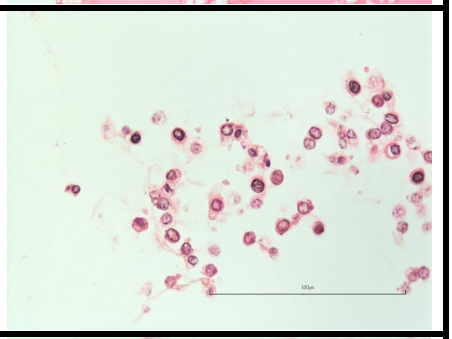
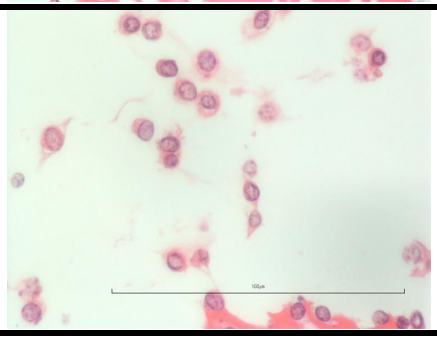
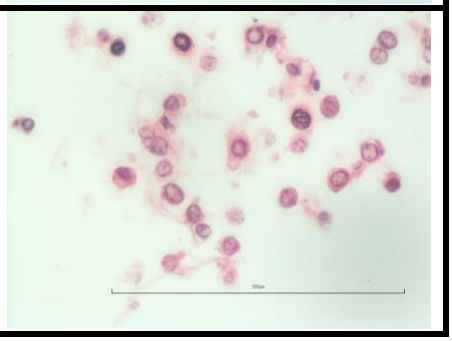
	CHITOSAN	CHITOSAN + HA
4X		
10X		
20X		
40X		
60X		

Table 8: HE stained samples at 2 weeks

A. Chitosan

- Scaffold appearance and colonisation

Scaffold appears highly eosinophilic.

At 4X magnification (see above in Table 1) chitosan scaffold appears intact and is evenly colonised by cells, with several cells in contact with the scaffold itself.

As it can be noticed in 10X and following magnifications, some cells are positioned deeply into the scaffold layers and dwell in its porosities (marked with arrowheads in figures 24 and 25).

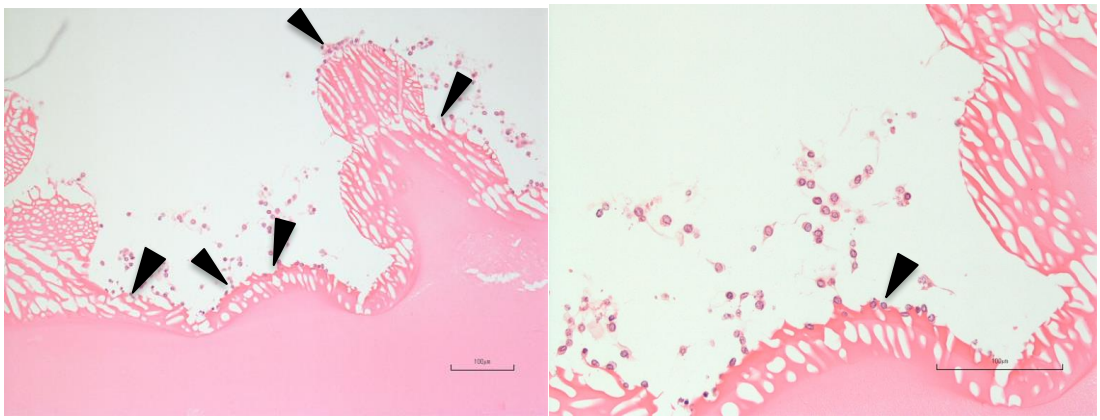


Figure 24 (left): Chitosan HE 10X and Figure 25 (right): Chitosan HE 20X. Black arrowheads indicate cells dwelling porosities. Cell distribution

Cells are mostly located in large, widespread aggregates of about 10-20 cells/100 μm^2.

- Quality and quantity of ECM

Some eosin-stained filaments (marked with black arrowheads in figure 26) originate from several cells; due to their filamentous aspect, they might be compatible with collagen fibres.

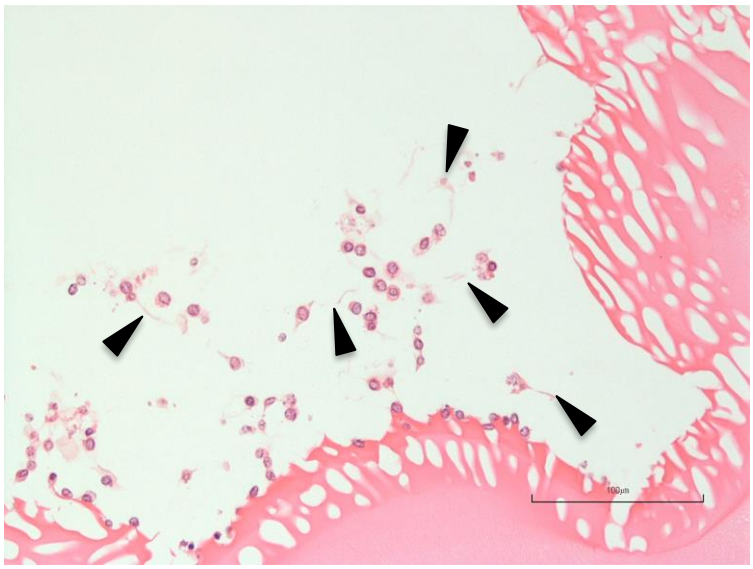


Figure 26: Chitosan HE 20X; black arrowheads mark eosinophilic filamentous material, probably collagen.

- Cell morphology

Most cells appear rounded (black arrowheads in figure 27) or teardrop shaped (red arrowhead in figure 27).

No major signs of cell degeneration can be noticed. Few nuclei appear denser and heavily stained (blue arrowhead in figure 27).

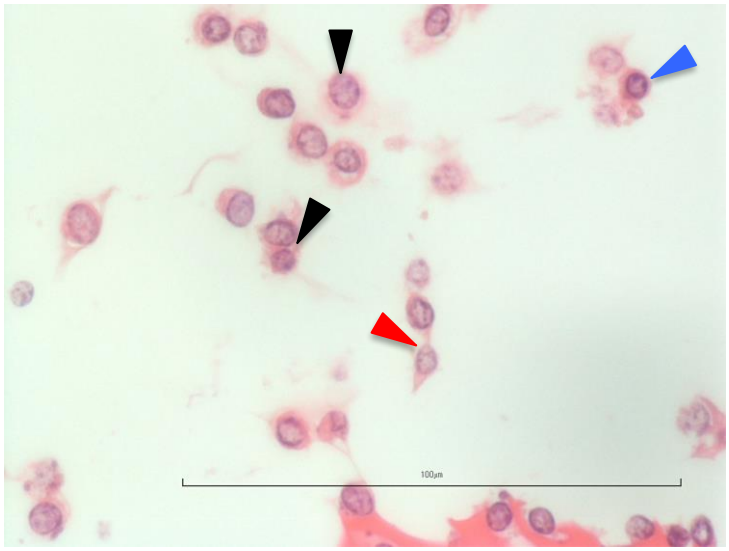


Figure 27: Chitosan HE 60X. Black arrowheads indicate round chondrocytes, red arrowhead indicated teardrop shaped chondrocyte, blue arrowhead indicates a cell with a dense nucleus.

B. Chitosan + HA

- Scaffold appearance and colonisation

The scaffold seems intact and unevenly colonised by cells, with some cells in contact with the scaffold itself.

- Cell distribution

Cells tend to distribute in large, dense aggregates of about 30-50 cells/100 μm^2.

- Quality and quantity of ECM

Most cells are surrounded by eosin-stained filamentous material (marked with black arrowheads in figure 28).

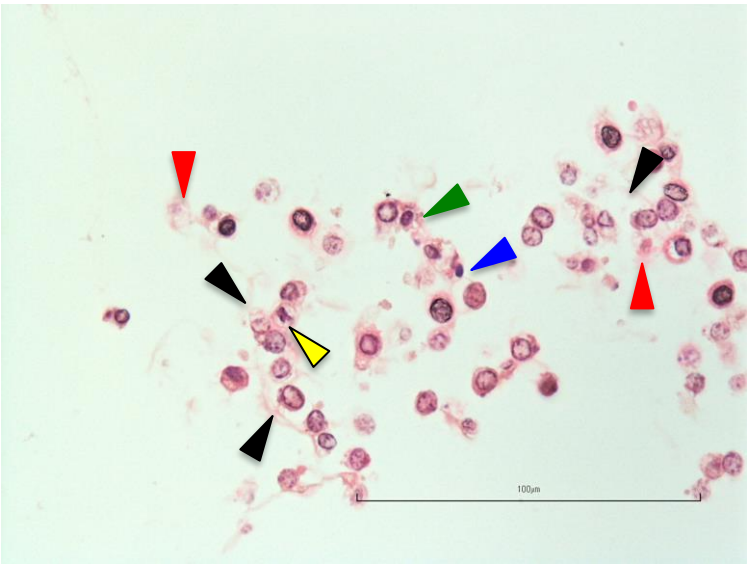


Figure 28: Chitosan + HA HE 40X. Black arrowheads: filamentous material; red arrowheads: perhaps degenerated cells; green arrowhead: traces of vacuolisation; yellow arrowhead: abnormal nuclear shape; blue arrowhead: pyknosis.

- Cell morphology

Cells have a rounded or teardrop shape. Cell morphology is not always even, with discrepancies in appearance (maybe degenerated cells marked with red arrowheads above in figure 28) and dimension; variation is also seen in nuclear shape, density and intensity (pyknosis marked above in figure 28 with a blue arrowhead, abnormal shape marked with a yellow arrowhead), cytoplasm homogeneity (traces of vacuolisation, marked with a green arrowhead in figure 28).

Haematoxylin and Eosin stained samples at 4 weeks

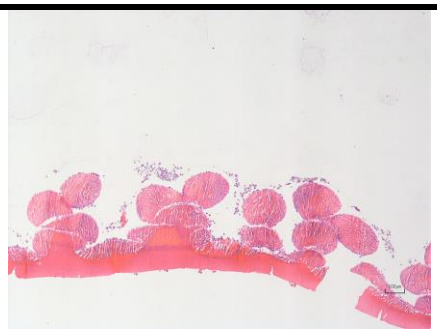
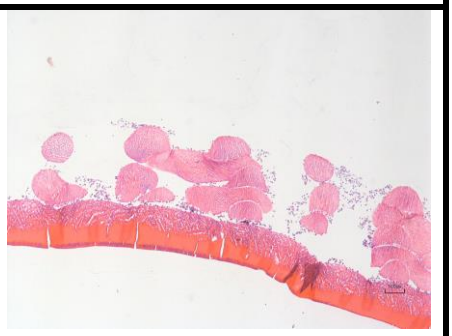
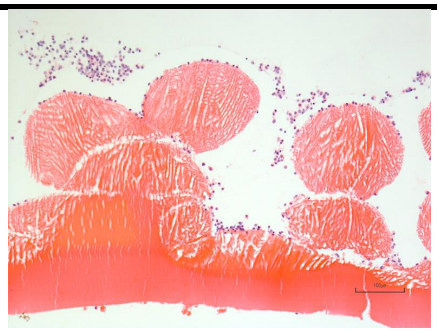
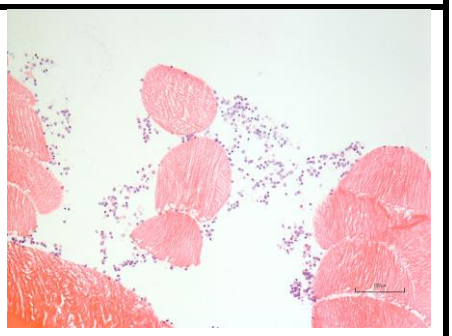
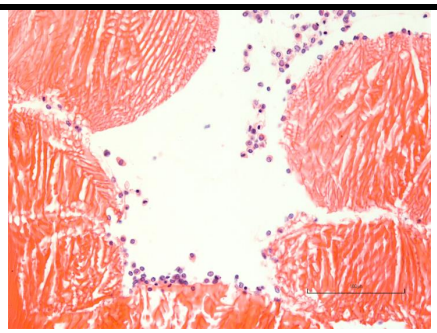
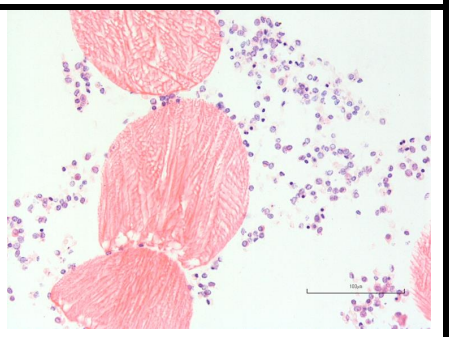
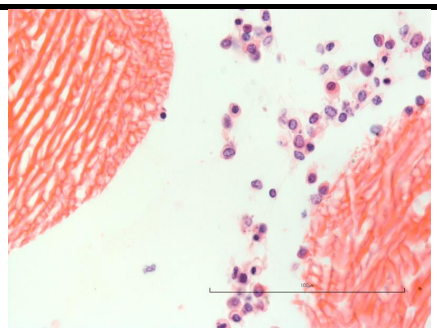
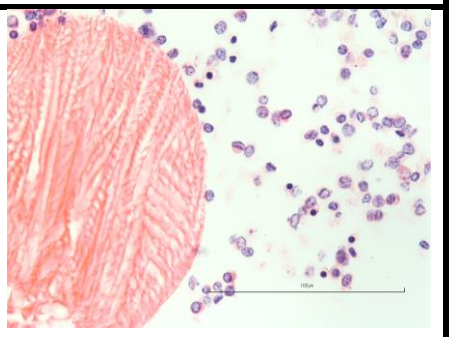
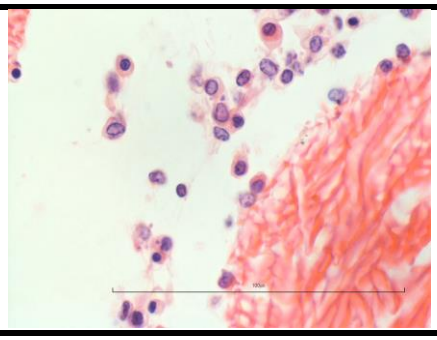
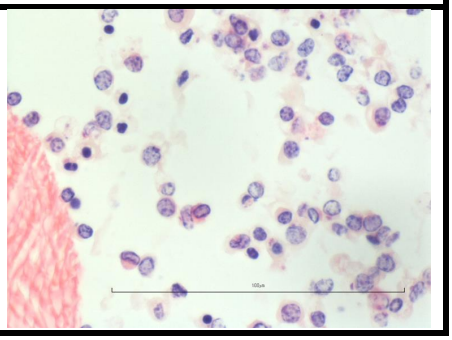
	CHITOSAN	CHITOSAN + HA
4X		
10X		
20X		
40X		
60X		

Table 9: HE stained samples at 4 weeks

A. Chitosan

- Scaffold appearance and colonisation

Scaffold seems intact with very few fragmentations due to microtome sectioning. Cells colonised the scaffold at all levels, often somewhat at a distance, some dwell into porosity (marked with black arrowheads in figure 29).

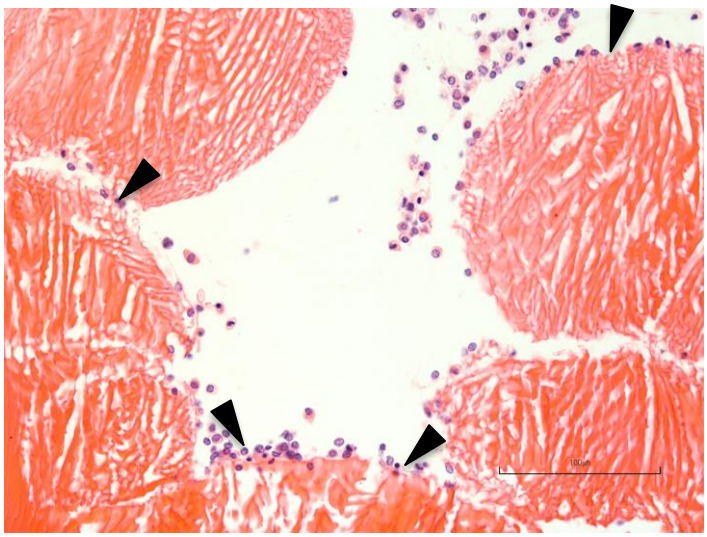


Figure 29: Chitosan HE 20X. Black arrowheads indicate some cells dwelling in scaffold porosities.

- Cell distribution

Cells tend to present in dense large aggregates of 20-40 cells/100 μm^2, unevenly distributed, with most cells in the superficial part of the scaffold (blue rectangle in figure 30).

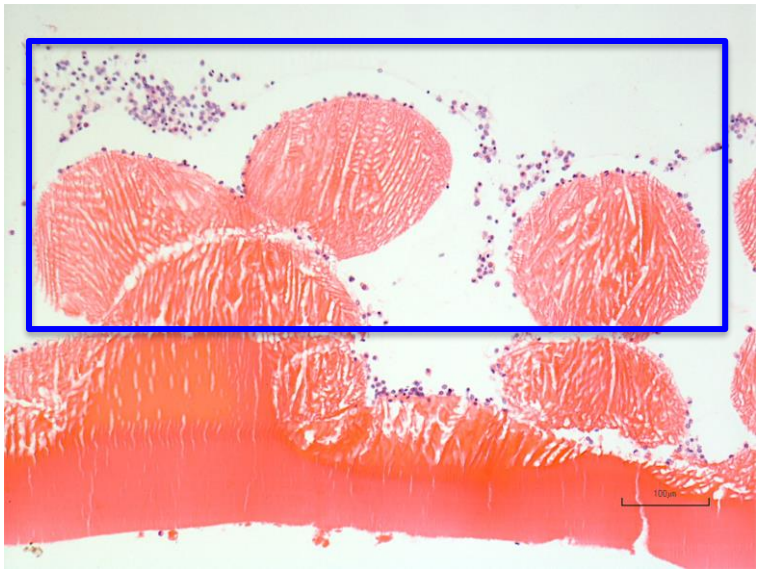


Figure 30: Chitosan HE 10X. Blue rectangle surrounds major cell aggregates in the superficial part of the scaffold.

- Quality and quantity of ECM

Most cells are surrounded by eosin-stained filamentous material.

- Cell morphology

Cells have a rounded or teardrop shape. Cell morphology is not always even, with some smaller cells and pyknotic nuclei (black arrowheads in figure 31).

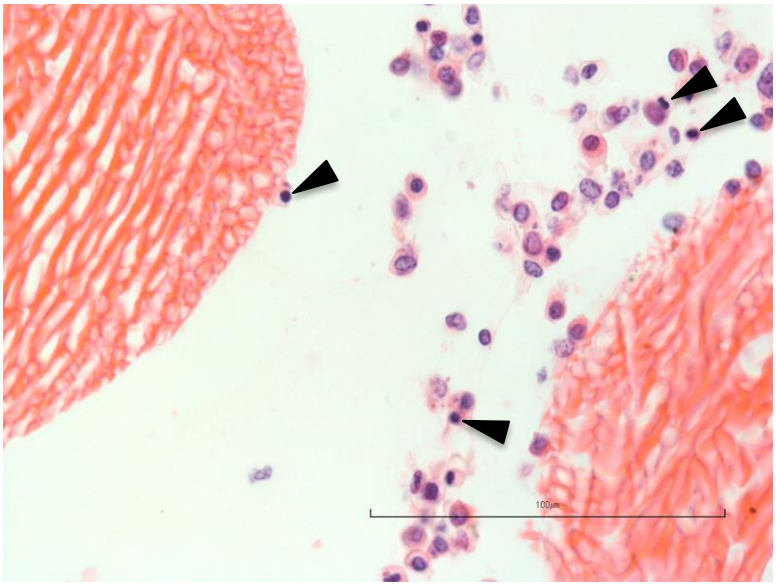


Figure 31: Chitosan HE 40X. Black arrowheads indicate nuclear pyknosis.

B. Chitosan + HA

- Scaffold appearance and colonisation

Scaffold seems intact with very few fragmentations due to microtome sectioning. Cells colonised the scaffold at all levels, getting close to its structure, few cells dwell into porosity.

- Cell distribution

Cells present in large widespread-packed aggregates of 50 cells/100 μm^2 and tend to be located deeper in the scaffold and closer to its structure (red rectangle in figure 32) compared to the only chitosan scaffold.

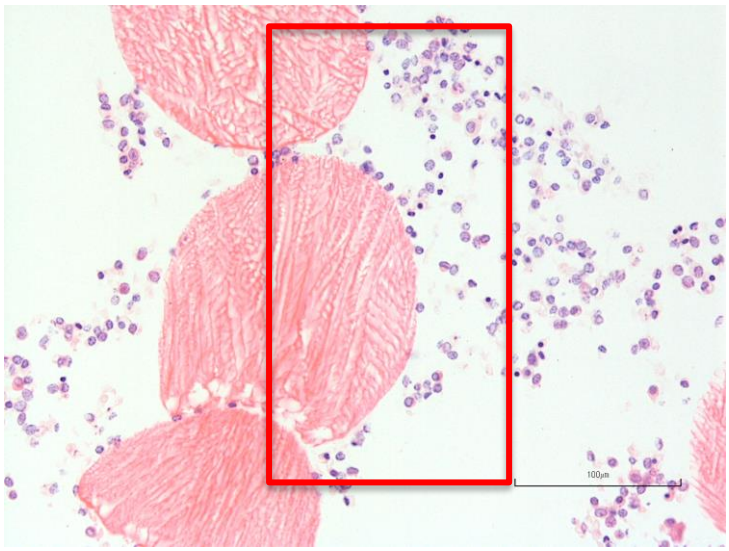


Figure 32: Chitosan + HA HE 20X. Red rectangle indicates the tendency of cells to aggregate close to the scaffold structure.

- Quality and quantity of ECM

Most cells are surrounded by eosin-stained filamentous material.

- Cell morphology

Cells have a rounded or teardrop shape. Some cells are smaller and have pyknotic nuclei (marked as black arrowheads in figure 33); some degenerated cells are seen (red arrowheads in figure 33).

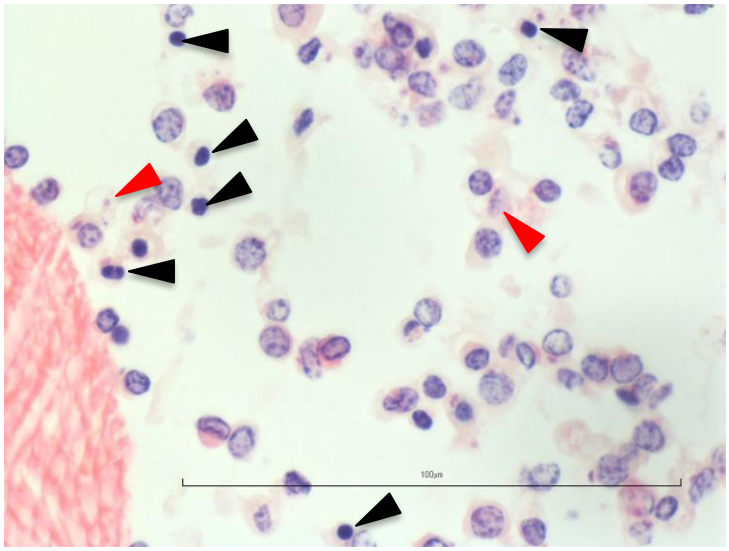


Figure 33: Chitosan + HA HE 60X. Black arrowheads indicate nuclear pyknosis. Red arrowheads indicate degenerated cells.

Masson's trichrome stained samples at 2 weeks

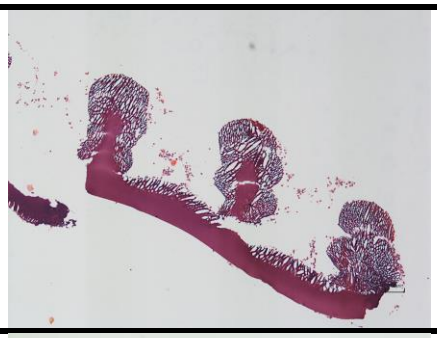
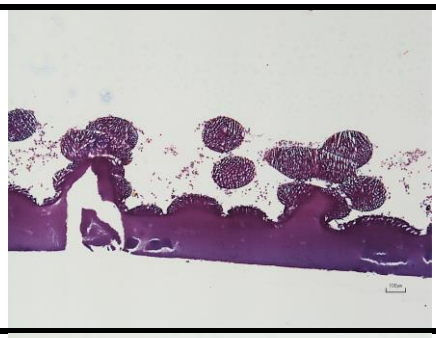
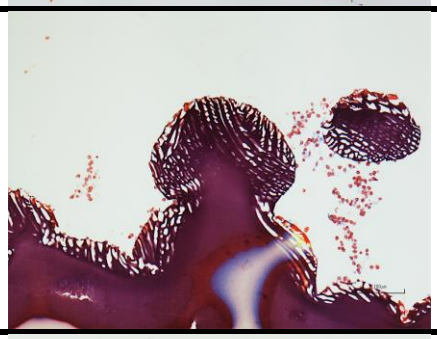
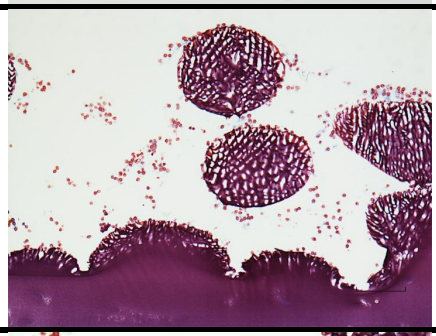
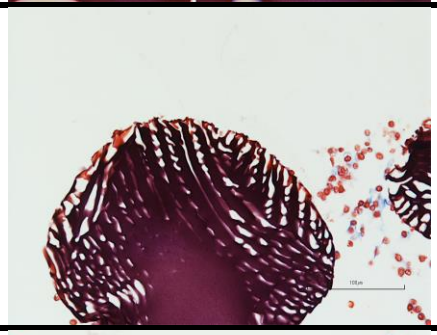
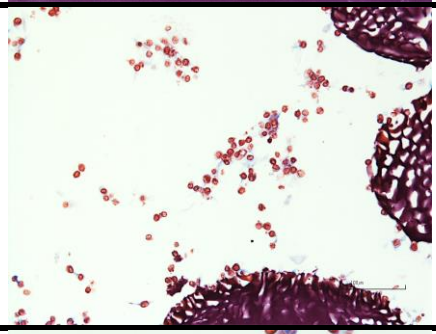
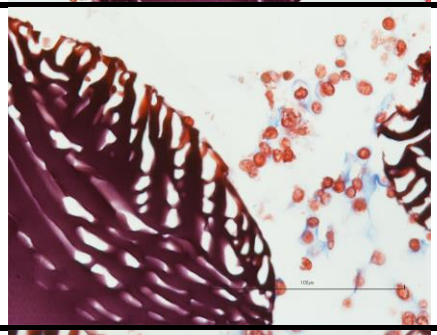
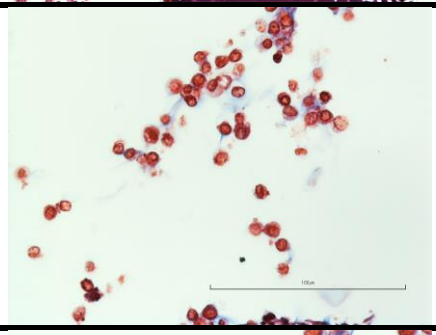
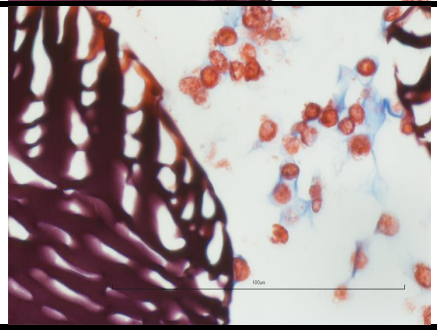
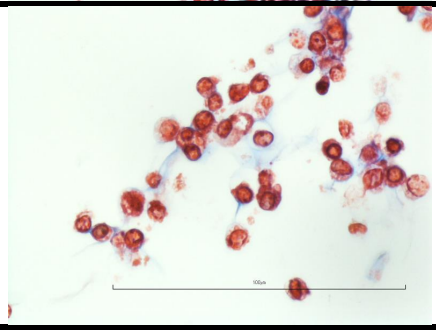
	CHITOSAN	CHITOSAN + HA
4X		
10X		
20X		
40X		
60X		

Table 10: MT stained samples at 2 weeks

A. Chitosan

- Scaffold appearance and colonisation

Scaffold is stained in deep purple-magenta in MT. It seems intact with some fragmentations due to microtome sectioning. A relatively small cell population colonised the scaffold at all levels, few cells dwell into porosity.

- Cell distribution

Cells present in medium-sized widespread aggregates of 20-40 cells/100 μm^2

- Quality and quantity of ECM

Almost all cells are surrounded by blue-stained filamentous material, more abundant and intense in certain cell aggregates (marked as black arrowheads in figure 34).

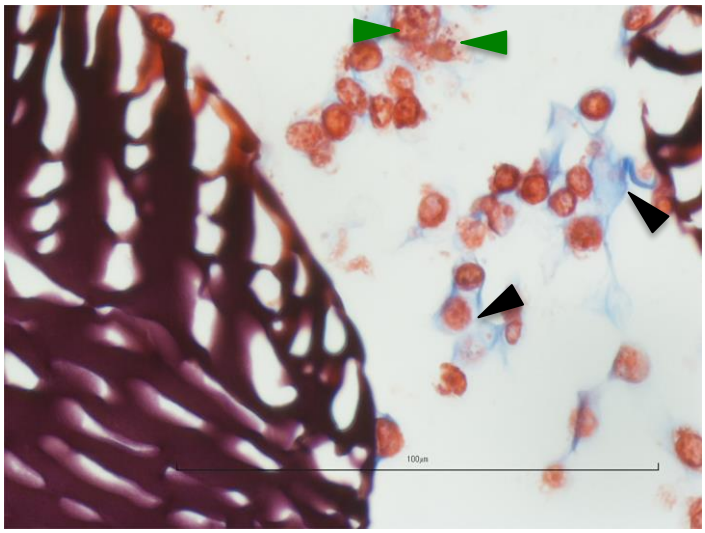


Figure 34: Chitosan MT 60X. Black arrowheads indicate blue-stained filamentous material. Green arrowheads indicate acidophilic cytoplasmatic granularity.

- Cell morphology

Cells have a rounded or teardrop shape. The cytoplasm presents some acidophilic granularity in several cells (marked with green arrowheads above in figure 34).

B. Chitosan + HA

- Scaffold appearance and colonisation

Scaffold appears intact with few fragmentations due to microtome sectioning. Cells colonised the scaffold at all levels, many took contact with scaffold structures or dwelled in porosities (red rectangles in figure 35).

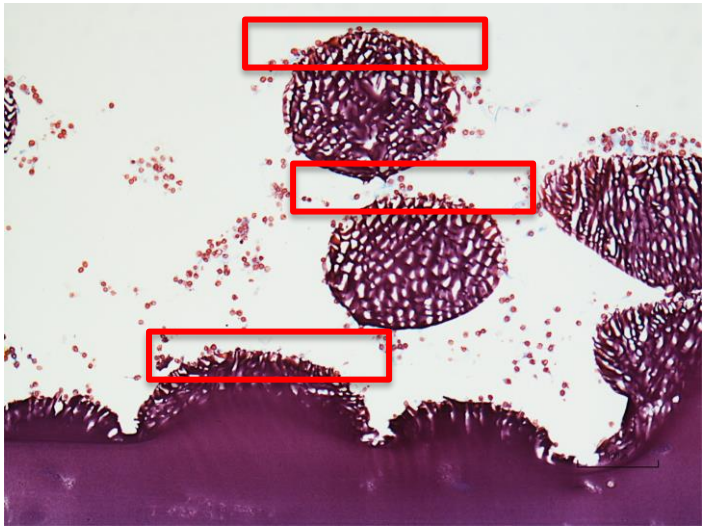


Figure 35: Chitosan + HA MT 10X

- Cell distribution

Cells are organised in large widespread to densely packed aggregates of 30-40 cells/100 μm^2.

- Quality and quantity of ECM

Most cells are surrounded by pale, blue-stained filamentous material.

- Cell morphology

Cells have a rounded or teardrop shape. The cytoplasm presents some acidophilic granularity in several cells (black arrowheads in figure 36). Few exhibit pyknotic nuclei (blue arrowhead in figure 36). Perhaps there are as well some degenerated cells (green arrowheads in figure 36).

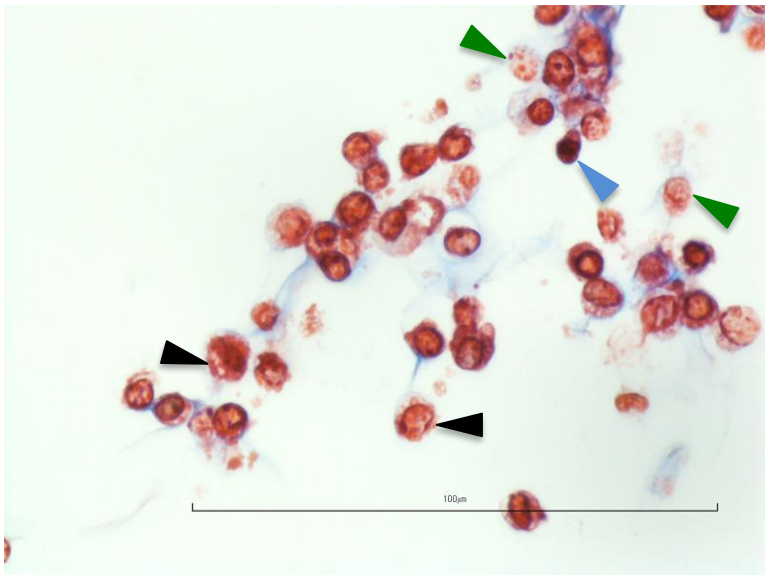


Figure 36: Chitosan+HA MT 60X. Black arrowheads: acidophilic cytoplasmatic granularity. Blue arrowhead: nuclear pyknosis. Green arrowheads: degenerated cells.

Masson's trichrome stained samples at 4 weeks

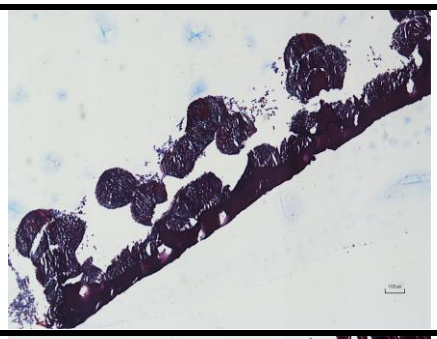
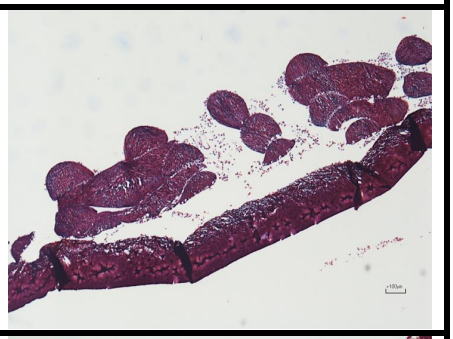
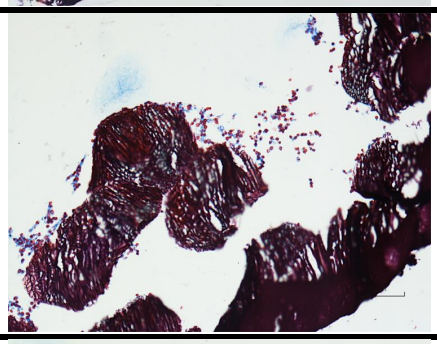
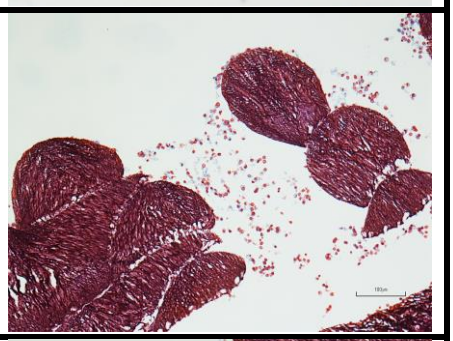
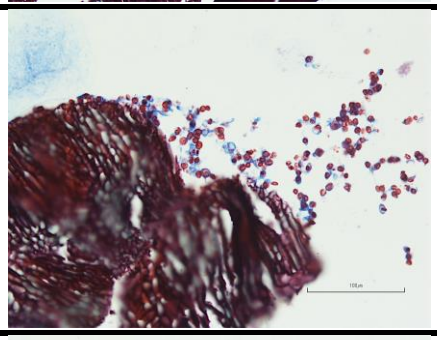
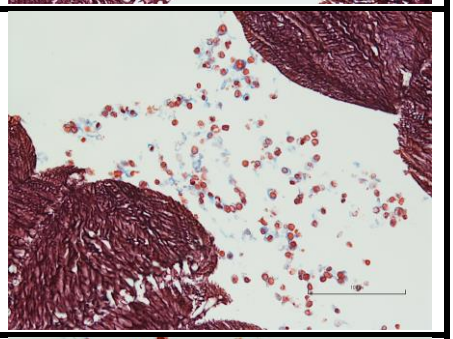
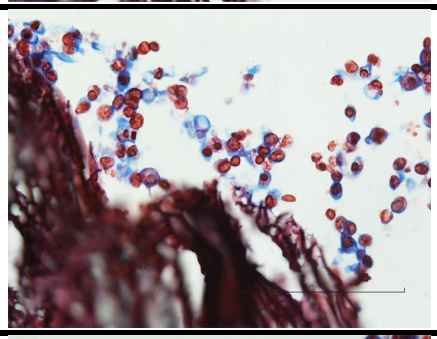
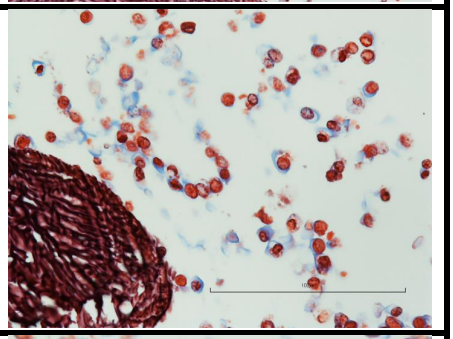
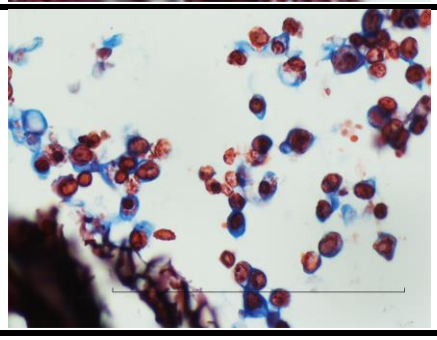
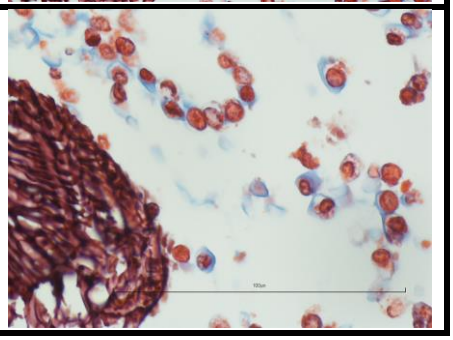
	CHITOSAN	CHITOSAN + HA
4X		
10X		
20X		
40X		
60X		

Table 11: MT stained samples at 4 weeks

A. Chitosan

- Scaffold appearance and colonisation

Scaffold seems overall intact, with some fragmentations due to microtome sectioning especially in the basal layers. A medium cell population colonised the scaffold at all levels, with the vast majority in superficial-medium part (red rectangle in figure 37), close to the structure and few cells residing into the scaffold porosities (blue rectangle in figure 37).

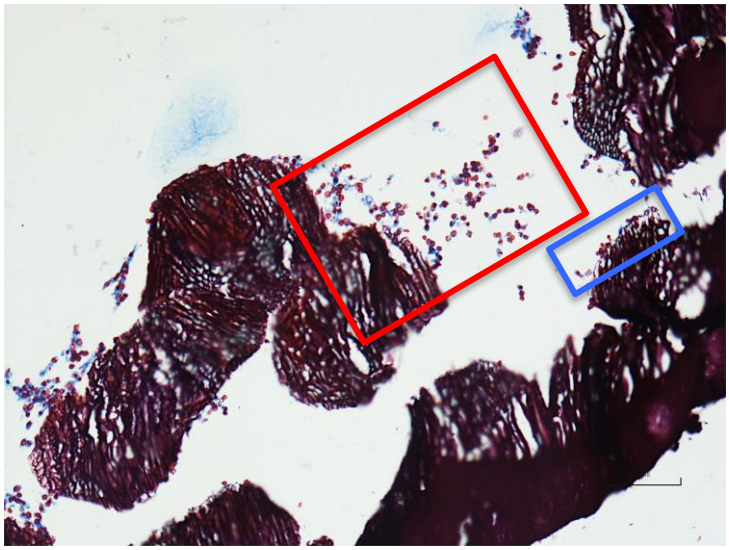


Figure 37: MT Chitosan 10X. Red rectangle surrounds vast majority of cells aggregated in the superficial part of the scaffold; blue rectangle indicates cells dwelling in scaffold porosities.

- Cell distribution

Cells present in small, quite packed aggregates of 40-50 cells/100 μm^2

- Quality and quantity of ECM

Almost all cells are surrounded by intense, rich blue-stained filamentous material, particularly abundant (some examples marked as black arrowheads in figure 38).

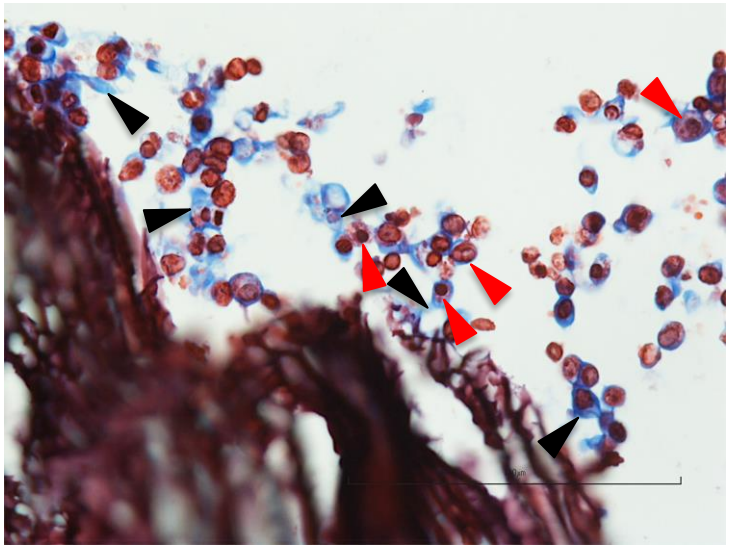


Figure 38: Chitosan MT 40X. Black arrowheads: blue-stained filamentous material. Red arrowheads: cells different in nuclear shape and/or dimension.

- Cell morphology

Cells have a rounded or teardrop shape. The cytoplasm presents some acidophilic granularity in few cells; some cells differ in dimension and nuclear shape (marked as red arrowheads above in figure 38).

B. Chitosan + HA
    - Scaffold appearance and colonisation

Scaffold appears intact with few fragmentations due to microtome sectioning and some overlapping in basal layers due to handling issues. Cells colonised the scaffold at all levels (4X), evenly distributed yet slightly more abundant in the medium part (red rectangle in figure 39).

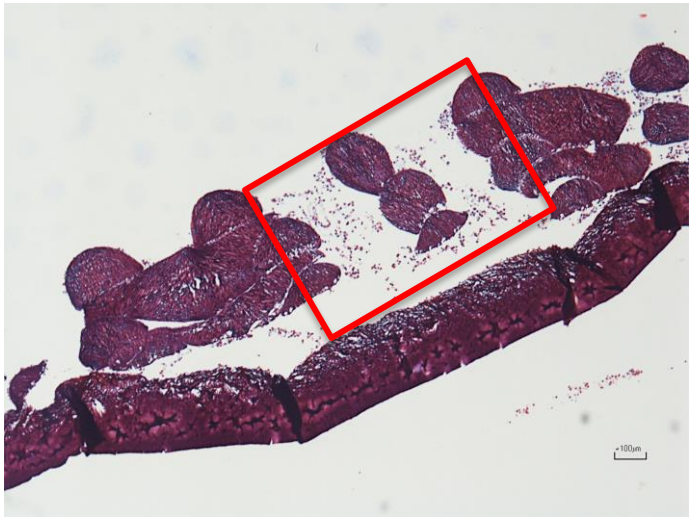


Figure 39: Chitosan + HA MT 4X. Red rectangle underlines cell aggregates concentrating in the medium scaffold layers.

- Cell distribution

Cells are mostly organised in large widespread aggregates of 20-30 cells/100 μm^2 (20X).

- Quality and quantity of ECM

Most cells are surrounded by abundant blue-stained filamentous material.

- Cell morphology

Cells have a rounded or teardrop shape. The cytoplasm presents some acidophilic granularity in several cells (black arrowheads in figure 40). Few exhibit different dimension, shape and pyknotic nuclei (blue arrowheads in figure 40).

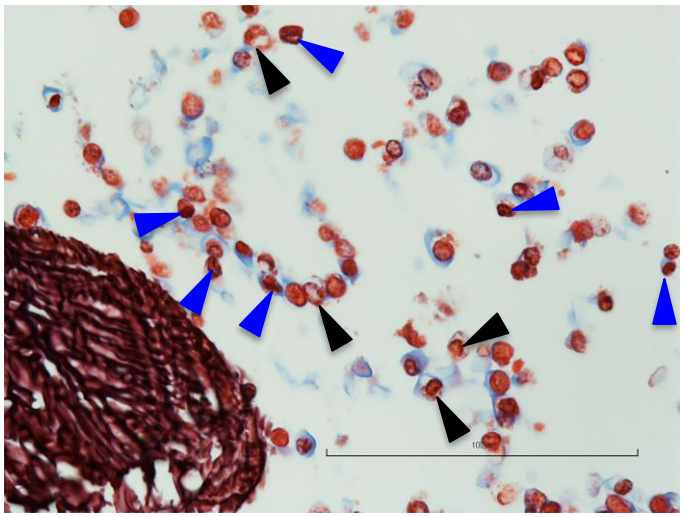


Figure 40: Chitosan + HA MT 40X. Black arrowheads: acidophilic cytoplasmatic granularity; blue arrowheads: altered shaped cells.

Alcian blue stained samples at 2 weeks

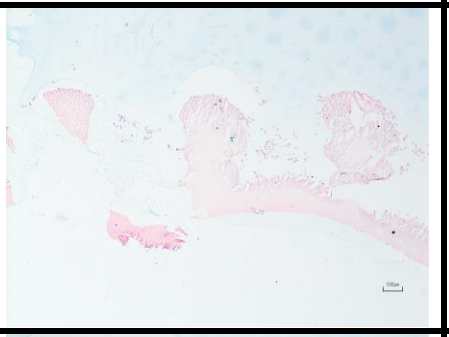
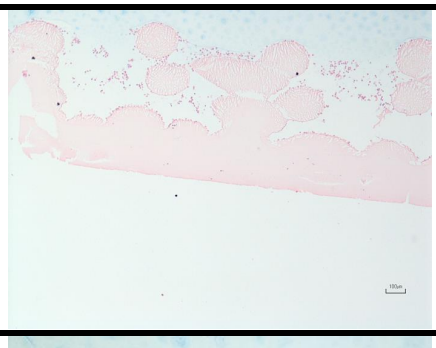
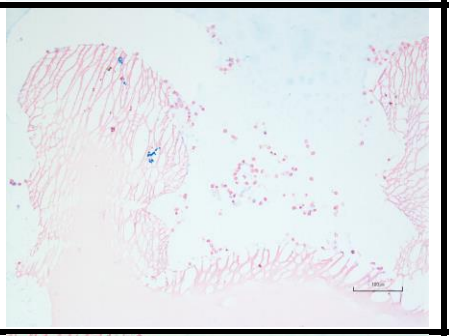
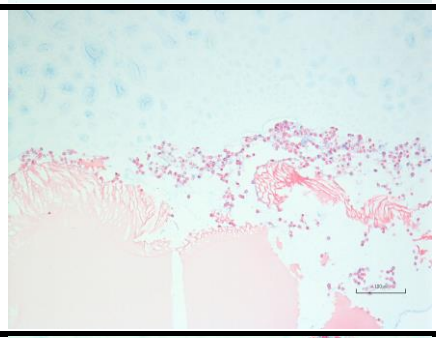
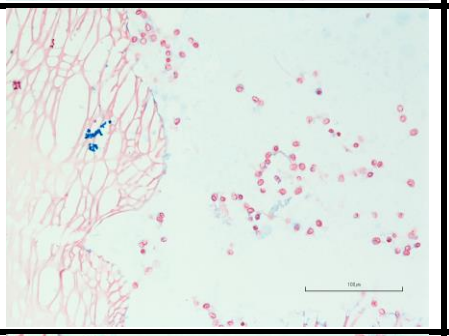
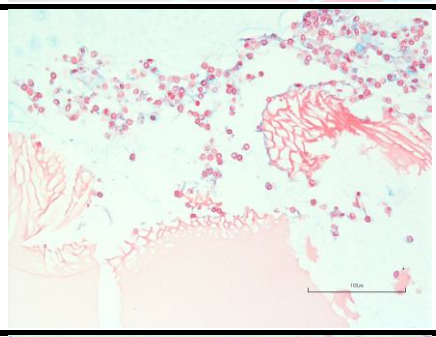
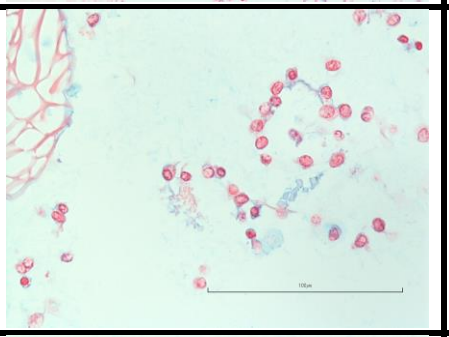
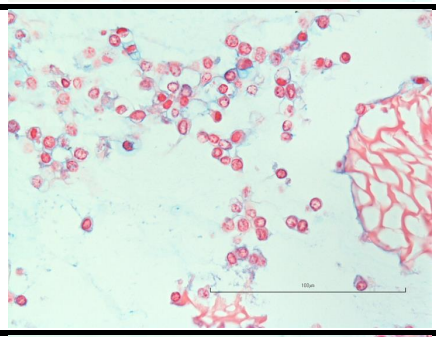
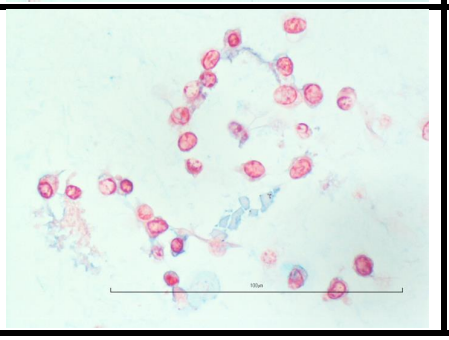
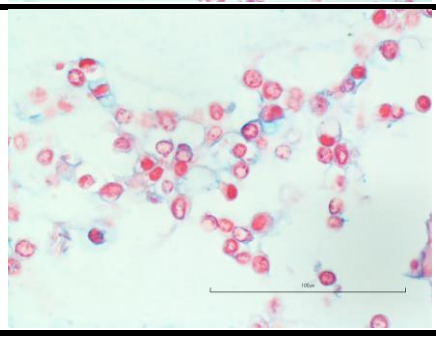
	CHITOSAN	CHITOSAN + HA
4X		
10X		
20X		
40X		
60X		

Table 12: AB stained samples at 2 weeks

A. Chitosan

- Scaffold appearance and colonisation

Alcian blue stains agarose in light blue and nuclear fast red gives a pink dye to the scaffold. The scaffold is intact. A medium-sized cell population colonised the scaffold at all levels, few cells dwell into porosity.

- Cell distribution

Cells present in large widespread aggregates of 20-30 cells/100 μm^2

- Quality and quantity of ECM

Most cells are surrounded by light blue-stained filamentous material, more abundant and intense in certain cell aggregates (black arrowheads in figure 41).

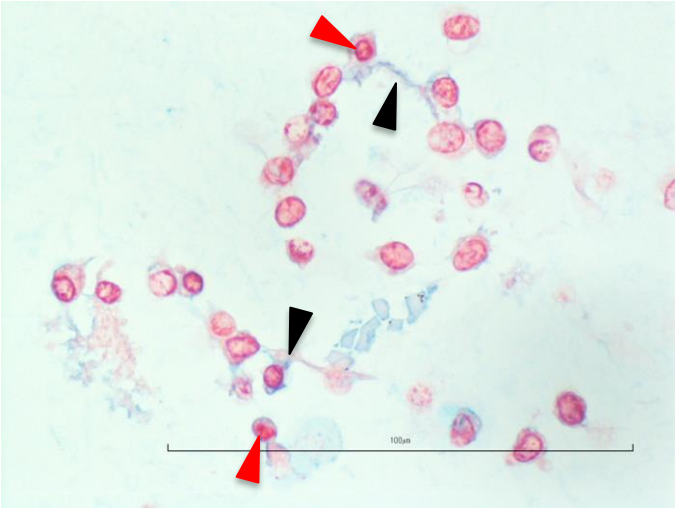


Figure 41: Chitosan AB 60X. Black arrowheads indicate blue-stained filamentous material. Red arrowheads indicate chondrocytes with dense nuclei.

- Cell morphology

Cells have a rounded or teardrop shape. Some cells appear different in dimension and have abnormal nuclear shape (red arrowhead above in figure 41).

B. Chitosan + HA

- Scaffold appearance and colonisation

Scaffold appears intact. Cells colonised the scaffold at all levels, most are located in the superficial-medium part (black rectangle in figure 42 and some took contact with scaffold structures or dwelled in porosities (red rectangles in figure 42).

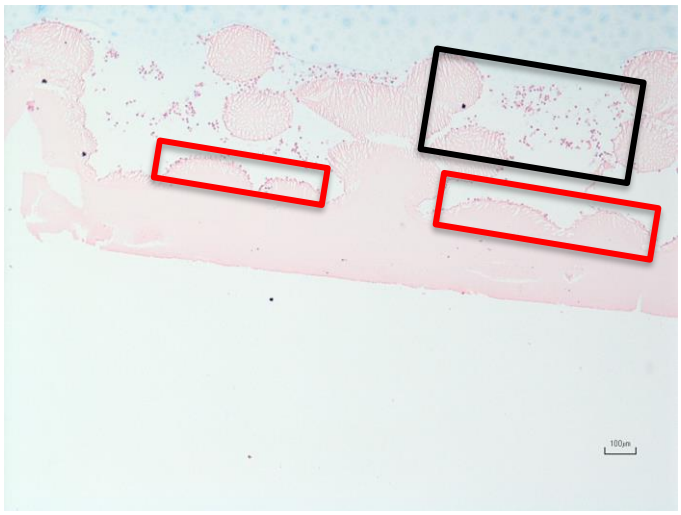


Figure 42: Chitosan + HA AB 4X. Black rectangle indicates a cell aggregate in the scaffold superficial layer, where most chondrocytes concentrate, while red rectangles indicate few cells dwelling in porosities.

- Cell distribution

Cells are organised in medium widespread to packed aggregates of 20-40 cells/100 μm^2.

- Quality and quantity of ECM

Most cells are surrounded by intense, light blue-stained filamentous material (black arrowheads in figure 43).

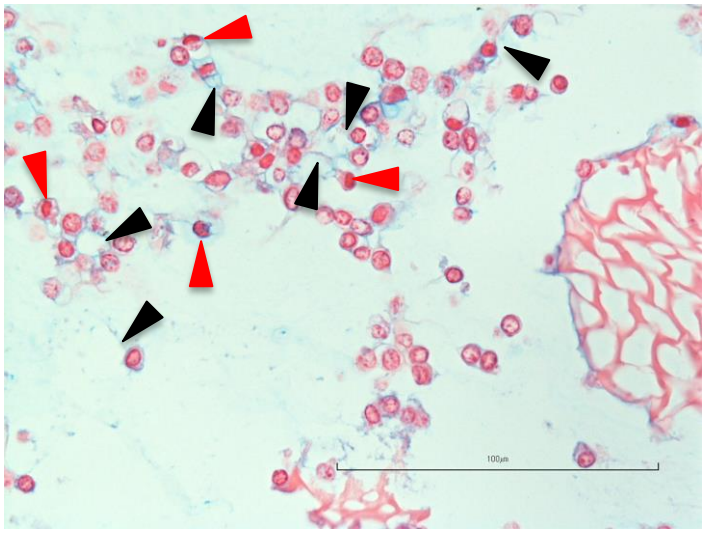


Figure 43: Chitosan + HA AB 40X. Black arrowheads indicate blue-stained filamentous material. Red arrowheads indicate altered morphology in chondrocytes.

- Cell morphology

Cells have a rounded or teardrop shape. Some exhibit dense nuclei or different dimensions (marked as red arrowheads above in figure 43).

Alcian blue stained samples at 4 weeks

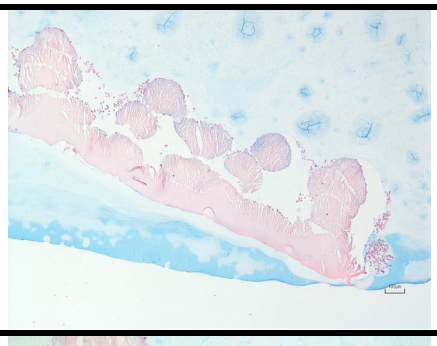
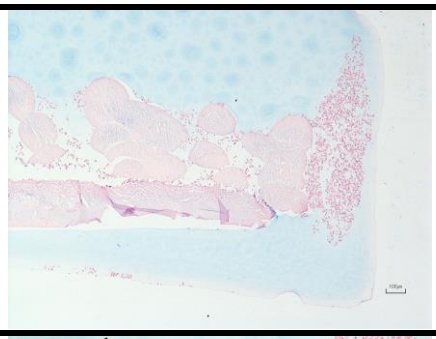
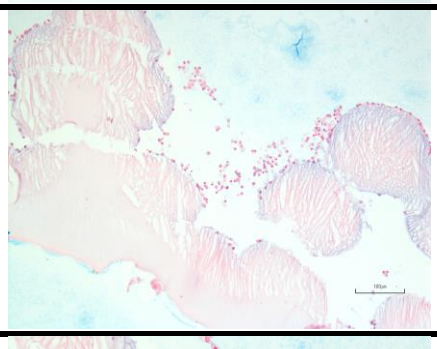
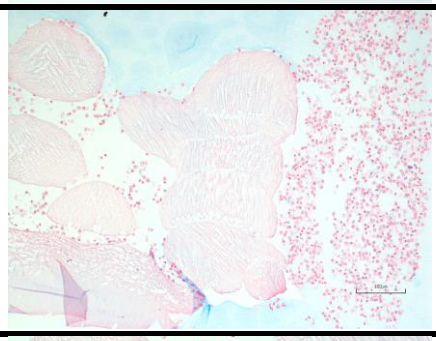
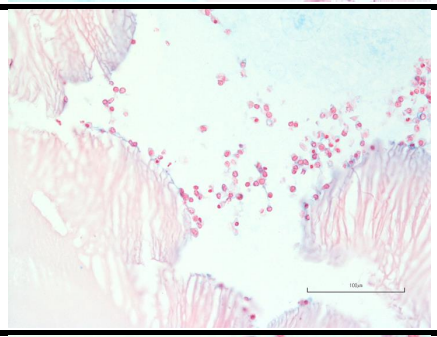
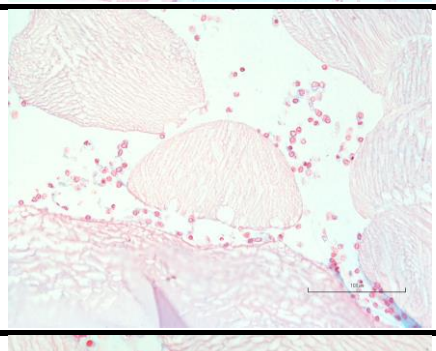
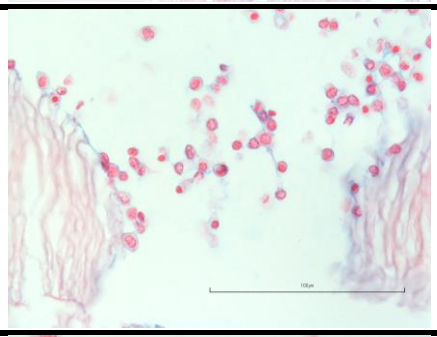
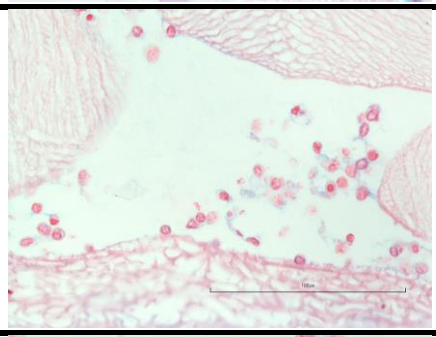
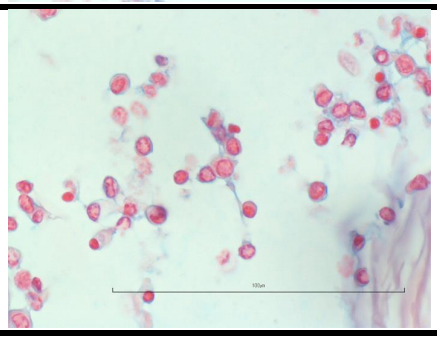
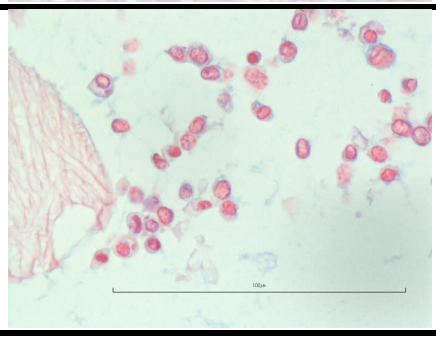
	CHITOSAN	CHITOSAN + HA
4X		
10X		
20X		
40X		
60X		

Table 13: AB stained samples at 4 weeks

A. Chitosan

- Scaffold appearance and colonisation

The scaffold is intact with little fragmentation and overlapping due to handling. A small cell population colonised the scaffold in some areas (black rectangle in figure 44), while others appear poorly populated (red rectangle in figure 44). Cells are located preferably close to scaffold structure.

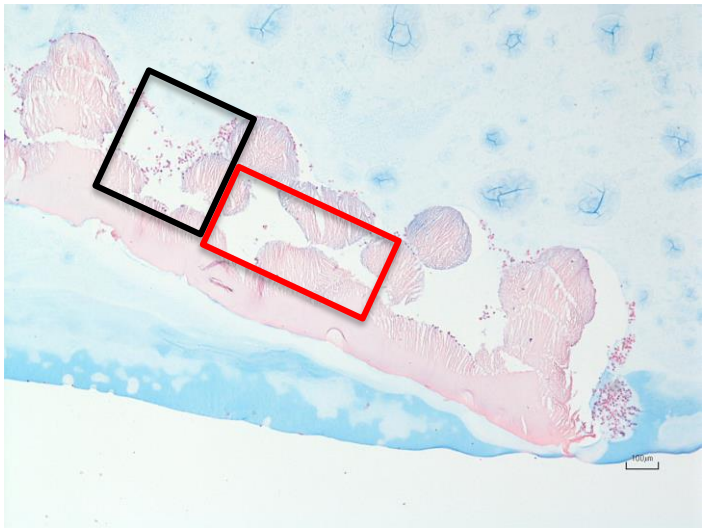


Figure 44: Chitosan AB 4X. Black rectangle: cell population. Red rectangle: scaffold area almost empty.

- Cell distribution

Cells present in small, quite packed aggregates of 30-50 cells/100 μm^2.

- Quality and quantity of ECM

Most cells are surrounded by light blue-stained filamentous material, more abundant and intense in certain cell aggregates (black arrowheads in figure 45).

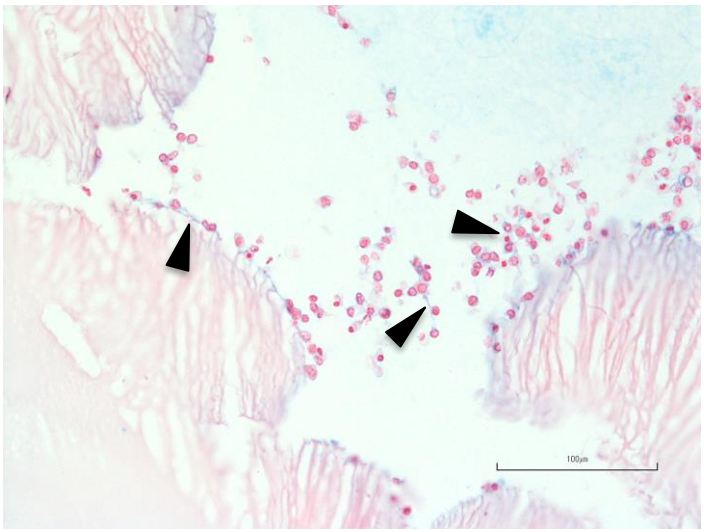


Figure 45: Chitosan AB 20X. Black arrowheads indicate blue-stained filamentous material.

- Cell morphology

Cells have a rounded or teardrop shape. Some cells appear different in dimension or nuclear shape (pyknosis) (marked as black arrowheads in figure 46).

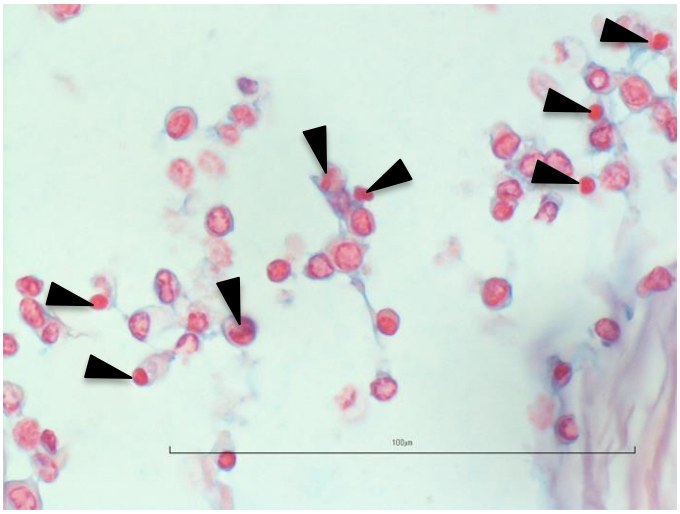


Figure 46: Chitosan AB 60X. Black arrowheads indicate nuclear pyknosis and abnormal cell shape.

B. Chitosan + HA

- Scaffold appearance and colonisation

The scaffold is intact with some fragmentation and overlapping due to handling. Cells colonised the scaffold at all levels, evenly distributed within the scaffold; one large aggregate of cells is present between scaffold and agarose layer.

- Cell distribution

Cells within the scaffold are organised in medium widespread aggregates of 20 cells/100 μm^2.

- Quality and quantity of ECM

Some cells are surrounded by pale, light blue-stained filamentous material (black arrowheads in figure 47).

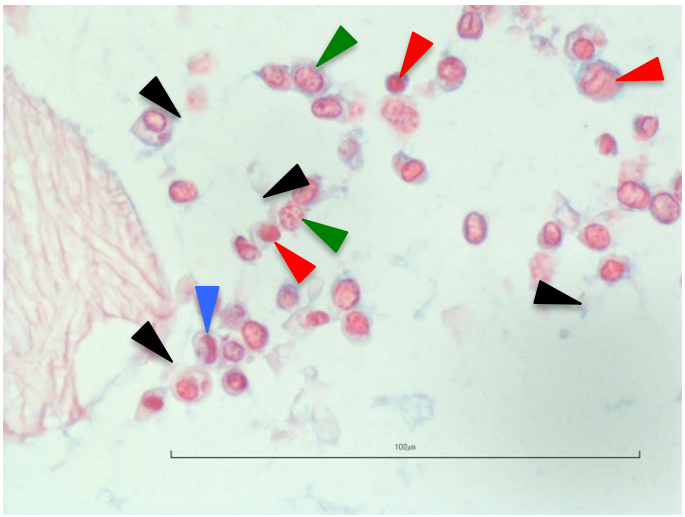


Figure 47: Chitosan + HA AB 60X. Black arrowheads: blue-stained filamentous material. Red arrowheads: pyknosis or altered cell shape. Green arrowheads: granular cytoplasm. Blue arrowhead: kidney-shaped nucleus.

- Cell morphology

Cells have a rounded or teardrop shape. Some exhibit dense, altered-shaped (kidney-shaped marked as a blue arrowhead above in figure 47), granular (green arrowheads above in figure 47) nuclei or different dimension or irregular cell shape (red arrowheads above in figure 47)

Collagen II IHC stained samples at 2 weeks

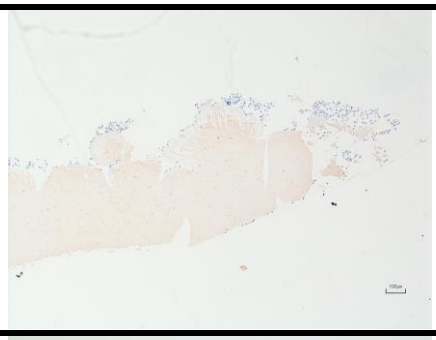
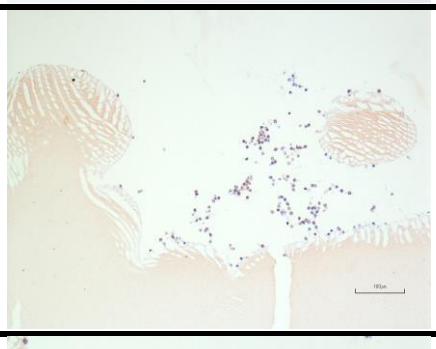
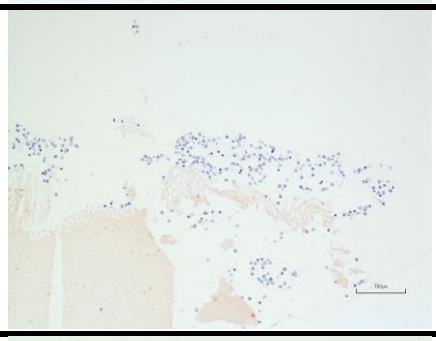
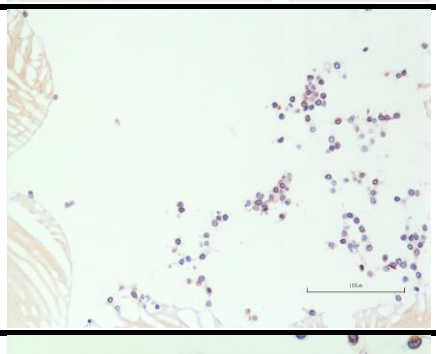
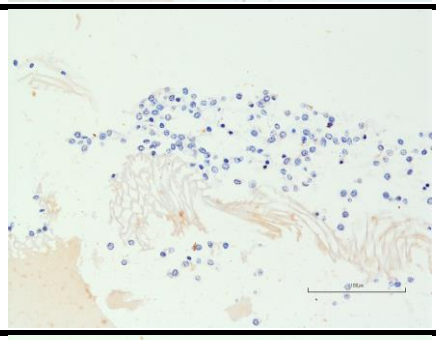
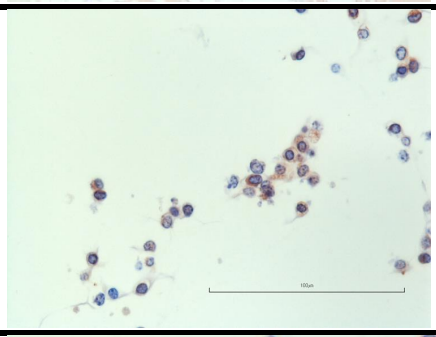
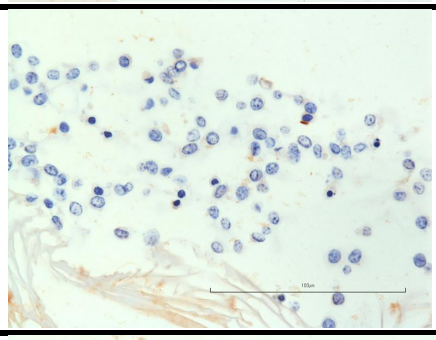
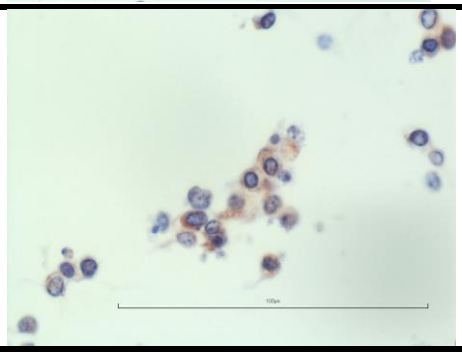
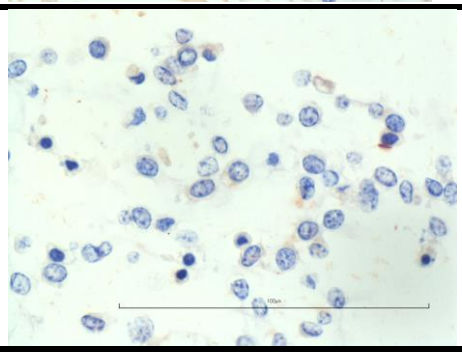
	CHITOSAN	CHITOSAN + HA
4X		
10X		
20X		
40X		
60X		

Table 14: IHC stained samples at 2 weeks

A. Chitosan

- Scaffold appearance and colonisation

The scaffold is stained in light brown-orange; it is intact with little fragmentation due to microtome sectioning. A small cell population colonised the scaffold in some areas, from surface to bottom. Some cells dwell in porosities.

- Cell distribution

Cells present in small, quite dense aggregates of 50 cells/100 μm^2.

- Quality and quantity of ECM

Most cells are surrounded by scarce, pale brown filamentous material (black arrowheads in figure 48).

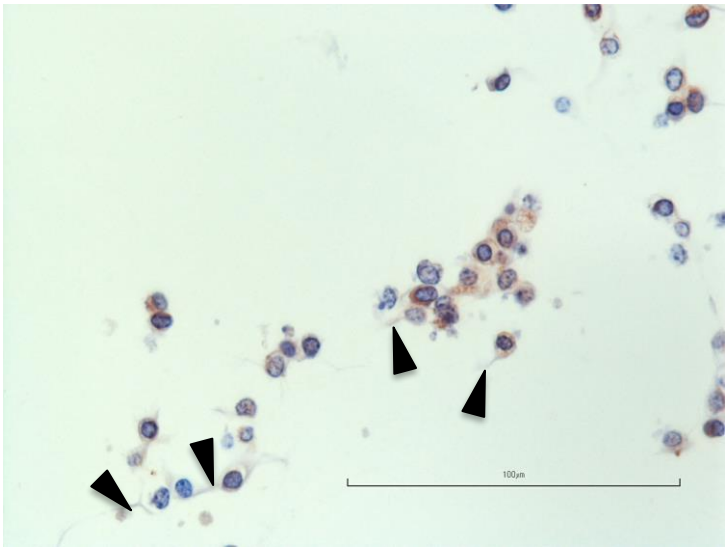


Figure 48: Chitosan IHC 40X. Black arrowheads indicate brown-stained filamentous material.

- Cell morphology

Cells have a rounded or teardrop shape. Cytoplasm has an intense brown-orange appearance.

B. Chitosan + HA

- Scaffold appearance and colonisation

The scaffold is intact with some fragmentation due to handling. Cells colonised the scaffold at all levels, locating preferably close to its structure.

- Cell distribution

Cells are organised in medium, packed aggregates of 50 cells/100 μm^2.

- Quality and quantity of ECM

Most cells are surrounded by brown filamentous material (black arrowheads in figure 49).

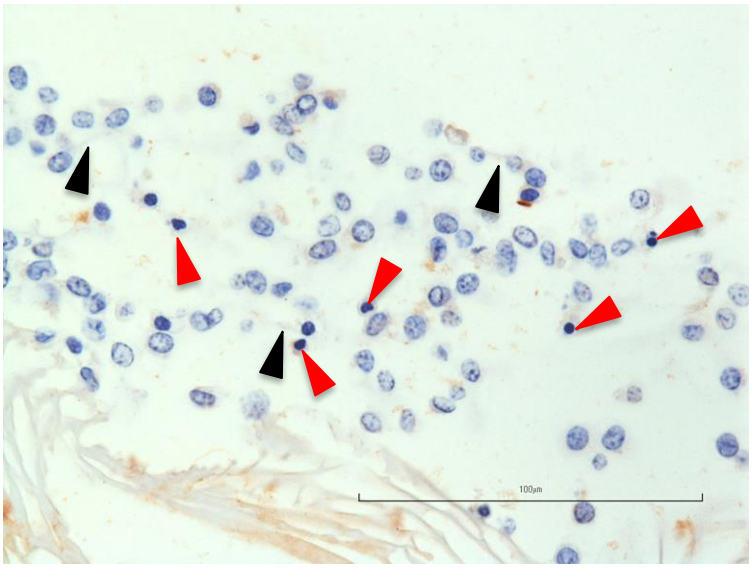


Figure 49: Chitosan + HA IHC 40X. Black arrowheads: brown-stained filamentous material. Red arrowheads: dense nuclei.

- Cell morphology

Cells have a rounded or teardrop shape. Some exhibit pyknotic or dense nuclei (red arrowheads in figure 49); few cytoplasms appear pale brown.

Collagen II IHC stained samples at 4 weeks

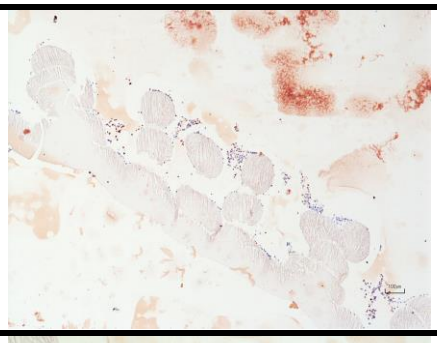
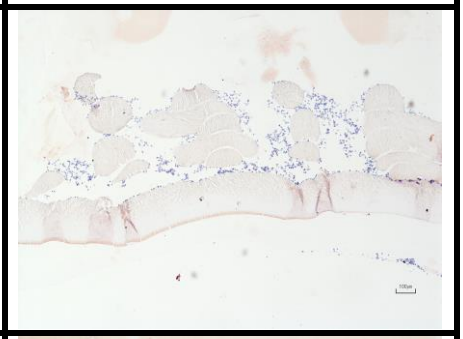
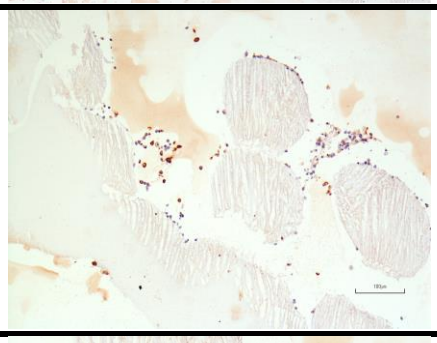
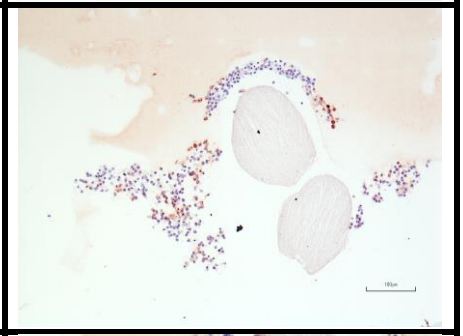
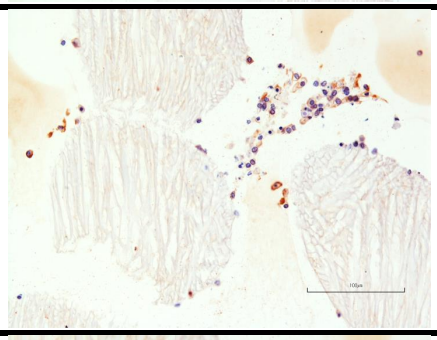
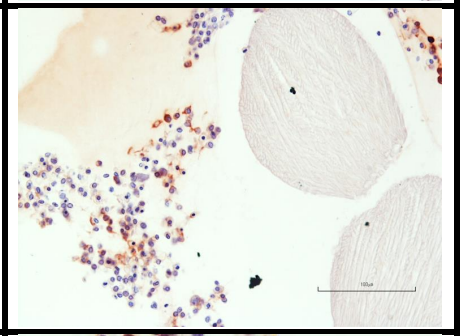
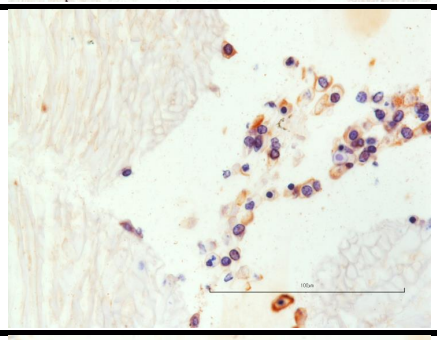
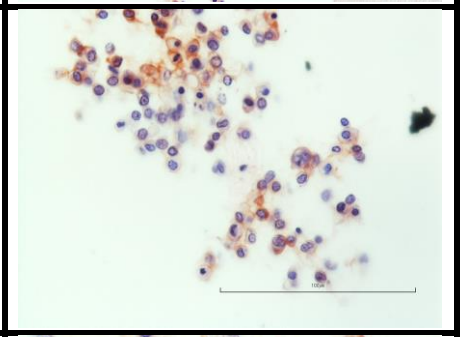
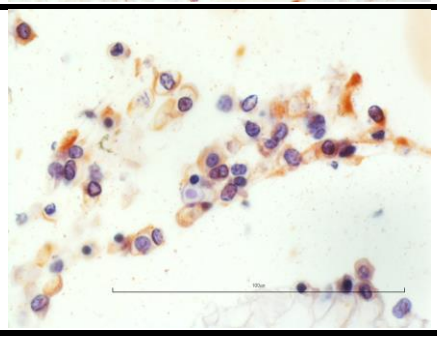
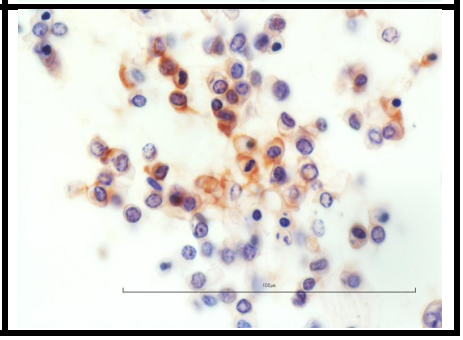
	CHITOSAN	CHITOSAN + HA
4X		
10X		
20X		
40X		
60X		

Table 15: IHC stained samples at 4 weeks

A. Chitosan

- Scaffold appearance and colonisation

The scaffold is intact with little fragmentation and overlapping due to handling. A small cell population colonised the scaffold in some areas, preferably in superficial part or deep porosities.

- Cell distribution

Cells present in small, quite dense aggregates of 40 cells/100 μm^2.

- Quality and quantity of ECM

Most cells are surrounded by intense, brown filamentous material (black arrowheads in figure 50).

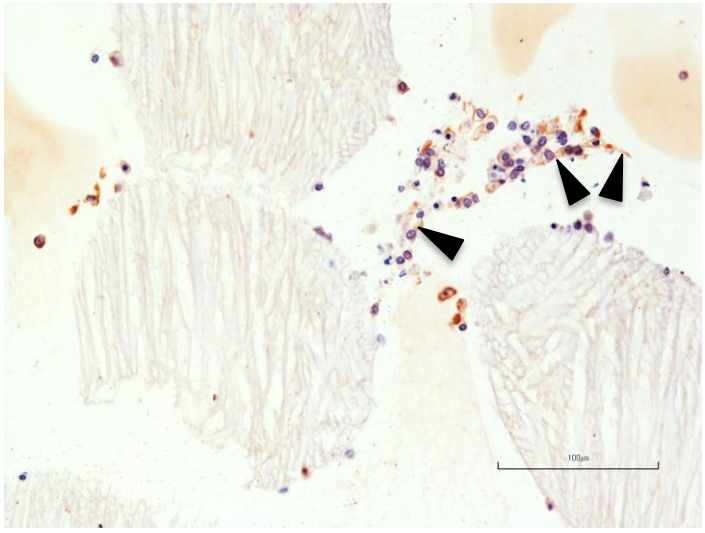


Figure 50: Chitosan IHC 20X. Black arrowheads: intensely brown-stained filamentous material.

- Cell morphology

Cells have a rounded or teardrop shape. Cytoplasms have an intense brown-orange appearance. Chondrocytes vary though in dimension and nuclear appearance (pyknosis marked as red arrowheads, lobulated shape in blue arrowhead in figure 51).

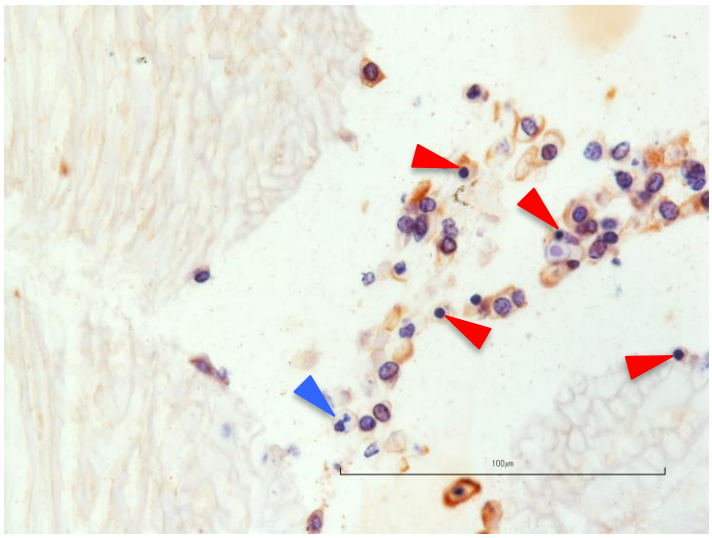


Figure 51: Chitosan IHC 40X. Red arrowheads: nuclear pyknosis. Blue arrowhead: lobulated nuclear shape.

B. Chitosan + HA

- Scaffold appearance and colonisation

The scaffold is intact with some overlapping in the basal layer due to handling. A large cell population colonised the scaffold at all levels.

- Cell distribution

Cells are organised in large, tight aggregates of 50 cells/100 μm^2.

- Quality and quantity of ECM

Most cells are surrounded by intense, brown filamentous material (black arrowheads in figure 52).

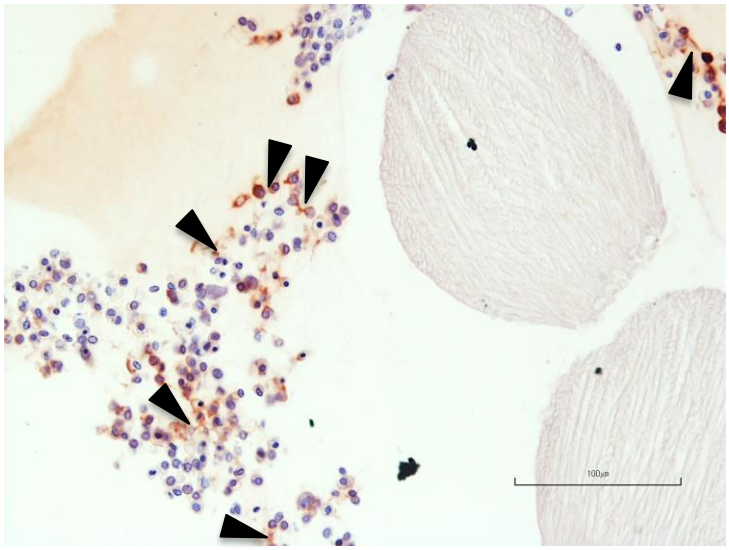


Figure 52: Chitosan + HA IHC 20X. Black arrowheads: intensely brown-stained filamentous material.

- Cell morphology

Cells have a rounded or teardrop shape. Cytoplasms have an intense brown-orange appearance (some inside black rectangle in figure 53). Several exhibit pyknotic or dense nuclei (red arrowheads in figure 53); few present altered nuclear shape (blue arrowheads in figure 53) and basophil bodies (green arrowhead in figure 53).

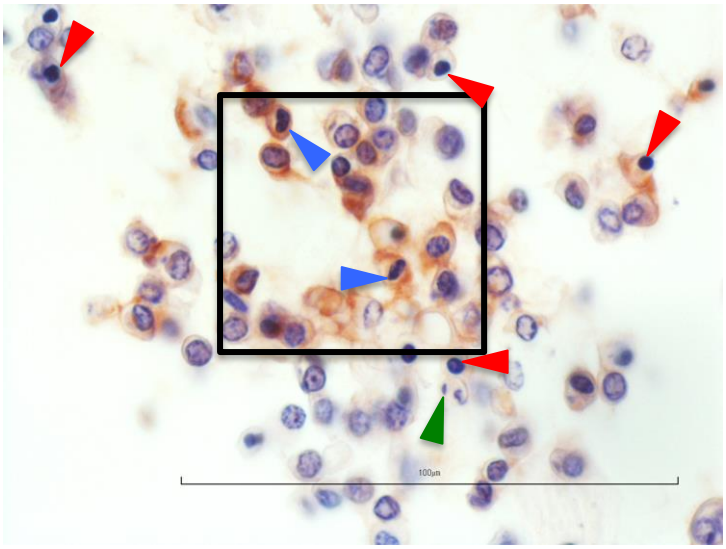


Figure 53: Chitosan + HA IHC 60X. Black rectangle indicates some very intensely stained cytoplasms. Red arrowheads: pyknotic or dense nuclei. Blue arrowheads: altered nuclear shape. Green arrowhead: a cytoplasmatic basophil body.

Discussion

Scaffold colonisation at equal times seems deeper and more even in the combined Chitosan + HA scaffold.

Comparing cell distribution from 2 weeks to 4 weeks time points, it can be noticed that cells tend to be more widespread in the scaffold at 2 weeks, whilst at 4 weeks they are quite packed. This might be due to cells moving and concentrating in zones where better conditions occurred (such as viability of nutrients, environmental stability, signalling from/to nearby cells), and/or cells avoiding potentially adverse conditions in certain niches. At 4 weeks, most cells would concentrate in the superficial to medium parts of the scaffold and fewer cells would be in the very deep parts, though a similar amount is located in deep porosities at 2 and 4 weeks.

ECM quality and quantity was investigated using specific staining in histology: the MT staining, collagen and some mucins appear blue, in AB staining, acid mucopolysaccharides and acid proteoglycans assume a light blue dye, in IHC, collagen II appears brown-orange. HE staining, where collagen appears pink, appears not specifically indicated for assessing ECM synthesis.

MT and AB stain connective tissue ECM non-specifically: MT emphasizes the protein components and AB the carbohydrate compounds. Regardless of time and scaffold type, chondrocytes productivity is positively assessed in both staining.

In MT, samples show a more intense positivity in Chitosan rather than Chitosan + HA scaffold and at 4 weeks rather than 2 weeks: in Chitosan scaffold at 4 weeks, blue appears in its most intense shade and abundance.

In AB staining, though all samples show some positivity, this is neither particularly evident, nor abundant, nor remarkably different on scaffold-type and time perspectives.

IHC shows positivity to collagen II synthesis in most cells.

Stability of a hyaline-like phenotype and the ability to synthetize ECM components are desired aspects in engineered cartilage.

The collagen II synthesis, assessed in our study, is a major element that allows us to consider that into this scaffold the cells are building ECM cartilage similar hyaline articular tissue.

Positivity to collagen II staining is more intense at 4 weeks, probably because cells had more time to increment their synthesis; at 2 weeks, chondrocytes and ECM in Chitosan

scaffold seem more intensely stained than in Chitosan + HA, but this difference is not still optically remarkable at 4 weeks.

Chondrocytes morphology is appropriate in the vast majority of cells: a rounded, teardrop shape is seen here, similarly to native chondrocytes in lacunae in hyaline cartilage. Major signs of degeneration, such as cytoplasm vacuolisation, nuclear pyknosis, abnormal nuclear shape, degenerated cells, can be seen in very few cells; perhaps more degeneration signs are seen at 4 weeks compared to 2 weeks, as the chondrocyte population matures.

Therefore, cells and ECM should progressively fill the voids as the scaffold biodegrades, meaning that target of colonisation should be characterised by cells spreading as much as possible in all available spaces, from surface to bottom. Colonisation achieved with Chitosan + HA would be preferable over Chitosan.

The final outcome of TE in cartilage repair is to achieve an implantable biopolymer, which should eventually resemble native hyaline cartilage.

Our study confirms that chitosan or functionalized chitosan is an important biopolymer for studying the cartilage regeneration *in vitro*.

As a promising biomaterial, chitosan has outstanding bioactivities, antimicrobial properties, non-toxicity, biocompatibility, biodegradability, and superior physical properties. It can be processed into diverse forms, including gels, films, fibers, and sponges. (Li, Hu, Yu, & Chen, 2018) In particular, the hydroxyl groups of chitosan make it easy to composite with other materials (Li, Hu, Yu, & Chen, 2018).

Bioactivity of chitosan is mainly reflected in its antimicrobial, anti-tumoral, and antioxidant properties. These bioactivities help with inflammation reduction when chitosan scaffolds are used for AC defect repairs.

Cell growth and death, tested to measure the cell viability, showed that cell viability increased after chitosan coating, which indicated that chitosan coating increased the biocompatibility (Li, Hu, Yu, & Chen, 2018).

Malafaya *et al.* (Malafaya, Santos, van Griensven, & Reis, 2008) comprehensively explored the *in vivo* biocompatibility of chitosan particles implanted in animals: after two weeks of implantation, connective tissue was growing and neovascularization had increased between the particles of the scaffold.

Li *et al.* (Li, Hu, Yu, & Chen, 2018) state that there are three biomaterial phases in tissues: tissue removal, tissue replacement, and tissue regeneration. The main purpose of the last phase is assisting or enhancing the body own repair capacity. Thus, biomaterials biodegradability is vital for regeneration after implantation in the body and for inducing

tissue self-regeneration. Chitosan is known to be degraded in vertebrates predominantly by lysozyme and certain bacterial enzymes in the colon, its rate in living organisms mainly depending on deacetylation degree and on average molecular weight (M_W), both affecting the affinity between enzyme and substrate (Li, Du, & Liang, 2007). Chitosan by-products exert no pyrogenic activity, toxicity, or mutation effect on implanted cells. Instead, chitosan and its degraded products could induce gene expression of cells for AC components, such as type II collagen (Li, Hu, Yu, & Chen, 2018).

Chitosan hydrogels for AC repair allow for the inclusion of cells and molecules for cartilage regeneration (Rodríguez-Vázquez, Vega-Ruiz, Ramos-Zúñiga, Saldaña-Koppel, & Quiñones-Olvera, 2015). The most combined inducer agent of cartilage regeneration is collagen type II, the main protein in cartilage ECM, which enhances the adhesion and formation of clusters of chondrocytes in vitro, a requirement for cartilage regeneration. Chitosan may also be combined with chondroitin sulphate, alginate and fibroin with similar results (Rodríguez-Vázquez, Vega-Ruiz, Ramos-Zúñiga, Saldaña-Koppel, & Quiñones-Olvera, 2015).

Moreover, chitosan can support the addition of an inducer. ECM synthesis and deposition is a key factor to recognize biocompatibility and normal cell function, in addition to a 3-D matrix for growth tissue by proliferation and differentiation of precursor cells (Rodríguez-Vázquez, Vega-Ruiz, Ramos-Zúñiga, Saldaña-Koppel, & Quiñones-Olvera, 2015).

A study by Why *et al.* (Whu, Hung, Hsieh, Chen, Tsai, & Hsu, 2013) evaluated *in vitro* and *in vivo* chitosan-gelatin 1:1 scaffolds, some crosslinked by a water-soluble carbodiimide or WSC ($C1G1_{WSC}$), for AC TE. These complex scaffolds possessed the proper degradation rate and mechanical stability in vitro. Chondrocytes were able to proliferate well and secrete abundant ECM. Implantation of chondrocytes-seeded scaffolds in the defects of rabbit articular cartilage confirmed that $C1G1_{WSC}$ promoted cartilage regeneration. The newly formed cartilage presented the histological feature of tide line and lacunae in 6.5 months. The amount of GAGs in $C1G1_{WSC}$ constructs harvested from the animals after 6.5 months was 14 wt.% of that in normal cartilage. The average compressive modulus of regenerated tissue at 6.5 months approached to that of normal cartilage, being much higher in blank control and typical of fibrous tissue. Type II collagen expression in $C1G1_{WSC}$ constructs was similarly intense as that in the normal hyaline cartilage. According to the above results, the use of $C1G1_{WSC}$ scaffolds may actually enhance the cartilage regeneration in vitro and in vivo; such scaffolds could be potential candidates for cartilage tissue regeneration.

To summarize, several studies have confirmed the excellent biocompatibility of chitosan in terms of *in vitro* chondrogenesis and maintenance of chondrocyte phenotypes.

The *in vivo* application of chitosan shows fewer studies and with more discordant data; this could depend on the chitosan form used (hydrogels, scaffolds, films, etc.) or on the association with other biopolymers that could improve its biocompatibility in the repair of the *in vivo* AC damage.

Blend scaffolds that combine chitosan with polymers, ceramics, or other materials have become more and important in the field of AC defect repair. Several materials are notably improved when composited with chitosan by altering its degree of deacetylation, average molecular weight, and form (Li, Hu, Yu, & Chen, 2018).

Chitosan composites provide physical, chemical and mechanical support, cell attachment, proliferation, and differentiation, with the corresponding biocompatibility to induce the bone and cartilage tissue regeneration (Rodríguez-Vázquez, Vega-Ruiz, Ramos-Zúñiga, Saldaña-Koppel, & Quiñones-Olvera, 2015).

Our study evidenced that chitosan represents an optimal biomaterial, alone or in combination, for fabrication of 3-D bioprinted scaffolds, to assess chondrocytes behaviour and ECM synthesis *in vitro*.

Conclusions and future directions

OA, due to its high prevalence and debilitating attitude, has a huge impact on health and wellbeing of humans and animals. Thanks to the research, its pathogenesis is getting more and more understood. Investigation in the field of molecular patterns involved in OA provides a further insight and it is the starting point for application of several new DMOADs, and perhaps for OA prevention rather than cure. Anabolic/catabolic balance, apoptosis, and signalling pathways triggering hypertrophic phenotype change, are key elements in describing what actually takes part in determining OA damages.

Equine medicine appears somewhat behind human medicine, despite the role of lameness and joint diseases in equines as well as in humans. Also, treating joints with mechanical loads typical not only of a horse, but also an athlete, is immensely challenging. While it is crucial to relieve the clinical signs of OA, it must be taken into account that the mere reduction of pain is insufficient, if not detrimental, in order to antagonize OA progression. Moreover, it could be useful to better understand the horse and rider biomechanics, in an attempt to educate horse and rider posture to avoid and cope with musculoskeletal injuries.

Tissue engineering represents an extremely promising field, especially for AC repair, since this has limited self-healing capacity: the ultimate frontier is to provide a brand-new cartilage that could fully substitute the damaged one.

Research on biomaterials and their forms has provided data on bioactivity, biocompatibility, biodegradation and architecture characteristics with the aim to pursue the best conditions possible for chondrocytes to maintain a hyaline-like phenotype and to synthetize abundant ECM similar to native cartilage one.

Chitosan is interesting not only because of its properties, but also for its abundance, easiness to obtain and to shape in many forms for TE. Chitosan 3-D printed scaffolds provide good mechanical properties while supporting chondrocyte colonisation and ECM production. In this experiment, chitosan-only and chitosan-HA scaffolds were seeded with cultured chondrocytes and histologically analysed through several staining techniques (HE, MT, AB, IHC): chondrocytes maintained an appropriate morphology while secreting an ECM containing both the carbohydrate and the protein (collagen II) components. As well as that, research has showed overall positive results, both *in vitro* and *in vivo*, particularly when using composite chitosan scaffolds.

Further investigation on biomaterials and their forms, and especially clinical trials in horses, are needed to assess new TE scaffold strategies for equine OA treatment.

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