Plowing of cartilage explants as mimetic system of the temporomandibular joint

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Abstract

Articular cartilage provides a low friction, wear-resistant and load-bearing surface for adjoining bones. Mechanical stress is one of the causes of cartilage degradation leading to progressive cartilage destruction and provoking the direct contact of articulating bones, with the ultimate consequences of movement impairment and severe pain.

In order to understand the pathomechanics of cartilage disruption due to mechanical stress, several in vitro models have been developed. Although studies based on these systems gathered insight into the biological response of cartilage to static or cyclic compression, their uniaxial design presents some limitations. Indeed, synovial cartilage is subjected to a more complex combination of mechanical stresses.

With the final goal of mimicking in vitro the complex loading patterns occurring in body joints and in particular in the temporomandibular joint, the aim of the present thesis is to study the effect of plowing, namely the simultaneous compression by an indenter (orthogonal to the cartilage surface) and motion of the indenter (parallel to the cartilage surface), on pristine cartilage obtained from bovine nasal septum (BNS).

A study concerning the mechanical and biochemical characterization of the BNS cartilage has been performed. Additionally, the suitability of BNS cartilage for in vitro loading experiments and the possibility to induce a biological effect on BNS cartilage by mechanical stress, have been tested by performing static uniaxial compression.

Plowing experiments, at different applied normal forces, demonstrated that the higher the applied normal force the higher is the developed tractional force and that the further cartilage deformation, measured as increase of the cartilage strips length, is also dependent on the magnitude of the applied normal force. Furthermore, plowing has been shown to induce gene-expression changes that are dependent on the tractional force, which, in turn, relates to the applied normal force.

Finally, it has been demonstrated that plowing with an applied normal force of 100 N and indenter speed of 10 mm/sec causes cell death of the chondrocytes closer to the surface as
well as matrix damage. In addition, plowing promotes in healthy chondrocytes the production and activation of catabolic enzymes. Together, these results suggest that the promoted pattern of gene transcription could induce a process of cartilage remodelling rather than cartilage degradation.
Riassunto

La cartilagine è un tessuto connettivo resistente alla pressione e alla trazione che si trova nelle articolazioni ed in altri organi dei quali costituisce un supporto meccanico. Nelle articolazioni ricopre parzialmente la superficie delle ossa aiutandole a sopportare pressioni e tensioni dovute al movimento corporeo. Lo stress meccanico è una delle cause principali di danno alla cartilagine che origina come piccole lesioni superficiali, ma che generalmente progredisce in più profonde abrasioni del tessuto cartilagineo. Queste abrasioni causano, ad uno stadio avanzato, il diretto contatto delle ossa articolari provocando quindi severi dolori e grandi difficoltà di movimento.

In passato, sono stati sviluppati diversi modelli in vitro con lo scopo di comprendere i processi e gli eventi che da uno stress meccanico portano a patologie della cartilagine. Questi apparati, nonostante abbiano fornito importanti risultati sulla risposta della cartilagine allo stress statico o ciclico, sono limitati dal fatto di essere uniassiali. Infatti, la cartilagine articolare è soggetta in vivo ad una combinazione di carichi che agiscono in più dimensioni (parallelamente e ortogonalmente al tessuto).

Lo scopo di questa tesi è di sviluppare un sistema capace di riprodurre in vitro in tessuto vivo i carichi articolari (in particolare quelli dell’articolazione temporomandibolare), e di studiare l’effetto del plowing (che consiste nella combinazione simultanea di compressione e di scorrimento di un condilo su una superficie cartilaginea) in cartilagine proveniente dal setto nasale bovino, tessuto che non è mai stato precedentemente sottoposto a carico meccanico.

Inizialmente, la cartilagine del setto nasale bovino è stata caratterizzata meccanicamente e biologicamente. Poi, alcuni campioni sono stati sottoposti ad esperimenti di carico statico al fine di confermare l’idoneità per test meccanici di questo tipo di cartilagine.

I primi esperimenti hanno rivelato che la forza di compressione applicata durante il plowing è responsabile della formazione di forze trazionali e anche della deformazione macroscopica della cartilagine. Inoltre, è stato dimostrato che nei condrociti il plowing causa variazioni di espressione genica, le quali dipendono anch’esse dall’intensità della forza applicata.
In conclusione, è stato dimostrato che il plowing con una forza normale di 100 N e velocità di scorrimento di 10 mm/sec causa morte cellulare e degradazione della matrice extracellulare a livello superficiale. Tuttavia, nei condrociti vitali esso induce sintesi/attivazione di enzimi catabolici che sono piuttosto coinvolti in un processo di rimodellamento che di degradazione.
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1 Introduction: cartilage development, structure and function.

“Cartilage is found where it is an advantage that the solid framework should be pliable and glutinous for the benefit of the flesh that surrounds them. This applies to the ears and the nostrils. Such projecting parts quickly get broken if they are brittle. Cartilage and bone are the same in kind and differ only by ‘the more and less’” (1).

Although Aristotle (384-322 BC) was not aware of the articular cartilage, he impressively provided the first description of the cartilaginous tissue.

Cartilage is an avascular, aneural, alymphatic connective tissue (2) placed in peripheral organs, such as nose and ear, where it has a function of support and in the body joints where it allows low friction movements of the body (3) and is responsible for absorbing mechanical shocks (4).

The focus of this introductory chapter is on the description of cartilage. After a brief section about tissue development (1.1.1), the chapter is organized by “zooming out” from a close microscopic analysis of the cartilage components (1.2), to a description of the cartilage properties and function (1.3, 1.4) and a final presentation of the macroscopic view of the cartilage in a body joint (1.5).

Section 1 of the present chapter focuses on a general overview of cartilage and provides a detailed description of the embryonic cartilage formation and development (1.1 and 1.1.1). Next, in section 2, an accurate description of the main cartilage components is reported (1.2) while in the third section the mechanical properties of the tissue are discussed (1.3). Section 4 provides insight into the mechanotransduction events occurring in cartilage. It focuses on the description of molecular ion channels, of integrins and of the intracellular signaling pathways of chondrocytes (1.4). In Section 5 a general introduction about body joints and more in detail on the temporomandibular joint (TMJ) is reported. Finally, in the section 6, a review of the state of the art about the devices developed and the knowledge gained in the mimicking joints in vitro is provided (1.6).
1.1 Cartilage: a quick look

Cartilage is an avascular, aneural and alymphatic connective tissue constituted of water (70-80%) and of a dense extracellular matrix (ECM) mainly composed of collagens and proteoglycans (PGs) (10-20% and 5-10% in turn) conferring tensile and compression resistance, respectively (5). The cells present in cartilage are the chondrocytes; they represent 1% of the cartilage volume and are responsible for ECM formation, maintenance and remodelling (6). As explained in more detail in the next section, chondrocytes originate during the early embryonic development as a result of tempo-spatial cellular interactions with the surrounding matrix, as well as with growth and differentiation factors (7).

1.1.1 Chondrogenesis: the process of cartilage formation

Chondrogenesis is the earliest phase of skeletal development. It begins with the mesenchymal cell (MC) condensation; it evolves into the chondrocyte differentiation and maturation and terminates with the formation of cartilage and bone during the endochondral ossification (Fig. 1) (7-10).

At early stages of limb development, the interactions between the apical ectodermal ridge and the zone of polarizing activity induce the arrangement of the proximal-distal and of the antero-posterior body axes (11), representing the driving forces of the limb organization. The triggering event of MC condensation (Fig. 1A), which consists of an increase in the cell density per volume, is the MC production of hyaluronan, collagen type I and type II as well as of cell-adhesion molecules such as neural cadherin (N-cad) and neural cell adhesion molecule (N-cam) (7). The further phenotypic cellular change of MC into chondrocytes is facilitated by the interaction of extracellular matrix (ECM) proteins such as tenascin and syndecan with cell-adhesion molecules exposed by the MC (Fig. 1B). This molecular interaction causes the disappearance of cell-adhesion molecules (N-cam and N-cad) from the MC membrane and the nuclear expression of the chondrocytic transcription factor SRY-box9 (Sox9) SRY-box5 and SRY-box6 (Sox5 and Sox6 respectively) (12).

At this point, the rate of chondrocyte proliferation and differentiation is regulated by the balance of the signaling fibroblast growth factor (FGF), hedgehog, the bone morphogenetic proteins (BMP2) and Wnt (13). Once the cells have assumed the chondrogenic phenotype, they
proliferate and produce collagen type II, IX and XI and aggrecan, inducing the formation of a cartilage template. Furthermore, the chondrocytes closer to the growing element and the cells of the perichondrium (an irregular connective tissue which surrounds the cartilage template) and of the periosteum (a connective tissue surrounding the bone) produce Runx2 which serves as positive regulatory factor for the differentiation into the pre-hypertrophic phenotype (Fig. 1C).

Now the hypertrophic chondrocytes that form the primary ossification center and the cells of the periosteum express the vascular endothelial growth factor (VEGF) and attract the blood vessels (Fig. 1D). The hypertrophic chondrocytes undergo apoptosis and furthermore, the osteoblasts and osteoclasts, which are the bone cells, invade the ECM left from the apoptotic cells and begin the mineral deposition. In contrast, the other chondrocytes at the edge of the structure, called epiphyseal chondrocytes, keep their phenotypes and produce some chondrogenic molecular markers such as Sox9, collagen type 2 and aggrecan.

During the late embryonic development, deeper epiphysical chondrocytes begin a process of hypertrophy similar to that described previously, terminating with the production of secondary ossification centers (Fig. 1E). Bone cells substitute the totality of chondrocytes while the only cartilage remains at the extremity of the structure originating thus the articular cartilage (14;15).

![Fig. 1 Chondrogenesis. A: MC condensation. B: Differentiation of MCs into chondrocytes. C: Formation of the first ossification center. Prehypertrophic chondrocytes (red), and hypertrophic chondrocytes (blue), are surrounded by the perichondrium (green) or periosteum (orange). D: Blood vessels invasion and bone formation in the ossification center. D: The secondary ossification centers are formed within the zone of the distal chondrocytes. Adapted from M. Wuelling & A. Vortkamp (16).](image)
1.2 Cartilage composition

As mentioned in the previous section, the main components of cartilage are water, proteoglycans, collagens and the chondrocytes in their chondrons. An introduction to the constituents of cartilage follows.

1.2.1 Water

Water is the main component of cartilage (70-80 % of the volume) (17) and its flow through the cartilage proteins allows the transport of nutrients to the chondrocytes (18). The inflow of water from the surrounding areas to the cartilage is induced by the swelling pressure. Indeed, proteoglycans, which are entrapped in the collagen network, have strong negative electric charges and thus they attract counter-ions (calcium and sodium), causing an increase in cartilage swelling pressure. However, cartilage swelling is normally controlled by the collagen network, which resists the inflow of water. When the collagen network is degraded, the amount of water in cartilage increases, causing a significant alteration of cartilage mechanical properties.

1.2.2 Proteoglycans

Proteoglycans are heavily glycosylated proteins mainly located in the connective tissues of the body. The most abundant proteoglycan in cartilage is aggrecan, which consists of a protein core with covalently bound glycosaminoglycan (GAG) side chains, namely chondroitin sulfate and keratan sulfate (19-21).

The aggrecan core protein consists of three globular, highly conserved regions called G1, G2 and G3, and of a less conserved domain named inter-globular domain (IGD), which is located between G1 and G2 (Fig. 2). Many aggrecans, in the presence of link proteins, can bind to a single, long hyaluronan chain (another non-branching glycoprotein) to form large aggregates (Fig. 3B). The binding regions for the keratan sulfate (KS) and the chondroitin sulfate (CS) are located between G2 and G3 (Fig. 2 and Fig. 3A) (22;23).
The G1 domain is responsible for setting the interactions between chondrocytes and ECM network by connecting the aggrecan to the link protein, and thus to the hyaluronic acid. The IGD domain is involved in the physiological turnover of aggrecan indeed it contains the proteolytic cleavage sites for a variety of matrix metalloproteinases (MMPs). The G2 domain is similar to the G1 domain (67% homology in the aminoacidic sequence) but contrarily to it, the G2 domain does not bind the link protein and contains keratan sulphate side chains. Since it has been shown that recombinant proteins containing G2 domain and the signal peptide for secretion are not secreted, it is believed that the G2 domain is involved in the quality control of the aggrecan (23) ensuring the correct folding of the protein. The KS domain is a highly repeated hexamer sequence, whereas the CS domain consists of 120 Ser-Gly dipeptide repeats frequently separated by acidic or hydrophobic residues. The G3 domain contains three different modules and it is essential for normal post-translational processing of the aggrecan core protein and the subsequent aggrecan secretion (23).
**Fig. 3 Proteoglycan structure.** A: Schematic view of proteoglycan monomer structure, showing chondroitin sulfate and keratan sulfate chains and the interaction of the monomer with hyaluronate chain and link protein. B: Molecular conformation of a typical aggrecan showing the size of the molecule. C: An electron micrograph of an aggrecan. Adapted from K. R. Flik et al. (S)

### 1.2.3 Collagens

Among the different identified collagen types (at least 28 different collagens occur in vertebrates, they are numbered I-XXVIII (24), type II collagen is the most abundant (85-90 %) in cartilage (25). The feature that makes collagen type II uniquely suited for cartilage is related to its high hydroxylysine content, which facilitates the glycosylation (26).

All collagen types are composed of three polypeptide chains (α-chains) arranged in a triple-helical conformation (27). The primary aminoacidic sequence of the polypeptide chains is rich in glycine and proline; moreover, the hydroxyproline ensures stability via hydrogen bonds along the length of the molecule. Additionally, hydroxylysine is involved in creating covalent crosslinks that stabilize the collagen fibrillar structure. The fibrillar network of the articular cartilage is composed of collagen type II, IX and XI (28). The basic structure of the collagen fibrils is a polymer of collagen type II fibers, heavily cross-linked one to another in a head-to-tail fashion.
Collagen type XI is abundantly located within the fibrils, covalently linked to collagen type II, and it is involved in the regulation of the fiber size. In contrast, collagen type IX is located on the exterior of the fibrils and its main function is to allow collagen fibrils to interact with proteoglycan (Fig. 4).

Collagen type VI is mostly concentrated around chondrocytes (29) but it is also interspersed loosely in spaces throughout the fibrillar matrix. Collagen type VI is mainly concentrated in fibrocartilages such as the meniscus and the intervertebral disc. Collagen type XII and XIV are two members of the fibril-associated collagens with interrupted triple helices (FACIT) collagen subfamily and they are involved in the cooperation and/or competition with various other fibril-binding proteins, such as decorin, biglycan and fibromodulin (26).

![Collagen fibrils](image.png)

**Fig. 4 Collagen fibrils.** Type II collagen is the main component while type IX and XI collagens are less representative. Adapted from D. R. Eyre et al. (28)

### 1.2.4 Chondrocytes and chondrons

The chondrocytes are the specialized cartilage cells responsible of the ECM building, maintenance and remodeling. Normally, the chondrocytes have a round/elliptical shape and they show typical features of metabolically active cells: rough endoplasmic reticulum, juxta-nuclear Golgi apparatus and conspicuous deposits of glycogen and lipid droplets (Fig. 5A).
**Fig. 5 Chondrocyte and chondron.** A: The chondrocyte (C) with its nucleus (n) and the membrane system (m) constituted by the Golgi cisternae and the endoplasmic reticulum. Glycogen and lipid vesicles (v) are visible at the cell periphery compartment. The pericellular matrix (PM) of the chondron appears as a dark grey layer surrounding the chondrocyte while the territorial matrix (TM) is visible as a lighter grey color around the PM. B: detail of the interface between chondrocyte and ECM in the chondron. The PM contains aggrecan monomers and collagen type IV. A fiber of collagen type II (II c) is detectable in the TM that is mainly composed of proteoglycans.

The chondron has been defined as a structural unit comprising the chondrocyte and its pericellular and territorial matrices (PM and TM respectively) (30). In a chondron, the PM contains high concentration of aggrecan monomer, hyaluronan and link protein, suggesting that this area is responsible for the supramolecular assembly of the hyaluronan-aggrecan-link protein complexes. Furthermore, also fibronectin and collagen type VI, which are known to interact with ECM proteins and with cell receptors, occupy the PM, indicating that here the ECM proteins-cell interactions occur (Fig. 5B). Around the PM is the TM, which contains the collagen-fibril network and is responsible for the mechanical protection of the cell (Fig. 5B).

### 1.3 Articular cartilage: the bone-protection layer

Depending on the molecular composition, three kinds of cartilage are distinguishable: hyaline cartilage, elastic cartilage and fibrocartilage (31). Hyaline cartilage contains mainly collagen type II and is present in body joints and in other organs such as the nasal septum, ears and air-tubes of the respiratory system. Elastic cartilage, mostly found in the outer ear, larynx and epiglottis, contains elastin fibers. Finally, fibrocartilage contains large quantity of collagen type I and is found in the pubic symphysis, in the intervertebral discs, in the knee meniscus and in the temporomandibular joint (TMJ) disc.
As earlier mentioned, hyaline (or articular) cartilage is located on the surface of adjoining bones. It works as a low-friction, weight-bearing and wear-resistant material that allows the movement of the body (32). In the articular cartilage, moving from the surface to the bone, four different zones are detectable namely superficial, middle, deep, and calcified. Interestingly, each region presents physical characteristics reflecting its mechanical function (5) (Fig. 6).

The superficial zone constitutes 10 to 20 % of the articular cartilage thickness and it corresponds to the articulating surface that provides a smooth gliding plane and resistance to the shear stresses. Here, chondrocytes are elongated and preferentially express proteins that have lubricating and protective functions (33) rather than GAGs. The numerous collagen fibrils present in this zone are oriented parallel to the surface and confer resistance to the shear stresses to this area. The middle zone encompasses 40 % to 60 % of the articular cartilage volume. This zone has a higher compressive modulus than the superficial zone and less organized collagen fibers but with larger diameter. The deep zone represents 30 % of the articular cartilage. It has spherical chondrocytes arranged in a columnar pattern, large-diameter collagen fibers oriented perpendicularly to the articular surface, and the highest concentration of PGs. A “tidemark” separates the deep zone from calcified cartilage, which rests directly on the subchondral bone. The last zone of articular cartilage, the calcified zone, is the area that anchors the overlying cartilage to the subchondral bone. It is characterized by small and randomly distributed chondrocytes in a matrix containing apatitic salts. The stiff calcified zone blocks the transport of nutrients from the underlying bone, rendering articular cartilage dependent on synovial fluid for nutritional support.
1.3.1 Mechanical properties of cartilage: the link between composition and mechanical properties of cartilage

The main components of cartilage, which are water, collagen and PGs, confer to this tissue its particular mechanical properties (34). Collagen, which represents 60-65 % of cartilage dry weight, is the tensile-resisting component while PGs, which occupy 10-15 % of the cartilage dry weight, are the compression–resisting components of cartilage. Collagen and proteoglycans form an interwoven solid matrix that allows the tissue to resist shear stresses as well as tension and compression (5).

The high negative charge density of the linear GAG chains results in electrostatic repulsion forces that significantly contribute to tissue compressive stiffness and osmotic fluid retention. Indeed, negative charges of neighboring GAGs repel each other, and in order to keep negative charges far apart, the PGs molecules are distended. When the negative charges are forced into proximity, during compression, the result is an increase in repulsion forces. Nevertheless, since the collagen network maintains proteoglycans in a compressed state, cartilage possesses a significant swelling pressure (35).

1.3.2 Viscoelastic behavior of cartilage

Cartilage, similarly to tendons and ligaments, exhibits viscoelastic properties-meaning a combination of viscous and elastic behaviors (36). A viscous substance is defined as a substance
that flows (continues to deform over time) under the action of an applied load that altered its initial status. Differently, elastic material deforms under the action of an applied load but returns to its original shape once the load is removed. A viscoelastic material is defined as an object that deforms under a load in a time-dependent fashion. The behavior of viscoelastic materials can be studied by performing conventional mechanical tests such as creep, stress-relaxation, and hysteresis (37) (Fig. 7).

During a creep test, mechanical stress\textsuperscript{1} is applied to a material and the strain\textsuperscript{2} or the deformation of the material is monitored over time (Fig. 7A). When mechanical stress is applied to an elastic material, it deforms instantaneously until the stress is released. Conversely, when a constant mechanical stress is applied to a viscoelastic material, it adapts over time until a dynamic equilibrium is reached.

In a stress-relaxation test, strain is applied to a material and the response of the material is monitored as mechanical stress (Fig. 7B). If the material is elastic, an immediate change in mechanical stress corresponds to an immediate change in strain. The stress then remains constant over time as long as the applied strain does not change. Viscoelastic solids exhibit as well an immediate change in stress in response to an applied strain; however, the stress does not remain constant but decreases exponentially until a nonzero plateau is reached, while the applied strain remains at a constant level.

For elastic material, the loading and unloading curves are identical (linear) but for a viscoelastic solid the loading curve is always above the unloading curve indicating that there is a loss of energy associated with the loading/unloading cycle. This effect is called hysteresis (Fig. 7C).

\textsuperscript{1} Mechanical Stress is defined as the force acting on a material divided by the area over which that force is distributed
\textsuperscript{2} Strain is a unit-less measure of shape changes and can be calculated as deformation divided by the original length of the material.
Fig. 7 Conventional mechanical tests used to study the mechanical properties of tissues. A: In a creep test the tissue is subjected to a constant stress and the deformation (strain) is observed. B: In a stress-relaxation test the tissue is subjected to a strain and the stress is monitored. C: during hysteresis a stress or strain is applied and the stress is observed as tissue response.
As shown in Fig. 8, cartilage presents a non-linear stress-strain response. The beginning of the loading is called the “toe region”. Here, after the loss of water, the cartilage begins to resist the load by increasing its stiffness. Later, the curve becomes linear and remains linear until failure of the cartilage matrix occurs. The slope of the curve, at any given strain value, provides a measure of stiffness (Young’s modulus) of the tissue. The three regions of the curve can be explained by what is occurring to the collagen during the tensile loading. During stretching, the collagen fibrils are re-aligned. Once the crimp has been removed from the collagen, further stretching causes the breakage of the molecule.

![Stress-strain behavior of cartilage](image)

*Fig. 8 Stress-strain behavior of cartilage. Short description of different regions.*

### 1.3.3 Common types of *in vitro* cartilage compression tests

*In vivo*, articular cartilage is subjected to a complex combination of compressive stresses (38), thus the understanding of the cartilage response to mechanical loading is of great importance. Three types of uni-axial compression tests (Fig. 9) are conventionally performed on cartilage explants *in vitro*: indentation, confined compression and unconfined compression (39).

In an indentation test, the uniaxial compression is performed by using an indenter having a surface smaller than the explant extension and thickness (Fig. 9A). An advantage of this test is that it can be performed on cartilage with its underlying bone.

In contrast, both confined and unconfined compressions are generally performed in cylindrical plugs of cartilage. During unconfined compression tests (Fig. 9B) a stress or strain is applied over the entire surface of the explant and even if the compression platen is made impermeable, a lateral expansion of the tissue and loss of liquid are permitted.
For the confined compression test (Fig. 9C), the cartilage specimen is placed within a confining chamber, usually with an impermeable bottom and a porous platen covering the top surface. Relative motion between the platen and bottom surface causes fluid movement, ideally only through the porous platen. Since the stiffness of the platen is high compared with the tissue, and the fluid is virtually incompressible, the major mechanism by which the tissue deforms is by exudation of the fluid through the surface that is in contact with the porous platen.

Fig. 9 Conventional compression tests used to study cartilage. A: Indentation test. B: Unconfined compression. C: confined compression

1.4 Cartilage mechanotransduction: the cell-matrix interactions

Mechanotransduction is a process by which a mechanical stimulus is converted into a biochemical signal, which causes changes in the cell behavior (40). It is still not well understood how cells sense mechanical forces and deformation, and convert and combine such signals with biochemical responses (41). Compression of cartilage provokes complex changes within the tissue that include matrix and cell deformation, hydrostatic pressure gradients, fluid flow, altered matrix water content and changes in osmotic pressure, ion concentration and fixed charge density (42). Chondrocyte mechanoreceptors, such as mechanosensitive ion channels (43) and integrins (44), are likely to be involved in recognition of these physiochemical changes.

1.4.1 The mechanosensitive ion channels

When a mechanical load is applied to a cell, an increase in cell membrane tension occurs. This event is sensed by the ion-channels that open and convert the mechanical force exerted on the cell membrane into electrical or biochemical signals (45;46). In chondrocytes, three main
types of ions channels have been described: voltage-gated sodium channels (VGSC), epithelial sodium channels (ENaC) and N-/L-type voltage-gated calcium channels (VGCC) (47).

The first step in a mechanotransduction cascade consists of the depolarization of the chondrocyte membrane. Indeed, when cartilage is subjected to a mechanical load, an influx of sodium through the VGSC and the ENaC occurs and causes the cell depolarization. Successively, since the VGCC and the stretch-activated ion channels (SAC) are opened, a further influx of calcium causes a membrane hyperpolarisation. The calcium-activated potassium channels are now opened and an efflux of potassium tries to restore the initial resting potential of the cell. The increase of the intracellular calcium level culminates in the propagation of calcium waves that activate signaling cascades.

1.4.2 Role of integrins in mechanotransduction

Integrins are a family of heteromeric transmembrane proteins, consisting of α and β subunits (48). The combination of α and β subunits determines the receptor specificity resulting in over 20 specific-receptor combinations (49). The location of integrins between the ECM and the cell cytoskeleton is ideally suited for their role as mechanoreceptors. The integrin receptor has an extracellular domain that provides a ligand-binding site while the cytoplasmic tail interacts with intracellular molecules and the actin cytoskeleton enabling the integrin receptor to translate mechanical signals into intracellular biochemical responses.

Among all the integrins expressed in adult cartilage (50;51), the α1β1, α2β1, α3β1 and α5β1 are the main mechanoreceptors. α1β1 and α2β1 bind to collagen and laminin (52-54); α3β1 binds to fibronectin and collagen type II (55) and α5β1 binds fibronectin in the extracellular matrix.

1.4.3 Intracellular signaling induced by mechanotransduction

The response of chondrocytes and in general, of cartilage to a mechanical load depends on the intensity and on the duration of the applied mechanical stresses. In general, upon a mechanical stimulus, one of the earliest events occurring in the chondrocyte within seconds to minutes is the change in the intracellular calcium levels. The newly formed calcium waves activate calcium-responsive proteins such as protein kinase C, Calmoduline (CAM) kinase and calcineurin, which trigger some other down-stream transcription factors (e.g. CREB and NF-AT).
Mechanotransduction mediated by the integrins consists of three main events: 1) the phosphorylation and activation of MAP kinase signaling pathways (denoted by MEKK and JNK) that lead to ERK1/2 phosphorylation (56); 2) the activation of PLC leading to IP3 generation and gating of intracellular calcium stores, and 3) the alterations in the actin cytoskeleton network. In general, the ultimate targets of the mechanotransduction signal cascade are specific genes that are able to adapt cartilage metabolism to the surrounding physical conditions.

1.5 Body joints: systems allowing the body movement

A joint is an anatomical structure where two connecting bones are held together (57). The main functions of a joint are to ensure the movement of the body and to provide mechanical support. Body joints can be classified by their structural composition or their anatomical function. Even if often the two classifications overlap, the structural classification describes the way in which bones are connected to each other, whereas the functional classification focuses on the degree of movements between the articulating bones.

According to structural classification, a joint can be defined as fibrous, cartilaginous and synovial (Fig. 10). In the first type of joints, the bones are linked by a dense connective tissue mainly represented by collagen whereas in a cartilaginous joint the two articulating bones are separated by cartilage. The synovial joints are characterized by the presence of a capsule surrounding the articulating bones containing a lubricating synovial fluid.

![Fig. 10 The three types of body joints](image)

The joints can also be functionally classified by the degree of mobility they allow (Fig. 10). A synarthrosis is a joint that does not allow any mobility. Most of these joints are also fibrous joints
(e.g. the sutures of the skull and the pelvis). An **amphiarthrosis** is a joint where a slight mobility is permitted. Most of these joints are cartilaginous joints (e.g. the vertebrae of the spine and the ribs). Lastly, the **diarthrosis** is a joint that permit a wide range of movements. All **diarthroses** are synovial joints (e.g. the shoulder, the hip, the elbow, the knee).

### 1.5.1 Joint diseases

Osteoarthritis (OA) and rheumatoid arthritis (RA) are the more important and well-known pathologies of the joints (58). Although the etiologies of these diseases are different, their common and irreversible result is cartilage destruction caused by elevated expression and activity of proteolytic enzymes (59). In physiological conditions, the production of proteolytic enzymes, required for the normal tissue remodeling and repair of the tissue (60;61), is accurately controlled by the chondrocytes. In contrast, in RA, the cells of the immune system (T and B lymphocytes, monocytes, macrophages, and dendritic cells) as well as the chondrocytes, release in the synovium an elevated content of cytokines, which in turn activate gene expression of cartilage-degrading enzymes (62;63). Similarly, in OA the catabolic enzymes are mainly produced by the chondrocytes in response to a prolonged, injurious mechanical stress (64).

### 1.5.2 Temporomandibular joint

The temporomandibular joint (TMJ) is the diarthroidial articulation comprising the condyle of the mandible and the glenoid fossa of the skull while a fibro-cartilaginous disc separates the two articulating surfaces (Fig. 11).

![Fig. 11 Anatomy of the TMJ. Fossa, condyle, and cartilage disc.](image)

The TMJ is a bilateral joint and, in physiological conditions, the left and the right joints always move rhythmically and simultaneously allowing mastication and speech (65).
During jaw opening and closing, which are mainly controlled by the facial muscles, the cartilage disc is simultaneously subjected to 1) a normal force, 2) rotation and 3) translation of the underlying condyle (Fig. 12). Indeed, mouth opening causes the sliding of the condyle-disc-complex along the glenoid fossa while at the same time the condyle rotates underneath the disc.

![Fig. 12 The TMJ during mouth opening. The anatomy is recorded by means of magnetic resonance imaging (MRI) and animated with in vivo motion data. In the upper part a sequence of sketches during the mouth opening is depicted. The lower part of the figure shows a detail of the glenoid fossa and the mandibular condyle. The cartilage disc occupies part of the space between fossa and condyle.](image)

The antero-posterior translation and the rotation of the condyle around the transversal axis are the primary movements of the TMJ, although medio-lateral translations and rotations around oblique axes also occur (66). TMJ simple movements can thus be distinguished in four types: opening/closing (abduction/adduction), protrusion (forward movement), retrusion (backward movement) and laterotrusion (lateral movements). Opening/closing occurs with larger rotational components than the other three types of movements, which are characterized by a minimum amount of rotation.

A typical loading modality occurring in the TMJ is the plowing, which originates from simultaneous condyle sliding and application of a normal force exerted by the condyle.
1.5.3 TMJ disorders

TMJ disorders (TMD) refer to a group of diseases with symptoms including pain, joint sounds (clicking, crepitus) and limited or asymmetrical jaw movements. Generally, the health and the quality of life of patients affected by TMD are deeply influenced (67;68). Among TMDs, disc displacement is the most common. Considering that the coordinated movement of the condyle and the disc is essential to maintain the integrity of the TMJ, when the disc is forced out of the correct position, a direct condyle-fossa contact occurs. The main symptom of disc displacement is a popping sound originating when the mouth opens up and then again when the mouth is closed as the disc is forced back into place. The disc displacement induces change of stress distribution in the disc itself and the increase of frictional coefficients between the articulating surfaces (69;70) resulting in the wear and tear on the TMJ disc (71) which could later lead to the onset of TMJ osteoarthritis (72).

1.5.4 Plowing effect

Plowing consists of the simultaneous compression (oriented normally to the surface), and motion (tangential to the surface) of a condyle onto cartilage (Fig. 13A). The combination of frictional and plowing forces occurring at the surface of the cartilage (Fig. 13B) causes a cartilage deformation that usually appears as a hump in front of the condyle (Fig. 13C). Moreover, the combination of frictional and plowing forces induces the formation of tractional forces (73). Plowing has been classified as one of the main friction mechanisms in all the synovial joints (74) and is a cause of OA onset (75).
**Fig. 13 Schematic example of the plowing effect.** A: Cartilage is represented as a grey surface while the white object represents the condyle. The red arrows indicate an applied loading consisting in a vertical force and in a motion on the horizontal plane. B: Detailed view of the interface between condyle and cartilage. The big arrow represents the frictional force whereas the small dashed arrows indicate the plowing forces. C: Cartilage deformation induced by the working forces is shown as a hump.

### 1.6 The literature review about cartilage loading

In order to understand the pathomechanics of cartilage degradation induced by mechanical stress, the response of cartilage to injurious mechanical loading has been extensively studied by means of several laboratory models (76). Several research groups have investigated the effect of impact loading or confined/unconfined compression on full thickness cartilage explants providing important results.

Jeffrey *et al.* showed that impact loading at 0.049 to 1.96 J causes macroscopic, physical changes on full thickness cartilage explants that became more flattened and fissured, depending on the severity of the impact. Interestingly they also demonstrated that impact loading on cartilage explants attached to their underlying bone is less harmful for the cartilage, suggesting the important shock-absorbing role of the bone (77). Lewis *et al.* and Quinn *et al.* demonstrated that injurious impact loading causes macroscopic cracks in cartilage explants and cell death (78;79).

Other studies have been focused on the investigation of the effect of impact loading on cartilage explants at later post-loading equilibration periods. Torzilli showed that 24 hours after impact stresses at different pressure (0.5 to 65 MPa), the PGs biosynthesis decreased, while the
water content increased, both with load-dependent magnitude trends (80). Natoli et al. studied the effect of impact stress at low (1.1 J) and high load (2.8 J) on cartilage explants attached to the underlying bone after 24 hours to 4 weeks post-loading equilibration. They showed that the cell death induced by mechanical stress increased over time and that the cartilage explants loaded with lower pressure presented a delayed biological response compared to the other loaded with higher pressure (81). Chan et al. studied the changes in the gene expression of full-thickness cartilage explants after impact loading at 30 MPa pressure. In particular, the authors reported that the genes coding for cytokine and chemokine receptors as well as for catabolic enzymes and mechanoreceptors were up-regulated 3 hours after the injury, while the genes coding for adhesion molecules and apoptosis were down-regulated (82).

Several studies have been focused on the effect of unconfined compression on cartilage explants. D’Lima et al. showed that unconfined compression of cartilage explants at 14 MPa induces GAG release and chondrocyte death (83). Similarly, Grodzinsky showed that uniaxial unconfined compression of cartilage plugs causes a GAGs loss over time and explained that it is due to the physical degradation of the cartilage and also to induced catabolic activities (84). Interestingly, it has been reported that during unconfined compression, an important parameter influencing the general mechanical stress is the rate of the strain. It has indeed been observed that the new synthesis of proteins as well as the cell death are strictly dependent on the rate of the applied strain (85). Grodzinsky reported also that static compression on articular cartilage explants causes specific time-dependent changes in chondrocytes gene expression and that cyclic uniaxial compression or shear stress regulates clusters of functionally related genes patterns (86;87).

Nevertheless, it has also been reported that moderate mechanical loading is necessary for the maintenance and the homeostasis of cartilage (88-91).
2 Aim of the thesis

Prolonged, injurious mechanical load is considered a major cause of cartilage damage, and thus a risk factor for cartilage destruction and occurrence of pathologies such as OA and RA (section 1.5.1) (92).

Mechanical injuries compromise cartilage homeostasis 1) directly, by causing physical damages, or 2) indirectly, by inducing the chondrocytes to over-produce catabolic enzymes.

The understanding of the molecular events leading to cartilage destruction may lead to alternative treatments for the cure of joint diseases (e.g. OA). As a matter of fact, intermediate processes still unknown could be discovered, and furthermore, new targets could be detected for the development of innovative drugs protecting cartilage from degradation.

The present study was motivated by the possibility of simulating in vitro the complex loading that cartilage undergoes in vivo in a joint. As described in section 1.6, several successful and important studies have already been performed by other researchers in order to reproduce mechanical cartilage loading. However, the uniaxial setup of these studies presents some limitations.

The main focus of this thesis is to mimic the plowing effects occurring in the TMJ and to measure mechanical and biological effects of this kind of loading on a pristine cartilage model, namely bovine nasal septum (BNS) cartilage. To achieve this goal, the work has been divided into sequential steps: 1) the mechanical and biochemical characterization of the cartilage model as described in Chapter 4; 2) the evaluation of BNS cartilage as a model tissue for loading experiments as reported in Chapter 5; 3) the plowing experiment of BNS cartilage at room temperature (Chapter 6); and 4) plowing of BNS cartilage with different applied normal forces performed at physiological temperature (Chapter 7).

Furthermore, Chapter 3 contains the experimental procedures including materials and methods. In Chapters 4, 5, 6 and 7 the motivation, results and discussion for each sequential step of the thesis work are introduced respectively within each chapter. However, a summary of the most relevant findings and a general outlook is located at the end of this thesis (Chapter 8).
3 Experimental procedures

All techniques and protocols used during the experimental studies are described throughout this chapter.

3.1 Software modeling and animation of the TMJ by dynamic stereometry

In previous work, three-dimensional modeling and animation software, so-called dynamic stereometry, has been developed at the “Laboratory of Physiology and Biomechanics of the Masticatory System” of the Center of Dental Medicine (ZZM) of the University of Zürich (93). This system measures TMJ disc deformations during mandibular function (93-95) by using elaboration of TMJ anatomies data acquired by magnetic resonance imaging (MRI) and jaw tracking (a system which allows to record mandibular movements dynamically with six degrees-of-freedom) (Fig. 14).

![Dynamic stereometry diagram](image)

**Fig. 14 Dynamic stereometry.** Temporomandibular joint models are reconstructed in three dimensions from magnetic resonance (MR) images and then animated with in vivo motion data recorded by a jaw tracking system.
3.2 The rolling/plowing explants test system (RPETS)

A rolling/plowing explant test system (RPETS) has been developed on the basis of the data describing TMJ disc loading acquired by using dynamic stereometry (“Laboratory of Physiology and Biomechanics of the Masticatory System” at the Center of Dental Medicine (ZZM) of the University of Zürich) (3.1). RPETS is able to mimic plowing effects resulting in vivo by the combination of compression and sliding of the mandibular condyle on the TMJ cartilage disc (Fig. 15) (96). RPETS is composed of a motor-driven indenter (mimicking the condyle) and of a tank (whose bottom corresponds to the temporal bone), where a cartilage strip (mimicking the TMJ disc) is placed. RPETS can move the indenter in the horizontal and vertical direction, and vary the applied forces and the sliding speeds. RPETS is equipped with linear variable differential transformers (LVDTs) for displacement measurements and with load-cells measuring forces in all directions. Furthermore, a software application based on LABVIEW 8.2™ (National Instruments, Austin, Texas) allows the user to program the experimental setup, to monitor loads in real time, and to record and store all experimental data.

Fig. 15 Perspective view of the rolling–plowing explant test system (RPETS). The motor-driven linear stages (1), the indenter (2) mounted on the moving arm, and the tank (3) are indicated. In the box above left, a detail of the tank containing the sample to test can be seen. The sample is glued onto a Plexiglas® plate and fixed by screws to the tank bottom.
3.3 Bovine nasal septum cartilage harvesting

In the present project, BNS cartilage has been chosen as cartilage model tissue for plowing experiments. The BNS is an abundant source of hyaline cartilage, pristine, not load bearing, and without orientation of cells or proteins (97). Furthermore, BNS cartilage has already been widely used by other researchers for different purposes: as a substrate for the study of enzymatic reactions or as a source of macromolecules such as GAGs (98-101).

BNS of 12-month-old calves—both female and male—was provided by a local abattoir within 4 hours of slaughter. After removal of surrounding bones and perichondrium, BNS appears with a quite regular shape (60 x 100 mm) but with variable thickness (3-8 mm) (Fig. 16).

Fig. 16 BNS cartilage. After removal of bones and perichondrium BNS cartilage appears as a large explant (40 x 100 mm). Scale bar = 10 mm.

Cartilage explants were shaped under sterile conditions by using a custom-made cartilage cutter that allows obtaining samples with constant thickness (2.15 ± 0.06 mm). Immediately after harvesting, cartilage explants were rinsed in Dulbecco’s Phosphate-Buffered Saline (D-PBS) and equilibrated overnight in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 10 mM Hepes buffer, 0.1 mM nonessential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B.

BNS cartilage explants were differently shaped, depending on the type of experiment to be performed. For mechanical and biochemical characterization (4.2.1), cartilage discs were obtained by using a biopsy punch (Ø 5 mm); for static loading experiments the cartilage was shaped in cuboidal explants (10 x 10 x 2 mm) (L x W x H) while, for plowing experiments, cartilage strips (60 x 17 x 2 mm) (L x W x H) were collected.
3.4 Cartilage-loading experiments

Two different types of loading have been set-up: static uniaxial compression and plowing. Static uniaxial compression consisted of a stress-relaxation test (3.4.1) whereas plowing consisted of simultaneous pressing the indenter orthogonally to the cartilage main surface while sliding it tangentially (3.4.2). A detailed description of the two loading setups follows.

3.4.1 Static-loading experiments

For the mechanical characterization of BNS cartilage (section 4.2.2), cartilage explants were subjected to confined or unconfined compression (as described in section 1.3.3).

A confined compression was performed by positioning the cartilage sample in a stainless-steel chamber (Ø 5 mm) placed over a stainless-steel porous filter allowing free fluid flow (Meyer Sintermetall AG, Studen, Switzerland; average pore diameter ≈ 25 μm). The compression was performed by using a non porous stainless-steel indenter (the clearance between chamber and indenter was 0.05 mm). After applying a preload of 0.5 N, a 10 % strain, reached with a ramp-rate of 0.001 mm/s, was applied to the cartilage explant and kept 1200 seconds for tissue relaxation.

The unconfined compression was performed by placing the cartilage specimen between two stainless steel plates and applying a 10 % strain at a ramp-rate of 0.001 mm/s and kept 1200 seconds for tissue relaxation. To avoid dehydration, the cartilage samples were immersed in PBS throughout the measurements.

Additional stress-relaxation tests were performed for studying the effect of static compression on gene expression of BNS cartilage (section 5.1.1). They consisted of unconfined compression aiming to reach a 50 % strain with a relaxation time of 1, 2 or 4 hours (section 1.3.2).

3.4.2 Plowing experiments

Plowing, which was performed in a sterile hood with a duration of 2 hours, consisted of the sliding of a cylindrical indenter (Ø 25 mm; stainless steel) at constant normal force on a cartilage strip (displacement of 40 mm; tangential speed: 10 mm/sec).
In more detail, plowing performed in our experiments was set up as a regular cyclic motion of the indenter on a cartilage strip as summarized in Fig. 17: A) vertical descent of the indenter until contact with the cartilage surface; B) vertical indentation to reach the desired normal force; C) horizontal displacement with constant speed while maintaining stable the normal force; D) raising of the indenter; E) return of the indenter to the initial position.

![Diagram showing the plowing process](image)

*Fig. 17 Schematic representation of the plowing on a cartilage strip. A) The indenter moves vertically until it contacts the cartilage; B-D) the indenter is displaced horizontally at the desired speed while keeping the applied normal force constant; D-E) the indenter releases the normal force, leaves the cartilage and returns to its initial position.*

3.5 Biological assays

The biological assays described in the following section aimed at 1) the determination of cartilage components such as water (3.5.1), GAGs (3.5.2) and collagen (3.5.3); 2) the study of chondrocytes gene expression changes (3.5.7) and 3) the microscopic evaluation of BNS cartilage (3.5.4, 3.5.5 and 3.5.6).

3.5.1 Determination of water content

Water content was measured by weighing each cartilage sample before and after freeze-drying and was expressed as the percentage of wet weight, using the following equation: % water = 100 \times (\text{wet weight} - \text{dry weight}) / \text{wet weight} (102).
3.5.2 GAGs measurements

GAGs content has been measured for two different purposes: 1) to investigate the general GAGs content in the BNS cartilage (section 3.5.2) and 2) to determine GAGs loss (as an indicator of cartilage degradation) into the media after cartilage plowing (section 7.3.4). In both cases, GAGs content was determined by means of the 1, 9-dimethylmethylene blue (DMMB) assay. Nevertheless, in the first case GAGs content was measured directly in the tissue whereas in the second case it was quantified in the culture media.

For the measurement of the GAGs content of the cartilage explants, the following protocol has been developed. Dry cartilage samples were digested at 64 °C for 18 hours in a solution containing 20 U/mL papain (Worthington Biochemical Corp., Lakewood NJ, USA) in 1 mM L-cysteine with 0.5 mM EDTA. After enzymatic digestion, the samples were centrifuged at 12,000 g for 5 min. Supernatants were collected and GAGs quantification was performed according to Barbosa et al. (103) with appropriate modifications. Briefly, 100 μL sample aliquots (papain digested cartilage) diluted at 1/1000 and Chondroitin-sulphate-A standard (10-100 μg/mL) were incubated with 1 mL of DMMB solution (16 mg/L DMMB in 0.2 M GuHCl, 1 g/L sodium formate and 1 mL/L formic acid) mixed continuously for 30 min in order to promote the formation of the GAGs-DMMB complex. After centrifugation at 12,000 g for 10 min, the precipitated complex was resuspended in 1 mL water and again centrifuged as before, in order to remove any dye excess. The complex was finally dissociated by adding 800 μL of decomplexation solution (50 mM sodium acetate buffer, pH 6.8, containing 10 % n-propanol and 4 M GuHCl). The absorbance of 200 μL solution was read at 656 nm using a spectrophotometer plate reader (Synergy HT multi-Mode Microplate Reader, BioTek Instruments Inc., Winooski VT, USA). Finally, GAGs concentrations were calculated on the basis of the Chondroitin-sulphate-A standard curve.

GAGs release into the media was assessed according to Jeffrey et al. (104). Briefly, 50 μL of appropriately diluted (1/100) medium samples as well as 50 μL of Chondroitin-sulphate-A standard (10-100 μg/mL) were mixed with 1 mL of DMMB solution (16 mg/L DMMB in 0.2 M Guanidine Hydrochloride (GuHCl)), 1 g/L sodium formate and 1 mL/L formic acid). The absorbance at 525 nm was immediately read in triplicate in a 96 well-plate using a spectrophotometer plate reader.
3.5.3 Collagen determination in BNS cartilage

Collagen content was quantified by means of the hydroxyproline assay, according to Reddy et al. (105) with some modifications. In more detail, 100 μL of diluted papain digested cartilage as well as 100 μL of standard hydroxyproline (Hyp) solution (2-40 μg/mL) were mixed with 100 μL of 4 N sodium hydroxide (1:1 v/v), gently mixed and hydrolyzed by autoclaving at 120 °C for 20 min. Autoclaved samples (100 μL) as well as standard solution (100 μL) were mixed with 425 μL of chloramine-T solution (0.056 M chloramine-T in 50% n-propanol) and incubated at room temperature for 25 min. Then, 475 μL of Ehrlich’s solution (1M p-dimethylaminobenzaldehyde in n-propanol/perchloric acid (2:1 v/v)) were added to the oxidized samples or to the standard solution and incubated at 64 °C for 20 min. Absorbance was read at 550 nm in a plate reader and the amount of Hyp in the samples was determined by comparing with a Hyp standard curve. Thereafter, since Hyp represents approximately 14.4 % of the aminoacidic composition of collagen, a factor of 6.94 was applied to calculate the total collagen content from the measured Hyp quantity.

3.5.4 Chondrocytes viability assay

Cartilage slices (500 μm) were rinsed in PBS and incubated for 20 min in DMEM containing 1 μg/mL calcein acetoxymethylester (AM) (Sigma-Aldrich Inc., St. Louis MO, USA) (“live—cell staining”) and 1 μM ethidium homodimer (Sigma-Aldrich Inc., St. Louis MO, USA) (“dead—cell staining”). The slices so treated were transferred into fresh DMEM and fluorescent images were acquired at 515 nm and 635 nm using an inverted fluorescence microscope (Axiovert 200, Carl Zeiss AG, Oberkochen, Germany) equipped with a digital camera.

3.5.5 Cartilage treatment for transmission electron microscopy

Cartilage explants were treated for transmission electron microscopy (TEM) as described by Hunziker et al. (106). The primary cartilage fixation was performed in a buffer solution containing 2 % glutaraldehyde and ruthenium (III) hexamine trichloride (RHT). After fixation, the samples were washed 3 × 5 min in the same buffer. Post-fixation was carried out in 1 % osmium tetroxide, again in the same buffer. After 2 hours, the tissue was washed three times in buffer and stored overnight at 4 °C. The tissue was then dehydrated in ethanol from 70 % to 95 %, each step during 15 min followed by a final dehydration in ethanol (100 %) 3 × 30 min. After 3 × 10
min in propylene oxide, the specimens were infiltrated with a mixture of propylene oxide and Epon 1:1 plus 0.6 % accelerator for 1 hour. Thereafter, the cartilage samples were kept for 2 days in Epon 812 with 1.2 % accelerator; polymerization took place at 60°C for 5 days.

3.5.6 Cartilage treatment for light microscopy

For the light-microscopy study, BNS cartilage explants were randomly collected and embedded in polymethyl methacrylate (PMMA) (Merck) using standard procedures (107). Cartilage trans-sections (5 μm) were cut from PMMA embedded blocks and stained with Goldner’s trichrome (Merck GmbH, Darmstadt, Germany).

3.5.7 RNA extraction and real-time quantitative polymerase chain reaction

The gene-expression changes induced by mechanical loading were measured by performing quantitative real-time polymerase chain reaction (RT-qPCR) after RNA extraction and reverse transcription. Cartilage explants were snap-frozen in liquid nitrogen and then stored at -80 °C. RNA extraction was performed according to Davidson et al. (108) with some modifications. Finely sliced cartilage sub-explants (~ 50 mg) were placed in Eppendorf tubes and homogenized twice for 1 min in 800 μL TRIZol reagent (Invitrogen Inc., Carlsbad CA, USA). After 5 min equilibration at room temperature, 200 μL of chloroform were added and the tubes were vigorously shaken, mixed and incubated for 2 min at room temperature. Following centrifugation at 9,500 g for 30 min at 4°C, the obtained aqueous phases were recovered, extracted with 200 μL of chloroform and treated as previously described. The recovered supernatants were transferred into 2 mL tubes, gently mixed with 500 μL of isopropanol, incubated for 10 min at room temperature and consecutively centrifuged at 9,500 g for 40 min at 4 °C. The supernatants were discarded and the pellets resuspended in 900 μL of lysis buffer (RNeasy Mini Kit®; Qiagen GmbH, Hilden, Germany) supplemented with 90 μL β-mercaptoethanol (Sigma-Aldrich Inc., St. Louis MO, USA). After adding 900 μL ethanol (75 %), the RNA was purified using Qiagen RNeasy mini kit (Qiagen GmbH, Hilden, Germany) whereas genomic DNA was digested with DNase kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of RNA was performed with random hexamer primers using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics AG, Rotkreuz, Switzerland), according to the manufacturer’s instructions. RT-qPCR of the obtained cDNA was performed on a 96 well plates by means of iCycler Real-Time Detection System® (iQ5; Bio-Rad
Laboratories Inc., Hercules CA, USA) and the reactions were carried out using QuantiFast™ SYBR® Green PCR kit (Qiagen GmbH, Hilden, Germany). The used primers were the same as reported by Fitzgerald et al. (86): they have been designed for the extracellular matrix proteins aggregan (Agg), collagen type I (Coll1), collagen type II (Coll2), fibronectin (Fn), for the catabolic enzyme stromelysin-1 (MMP-3) and its inhibitor, i.e. the tissue inhibitor of metalloproteinase (TIMP-1), and for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and ribosomal RNA 18S (18S rRNA) (Table 1). Housekeeping genes and control samples were run on every plate for normalization purposes. The gene regulation was calculated as a multiple by using the comparative threshold cycle (Delta-Delta Ct) method (109). Thus, a gene was considered up-regulated when the multiple of expression was higher than 2 fold and down-regulated for values lower than 0.5 fold.

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<th>Reverse Primer (5’ – 3’)</th>
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</table>
4 Mechanical and biochemical characterization of bovine nasal septum cartilage

4.1 Abstract

BNS cartilage is hyaline, not load-bearing and shows no orientation of cells or fibers (97). Therefore, it can be considered a good candidate for performing in vitro loading experiments. The fact that, in physiological conditions, BNS cartilage has only a support function, and thus it is not subjected to mechanical loading, suggests that it is pristine and thus, as earlier mentioned, a convenient model tissue for in vitro cartilage loading experiments.

The present chapter focuses on the mechanical and biochemical characterization of BNS cartilage. Mechanical (static confined and unconfined stress-relaxation) and biochemical assays (microscopic assessment of the tissue; water, glycosaminoglycans and collagens contents) of BNS specimens have been performed by conventional assays: light microscopy, transmission electron microscopy (TEM), lyophilization, 9-Dimethyl-methylene blue (DMMB) assay and Hydroxyproline (HyP) assay, respectively.

4.2 Experimental procedures

4.2.1 Explants harvesting from bovine nasal septum cartilage

Three BNS samples from 12—months—old—calves, both female and male, were provided by the local abattoir within 4 hours of slaughter. Under sterile conditions, after removal of surrounding bones and perichondrium, each BNS was divided into nine regions, following the dorso-ventral and antero-posterior axes (Fig. 18B). Each region was washed in phosphate-buffered saline (PBS) (Invitrogen Inc., Carlsbad CA, USA), and equilibrated overnight in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 10 mM Hepes buffer, 0.1 mM nonessential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B.

Each region was cut by means of a custom-made microtome blade cutter in order to obtain explants of constant thickness (2 mm). From each region, four explants (average ø 4.72 ± 0.03 mm; average thickness 2.15 ± 0.06 mm) were obtained by means of 5-mm ø sterile biopsy
punches. One cartilage sample was stored in PBS at 4 °C until mechanical tests were performed, whereas three explants were collected for biochemical characterization.

**Fig. 18 BNS cartilage harvesting.** A) After removal of bones and perichondrium, BNS cartilage appears as a large explant (40 x 100 mm). B) The BNS cartilage has been divided into 9 regions along the postero-anterior and dorso-ventral axes. Scale bar = 10 mm.

### 4.2.2 Mechanical characterization

As described in Chapters 1 and 3, (sections 1.3.3 and 3.4.1 respectively) BNSs cartilage explants (10 x 10 x 2 mm) (L x W x H) were subjected to static confined and unconfined stress-relaxation tests by using a universal testing machine (Zwick/Roell Z010, Zwick GmbH, Ulm, Germany) using a built-in software (test-expert V 11.02, Zwick/Roell, Ulm, Germany).

One day after harvesting, confined compression tests were performed by positioning the sample in a stainless-steel chamber (Ø 5 mm), which was placed over a stainless-steel porous filter to allow free fluid flow (Meyer Sintermetall AG, Studen, Switzerland; average pore size = 25 μm) (Fig. 19A). A non-porous stainless-steel indenter was used to apply compression and the clearance between chamber and indenter was 0.05 mm. Initially, the cartilage sample was subjected to a preload of 0.5 N, and then a 10 % strain at a ramp rate of 0.001 mm/s was applied. It was followed by 1200 seconds of relaxation.

The second day after harvesting, unconfined compression tests were performed on the same samples by placing each specimen between two stainless-steel plates and applying a 10 %
strain at a ramp-rate of 0.001 mm/s followed by 1200 seconds of relaxation (Fig. 19B). To avoid dehydration, the cartilage discs were immersed in PBS throughout the measurements.

**Fig. 19 Protocols for confined and unconfined stress-relaxation tests of BNS cartilage.** A) During confined compression, the cartilage sample is placed in a stainless-steel chamber which is located on a stainless-steel porous filter. A non porous stainless-steel indenter is used to apply a ramp-strain compression and then relaxation is allowed. B) During unconfined compression, the cartilage explant is placed between two stainless steel plates and subjected to ramp-strain compression and relaxation.

### 4.2.3 Biochemical characterization

*Light Microscopy and TEM.* For the light-microscopy study, BNS cartilage explants were randomly collected and embedded in polymethyl methacrylate (PMMA) (Merck GmbH, Darmstadt, Germany) using standard procedures (107). Cartilage trans-sections (5 μm) were cut from PMMA embedded blocks and stained with Goldner’s trichrome (Merck GmbH, Darmstadt, Germany) (section 3.5.6).

For the TEM, cartilage explants were sequentially fixed with 2.5 % glutaraldehyde supplemented with ruthenium (III) hexamine trichloride (RHT). The post-fixation was carried out in 1 % osmium tetroxide, whereas the successive tissue dehydration was performed in ethanol from 70 to 100 %. The specimens were infiltrated with a mixture of propylene oxide and Epon 1:1 plus 0.6 % accelerator for 1 hour and the polymerization took place at 60 °C for 5 days (detailed protocol in section 3.5.5).

*Water, GAGs and collagen content.* Water, GAGs and collagen content were determined, for each of the nine BNS regions, by means of lyophilization, DMMB assay and hydroxyproline assay respectively, as reported in Chapter 3 (paragraphs 3.5.1; 3.5.2 and 3.5.3 respectively).
4.2.4 Statistical analysis

Analysis of variance (ANOVA) was used to assess the independent effects of cartilage source (3 different nasal septa) and location within the nasal septum (through the postero-anterior and the dorso-ventral axes), on mechanical properties, water, GAGs and collagen content. Differences of statistical significance were based on values of $p < 0.05$.

4.3 Results and discussion

4.3.1 Mechanical characterization

Both in confined and unconfined compression, the elastic modulus ($E$) was measured either immediately after strain application ($E_0$), or at the end of the relaxation phase ($E_R$). $E_0$ and $E_R$ were similar in all 9 regions (Fig. 20). Furthermore, as shown in Table 2, the average value of $E_0$ was $4.07 \pm 0.57$ MPa for confined compression, and $4.54 \pm 0.96$ MPa for unconfined compression. The average value for $E_R$ was $2.27 \pm 0.36$ MPa for confined compression and $2.11 \pm 0.29$ MPa for unconfined compression.

When the elastic moduli were derived from confined compression, they were slightly, but not significantly lower than the modulus values of the unconfined test ($p > 0.05$). The limited number of cartilage samples did not allow a fully ANOVA to determine the variation on mechanical properties in the different regions. Nevertheless, a 2-tailed t-test for both confined and unconfined compression showed significant $E_0$ and $E_R$ differences between the dorsal and the ventral regions. In more detail, the differences of $E_0$ and $E_R$ were significant between the dorso-medial region and the ventral-anterior region and between the dorso-anterior region and the ventral-anterior regions ($p < 0.05$) (Fig. 20).
Fig. 20 Mechanical characterization of BNS cartilage. Instantaneous Young’s modulus ($E_0$) and relaxation Young’s modulus ($E_R$) measured during confined and unconfined stress-relaxation in the nine BNS regions collected moving through the dorso-ventral and antero-posterior axes. Each type of compression was from a separate BNS, (n=3) ( * indicates that $P < 0.05$).

Table 2 Average values and standard deviations of the $E_0$ and $E_R$ measured during confined and unconfined stress-relaxation of BNS cartilage plugs.

<table>
<thead>
<tr>
<th></th>
<th>CONFINED COMPRESSION</th>
<th>UNCONFINED COMPRESSION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVERAGE (MPa)</td>
<td>STANDARD DEVIATION</td>
</tr>
<tr>
<td>$E_0$</td>
<td>4.07</td>
<td>0.57</td>
</tr>
<tr>
<td>$E_R$</td>
<td>2.27</td>
<td>0.36</td>
</tr>
</tbody>
</table>

The reported results are comparable with those described by Gaon et al. (110). They collected 3 regions from the porcine nasal septum moving through the dorso-ventral axis and found a decrease in the Young’s modulus (5.5 MPa, 4.8 MPa and 4.8 MPa).

Other topographical stiffness variations have been also measured in the cartilage of the human knee (111) and in the disc of the temporomandibular joint of the pig (112). The authors explained that these stiffness variations are probably due to the adaptation of cartilage to the different load distribution in these joints. In the case of nasal cartilage, which is not subjected to mechanical stresses, the topographical variation of stiffness observed through the dorso-ventral axis could be due to the influence of neighboring bone structures (bone stiffness is in the order of GPa). Indeed, contrarily to the ventral part, the dorsal part of the BNS cartilage is not covered with perichondrium but it is directly in contact with bony structures of the skull.
4.3.2 Biochemical characterization

*Light Microscopy and TEM.* In contrast to articular cartilage, where cells follow a precise arrangement pattern (section 1.3), in BNS cartilage the chondrocytes are randomly distributed in the ECM (Fig. 21A and B). Additionally, BNS cartilage presents features of a metabolically active tissue, as indicated by the presence of chondrocytes in division (they appear as two cells very close one to each other) (Fig. 21C). Moreover, the difference in staining observed between the pericellular matrix (PM) and the territorial matrix (TM) (Fig. 21B and D) suggests that these two portions of ECM have different compositions and functions, similarly to other cartilage types (section 1.2.4). Furthermore, as showed in Fig. 21D, E and F, TEM allowed a closer inspection of the chondrocyte. Fig. 21D shows a chondrocyte (C) placed in its PM. The nucleus (n) and the membrane system (m) constituted by the Golgi cisternae and the endoplasmic reticulum are visible. Additionally, glycogen and lipid vesicles (v) are detectable in the peripheral compartment of the cell. In TEM, the PM of the chondrocyte appears as a dark-grey layer surrounding the chondrocyte whereas the territorial matrix TM is visible as a light-grey color around the PM. In Fig. 21E two chondrocytes deriving from an earlier division process are shown while Fig. 21F reveals some details of the interface chondrocyte-PM. In this picture, a collagen type II (cII) fiber is visible.
Fig. 21 Light microscopy and TEM of BNS cartilage. A) Chondrocytes (black ellipsoidal shapes) are randomly distributed in the ECM. B) The pericellular matrix (PM; white shadows around the chondrocytes) is less dense than the territorial matrix (TM; light blue background). C) Detail showing dividing cells. D) A chondrocyte (C) in the (PM); nucleus (n), Golgi and endoplasmatic reticulum membrane system (m), glycogen and lipid vesicles (v) are indicated. E) Two dividing cells. F) The interface between chondrocyte (C) and pericellular matrix (PM); a type II collagen fiber (cII) is visible.

Water, GAGs and collagen contents: In order to measure water, GAGs and collagen contents and distributions in the BNS, three explants for each of the nine regions were collected. Water, GAGs and collagen were measured respectively by means of freeze-drying, DMMB and hydroxyproline assays.

Water content was 78 ± 1.4 % of the cartilage wet weight and it showed a slight tendency to decrease moving through the postero-anterior axes and a slight increase through the dorsal-ventral axes (Table 3).

Table 3. GAGs, collagen and water content average values and respective standard deviations.

<table>
<thead>
<tr>
<th>Region</th>
<th>GAG (μg/mg)</th>
<th>Standard deviation (GAGs)</th>
<th>Coll (μg/mg)</th>
<th>Standard deviation (Coll)</th>
<th>Water (%)</th>
<th>Standard deviation (% water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DORSAL</td>
<td>388.6</td>
<td>69.0</td>
<td>251.5</td>
<td>27.9</td>
<td>77</td>
<td>1.5</td>
</tr>
<tr>
<td>MEDIAL (DORSO-VENTRAL)</td>
<td>375.8</td>
<td>66.4</td>
<td>249.4</td>
<td>27.0</td>
<td>78</td>
<td>1.4</td>
</tr>
<tr>
<td>VENTRAL</td>
<td>415.7</td>
<td>39.1</td>
<td>253.7</td>
<td>33.0</td>
<td>79</td>
<td>0.8</td>
</tr>
<tr>
<td>POSTERIOR</td>
<td>429.6</td>
<td>11.8</td>
<td>218.7</td>
<td>1.4</td>
<td>79</td>
<td>0.5</td>
</tr>
<tr>
<td>MEDIAL (POSTERO-ANTERIOR)</td>
<td>423.8</td>
<td>17.2</td>
<td>267.5</td>
<td>6.2</td>
<td>79</td>
<td>0.8</td>
</tr>
<tr>
<td>ANTERIOR</td>
<td>326.7</td>
<td>38.6</td>
<td>268.4</td>
<td>11.9</td>
<td>77</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Interestingly, as showed in Fig. 22 and Table 3, GAGs showed a similar trend to water. In contrast, the collagen content had a slight increase moving through the postero-anterior axis. Nevertheless, since the variations are in the same range of magnitude, they can be considered not relevant. Additionally, considering that the average values for GAGs and collagen contents per mg of cartilage are $393 \pm 54 \mu g/mg$ and $251 \pm 25 \mu g/mg$, respectively, the observed differences can be considered unimportant for loading experiments.

In agreement with previously reported results, GAGs and collagen contents were $393 \pm 54 \mu g/mg$ (of cartilage) and $251 \pm 25 \mu g/mg$ (of cartilage), which correspond to $39 \pm 5.0 \%$ and $25 \pm 2.5 \%$ of the cartilage dry weight (113).

![GAGs and collagen content in BNS cartilage](image)

**Fig. 22 GAGs and collagen content in BNS cartilage.** GAGs content decreases moving through the posteroanterior axes and, in contrast, collagen content tends to increase moving along the same axis.

### 4.4 Conclusion

Mechanical and biochemical characterization suggested the possibility of using BNS cartilage as a model tissue for loading experiments.

The instantaneous and relaxation elastic moduli (E₀ and Eᵣ respectively) were determined for both confined and unconfined compression. In spite of the different experimental setting, E₀ and Eᵣ were similar: respectively $4.07 \pm 0.57$ and $2.27 \pm 0.36$ for confined compression and $4.54 \pm 0.96$ and $2.11 \pm 0.29$ for unconfined compression. Nevertheless, a slight decrease of E₀ and Eᵣ was measured along the dorso-ventral axis.

Histological features of BNS cartilage were revealed by light microscopy and TEM. In particular, chondrocytes are randomly distributed and present typical characteristics of metabolically active cells.
Water represents 78 ± 1.4 % of BNS cartilage wet weight, whereas GAGs and collagen make up 39 ± 5 % and 25 ± 2.5 %, respectively, of BNS cartilage dry weight.

Although GAGs and water contents presented a slight tendency to decrease along the postero-anterior axis and a slight increase in the dorso-ventral axis, the collagen showed an opposite trend. However these latter variations can be considered irrelevant.

BNS cartilage has already been widely used as substrate for the study of enzymatic reactions or as a source of macromolecules such as GAGs (98-101). In the present study we show that because of its mechanical and biochemical characteristics, BNS cartilage can also be considered as a good model tissue for in vitro loading experiments since it is quite homogeneous.
5 BNS cartilage gene-expression changes induced by static uniaxial compression

In this chapter, changes in cartilage gene expression caused by static uniaxial compression are reported. BNS cartilage explants have been subjected to unconfined compression by using the RPETS at room temperature (RT; 23 °C) or at physiological temperature (37 °C).

The mechanical injury performed at RT consisted of unconfined compression stress to 50 % of the initial thickness of cartilage explants, at an indenter speed of 0.01 mm/sec, followed by 1, 2 or 4 hours of tissue relaxation. The changes in gene expression were studied upon completion of relaxation.

The unconfined compression performed at 37 °C consisted of a strain to 50 % of the initial thickness of cartilage explants, at an indenter speed of 0.01 mm/sec, followed by 2 hours of relaxation. In this case, changes in gene expression were measured after 2 and 24 hours post-relaxation.

The aims of the present study were: 1) to investigate the mechanical response of cartilage to static uniaxial compression (stress-relaxation tests); 2) to evaluate the effect of relaxation duration on gene expression; 3) to assess the effect of post-relaxation equilibration time and 4) to study the effect of temperature during static uniaxial experiments.

5.1 Experimental procedures

As previously described in Chapter 3 (section 3.3), cartilage explants were collected under sterile conditions from BNS of 12 months old calves.

Immediately after harvesting, cartilage explants (10 x 10 x 2 mm) (L x W x H), were rinsed in Dulbecco’s Phosphate-Buffered Saline (D-PBS) and equilibrated overnight in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 10 mM Hepes buffer, 0.1 mM nonessential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B.
5.1.1 Mechanical tests

In order to perform static uniaxial compression of BNS cartilage explants, a suitable RPETS indenter was used. As shown in Fig. 23, a cube-like aluminum indenter covered completely the surface of the cartilage sample while immersed in culture medium. Cartilage explants were collected from the middle part of three BNSs and for each static compression, a further control cartilage sample was placed in the same culture plate where the loading occurred.

![Fig. 23 RPETS configured for static compression of cartilage explants. The indenter is a cube-like unit; during loading, cartilage is placed in a cell-culture-plate.](image)

Two different experimental settings for unconfined compression (section 1.3.3) of cartilage explants were performed. The first set of experiments was performed at RT (∼ 23 °C) and consisted of a strain aiming to reach 50 % of the initial sample thickness with an indenter speed of 0.01 mm/sec. In order to observe the effect of the duration of relaxation, once the desired deformation was obtained, explants were allowed to relax during 1, 2 or 4 hours (section 1.3.2). Upon completion of relaxation, cartilage samples were snap frozen in liquid nitrogen and stored at -80°C until subsequent RNA extraction.

The second set of unconfined cartilage compression experiments was performed at 37 °C and, similarly to the first set of experiments, it consisted of a 50 % strain of cartilage explants, but was followed by only a 2 hours relaxation period. Upon relaxation completion, cartilage explants were either immediately collected or equilibrated for 2 or 24 hours, in order to study the effect of post-relaxation equilibration time on gene expression. Once equilibration was completed, samples were snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction was performed.
5.1.2 RNA extraction and gene-expression

RNA extraction, reverse transcription of mRNA and gene expression quantification by RT-qPCR were performed as previously described in Chapter 3 (section 3.5.7). The genes studied were Aggrecan (Agg), Collagen type I (Coll1), Collagen type II (Coll2), Fibronectin (Fn), Stromelysin-1 (MMP-3) as well as its inhibitor (TIMP-1), whereas the housekeeping genes were glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and ribosomal RNA 18S (18S rRNA). Housekeeping genes and control samples were run on every plate for normalization purposes. Gene regulation was calculated as the multiple of changes by using the comparative threshold cycle (Delta-Delta Ct) method. Thus, a gene was considered up-regulated when the multiple of expression was higher than 2 fold and down-regulated for values lower than 0.5 fold.

5.1.3 Statistical analysis

All the compression experiments were carried out in triplicate. Each statically loaded cartilage sample and the respective control sample were from a different nasal septum. Results are expressed as the mean ± S.E. Statistical differences were analyzed using Student’s t test.

5.2 Results and discussion

5.2.1 Mechanical response of BNS cartilage to stress-relaxation

The first set of unconfined compression experiments consisted of the application of a stress, aiming to deform the initial cartilage sample thickness to 50 % followed by different relaxation durations. In Fig. 24 an example of the loading-force, monitored over time, is reported. The maximal force needed to reach the desired tissue deformation was 77.1 ± 0.8 N and was reached in 100 ± 0.5 sec.
Since cartilage is a viscoelastic material, it is more deformable under the action of a stress applied more slowly. However, the stress in the tissue decreases until a non-zero plateau was reached (section 1.3.2). Clearly, as shown in Fig. 24, the tissue-relaxation rate started to slow down after 2 hours and the plateau was almost reached after 4 hours. In Fig. 24 the three curves do not overlap exactly because they originate from three different cartilage samples.

5.2.2 Effect of relaxation duration on gene expression

Generally, among the studied genes, Coll1, MMP-3 and TIMP-1 were up-regulated after static compression. In detail, Coll1 was already up-regulated at an early relaxation time (1 hour); MMP-3 was up-regulated after 2 hours relaxation and finally TIMP-1 was up-regulated only after 4 hours relaxation.

Additionally, post hoc tests showed gene regulation changes dependent on relaxation time. Indeed, Agg, Coll2 and MMP-3 showed significant differences ($p = 0.03$; $p =0.01$ and $p= 0.05$ respectively) of gene regulation over the relaxation time (Fig. 25).

These results show the effect of stress changes due to relaxation in BNS cartilage and how its gene regulation is affected.
5.2.3 Effect of post-loading equilibration on gene expression

The effect of post-loading equilibration duration was investigated after unconfined stress-relaxation of 2 hours performed at 37 °C.

As shown in Fig. 26, a clear decrease in gene regulation was visible during post-loading equilibration for MMP-3 (3, 2.7 and 1.9 fold) and TIMP-1 (2, 1.14 and 1.2 fold). The other genes were in general not regulated, except Coll1 after 24 hours. These results suggest that not only the relaxation time, but also the post-loading equilibration period has an influence on gene-expression levels in BNS.
5.2.4 Effect of temperature during static compression

In order to investigate the influence of the explant temperature, results of the experiments analyzed in sections 5.2.2 and 5.2.3 have been merged. Thus, gene expression analyzed after 2 hours relaxation at RT and gene expression after 0 h post stress-relaxation equilibration at 37°C were compared.

In general, the effect of temperature was not very clear results, even though, for most of the observed genes, the higher temperature seems to induce a slightly greater up-regulation (Fig. 27). One reason could be the absence of a strong gene up-regulation. Nevertheless, the Fn gene appears to be a good example to study the temperature influence. Indeed, Fn expression for BNS cartilage loaded at 37 °C was significantly increased compared to the compression experiment performed at RT (1.5 and 0.6 fold respectively).

![Fig. 27 Influence of the temperature on the gene expression](image)

5.3 Conclusions

BNS cartilage has a mechanical behavior typical of a viscoelastic material: it deforms easily under the action of a slowly applied stress and its deformation decreases until a non-zero plateau is reached (Fig. 24). Tissue relaxation starts to slow down after 2 hours while finally the plateau is reached after 4 hours of equilibration.

A 50 % deformation of the initial sample thickness caused significant Coll1, MMP-3 and TIMP-1 up-regulation which depends on relaxation duration. Furthermore, the results suggest
that the equilibration time post-relaxation also influences chondrocytes gene regulation, which decreases over time.

Although temperature seems not to influence the regulation of gene expression significantly, Fn appears as an exception; indeed, for cartilage loaded at 37 °C, Fn expression increased compared to the compression experiment performed at RT (1.5 and 0.6 fold respectively) (Fig. 27).

In this chapter the biological response of BNS cartilage to mechanical loading has been reported. Consequently, the possibility of using BNS cartilage as model tissue for loading experiments has been confirmed.
6 Coupling plowing of cartilage explants with gene expression in models of synovial joints

The content of this chapter has been submitted to the “Journal of Biomechanics” (April 2011). Most of this work was done by myself, including the experiments and paper writing. Nevertheless, the contribution of the co-authors has also been important: V. Colombo developed the RPETS and collaborated in the interpretation of the mechanical results; F.E. Weber contributed to the interpretation of the results on the biological effects of plowing of pristine cartilage; N.D. Spencer and L.M. Gallo contributed to the study design, the analysis and interpretation of the data and to the correction of the manuscript.

6.1 Abstract

Articular cartilage undergoes complex loading modalities generally including sliding, rolling and plowing (i.e. the compression by a condyle normally to the tissue surface under simultaneously tangential displacement, thus generating a tractional force due to tissue deformation). Although in vivo in studies it was shown that excessive plowing can lead to osteoarthritis, little quantitative experimental work on this loading modality and its mechanobiological effects is available in the literature. Therefore, a rolling/plowing explant test system was developed to study the effect on pristine cartilage of plowing at different perpendicular forces.

Cartilage strips harvested from the bovine nasal septum of 12-months-old calves were subjected for 2 hours to a plowing-regime with indenter normal force of 50 or 100 N and a sliding speed of 10 mm s⁻¹. 50 N produced a tractional force of 1.2 ± 0.3 N, whereas 100 N generated a tractional force of 8.0 ± 1.4 N. Furthermore, quantitative-real-time polymerase chain reaction experiments showed that TIMP-1 was 2.5x up-regulated after 50 N plowing and 2x after 100 N plowing, indicating an ongoing remodeling process. The expression of Collagen type I was not affected after 50 N plowing but it was up-regulated (6.6x) after 100 N plowing suggesting a possible progression to an injury stage of the cartilage, as previously reported in cartilage of osteoarthritic patients. We conclude that plowing as performed by our mimetic system at the chosen experimental parameters induces changes in gene expression depending on the tractional force, which, in turn, relates to the applied normal force.
6.2 Introduction

Synovial joints are systems comprising bones, cartilage, tendons and ligaments. These elements interact and cooperate allowing joint movement, which is also facilitated by synovial fluid—the joint lubricant. Within the mechanical system, each element has a specific function: bones provide support, cartilage — placed on adjacent bones — provides a sliding surface and adsorbs shocks, tendons link muscles and bones, while ligaments stabilize the joint (114).

It has been demonstrated that in pathological conditions such as in osteoarthritis (OA), elevated expression and activities of proteolytic enzymes cause destruction of articular cartilage and ultimately of the underlying subchondral bone (58;115). Patients affected by this degenerative disease suffer from severe pain and movement impairment with a general decrease in the quality of life (116).

Since injurious mechanical stress has been recognized as one of the causes of OA development, cartilage overloading has been the focus of a number of studies aimed at mimicking unfavorable mechanical conditions that cartilage could experience (86;91;92;117;118). Although these studies provided insight into the biological response of cartilage to static and cyclic loading of different duration, normal force magnitude and frequency, their uni-axial design presents some limitations.

Drop-tower devices have been built and described by Jeffrey et al. (77) and Repo and Finlay (119). A common disadvantage of these devices was that the strain rate during compression could not be measured. Later, other experiments have been performed by using systems with computer-controlled motor-driven plates that were able to control load or displacement during injury (83;120-122).

Indeed, in vivo, articular cartilage undergoes more complex loading modalities than pure uni-axial compression, including predominantly a combination of sliding, rolling and plowing (123;124). Plowing has been earlier described as the deformation of synovial cartilage due to the combination of compression and tangential motion of the contacting bones (125). In in vivo studies in dogs, it was shown that excessive plowing can be considered as a cause of OA onset (75). Moreover, it has been reported that plowing is the dominant mechanism in synovial cartilage leading to tangential tractional forces (74). However, little quantitative experimental work on plowing and its mechanobiological effects has been reported in the literature.
Plowing has been extensively investigated in the temporomandibular joint (TMJ), in particular in its fibrocartilaginous disc, which is subject to compression by the underlying mandibular condyle that in general translates and rotates simultaneously (126-132). In our previous work, the complex TMJ disc deformation, and thus indirectly its loading, has been studied quantitatively from in vivo data by coupling magnetic resonance imaging (MRI) and jaw-tracking of human subjects (93-95;133).

This has led to the idea of replicating this information experimentally on live tissue in a bench-top apparatus, a so-called rolling/plowing explant test system (RPETS), in order to study the biological reaction of cartilage to this type of loading (96;134;135). The RPETS is able to mimic plowing effects occurring in vivo that result from the combination of an applied normal force and the sliding of a condyle on a cartilage sample. The cartilage sample is placed on the bottom of a tank and kept alive by a culture medium. For a first set of experiments, bovine nasal septum cartilage was chosen as tissue model since it is abundantly available from abattoirs, it delivers sample of convenient size and shape and is pristine. RPETS settings and parameterization have been the focus of this study in order to detect whether gene expression in bovine nasal septum cartilage is dependent on tractional forces due to plowing.

The objectives of this chapter are therefore to apply plowing at different normal forces to pristine cartilage collected from bovine nasal septa and analyze differences in resulting tractional forces and gene expression.

6.3 Experimental procedure

6.3.1 Tissue harvesting and experimental setup

BNS of 12-month-old calves were provided by a local abattoir within 4 hours of slaughter. Under sterile conditions and using a custom-made cutter, cartilage strips (60 x 17 x 2mm) (L x W x H) and control cartilage explants (20 x 30 x 2 mm) were collected from BNS cartilage. Control samples and cartilage strips were washed in Dulbecco’s Phosphate-Buffered Saline (D-PBS) and equilibrated overnight in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM Hepes buffer, 0.1 mM nonessential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B.
In order to perform plowing, the cartilage strips were glued at the extremities to a Plexiglas® support by means of cyanoacrylate glue. The plowing was performed, by using RPETS, in DMEM for 2 hours at room temperature (≈23 °C) in a sterile hood (Fig. 28A). It consisted of the simultaneous application of a normal force on the vertical axes and of the horizontal sliding (displacement: 40 mm, tangential speed: 10 mm/sec and a constant normal force: 50 or 100 N) of a fixed, cylindrical indenter (Ø 25 mm; stainless steel) on a cartilage strip (Fig. 28B). Plowing was performed as a regular cyclic motion of the indenter (Section 3.4.2;) summarized as the sequence of the following steps: a) vertical descent of the indenter to contact the cartilage; b-d) vertical indentation to reach the desired normal force and horizontal displacement with constant speed while maintaining stable the normal force; d-e) returning of the indenter to the initial position.

**Fig. 28 Plowing of cartilage strip.** A: the RPETS tank containing the medium. The cartilage strip is glued by the extremities to a Plexiglas® support while a free swelling cartilage sample is used as control. Both, cartilage strip and control are completely immersed in culture medium. B: During the plowing the indenter slides in a unidirectional manner on the cartilage strip while keeping constant the applied normal force.

During plowing, the control cartilage sample is placed, as free swelling explant, in the same medium in which the loading occurred. In order to monitor macroscopic changes of cartilage, the length, the width and the thickness of the strips were measured before and after plowing. After plowing, cartilage strip and control were treated for RNA extraction; the further gene expression was studied by means of RT-qPCR.

### 6.3.2 RNA extraction and gene-expression study

Immediately after plowing, 3 cartilage explants were collected from the central region of the strip, and from the control samples. After snap-freezing the cartilage in liquid nitrogen, the RNA extraction, the reverse transcription of the mRNA and the gene expression study by RT-
qPCR were performed as described in the Chapter 3 (section 3.5.7). The studied genes were aggrecan (Agg), collagen type-I (Coll1), collagen type-II (Coll2), fibronectin (Fn), stromelysin-1 (MMP-3) as well as its inhibitor (TIMP-1), whereas the housekeeping genes were glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and ribosomal RNA 18S (18S rRNA).

Housekeeping genes and control samples were run on every plate for normalization purposes. The gene regulation was calculated as fold of changes by using the comparative threshold cycle (Delta-Delta Ct) method. Thus, a gene was considered up-regulated when the fold of expression was higher than 2 and down-regulated for values lower than 0.5.

6.3.3 Statistical analysis

All the plowing experiments were carried out in replicate; 4 strips were plowed at 50 N while 5 strips were subjected to 100 N plowing. Each strip and the respective control explant were from a different nasal septum. For the RT-qPCR the plowed samples were collected in triplicate from each strip, thus yielding 12 samples for 50 N and 15 samples for 100 N. Results are expressed as the mean ± S.E. Statistical differences were analyzed using Student’s t test.

6.4 Results

As showed in Fig. 29, plowing of cartilage strips caused the development of tractional forces (TF), which are defined as the result of frictional and sliding forces produced by the cartilage deformation. The plowing with applied normal force 50 N induced a TF of 1.2 ± 0.3 N (Fig. 29A, black line), while the plowing with 100 N applied normal force induced a TF of 8.0 ± 1.6 N (Fig. 29B, blue line).

From a macroscopic point of view, an increase in the whole cartilage strip length (Δℓ) was observed at both the applied normal forces: strips plowed at 50 N had a Δℓ of 1.0 ± 0.06 mm while strips plowed at 100 N had a Δℓ of 1.9 ± 0.29 mm. These absolute length increases corresponded to relative elongation of 1.5 ± 0.2 % and 3.0 ± 0.7 % respectively.

Plowing with 50 N applied normal force did not induce significant changes in the chondrocyte gene expression, with the exception of TIMP-1, which was slightly up-regulated (2.5 fold) (Fig. 30). At the opposite, strips plowed at 100 N normal forces showed up-regulation (6.6 fold) of Coll1 and slight up-regulation of both MMP-3 (2.5 fold) and TIMP-1 (2.1 fold) (Fig. 30).
The other studied genes (Agg, Coll2 and Fn) did not appear to be affected by plowing at either of the applied normal forces.

**Fig. 29 Plowing profiles during an arbitrary chosen period of 180 sec.** A: the applied normal force is 50 N (pink line); the lower black line is the tractional force measured in the horizontal axis. B: the applied normal force is 100 N (green line); the blue line is the tractional force. In C the tractional forces for plowing at 50 N (black) or 100 N (blue) are compared.

**Fig. 30 Gene expression study** Cartilage gene expression changes after plowing at 50 N (white bars; n = 4 experiments) or 100 N (grey bars; n = 5 experiments) measured immediately after mechanical stress. Plowing performed with higher normal forces caused stronger changes in the gene expression than plowing with lower normal force. Each experiment was from a separate BNS, and for each experiment three similarly treated explants were pooled for RNA extraction. Expression levels are normalized to those of the control, which was non-loaded free swelling explant.
6.5 Discussion

Cartilage strips plowed with a normal force of 50 N exhibit up-regulation (2.5 fold) of TIMP-1. This finding can be correlated with results reported by others about up-regulation of TIMP-1 after cyclic compression of cartilage explants (86). In general, TIMPs prevent cartilage breakdown by tightly binding active MMPs (114;136). It has been reported that in lightly damaged healthy cartilage the levels of MMPs are reduced, whereas those of TIMP are increased (115). The result obtained in the present study for TIMP-1 up-regulation could thus indicate that at 50 N plowing, the cartilage breakdown does not occur but rather that cartilage regulates its metabolic activity by blocking the possible ongoing catabolism and undergoing a normal process of remodeling (137;138).

Moreover, cartilage strips plowed with a normal force of 100 N showed slight up-regulation of both MMP-3 (2.5 fold) and TIMP-1 (2.1 fold) and a significant up-regulation of Coll1. It is known that in OA, chondrocytes are subjected to a “dedifferentiation” process, consisting of a change to a fibroblast-like phenotype. This change is characterized by the synthesis of Coll1 as a replacement for Coll2 (139;140). This suggests that plowing with applied normal force of 100 N has a significant destructive effect on cartilage explants. Furthermore, since plowing with a force of 100 N induced the production of a tractional force reaching 8 N, we conclude that the tractional force plays an important role in cartilage injury during plowing. Interestingly, we also observe that the length of strips plowed at 100 N increased by around 2 mm, meaning that the strips are deformed beyond the elastic region. This finding is in agreement with Tanaka and van Eijden, who studied the physiological stretching to which the TMJ disc is subjected in vivo (132) suggesting that we have reached the ultimate strength as measured in the TMJ disc.

6.6 Conclusion

In the present study the effect of plowing with different applied normal forces on pristine cartilage (bovine nasal septum) has been reported. The cartilage deformation, measured as increase in the cartilage strips length, was dependent on the magnitude of the applied normal force. Moreover, profiles of the tractional forces generated during plowing have been described as: the higher the applied normal force the higher the generated tractional forces. Furthermore it has also been reported that plowing with higher applied normal force seems to harm for the
cartilage explants as indicated by the up-regulation of type I collagen and of the catabolic enzyme MMP-3.
7 Mechanical plowing induces remodelling of cartilage

This work has been submitted in Journal of Biological Chemistry (May 2011). Most of this work was done by myself, including experiments and paper writing. Nevertheless, the contribution of the co-authors has also been important: C. Ghayor contributed to the discussion of the biological results; F.E. Weber contributed to the interpretation of the biological results and to the writing of the paper; L.M. Gallo and N.D. Spencer contributed in the study design, the establishment of the project steps, the discussion of the results and to the correction of the manuscript.

7.1 Abstract

To investigate the response of cartilage to dynamic mechanical stress, the effect of plowing on cells viability, gene expression and glycosaminoglycan (GAGs) release has been studied. Cartilage strips were subjected to mechanical plowing using a cylindrical indenter, applying in the vertical axis a constant normal force and moving at constant speed in the horizontal axis.

After plowing, cell viability assay and qRT-PCR showed that plowing induces cell death superficially and matrix metalloproteinase 3 (MMP-3) up-regulation, both dependent in magnitude on the applied normal force. The addition of a transcription inhibitor, actinomycin-D, before or after plowing confirmed that plowing was responsible for the observed MMP-3 up-regulation. The transcription of other genes involved in the cartilage turn-over like TIMP-1 (a tissue inhibitor of metalloproteinase 1), Aggrecan, type I Collagen and type II Collagen and of Fibronectin were shown not to be significantly affected by plowing.

However, actinomycin-D treatment revealed that plowing induces a strong increase in TIMP-1 and type I Collagen mRNA contents. A net gain of these transcripts was detectable solely when mRNA degradation was blocked by actinomycin-D treatment suggesting that plowing influences mRNA degradation for TIMP-1 and type I Collagen. It was found that actinomycin-D treatment also influences the gene regulation of Aggrecan, type II Collagen and Fibronectin.

Furthermore, the dimethyl-methylene blue (DMMB) assay revealed that over time, 100 N plowed cartilage explants exhibit an enhanced GAGs release. The post-loading application of
GM6001, a metalloproteinase inhibitor, showed that plowing induces GAGs release by the activation of catabolic enzymes.

7.2 Introduction

Since injurious prolonged mechanical stress is considered as a cause of cartilage failure, in order to understand the pathomechanics of cartilage degradation occurring in pathological synovial joints, experimental models mimicking in vivo mechanical stresses on cartilage have been developed (1.6 and 6.2). Although these models reproduce in vitro the mechanical stresses that cartilage experiences in vivo and allowed gathering insights into the biological response of this complex tissue to mechanical injuries, their uni-axial design presents some limitations.

In this chapter the effect of plowing on cells viability, gene expression and glycosaminoglycan (GAGs) release has been studied. Cartilage strips were submitted to mechanical plowing at 37°C, using a cylindrical indenter, applying in the vertical axis a constant normal force and moving at constant speed in the horizontal axis.

7.3 Experimental procedures

7.3.1 Cartilage explants harvesting and experimental setup

As previously described in the section 0, under sterile conditions, control cartilage explants (20 × 30 × 2 mm) (L × W × H) and cartilage strips (60 x 17 x 2 mm) (L x W x H) were harvested from BNS of 12 months old calves provided by the local abattoir within 4 hours after slaughter. Cartilage samples were obtained after removal of surrounding bones, perichondria and surplus cartilage and shaped using a custom-made two parallel blades cutter. Control samples and cartilage strips were washed in Dulbecco’s Phosphate-Buffered Saline (D-PBS) and equilibrated overnight in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (FBS) (10%), Heps buffer (10 mM), nonessential amino acids (0.1 mM), penicillin (100 units/mL), streptomycin (100 μg/mL) and amphotericin-B (0.25 μg/mL).

Before plowing, cartilage strips were glued at both ends to Plexiglas® supports using cyanoacrylate glue. As described in the section 3.4.2, the strips were subjected to plowing for 2 hours at 37°C in DMEM by using a cylindrical indenter (Ø 25 mm; aluminum) moving in the horizontal axis with a constant speed of 10 mm/sec and contemporary compressing the cartilage
in the vertical axis by applying normal forces of 25, 50 or 100 N respectively. During the plowing, control cartilages were placed as free swelling explants in the same medium where the plowing was carried out.

After plowing, ≈ 500 μm cross-sections were collected from control and plowed strips. In addition, three explants (M1, M2, and M3) were collected from the middle part of the strip (Fig. 31). Controls and M regions were cut each in sub-explants (≈ 50 mg each) and incubated in DMEM at 37°C, 5% CO₂ for different equilibration durations (2, 4, 24 hours).

![Diagram](image)

**Fig. 31 Protocol for plowing and samples collection.** A: cartilage strip is plowed by a cylindrical indenter moving in the horizontal axis with a speed of 10 mm/sec and applying in the vertical axis normal forces of 25, 50 or 100 N; a free swelling cartilage explant is used as control; B and C: after plowing, three explants (M1, M2 and M3) are collected from the middle part of the strip; D: cartilage sub-explants are collected from the M regions and from free swelling control cartilage and incubated for different durations (2, 4 and 24 hours).

### 7.3.2 Chondrocytes viability assay

The chondrocytes viability assay was performed, as in detail described in the section 3.5.4, by incubating cartilage slices with calcein acetoxymethylester (AM) (live cell staining) and ethidium homodimer (dead cell staining).
7.3.3 RNA extraction and quantitative real time polymerase chain reaction

The gene expression changes induced by plowing have been studied by performing quantitative real time polymerase chain reaction (RT-qPCR) upon RNA extraction and reverse transcription respectively. The protocols have been extensively described in the section 3.5.7. The studied genes are the extracellular matrix proteins aggrecan (Agg), collagen type-I (Coll1), collagen type-II (Coll2), fibronectin (Fn), the catabolic enzyme stromelysin-1 (MMP-3) and its inhibitor the tissue inhibitor of metalloproteinase (TIMP-1). The housekeeping genes are glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and ribosomal RNA 18S (18S rRNA). Housekeeping genes and control samples were run on every plate for normalization purposes. The gene regulation was calculated as fold of changes by using the comparative threshold cycle (Delta-Delta Ct) method. Thus, a gene was considered up-regulated when the fold of expression was higher than 2 and down-regulated for values lower than 0.5.

7.3.4 GAGs release

After different post-plowing equilibration durations (1-4 days) 1 mL samples media and cartilage sub-explants were collected and stored at -20°C until when the DMMB assay was performed (141). GAGs measurements in the media were carried out according to Jeffrey et al. (104) as described in the section 3.5.2.

7.3.5 Inhibition experiments

To confirm that the change of chondrocytes gene expression was induced by plowing, cartilage strips and control explants were incubated in medium supplemented with 30 µM transcription inhibitor, actinomycin-D, for 2 hours before or separately, for 2 hours after plowing with applied normal force of 100 N. In the first case, the gene expression was measured upon plowing completion, while in the second case the gene expression was measured upon the 2 hours post-plowing equilibration in actinomycin-D.

To confirm that plowing causes GAGs release by activating catabolic enzymes, MMPs inhibition experiments using GM6001 have also been performed. Briefly, cartilage strips were incubated during 4 hours before loading in serum free DMED supplemented with 1% sodium selenite, insulin, transferrin and ethanolamine (SITE) and containing 10 µM GM6001. After plowing, which was performed in serum free medium, cartilage sub-explants were collected.
from both control cartilage and strips and incubated again in DMEM containing GM6001 and SITE for 1-4 days.

7.3.6 Statistical analysis

All the experiments were carried out in triplicate, each time from a different nasal septum. Results are expressed as the mean ± S.E. Statistical differences were analyzed using two-way ANOVA and Student's t test.

7.4 Results

7.4.1 Cell viability

After plowing of cartilage strips, the chondrocytes viability was assayed using the calcein AM and ethidium-homodimer staining (Fig. 32). The overall viability of the chondrocytes in the cartilage strips exceeded 95% for all the forces applied (25, 50 or 100 N); at the surface and at the bottom of the implants a zone of dead chondrocytes was observed. The thickness of the dead zone resulted to be related to the magnitude of the applied normal force during plowing: strips plowed with 50 N and 100 N normal forces had more dead cells (Fig. 32A and Fig. 32B) compared to the strips plowed at 25 N (Fig. 32A).

Fig. 32 Chondrocytes viability after plowing at different applied normal forces. A, B and C: Cartilage cross-sections of strips plowed with applied normal force of 25, 50 and 100 N respectively (Scale bar = 500 µm). a, b and c are details of A, B and C respectively (Scale bar = 50 µm).
7.4.2 Effect of plowing on cartilage gene transcription after 2, 4 and 24 hours post-plowing equilibration

Upon plowing completion, cartilage explants were equilibrated for 2, 4 or 24 hours and at each time point, the effects of plowing on the gene expression were measured using qRT-PCR (Fig. 33). The studied genes were Stromelysin-1 (MMP-3), fibronectin (Fn), type I Collagen (Coll1), type II Collagen (Coll2), Aggrecan (Agg) and tissue inhibitor of metalloproteinase (TIMP-1). After 2 hours equilibration, cartilage explants subjected to 25 N plowing revealed only a slight change in the MMP-3 mRNA synthesis (1.8 fold up-regulation) and this value slightly increased (2.3 fold) after 4 hours but it dropped to 0.9 fold after 24 hours equilibration. Furthermore, the cartilage strips subjected to 50 N plowing had 4.7 fold up-regulation of MMP-3 after 2 hours equilibration and this value decreased over time (4.1 fold after 4 hours and 2.4 fold after 24 hours equilibration). The up-regulation of MMP-3 for strip subjected to 100 N plowing was stronger: 6.3 fold after 2 hours, 5 fold after 4 hours and 3.2 fold after 24 hours post-plowing equilibration. These results suggest that plowing of cartilage strips at different applied normal forces induces a force-dependent MMP-3 regulation.

The plowing did not influence the net gene expression of Fn, and type I and II Collagens, whose levels remained of around 1 fold regulation (Fig. 33).

Aggrecan mRNA was influenced only by 100 N plowing: it was slightly down-regulated (2 fold) after 2 hours equilibration and this down-regulation remained almost constant over time reaching only 2.5 fold after 24 hours. On the contrary, TIMP-1 transcription was affected only by plowing with applied normal force of 25 N. After 2 hours post-plowing equilibration TIMP-1 was 2.6 fold down-regulated and after 24h the down-regulation was of 2 fold.
Fig. 33 Study of the gene regulation after plowing with applied normal forces 25, 50 and 100 N. Black bars: gene expression after 2h post-plowing equilibration; grey bars: gene expression after 4h post-plowing equilibration; white bars: gene expression after 24h post-plowing equilibration. Expression levels, (normalized against the GAPDH and 18S genes), are normalized to that of controls which were not loaded free swelling explants. Each experiment was from a separate BNS (n=3), and for each experiment three similarly treated explants were pooled for RNA extraction.
7.4.3 Transcription inhibition experiments

In order to determine if the plowing directly affects the regulation of cartilage genes, the transcription inhibitor actinomycin-D was added to the culture media either 2 hours before or for 2 hours after 100 N plowing (Fig. 34) The gene regulation was measured in three sets of separate experiments: i) upon plowing completion; ii) immediately after the plowing of cartilage strips previously treated for 2 hours with actinomycin-D; iii) after 2 hours actinomycin-D exposure of plowed strips.

Upon plowing completion and without inhibitor treatment, MMP-3 was 8.6 fold up-regulated (Fig. 34A) but when cartilage strips were treated with actinomycin-D for 2 hours before plowing, no MMP-3 up-regulation was observed upon plowing completion (Fig. 34A). In contrast, when the treatment with the transcription inhibitor was performed during 2 hours after plowing, the MMP-3 was 7.8 fold up-regulated (Fig. 34B). This value was not different from the MMP-3 expression of plowed cartilage measured after 2 hours equilibration without actinomycin-D (P > 0.05) (Fig. 34B). These results suggest that the MMP-3 up-regulation is dependent on new mRNA synthesis which occurs during plowing and not in changes of the MMP-3 mRNA stability. Besides MMP-3 gene regulation, actinomycin-D treatment of cartilage revealed that plowing also influences TIMP-1 expression. Upon plowing completion, TIMP-1 expression was 1.7 fold up-regulated (Fig. 34A) and similarly, when cartilage strips were pre-incubated for 2 hours in media containing actinomycin-D and then subjected to 100 N plowing the gene expression of TIMP-1 was 2 fold up-regulated (Fig. 34A).

Moreover, cartilage incubated in actinomycin-D for 2 hours after plowing, had a strong (7.5 fold) TIMP-1 up-regulation (Fig. 34B). Since qRT-PCR of plowed cartilage strips not treated with inhibitor, revealed that TIMP-1 expression measured after 2 hours equilibration, was not affected by plowing (1 fold as shown in Fig. 34B), these results suggest that when transcription is enabled, the TIMP-1-mRNA content is reduced. As shown in Fig. 34A and Fig. 34B, the mRNA regulation of type I Collagen showed a trend of regulation similar to TIMP-1 indicating that plowing also reduces the stability of type I Collagen mRNA.

The other studied genes, Agg, Fn and Coll2, which normally were slightly or not affected by plowing when no inhibitor was added, showed a new response. Indeed, when the incubation in
actinomycin-D was done either before or after plowing their levels of expression remained at around 2 fold up-regulations.

7.4.4 Effect of plowing on the GAGs release

In order to investigate the cartilage degradation caused by plowing, the GAGs release into the medium has been measured using a DMMB assay after 1 to 4 days of post-plowing equilibration (Fig. 35). As shown in Fig. 35A, either control or loaded samples showed an increasing GAGs release over time but the samples subjected to plowing showed an enhanced GAGs release compared to the control (P<0.05 at all the observed time points). To determine if the post-plowing GAGs release was either mechanically or enzymatically induced, MMPs activity was inhibited by GM6001 treatment before plowing.

![Diagram](image)

Fig. 34 Actinomycin-D treatment of cartilage. Gene transcription after 100 N plowing of cartilage strips not treated or treated with actinomycin-D. A: White bars: gene expression measured immediately after plowing with 100 N applied normal force without actinomycin-D treatment; grey bar: before plowing cartilage strips are incubated with actinomycin-D during 2 hours and the gene expression is measured upon plowing. B: white bars: gene expression measured after 2 hours equilibration of cartilage strips plowed at 100 N applied normal force without actinomycin-D treatment; black bars: immediately after plowing cartilage strips are incubated during 2 hours with actinomycin-D. For all the experiments, the expression levels are normalized to those of controls which were not loaded free swelling explants treated or not with actinomycin-D respectively. Each experiment was from a separate BNS (n=3), and for each experiment three similarly treated explants were pooled for RNA extraction.
Fig. 35 GAGs release GAGs release into the medium from 100 N plowed cartilage and control on days 1-4 post plowing (n=3 experiments for each condition and for each time point). A: GAGs release for both loaded and control explants \((P < 0.05)\); B: Effect of treatment with MMPs activity inhibitor (GM6001) on GAGs release. Full squares represent the GAGs release from strip not treated with GM6001 while full triangles are the strip treated with GM6001; C: GAGs release is linear over time as indicated by the \(R^2\) values; D: GAGs releases from control samples treated or not with GM6001 are not statistically significant \((P>0.05)\); the post-plowing GAGs release for cartilage strip treated with GM6001 or strip not treated with GM6001 are also reported.

As shown in Fig. 35B, the cartilage not treated with MMPs inhibitor (filled square) had higher GAGs release and this difference is confirmed also at later time points. The loaded samples, independently of MMPs inhibitor treatment, showed a linear GAGs release during time with excellent correlation factors \((R^2\text{ values } \geq 0.99,\text{ in Fig. 35C})\). The GAGs release from the loaded samples treated with MMPs inhibitor was lower than the release from the not treated samples (Fig. 35D). The GAGs release from the control samples, irrespective of MMPs inhibitor treatment, showed no statistically significant difference (Fig. 35D).

These results suggest that the GAGs release is due to a mechanical and an enzymatic component and that under plowing conditions the enzymatic component is more dominant.

7.5 Discussion

In the present chapter we reproduced in vitro the “plowing-effect” occurring in the TMJ cartilage disc and demonstrated that it compromises the cartilage stability and maintenance by causing cell death at the surface of the explants, inducing changes in the chondrocytes gene expression and activating catabolic enzymes.
It should be noted that the plowing parameters used in this study were comparable to physiological conditions. The indenter speed has been chosen after evaluation of TMJ recordings performed during rhythmic jaw opening and closing (133). Interestingly, this speed value can be also compared with that estimated in other joints, such as in the knee during walking (142). The applied normal forces were chosen according to the study of Seller and Crompton showing that 100 N corresponds to the condylar TMJ force during biting (143). BNS cartilage is chosen as model tissue because although it is similar to the articular cartilage it has not the typical zonal distribution of cells and proteins but rather a homogeneous distribution of its components.

It has been found that plowing of cartilage strips induces cell death increasing with the applied normal force magnitude. The fact that dead cells were mainly detected at the surfaces of the explants suggests that the chondrocytes located closer to the surface are more exposed to the mechanical stress and therefore more vulnerable than those in the deeper zones. Since the cell viability assay was performed within 30 minutes after the plowing, and as it is known, that all the apoptotic processes such as cell membrane degradation and DNA fragmentation occur 2 hours after the beginning of the apoptosis process (144), we could conclude that the observed cell death can be attributed to necrosis.

Furthermore, qRT-PCR revealed that MMP-3 up-regulation, like the observed superficial cell death, is dependent on the magnitude of the applied mechanical stress and that it decreases over a 24 hour post-plowing equilibration period (Fig. 33). At the opposite, the mRNA content for Fn, type I and type II collagens remained unchanged compared to the control values. All these genes code for proteins of the ECM and their transcription is not induced by plowing with 25, 50 or 100 N. Interestingly, only plowing with applied normal force of 100 N induced a slight down-regulation of Aggrecan, another ECM component. These results indicate that at 100 N applied normal force catabolic and anabolic events are induced since mRNAs of catabolic enzymes like MMP-3 are up-regulated and the mRNA contents of ECM proteins are reduced. Since active MMP-3 digests collagens, PGs, and other ECM proteins and additionally activates the pro-forms of other MMPs and aggrecanase II (145-147), our results suggest that plowing causes an increase in the catabolic activities starting at 25N normal force and a decrease of the anabolic activity of the tissue starting between 50 and 100 N.

It is known that, cartilage has a poor intrinsic healing capacity (148). Nevertheless, after an injury, the healthy chondrocytes promote a remodeling process consisting in the elimination of
the damaged matrix and in the re-building of new ECM (149). We could thus suppose that in the plowed cartilage, the viable chondrocytes start to remodel the matrix by producing MMP-3 as showed with qRT-PCR experiments to clear space for cell ingrowth and/or the deposition of newly synthesized ECM proteins.

Application of the transcription inhibitor actinomycin-D before plowing reduced the MMP-3 mRNA content to its control level (Fig. 34A) indicating that early events during the 2 hours plowing period induce an increase in MMP-3 mRNA transcription. Under the same conditions, the mRNA of TIMP-1, Coll1, Agg, Fn and Coll2 were increased by a factor of two. Assuming that in the presence of actinomycin-D the transcription was fully inhibited, as in the case of MMP-3, these results suggest that during plowing all these mRNAs were to a certain extent prevented from degradation and that this process is in turn dependent on transcription. Considering that all these mRNA content data are related to controls not undergoing plowing it is suggested that plowing has an additional, so far unknown, stabilizing effect on mRNAs for TIMP-1, Coll1, Agg, Fn and Coll2 which depends on an active transcription. It could also be that actinomycin-D itself stabilizes certain mRNA species (150-152). The degradation of mRNA is an essential determinant in the regulation of the gene expression, and it can be modulated in response to environmental signals by cis-acting elements and trans-acting factors that contribute to mRNA regulation decay (153;154). Additionally, it has already been reported that actinomycin-D has a stabilizing effect on mRNA transcription of TIMP-1 by affecting trans-acting factors involved in TIMP-1 mRNA degradation (155).

This stabilizing effect or artefact of actinomycin-D could also account for the 2 fold increase in Agg, Coll2, and Fn mRNA when applied for 2 hours upon plowing completion. The 7-8 fold increase in mRNA content for TIMP-1 and Coll1, however, suggests that both species increased during plowing and in absence of actinomycin-D decline to control levels during the 2 hours equilibration period. This hypothesis would demand for a half-life of TIMP-1 and Coll1 mRNA after plowing of less than 1 hour. In contrast, the half-life time of MMP-3 mRNA is around 24 hours as deduced from Fig. 33. Whether or not plowing can induce factors able to modulate the half-life of specific mRNA species like TIMP-1 or Coll1 cannot be decided upon these results.

In essence we have observed 3 patterns of transcription regulation. The first is of the MMP-3 mRNA type whose transcription is induced during plowing; the second includes TIMP-1 and Coll1 whose mRNAs decay occurs during early post plowing equilibration period and the
third concerns Agg, Fn and Coll2 whose basal transcription is stabilized or not affected by plowing.

Furthermore, we have shown that the GAGs release of plowed cartilage strips is higher than the release of control explants \((84; 117)\) and that this release is due to both mechanical damage and enzymatic activity. Since the GAGs release has been observed also after treatment of plowed cartilage with MMPs inhibitor, we could state that plowing provokes mechanical degradation of the cartilage matrix. This finding is also supported by two macroscopic events which are the bending of the strip during plowing (due to the fact that the strip is glued by the extremities to the support) and the increase of the cartilage strips length. Both observations suggest that plowing could damage the collagen fibers causing in turn, a GAGs release \((84)\). Nevertheless, given that already after 1 day equilibration, the GAGs release from plowed cartilage was 30% higher than in plowed cartilage treated with GM6001, we could conclude that GAGs loss is also due to the catabolic activity of MMPs that are activated during plowing. Thus, the increase in MMPs mRNA induced by plowing as in the case for MMP-3 yields an increase in MMP-activity.

When cartilage explants are subjected to a static compression with applied pressure above 0.5 MPa, the chondrocytes metabolism is irreversibly compromised already after 1 hour compression \((156; 157)\). Following the measurement of the contact area between the condyle and the cartilage, the pressure during 100 N plowing has been calculated to be 2.5 MPa. This finding suggests that plowing with high applied pressure is not as harmful as a static compression. Contrarily to what happens during a static compression, where the flow of gases and nutrients are limited, during plowing the sliding of the indenter mixes the surrounding medium thus facilitates the exchange of gases and molecules ensuring a better cartilage homeostasis but moreover squeezes fluid out of the cartilage which will be replenished by new medium.

7.6 Conclusion

The response of cartilage explants to mechanical injuries has been extensively investigated \textit{in vitro} but, to our knowledge, plowing experiments where the indenter contemporary applies a compression and slides on the cartilage explants have not been reported so far.
On the basis of the obtained results, we suppose that plowing with applied normal force of 100 N and indenter speed of 10 mm/sec causes cell death of the chondrocytes closer to the surface as well as matrix damage observed as GAG loss. In addition, plowing promotes, in the healthy chondrocytes the production and activation of catabolic enzymes, such as MMP-3 and shows 2 hours after plowing no effect on anabolic genes such as aggrecan, type I and type II collagen and of fibronectin. This pattern of gene transcription could promote ECM degradation aiming to facilitate the migration of viable cells into the damaged sites to regenerate the damaged matrix.
8 Summary and outlook

8.1 Summary

Cartilage is a fascinating connective tissue with outstanding biological and mechanical properties. The most abundant proteins of cartilage, GAGs and collagen, provide compressive and tensile resistance to the tissue, respectively, and confer viscoelastic properties to it by entrapping water (section 1.3.2).

In body joints, cartilage is located on the surface of adjoining bones where it absorbs mechanical shocks and works as a low friction, weight-bearing and wear-resistant material that allows a facile movement of body joints. Furthermore, cartilage is also present in other body locations, such as ear and nose where it has mainly a support function.

Cartilage pathologies such as osteoarthritis (OA) and rheumatoid arthritis (RA) are characterized by progressive tissue degradation. Since cartilage has a poor intrinsic healing capacity, the ultimate solution for patients affected by these diseases is total joint replacement.

Much interest has been focused on the understanding of mechanobiological processes triggering cartilage degradation. With this in mind, and considering that mechanical loading is one of the main causes of cartilage failure, several joint-mimetic systems have been developed (section 1.6), and have thus provided relevant knowledge about cartilage response to static or cyclic uniaxial loading. Nevertheless, articular cartilage under physiological conditions undergoes a more complex stress pattern.

Since plowing is one of the loading modalities occurring in the temporomandibular joint (TMJ), the motivation of this thesis was to produce a system mimicking TMJ loading and to study the effect of plowing on pristine cartilage (bovine nasal septum cartilage, BNS).

In an interdisciplinary context including mechanics and biology, this work has been divided into sequential steps: 1) mechanical and biochemical characterization of BNS cartilage (Chapter 4); 2) evaluation of BNS cartilage as model tissue for loading experiments (Chapter 5); 3) preliminary plowing experiment of BNS cartilage at room temperature (Chapter 6); and 4) plowing of BNS cartilage with different applied normal forces at physiological temperature (Chapter 7).
Chapter 4 focuses on the mechanical and biochemical characterization of BNS cartilage. Instantaneous and relaxation elastic moduli (E₀ and Eᵣ respectively) were determined for both confined and unconfined compression. In spite of the different experimental settings, E₀ and Eᵣ were similar: 4.07 ± 0.57 respectively 2.27 ± 0.36 for confined compression and 4.54 ± 0.96 respectively 2.11 ± 0.29 for unconfined compression. Histological features of BNS cartilage were revealed by light microscopy and TEM. In particular, it has been described that chondrocytes are randomly distributed and present typical characteristics of metabolically active cells. Moreover, it has been demonstrated that water represents 78 % of BNS cartilage wet weight, whereas GAGs and collagen make up 39.0 ± 5.0 % respectively 25.0 ± 2.5 % of BNS cartilage dry weight. Although GAGs and water contents showed a slight tendency to decrease through the postero-anterior axis and a slight increase in the dorso-ventral axis, collagen showing an opposite trend, these variations can be considered as irrelevant.

Chapter 5 focuses on preliminary static-loading experiments of BNS cartilage to study possible changes in gene expression. First, it has been shown that BNS cartilage, when subjected to a 50 %-strain unconfined compression and subsequent relaxation, has a mechanical behavior typical of a viscoelastic material: it deforms easily under the action of an slowly-applied stress and, after holding the strain, its stress decreases until a non-zero plateau is reached. Tissue relaxation starts to decrease after 2 hours, whereas the plateau is reached after 4 hours of equilibration. Additionally, it is reported that 50 % deformation of the initial sample thickness caused significant Coll1, MMP-3 and TIMP-1 up-regulation depending on relaxation duration. Furthermore, the results suggest that the equilibration time post-relaxation also influences chondrocytes gene regulation, which decreases over time. Although temperature seems not to influence gene expression significantly, Fn appears as an exception; indeed, for cartilage loaded at 37 °C, Fn expression increased compared to the compression experiment performed at RT (1.5 and 0.6 fold respectively).

The effect of plowing with different applied normal forces on bovine nasal septum cartilage has been extensively investigated and is reported in Chapter 6. Cartilage deformation, measured as an increase in cartilage strips length, has been shown to be dependent on the magnitude of the applied normal force. Moreover, profiles of tractional forces generated during plowing have been described: the higher the applied normal forces, the higher the generated tractional forces. Furthermore it has been described that plowing with higher applied normal force could be harmful for cartilage explants, as indicated by the up-regulation of Collagen type I
and of the catabolic enzyme MMP-3. Altogether, these results confirm that in our experimental setup, the tractional forces developed during plowing are correlated to the applied normal force.

Finally, in the last part of the manuscript (Chapter 7) the effect of plowing with different applied normal forces on cell viability, gene expression and GAGs release is described. It has been shown that plowing with an applied normal force of 100 N and indenter speed of 10 mm/sec causes chondrocytes death close to the surface as well as matrix damage consisting of GAG loss. In addition, plowing promotes in healthy chondrocytes the production and activation of catabolic enzymes, such as MMP-3 and shows two hours after plowing no effect on anabolic genes such as Aggrecan, Collagen type I and type II and Fibronectin. This pattern of gene transcription could promote extracellular matrix degradation aiming at facilitating the migration of viable cells into the damaged sites for the regeneration of the damaged matrix.

8.2 Outlook

The results reported on this thesis provide different impetus for further investigations. Besides the mechanical and biochemical effects caused by mechanical stress, the study of the cartilage surface would also be of tribological importance. Indeed, AFM measurements of the surface of plowed-cartilage could reveal changes in the smoothness of the tissue and thus, provide insight into the microscopic superficial cartilage degradation caused by mechanical stress.

Furthermore, although the effect of plowing on gene regulation for several cartilage structural protein (such as aggrecan an collagens), as well as for proteins involved in the mechanotransduction (fibronectin) and for catabolic enzymes and its inhibitors, has been investigated in this work, further patterns of genes may be explored, for example, by using the DNA gene-chip technique.

The study of different plowing combinations (with varying durations, indenter speeds and applied forces) and the use of indenters with different geometries (saddle-like or spherical) would be of interest in the reproduction of the TMJ and of other body joints. Additionally, investigation on the biological effect of the rolling and on the possible combination of the rolling and the plowing would provide supplementary knowledge in the processes of cartilage degradation due to mechanical stresses.
Furthermore, in order to improve the reproduction of the body joints, other technical refinements could be made: to cover the surface of the indenter with cartilage, and to use hyaluronic acid instead of culture medium. In this way, a more accurate reproduction of the friction forces developed during dynamic loading would be reproduced.

Finally, since it has been reported that shear stress and cyclic uniaxial loading enhance the functional properties of tissue engineered cartilage, it would be of interest to study the effect of plowing on artificial construct of cartilage.
References


