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PhD Thesis

# New insights into mutable collagenous tissues: an inspiring model for tissue regeneration

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*To My Parents... always supportive, constantly caring, eternally loving.*

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# Abstract

The biomimetic approach represents a new strategy pursued in the field of human regenerative medicine, since existing biomaterials lack the inherent adaptability of natural tissues; in particular, they do not truly mimic the dynamic microenvironment of tissues and organs.

Echinoderms are a good example of this ability, since they possess dynamic connective tissues called Mutable Collagenous Tissues (MCTs), able to undergo changes in their mechanical properties (stiffness, tensile strength and viscosity) in a short physiological time scale. This phenomenon is called mutability, and is initiated and modulated by the nervous system, especially by secretions of a specific cell type, the juxtaligamental cells (JLCs). Several studies reveal that MCTs are one of the key elements of echinoderm regenerative capacities, since they provide a dynamic ECM with an optimal growth-promoting environment for tissue repair and regeneration. However, the mechanisms that are behind the capabilities of MCTs to assume distinct mechanical states are still enigmatic. Thus the main aim of this work was to contribute for the understanding of those mechanisms.

The model studied in this work was the compass depressor ligament (CDL) of sea urchin *Paracentrotus lividus*, due to its easy extraction and typical MCT structure in comparison to others MCTs, with the advantage of not presenting calcite ossicles.

The first part of this work consisted on the investigation of the CDL ECM key-components, including fibrillar proteins but also the JLCs. Their structure and arrangement were studied in order to understand how natural MCTs actually function. Electron microscopy techniques were used to obtain a three-dimensional view of the CDL architecture at the micro- and nano-scales, and to clarify the micro-organization of the ECM components when the tissue changes from the compliant to the standard state or from the standard to the stiff state. With this investigation we expand the current knowledge of the relationship between organization of CDL ECM and its different mechanical states.

The biochemical changes that the CDL undergoes during its reversible tensility were investigated in detail. Confocal Raman microscopy, and Fourier Transform Infrared spectroscopy were used to investigate the possible similarities between CDL ECM and mammalian ECM components. The possible remodelling of a new ECM, as well as the contribution of water to CDL mutability, were hypothesized and evaluated, since these phenomena normally occur in mammalian adaptable connective tissues (such as the uterine cervix). We found that the fibrillar collagen has strong similarities with collagen type I and that glycosaminoglycans (GAGs) are from the sulphate family. Also, we concluded that CDL mutability involved subtle adjustments of protein components and tissue hydration, most likely without synthesis of a new ECM.

In view of the fact that mammalian ECM homeostasis is balanced by local protease activity involving matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), the potential function and involvement of MMPs in CDL mutability was also investigated. This work has provided the first evidence that MMPs may be involved in the mechanism by which echinoderm MCT undergoes changes in tensile properties. Gelatin zymography has revealed the presence of a consistent pattern of MMP activity that varies quantitatively according to the mechanical state of the ligament. Biomechanical results also demonstrate that MMPs are involved in CDL mutability, since an increase in CDL stiffness occurs upon stimulation with an MMP inhibitor. Similarly to mutability the stiffening action of the inhibitor was reversible.

Another major contribution of this work was the integrated morphological, biochemical and biomechanical investigation that was performed in CDL, comparing different mechanical states that mimic the mutability of the tissue *in vivo*. The acquired knowledge regarding to CDL structure, biochemistry and organization, as well as the contribution to understanding the mechanisms that promote such dynamic and reversible environment, may be inspiring for biomaterials scientists. As these structures are commonly present at the autotomy planes of echinoderms, enhancing tissue regeneration, the knowledge obtained in this thesis may open new and exciting strategies in regenerative medicine.

# Resumo

O bio-mimetismo tem surgido nos últimos anos como uma nova estratégia para abordar as questões relacionadas com a medicina regenerativa, numa tentativa de contornar os problemas de ausência de capacidade natural de adaptação dos biomateriais. De facto, a maior parte dos biomateriais actualmente utilizados não conseguem adaptar-se ao ambiente estruturalmente dinâmico que caracteriza os tecidos e órgãos naturais.

Os equinodermes constituem um bom exemplo desta capacidade de evolução dinâmica, já que possuem Tecidos de Colagénio Mutáveis (MCT na sigla inglesa) que podem sofrer alterações muito rápidas das suas propriedades mecânicas (rigidez, resistência à tracção, e viscosidade) num período curto de tempo. Este fenómeno, designado por mutabilidade, é iniciado e governado pelo sistema nervoso do animal, através da secreção de proteínas pelas células justaligamentais (JLCs na sigla inglesa). Diversos estudos já demonstraram que a existência de MCTs constitui um elemento chave que explica as capacidades de regeneração dos equinodermes, já que deles resulta uma matriz extra-celular (ECM na sigla inglesa) dinâmica, ambiente ideal para promover as condições de crescimento necessárias para a regeneração de tecidos. No entanto, os mecanismos que explicam as capacidades de os MCTs assumirem estados mecânicos distintos, ainda não são conhecidos, tendo sido objectivo deste trabalho contribuir para a sua compreensão.

O modelo escolhido para ser estudado neste trabalho foi o ligamento depressor do compasso (CDL, do ingês) do ouriço do mar da espécie *Paracentrotus lividus*. Esta escolha justifica-se pelo facto de este tecido ser fácil de extrair do animal, apresentando uma constituição semelhante à de outros MCTs, com a vantagem de não apresentar ossículos de calcite.

A primeira parte deste trabalho consistiu no estudo dos elementos-chave que constituem a ECM do CDL, nomeadamente proteínas fibrilares e as JLCs. De modo a entender o funcionamento dos MCTs, foi estudada com detalhe a estrutura e organização da ECM. Técnicas de microscopia electrónica foram utilizadas de forma

a se obter uma visão tri-dimensional da arquitectura do CDL nas escalas nano e micrométrica, e para clarificar a micro-organização dos componentes da ECM, quando os tecidos mudam do estado relaxado para o estado de controlo, ou do estado de controlo para o contraído. Com esta investigação foi possível progredir no estudo da organização da matriz extracelular do ligamento e na possível correlação entre a estrutura da sua matriz e as diferentes propriedades mecânicas do tecido.

As modificações bioquímicas do micro-ambiente que constitui o CDL durante estas alterações mecânicas reversíveis foram investigadas em detalhe. Microscopia confocal de Raman, espectroscopia de infravermelho com transformada de Fourier foram as técnicas utilizadas para investigar as possíveis semelhanças entre os componentes da matriz do ligamento e aquela que constitui os tecidos de mamíferos. Foi também avaliada a hipótese de uma nova matriz extracelular ser produzida durante o processo de mutabilidade, e investigada a possível contribuição da água para este processo, já que estes dois mecanismos estão presentes nos tecidos conjuntivos com propriedades mecânicas adaptáveis dos mamíferos (útero durante a gravidez). Verificou-se que o colagénio fibrilar presente no CDL tem fortes semelhanças com o colagénio tipo I presente nos mamíferos. Também, os glicosaminoglicanos (GAGs) foram identificados como pertencendo à família dos sulfatos. Foi assim possível concluir que o fenómeno de mutabilidade envolve pequenas variações na secreção de proteínas e no nível de hidratação dos tecidos, não envolvendo a síntese de uma nova ECM.

O eventual envolvimento e mecanismo de acção das metaloproteínases (MMPs) no fenómeno de mutabilidade dos equinodermes foi também investigado já que a homeostasia da ECM nos tecidos de mamíferos é controlada pela actividade local das protéases, envolvendo MMPs e inibidores tecidulares de metaloproteínases (TIMPs). Foi possível evidenciar, pela primeira vez, que as MMPs podem ter um papel a desempenhar no processo de mutabilidade dos equinodermes. De facto, testes de zimografia em géis de gelatina mostraram que a atividade das MMPs varia quantitatitvamente conforme o estado mecânico dos ligamentos. Os resultados biomecânicos também demonstraram que as MMPs estão envolvidas no fenómeno de mutabilidade já que houve um aumento da rigidez quando um inibidor de MMPs foi

utilizado e visto que a acção do inibidor foi reversível tal como o próprio fenómeno de mutabilidade.

A originalidade deste trabalho advém do facto de se ter procedido a uma investigação detalhada e extensiva dos aspectos morfológicos, bioquímicos e biomecânicos que governam os diferentes estados mecânicos do CDL em condições semelhantes às que ocorrem *in vivo*. O conhecimento adquirido, no que respeita à estrutura, bioquímica e organização do CDL, conjugado com o entendimento dos mecanismos fundamentais que promovem um ambiente dinâmico e reversível naqueles tecidos, podem, no futuro, inspirar os cientistas que trabalham na área dos biomateriais. De facto, como estas estruturas estão em geral presentes nos planos de autotomia dos equinodermes, favorecendo o processo de regeneração, os resultados desta tese podem ser úteis para avanços vindouros no campo da medicina regenerativa.

# Résumé

L’approche biomimétique représente une nouvelle stratégie poursuivie dans le domaine de la médecine régénérative, les biomatériaux existants n'ayant pas la capacité d'adaptation inhérente aux tissus naturels. En particulier, ils ne sont pas apte à reproduire l’évolution et changements qui se produisent continument dans l'environnement tissulaire au cours des phénomènes de régénération.

Les échinodermes sont un bon exemple de cet aspect dynamique, présent dans les mécanismes d’évolution,, car ils possèdent des tissus conjonctifs appelées en anglais ‘’MCT’’ (Mutables Collagènes Tissus) , capables de subir , sur des temps physiologiques de courtes durée des changements importants au niveau des propriétés mécaniques ( tels que: la rigidité, la résistance à la traction, la viscosité). Ce phénomène est appelé la mutabilité.  Il est initié et modulée par le système nerveux, en particulier par les sécrétions d'un type spécifique de cellules, les cellules juxtaligamentales.

Plusieurs études révèlent que les MCT sont un des éléments clés des échinodermes concernant les capacités de régénération au travers à la fois, de la matrice extracellulaire (ECM) mais également en favorisant un environnement optimal vis-à-vis des étapes de croissance, réparation et régénération. Cependant, les mécanismes qui gouvernent les capacités des ‘’ MCT ‘’à assumer différents états mécaniques sont pour l’heure encore énigmatiques.

Le modèle étudié dans ce travail a été celui du ligament dépresseur (CDL) chez l’oursin de mer (*Paracentrotus lividus*), en raison d’une facilité d’extraction facile et d’une constitution et structure équivalente aux autres MCT. Il présente en outre l'avantage de ne pas présenter d’osselets calcite.

La première partie de ce travail a porté sur la CDL ECM, protéines fibrillaires et cellules, composants clés. Structure et disposition ont été étudiées afin de comprendre comment fonctionnent réellement les ‘’MCT’’ naturels. Les techniques

de microscopie électronique ont été utilisées aux échelles micro et nano afin d'obtenir une vue tridimensionnelle de l'architecture des CDL, et de clarifier les organisations des composants ECM associées à la modification des tissus en relation avec les différents états étudiés dans ce travail.

Les changements biochimiques que subit le microenvironnement du CDL au cours d'une sollicitation de type charge- décharge en traction ont été également étudiés en détail. La Spectroscopie Raman, FTIR et des techniques de spectrométrie de masse ont été utilisées pour rechercher et étudier les similitudes biochimiques existantes entre CDL, ECM et les mammifères. En outre, des travaux visant à identifier les molécules effectivement impliquées dans la mutabilité CDL ont été menés. La synthèse possible d'une nouvelle ECM ainsi que la contribution de l'eau à la mutabilité des CDL ont été émis comme hypothèses possibles et ont été évaluées, en effet il a été constaté que le processus d'adaptabilité des tissus conjonctifs est un phénomène présent normalement chez les mammifères.

Chez ces derniers, l'homéostasie (ECM) est équilibrée par l'activité au niveau local de la protéase impliquant les métalloprotéinases matricielles (MMP) et leurs inhibiteurs tissulaires (TIMP). Pour la première fois la fonction de potentiel et l'implication des MMPs dans le phénomène de mutabilité échinodermes a été évaluée.

L'originalité de ce travail réside dans le choix et la mise en œuvre d'une approche à la fois multi-échelles et pluridisciplinaire appliquées aux études morphologiques, biochimiques et biomécaniques des CDL, ces derniers étant considérés comme un modèle de la mutabilité des tissus in vivo. Les connaissances acquises sur la structure des CDL, la biochimie et de l'organisation ainsi que les mécanismes fondamentaux qui favorisent de tels environnements dynamiques et réversibles, devraient constituer une étapes dans la compréhension de la régénération tissulaire domaine phare de la médecine régénérative.

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# List of abreviations

| | |
|---|---|
| MCTs | Mutable collagenous tissues |
| JLCs | Juxtaligamental cells |
| ECM | Extracellular matrix |
| CDL | Compass depressor ligament |
| GAGs | Glycosaminoglycans |
| MMPs | Matrix metalloproteinases |
| TIMPs | Tissue inhibitor metalloproteinases |
| PGs | Proteoglycans |
| SURF | Sea urchin fibrillar module |
| NSF | New stiffening factor |
| HA | Hyaluronic acid |
| TEM | Transmission electrom microscopy |
| CSEM | Cryo-scanning electron microscopy |
| FIB/SEM | Focused ion beal/scanning electron microscopy |
| FEG/ESEM | Field emission gun-environmental scanning electron microscopy |
| FT-IR | Fourier transform infrared |
| ATR | Attenuated total reflectance |
| LWD | Low working distance |
| MW | Molecular weight |
| MALDI-TOF/TOF | Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight |
| MS | Mass spectrometry |
| IR | Infrared |
| MRI | Magnetic resonance imaging |
| PPSW | Propylene phenoxetol in seawater |
| AChSW | Acetylcholine chloride in seawater |
| SW | Seawater |
| DMA | Dynamic mechanical analyser |
| E* | Stiffness |
| Tan δ | Damping |
| E´´ | Loss modulus |

| | |
|---|---|
| E´ | Storage modulus |

# Chapter I

## Aims and thesis synopsis

The potential use of echinoderms as biomimetic models for regenerative medicine remains largely unexploited. In fact, only a few biomedical studies have focused on adult echinoderms, which have interesting regenerative capabilities and are phylogenetic closed to chordates [1-9]. Sea-urchins, the model used in this thesis, present in their anatomy a distinguishing connective tissue, comparing to others phyla, which is considered as a key factor contributing to the early evolutionary success of echinoderms [10,11]. It was recognised 100 years ago as "something unusual" in echinoderms, since it has the ability to change reversibly the mechanical properties in a few seconds [10-15]. This interesting connective tissue was studied in this thesis since it is known that the ECM of tissues and organs is undoubtedly dynamic from the chemical, structural and biomechanical points of view [5,16-18]. The ECM of the compass-depressor ligament (CDL) of sea-urchins was considered an ideal tissue to understand the fundamental mechanisms associated to echinoderm ECM dynamicity and reversibility. The CDL of sea-urchin was selected as the model of study since has the advantage of having a classic MCT organization [10,19]. A better understanding of mutability phenomena is likely to provide a unique opportunity to develop new concepts that can be applied in the design of a dynamic biomaterial for tissue regeneration. Thus the main goal of this thesis was to contribute to the identification of the fundamental ECM components of the CDL, their chemistry, structure and organization, which are responsible for its mechanical adaptability. The study was performed in CDLs in different mechanical conditions that mimic the *in vivo* CDL mutability.

In chapter II, a review of the current knowledge regarding the mechanically adaptable connectives tissues of echinoderms is presented. Particular attention was given to the MCT architecture and the role of MCT components in mutability. As mammals present a type of mechanical adaptable tissues (the uterine cervix during pregnancy), a comparison regarding both structures and mechanisms was established, in order to elucidate the possible mechanisms associated with reversible tensility [20-23]. The possible contribution of MCT to the regeneration phenomena was also reviewed in this chapter.

The results obtained during the experimental work are presented in the form of papers (chapter III, IV and V), which were submitted/published in international scientific journals.

In Chapter III the basic biology of CDL in the different mechanical conditions that mimic in vivo CDL mutability, particularly the key-components and the fundamental interactions involved in CDL reversible tensility were studied. Information on the structural organization of the tissue as a whole and the spatial interconnections between the different components was achieved through extensive morphological characterization, using different electron microscopy techniques. (Paper published in PLoSOne).

The biochemical modifications that CDL experience during mutability are presented in detail in chapter IV. Spectroscopy techniques were used to identify the specific molecular components that contribute to CDL biochemical environment. The possible remodelling of a new ECM matrix, as well as the potential contribution of water to the CDL mutability phenomenon, was also evaluated. A mass spectrometry study was performed in order to identify possible key-proteins involved in the mechanism. (Paper submitted to Biointer*phases* journal).

Adaptable connective tissues are also present in mammals (uterine cervix during pregnancy), in which ECM homeostasis is balanced by local protease activity [16,20-26]. Although hypothesized, there have been no previous attempts to detect the presence of matrix metalloproteinases (MMPs) in MCTs. New insights into the biomechanics of CDL, as well as the possible involvement of MMPs into the mechanism that govern tissue reversibility are presented in chapter V (Paper submitted to PLoSOne).

Chapter VI presents a general discussion on the results obtained and suggests new avenues for future research.

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# Chapter II

## Echinoderms mutable collagenous tissues

# 1. Echinoderms

Echinoderms (Phylum Echinodermata) are a phylum of marine animals belonging to a branch of the animal kingdom known as deuterostomes that are globally distributed around the World [1]. Their name is derived from the Greek (*echinos*, "spiny"; *derma*, "skin") meaning spiny skin and around 13000 echinoderms fossil species are known to exist, being the oldest identified as having lived in the Cambrian period. At present there are 7000 living species divided in the five classes of echinoderms: Echinoidea (sea urchins, sand dollars and sea bisbuits), Asteroidea (sea stars), Crinoidea (sea lilies and feathers stars), Holothuroidea (sea cucumbers) and Ophiuroidea (brittle stars and basket stars) [1,2].

The phylogenetic relationships among the five classes have been extensively controversial, but it is generally accepted that the class Crinoidea branched first and that the Echinoidea and Holothuroidea are sister clades [1,2]. Echinoderms are positioned among the Deuterostomia, being the few invertebrates placed in the same evolutionary branch of vertebrates, that share common cellular and molecular mechanism with chordates [1,3-6].

The distinctive morphological characteristics of Echinodermata phylum are: a pentamerous radial symmetry (in adults) with a calcareous endoskeleton with spines or calcareous spicules. They have a complex subepithelial radial nervous system without head or brain, usually with two or three networks positioned at different levels of the body, however without specialized sense organs. They also own a water-vascular system that extends from the body surface as a series of tentacle-like projections (canals) that are essential for gas exchange purposes, and are normally involved in functions such as feeding, locomotion and cellular respiration. Echinoderms have a complete digestive tube, divided into a throat, stomach, intestine and rectum (anus absent in ophiuroids). The circulatory system when present composes the haemal system (contributing little if any role in circulation) that is surrounded by extensions of coelom (the main body fluids are the coelomic ones) [1,2]. They have a diverse immune system with large repertoire of innate pathogen

recognition proteins [1,2,7]. Most echinoderms have separate sexes where reproduction occurs by external fertilization [1,2].

The extracellular matrix (ECM) in invertebrates like in vertebrates is the non-cellular component present in all tissues and organs that provide crucial physical support for the cellular elements but also biochemical and biomechanical cues required for tissue homeostasis [8-12]. Although it is a characteristic of all metazoans, the ECM has evolved into a variety of tissues where substantial variations in composition, molecular size and structure characterize the diversity of all evolutionary processes [10,13,14]. The pathway by which ECM constituents are secreted from cells is highly conserved and clearly precedes the metazoan. Although some of the ECM proteins were highly conserved through evolution (e.g. collagen, integrins, matrix metalloproteinases), many unique features are found among the invertebrate molecules of the ECM [2,7,8,10-12,14].

## 2. The uniqueness of the echinoderm phylum

Echinoderms possess peculiar and unique connective tissues, called Mutable Collagenous Tissues (MCTs). These tissues are considered as intelligent connective tissues due to their capacity of undergoing reversible changes in their passive mechanical properties (e.g. tensile strength, stiffness, viscosity) in a short time span, through a non-muscular mechanism [15,16].

They appear in the form of dermal connective tissues, interossicular ligaments, tendons and connective tissue, performing analogous mechanical functions as collagenous connective tissues present in equivalent positions in vertebrates' bodies [15]. They are present in all echinoderms classes in a diversity of functional locations where their structure can show one of three patterns of tensile change: (1) only reversible stiffening and destiffening (e.g. MCT stiffens to maintain body posture and softens to allow body movements due to water currents or even gravity) [16-19]; (2) reversible stiffening and destiffening, but they can also show irreversible destabilisation (always associated with detachment of body parts: autotomy

mechanisms) [20]; (3) only irreversible destabilisation (again always associated with autotomy) [21,22].

Although some echinoderm structures were defined as MCTs, only the ones present in Table 1 were experimentally tested (mechanical tests were used to assess the biomechanical properties of the tissues) and the results were already published [23].

**Table 1:** MCTs structures present in all echinoderms classes (adapted from Wilkie *et al*.) [23].

| Class | Reversible | Irreversible | Reversible - Irreversible |
|---|---|---|---|
| ***Asteroidea*** | - Spine ligaments<br>- Aboral dermis<br>- Longitudinal interambulacral ligaments outwith autotomy region | - Aboral dermis<br>- Longitudinal interambulacral ligaments within autotomy region | |
| ***Crinoidea*** | - Cirral ligament<br>- Synarthrial ligaments of arm<br>- Ligaments of the stalk | | - Syzygial ligaments of arm<br>- Synostosal ligaments of stalk |
| ***Echinoidea*** | - Central spine ligament<br>- Capsular spine ligament<br>- Periodontal ligament<br>- Compass depressor ligament<br>- Compass-rotula ligament<br>- Peristomial membrane<br>- Tube feet | | |
| ***Holothuroidea*** | - Body wall dermis | - Introvert dermis | -Pharyngeal retractor muscle-longitudinal body wall muscle tendons |
| ***Ophiuroidea*** | - Proximal oral arm plate ligaments<br>- Oral shield plate ligaments | - Intervertebra ligaments<br>- Distal oral arm plate<br>- Disc dermis | - Autotomy tendons of intervertebral muscles |

The magnitude of the reversible changes that MCTs can accommodate were analysed and quantified using several mechanical testing methods, namely creep,

stress-relaxation, stress-strain, and dynamic stress-strain tests [15,16,23]. The mechanical adaptability of all these MCTs was experimentally tested *in vitro* by stimulation with chemical agents, such as calcium, potassium, magnesium, neurotransmitters (e.g. acetylcholine that increases reversibly the stiffness of MCTs) and anaesthetics (e.g. propylene phenoxetol that decrease reversibly MCTs stiffness) in order to mimic the *in vivo* situation [15,16-19,24-29]. Treatments with neuro-active agents, such as $K^+$, mimic *in vitro* the irreversible changes in MCT mechanical properties, since they cause an immediate decrease in viscosity with subsequent tissue rupture [15,21,22].

It is important to note that MCT variable tensility is also strongly affected by the extracellular ionic environment; their variable tensility is pH-dependant. Holothurian dermis has lower viscosity at pH between 6 and 8, and higher viscosity below and above this range [24,26]. It seems that alterations in the number of charged groups in the crosslinking agents influence their mutual interactions and their possible configuration [15,16,24-27].

The responsiveness of MCTs to neurotransmitters and to excess of cations suggests that MCT tensility is nervously mediated, in which the neural input seems to involve a cholinergic hyponeural motor system [15,29-31].

The functional significance of MCTs is high. They are involved in locomotion, maintenance of posture with low oxygen consumption (maintenance of posture is normally performed by muscles in others animals) and self-induced defensive detachment of body parts (autotomy) that is followed by regeneration [15-17,21,32,33]. The non-autotomy associated MCTs present reversible changes in tensile strength, allowing the entire animal or individual appendages to fix posture (stiff condition). On the contrary in the compliant condition the entire body or its appendages have freedom to move [15]. All examples of echinoderm autotomy found in all echinoderms classes present a rapid destabilization of collagenous structures (completely lose tensile strength) to allow a part of the body to be released in an emergency (e.g. predator attack). This characteristic may led echinoderms to employ asexual reproduction by fission [34,35].

## 3. Mutable collagenous tissues in sea-urchins

Sea-urchins, heart urchins (spatangoids), and sand dollars (clypeasteroids) are example of animals that belong to the *Echinoidea* class (Fig. 1A) [1]. The external appendages of sea urchin, such as the catch apparatus (or the capsular ligament) of the spine-test joint and the tube feet (Fig. 1B) are considered mutable collagenous tissues [15,16,36].

The catch apparatus was the first echinoid MCT to be described in 1967, however without being considered as an MCT, since Del Castillo *et al.* have presented a model in which the stiff properties of the tissue was attributed only to the muscle cells [37]. Subsequently Wilkie *et al.* proposed a new model suggesting that the catch apparatus is a collagenous connective tissue with a small proportion of muscles cells, and that the function of this tissue is entirely dependent on the collagen structure of its extracellular matrix [37,38].

The tube feet, another example of MCT recently studied by Santos *et al.*, belongs to the echinoderm water-vascular system and can present a variety of forms and functions [36,39,40]. Indeed, this organ is involved in many different activities such as locomotion, feeding or strong fixation to the substratum where they resist the tensions imposed on the animal by hydrodynamics. They work in a traction system where they attach to substrates and contract, thus pulling the sea urchin to detach easily and voluntary [2,33,36,39-42].

The internal anatomy of a sea urchin is dominated by a large coiled digestive system, which consists basically of a tube joining the lower mouth to the anus on the upper surface. In regular echinoids the mouth opens into an oesophagus that initially runs through the centre of the Aristotle's lantern [1,2]. The Aristotle's lantern is the masticatory apparatus to which the teeth are attached. This apparatus is a complex but versatile system composed by skeletal pieces, elevator muscles, ligaments, and compass depressors, all these structures being involved in all the main motor activities such as feeding, digging, scraping and locomotion [2].

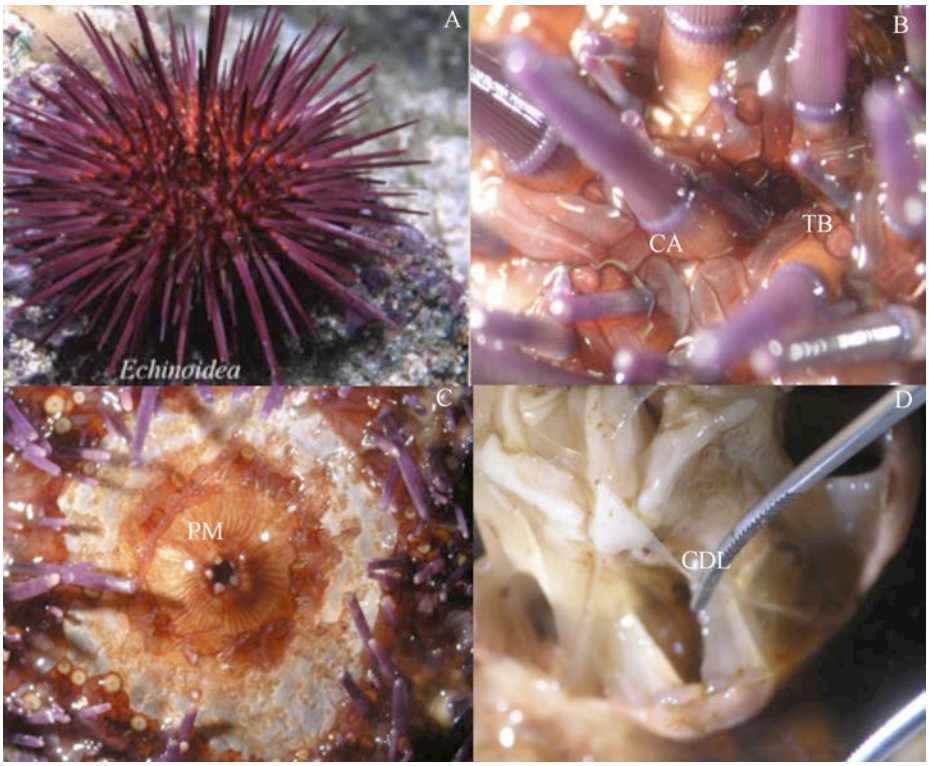


**Figure 1: MCTs in Echinoidea:** (A) sea urchin *Paracentrotus Lividus*, (B) tube feet (tb) and the catch apparatus (CA), (C) peristomial membrane (PM) and (D) the compass depressor ligament (CDL).

The functional morphology investigation of the Aristotle's lantern as well as its biomechanical behaviour was studied in detail, pointing to the existence of two tissues in direct contact with this masticatory apparatus that has variable tensility [17-19,28]. The peristomial membrane is one of them. It is a flexible tissue of the buccal apparatus that surrounds the mouth (Fig. 1C) and connects the lantern to the test of the animal, playing a fundamental role in lantern's movements. Their variable tensility is helpful not just in motor activities but also adapts its mechanical properties depending of the volume of coleomic fluid inside of the test [17-19,28].

Another MCT that is present into the Aristotle's lantern is the compass depressor ligament (Fig. 3D), that was considered as an MCT after the ultrastructure studies performed by Lanzavecchia *et al.* on the lantern [17,43]. Each lantern contains ten CDLs that extend from the distal lobes of the compasses to the interambulacral processes of the perignathic girdle (inner edge of the test) at its junction with the flexible peristomial membrane [15,17,18,28,44,45]. Their variable tensility helps the animal to stabilize the position of the lantern and to regulate the internal pressure. It is

also known that it works as a ventilator, since the elevation and depression of the compass and movement of associated fluids oxygenate the muscles [15,17].

MCT also confers mechanical adaptability to structures that lack some kind of musculature (e.g. sea-lily stalk) or can also work in parallel with muscular components (e.g. asteroids and echinoids spine ligaments, echinoid peristomial membrane) [15,23]. Although CDLs were considered to be muscle due their ability to contract, ultrastructure investigations revealed that CDLs from *P. lividus* are basically collagenous ligaments that present in their inner surface a thin myoepithelium (with peritonocytes and myocites) that represent only 8% of the total area [17,23]. The presence of muscles cells complicates the interpretation of CDL variable tensility. However, for being responsible for the maximally stiffened state of CDL muscles would have to develop a high tensile strength, superior to that of the strongest muscle known (the retractor muscle of *Mytilus edulis*) [17,23]. Furthermore, muscle cells have not been detected in the majority of confirmed MCTs that show variable tensility [17,23]. Although some control pathways regulating contractile and collagenous have common features there is no evidence for the participation of muscles in the mechanical adaptability of MCTs [15,23].

## 4. General MCT architecture

The mutable collagenous tissues can be considered as composite materials, constituted by a dense extracellular matrix of collagen fibrils, fibrillin microfibrils, proteoglycans (insoluble and soluble PGs), water and a number of specific constitutive and regulative proteins (effector proteins) already identified and characterized in holothurian dermis (see Fig. 2) [15,29]. As MCTs are present in several anatomical locations, they perform the same mechanical functions as the collagenous connective tissue at analogous locations in the bodies of vertebrates, which can be generalized as resisting, transmitting and dissipating energy, while the interfibrillar matrix gives resistance to compression maintaining the tissue hydrated [8,12,15,46].

Besides collagen, other supramolecular assemblies are present in all studied MCTs. This is the case of the loose networks of microfibrils endowing tissues with elasticity [15,29]. An extensive morphological, biochemical and immunological characterization of these structures from *C. frondosa* reveal that they resemble fibrillin-containing microfibrils of mammalian connective tissues [47-51]. The microfibrils network extracted from holthurian dermis tested by tensile testing shown to be extensible reversible up to 300% of their inicial length [48]. It seems that this protein help MCT in a compliant state to return its initial dimensions after it has undergone deformation [15,47,48].

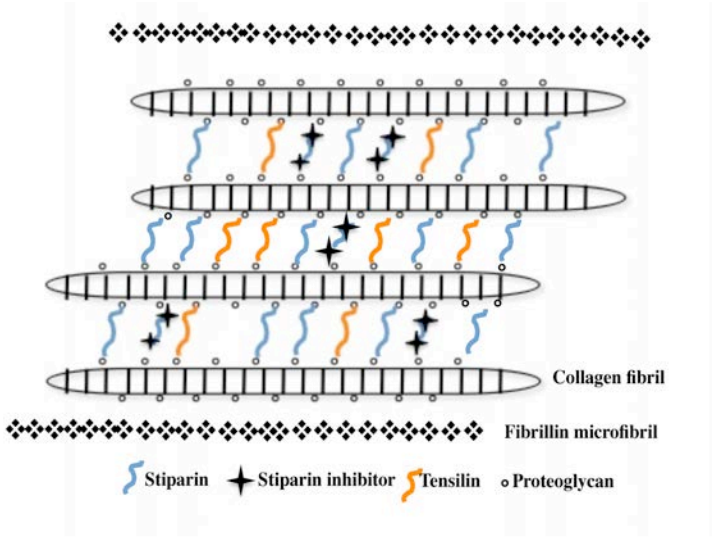


**Figure 2:** MCT organization: MCT is constituted by parallel aggregations of collagen fibrils to which proteoglycans and glycosaminoglycans are attached serving as binding sites for molecules involved in interfibrillar cohesion (stiparin and tensilin). Collagen fibrils are delimited by fibrillin microfibrils that helps the tissue to re-establish its initial mechanical properties after undergone deformation [15].

Although other cell types are present (e.g. fibroblast-like, phagocytes and myocytes), all confirmed MCTs are permeated by or in contact with the juxtaligamental cells (JLCs) [15,17,18,33,52-54]. It has been known that JLCs are a part of the nervous system due to their close association with neuronal processes (functional contact with axons sometimes at chemical synapse-like junctions) providing MCT innervation [15,17,52,54]. Neural inputs to MCTs seem to include both cholinergic and aminergic components. The activities of these cells are controlled at least partly by cholinergic pathway, although there is evidence of the presence of aminergic innervation due to the presence of axon-like structures

[15,17,52,54]. In most MCTs structures it is possible to identify more than one population of granules that is distinguishable by shape (circular or oval profiles), size and variable electron density [55]. These cellular elements are considered as the effector cells responsible for MCT tensility, since they terminate within MCT, they are in close contact with the nervous system and in the sea cucumber model (dermis of sea cucumber *Cucumaria frondosa*) it was identified molecules in their granules that influence the interfibrillar cohesion [15,56,57]. Also in the sea cucumber model, more than one populations of JLCs was distinguishable morphologically, suggesting that one cell type could be responsible for the release of the stiffening protein and another for the de-stiffening protein resulting in the stiff and compliant mechanical state respectively [55]. The only case that it was observed possible exocytosis and reduction in granule electron density, granule number and size was in JLCs located in MCTs after autotomy. However it was not possible to evaluate if it was a result of the tissue disintegration due to autotomy [53].

## 5. ECM constituents and their role in the molecular mechanism of mutability

### 5.1 Collagen and fibrillin

Based on current evidence, it appears that most MCTs consist of parallel aggregates of discontinuous, spindle-shaped collagen fibrils with paraboloidal tips to which PGs are covalently or non-covalently attached [15,29,58-64]. This seems to be the perfect arrangement for fibrils that strengthen a discontinuous fibre composite avoiding shear-stress concentration near its ends [15,59,65].

Although the main fibrillar extracellular components are common in the matrix of all MCTs structures, their organization and spatial arrangement can be diverse. In sea urchins for example, the compass depressor ligament as well as the spine ligament present a structure with predominantly parallel fiber array. However the peristomial membrane shows distinct layers with orthogonal fiber arrays [15,17,18]. This structural diversity of MCTs is comparable to that of mammalian connective tissues [15,17,18,66,67].

Similarities were also found between vertebrate collagen type I and sea urchin collagen fibrils regarding d-banding pattern (67nm), amino-acids composition, cross-link chemistry and gene organization [15,59,61,62,65,68-73]. Although the work performed by Trotter *et al.* suggests some close resemblance between the d-banding pattern of collagen from sea-urchin spine ligament, holothurian body wall and mammalian type I fibrils have some differences in the staining intensity, suggesting that dissimilarities might occur at the amino acid sequences [15,59,60,68]. Also some differences were found regarding the chain composition of echinoid and holothurian collagen molecules, their solubility and amino acid composition [15,60,74]. Although some differences were achieved, none of them can be correlated with MCT variable tensility.

Interesting Exposito *et al.* have demonstrated the presence of heterotypic fibrils with collagen molecules that undergo distinct maturation in their N-propetide domain. This domain seems to be specific of echinoderm phylum and consist of a 140 amino acid long motif (sea urchin fibrillar module (SURF) [72,73,75,76]. Although they seem to be exclusive of echinoderms and are present in MCTs structures, their contribution to the variable tensility is still speculative since SURF molecules also appear in several tissues that are not mutable [73,75,76].

During the last decade, numerous investigations have characterized the fibrillar collagen chains in hydra, worms, and sea urchin [73]. These data support the concept that in invertebrates and vertebrates the triple helix is conserved, despite the presence of some imperfections and sometimes low levels of sequence identity [73]. Sea urchin collagen literature identifies the presence of two fibrillar α chains (1 α and 2 α) involved in the formation of heterotrimeric molecules [$(\alpha 1)_2$ α2] that are characteristic of mammalian type I collagen [66-70,72-78]. Phylogenetically, echinoderm striated collagen fibrils are also close to mammalian type I fibrils. The phylogenetic tree of the collagen genes presented by Wada *et al.* demonstrate the proximity of sea urchin collagen genes to those coding collagens of mammalian type I fibrils [15,73,76,79].

The variable tensility of MCT does not involve changes in the mechanical properties of the collagenous fibrils. All the ultrastructural studies performed were unsuccessful to prove that MCT tensility is accompanied by modifications in fibrils diameter or even by changes in d-banding pattern. Futhermore, the fusiform shape of the fibrils is not related with MCT variable tensility [15,29].

Fibrillin microfibrils were already biochemically and ultrastructuraly characterized by Thurmond and Trotter. However, their involvement in the mutability phenomenon is inconclusive since they appear in connective tissues that are not mutable [15,47-51].

### 5.2 Ground substance and molecular interactions involved in the interfibrillar cohesion

MCTs can be maintained stiff or compliant due to the ground substance molecules that interconnect fibrils, this process being strongly influenced by the extracellular ionic composition and concentration, suggesting that the capacity of the fibrils slide past each other can be controlled through alterable interaction of specific charges that constitute the extracellular matrix [15,16,24-27,29]. They are extremely sensitive to the ionic surrounding medium; their tensile strength is pH dependent and the stiffness decrease with monovalent cation concentration (e.g. $K^+$). It is possible that monovalent cations mask GAG anionic sites reducing collagen-GAG and GAG-GAG interactions. Divalent cations ($Ca^{2+}$ and $Mg^{2+}$), have an opposite influence on MCT stiffness regarding the monovalent ones, it is possible that they can act as divalent cross-linkers. However the current hypothesis suggests that their role on the variable tensility is more related with their action on the cellular components (JLCs) rather than the ECM itself [15,24,27,29,80].

As in mammalian connective tissues, one of the molecules involved in interfibrillar cohesion are proteoglycans (PGs) that are covalently or non-covalently attached to collagen fibrils, serving as binding sites for the effector molecules responsible for interfibrillar cohesion [8,81-84]. PGs with their typical glycosaminoglycans (GAGs) side chains were already characterized in the echinoid spine ligament, where GAGs belong to the chondroitin-dermatan sulphate family

[29,59,60,81,84-86]. In holothurian dermis, highly sulphated fucose GAGs were also observed and associated with collagen fibrils [59,61,81].

It seems that the integrity of the ECM of MCT in contact with extracellular medium with a ionic composition of sea-water depends on the electrostatics interactions that are very important to maintain the interfibrillar cohesion [16,24-27]. MCT tensility seems to be adjusted through changes in the type or even density of interactions between adjacent PGs molecules or between PGs and collagen fibrils. While PGs-PGs interactions are poorly characterized, it is agreed that collagen-PGs interaction are mainly electrostatic where the softening of MCT may result from the weakening or even suppression of PG-collagen interaction, which allows the interfibrillar slippage to occur [15,16,27,29].

### 5.3 Effector molecules

In terms of molecules responsible for interfibrillar cohesion, clear interfibrillar bridges were already visualised (with electron microscopy techniques), and some of the proteins involved were already identified in the sea cucumber model [15,29,55-57,87,88]. The one that is more likely to have a role in the standard tensile state is stiparin, the most abundant glycoprotein that interacts with collagen fibrils via a surface–bound PG [57]. MCT re-stiffening from the 'compliant' state has been ascribed to tensilin, a glycoprotein that binds to collagen fibrils via surface GAGs forming interfibrillar bridges between collagen fibrils, preventing interfibrillar slippage [56-58]. Tensilin was isolated from *Cucumaria frondosa* with agents causing cell lysis, indicating that it is secreted from cells, however it was also localized extracellularly (it depends on the mechanical state of the tissue) [15,55,56,58]. The hypothesis at the moment is that collagen fibrils are held together by stiparin through weak bonds (since stiparin is easily extracted with seawater) that facilitates the action of effector molecules such as tensilin (see Fig.3). Another stiffening agent has been recently identified. The MCT stiffening from the standard to the maximally stiffened state was recently attributed to a novel stiffening factor (NSF). However it still essential to elucidate if the stiffening activity of NSF is due to its direct action on extracellular components or to effects on cells [87].

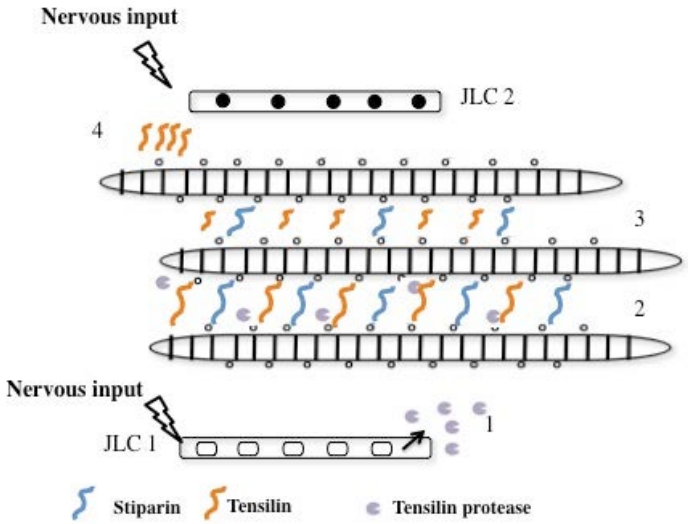


**Figure 3: The hypothesis of tensilin-tensilin protease:** compliant MCT results from the release of tensilin protease from juxtaligamental cell (JLC 1) (1), which has the ability to cleave tensilin near its GAG-binding site (2). Fibrils are able to slide past each other becoming the tissue in the compliant condition (3). The standard condition results from the release of fresh tensilin from a second type of juxtaligamental cell (JLC 2) to the extracellular matrix (4) [15].

The potential destiffening agent has not yet been identified. It has been wondered that enzymes might have such role. The hypothesis of tensilin–tensilin protease proposed by Wilkie *et al.* appears plausible, since the C-terminus of tensilin (that contains a collagen-binding domain) undergoes proteolysis *in vitro*. It has been suggested that tensilin-induced stiffening is reversed *in vivo* by a specific protease. Also, as the amino acid sequence of tensilin has 21-36% homology with a tissue inhibitor metalloproteinase (TIMP) raises the possibility that the mutability mechanism may have evolved from a MMP-TIMP system [15]. It seems that once released in the extracellular matrix this protease will cleave tensilin near the GAG binding site, allowing fibrils to slide past each other and resulting in a compliant condition. Reversibility is obtained by the release of “fresh” tensilin to the ECM that will restore or perform new bonds becoming the tissue in its initial mechanical properties [15].

A “stiparin inhibitor” and a “ plasticiser factor” were also identified as responsible for the transition from the standard to the compliant state [15,55,88]. Till now, the factor(s) that return MCT from the maximally stiffened state to the standard state were not identified. It can be hypothesised that, different mechanisms and proteins effectors can be involved in the different transitions (see Fig.4).

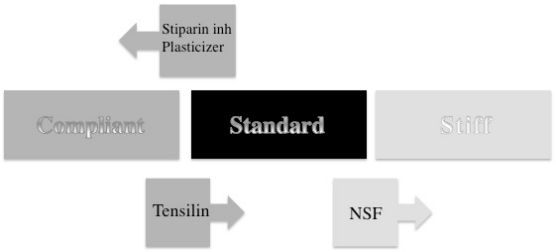


**Figure 4:** Effector molecules already identified in the sea cucumber model and involved in the transitions compliant-standard, standard-stiff and standard-compliant state. NSF- novel stiffening factor; Stiparin inh- stiparin inhibitor.

### 5.4 The involvement of calcium ions in MCT mutability

The mechanism of the nervous mediated changes in MCT tensility is not completely understood. However, it was proposed that calcium could be an effective agent for handling the tensile properties of echinoderm MCTs [15,89]. The pioneer work of Wilkie *et al.* demonstrates that the viscosity of intervertebral ligament of a brittle star was calcium dependent [15,24,54]. It is known that the mechanical properties of MCTs are affected by changes in the extracellular ionic concentration as it was referred before. Increasing $Ca^{2+}$ concentration cause MCT stiffening. On the contrary, bathing in a $Ca^{2+}$ chelating solution reduces stiffness [15,90]. This result suggests that calcium ions could contribute to the interfibrillar cohesion like a crosslinking agent, like in mammalian connective tissues. It is thought that $Ca^{2+}$ maintains the linkage between collagen fibrils and PGs, and that JLCs could modulate the amount of extracellular calcium inducing changes in tissue stiffening [15,29,80]. However, MCTs also become stiff even in the presence of $Ca^{2+}$ chelating medium where their cells are lysed, suggesting the hypothesis that there are others molecules (effectors proteins) that are being released from the cells to the ECM [89-91]. Trotter and Koob *et al.* demonstrate that $Ca^{2+}$ is important only in the cellular regulation of MCT stiffness and for the first time they extracted from MCT cells an organic factor that contribute to tissue stiffening [89]. This results as led to a search on the effector proteins (described already in 5.3) that contribute to the mutability phenomenon and holothurian *C. frondosa* was used as a model for this propose [55-57].

At present recent evidence favours the view that the influence of $Ca^{2+}$ concentration in the manipulation on MCT tensility is due mainly to direct effects on

the cells rather than the ECM itself. Calcium could be a homing signalling that activates the JLCs that modulates tensility by secreting effector molecules that alters the interfibrillar cohesion [15,29].

## 6. The concept of mutability: from invertebrates to vertebrates

Connective tissues with adaptable mechanical properties are not exclusive of echinoderms, since through the animal Kingdom there are a few examples. An analogue of MCT is the cuticle of some insects that show reversible extension when the animal feeds. The mechanism of cuticular plasticization in feeding is in a short physiological time scale and is related to hydration involving the transport of $H^+$ ions into the sub-cuticular space by cells in the hypodermis [15,92].

Indeed, one of the most remarkable and well known example comes from mammals (including humans) involving the collagenous structures associated with the female reproductive tract in pregnancy that transforms from a stiff cervix, that maintains the fetus in the uterus, to a very compliant state, with a maximal loss of tensile strength, at the time of birth, to allow the passage of the fetus [93-99].

Cervical mechanical adaptability also called remodelling, is divided into distinct phases called softening, ripening and postpartum repair and is orchestrated by a specific endocrine microenvironment, which will influence cell functions as well as concentration and structure of ECM constituents. A completely remodelling of the cervix occurs after labour [94,96-98].

The extracellular matrix of the uterine cervix is mainly constituted by fibrillar collagen, proteoglycans, hyaluronan, elastic fibers (elastin) and water. Although both ECMs (MCTs and cervix stroma) share common constituents, the time scale of action is completely different [94,97-99]. The strength of cervix matrix depends on the content and organization of fibrillar collagens type III and I. Cervical softening is associated with the disorganization of the collagen network where the mature and cross-linked collagen matrix is replaced by a less mature collagen matrix with a lower degree of crosslinking that results in a disorganized packing of fibrils with large

spaces between them. This disorganization of the ECM leads to a loss of tissue integrity with a progressive increase in tissue compliance [95-97,99-101].

The softening phase is associated with changes in cervical proteoglycan composition and metabolism. The increase in tissue hydration as a result of the increase in GAGs (unsulphated, HA, sulphated) concentration and composition is the process initiated during softening that is then prolonged through ripening. The increase in hyaluronic acid (HA), auguments the interfibrillar spacing between collagen fibrils, facilitating disorganization of collagen network and providing an increase in viscoelasticity of the cervix [94,95,97,99-101]. At the end of pregnancy the increase of tissue compliance and viscoelasticity is a result of the increase of synthesis of high molecular weight HA that possibly forms cross-links to versican leading to tissue distensibility, hydration and disorganization of collagen matrix [94,97,99]. Also in the ripening process, enzymatic degradation of the ECM via matrix metalloproteinases (MMPs) together with increased expression of the tissue inhibitors of metalloproteinases (TIMPs) (these apparently balancing the degradation process) seems to occur [95-97,102-105]. The variable tensility result from changes in the biochemical and structural composition of the cervix and by an MMP-dependant degradation of collagen fibrils and others ECMs components.

Prior to or during the onset of labour there is an increase on hyaluronidase and ADAMTS1 expression which seems to be responsible for the completely loss of cervical tensile strength that allows the birth. The postpartum repair is characterized by an important increase in transcription of genes involved in matrix repair, which ensures the recovery of tissue integrity. This active remodelling is also characterised by the infiltration of tissue monocytes, which differentiate to produce macrophages with different phenotypes, allowing the matrix clean up, preventing overactivation and promoting tissue repair [94,97,99,106].

## 7. Is there any possible link between mutability and regeneration?

Adult echinoderms show pentaradial symmetry and absence of a clear anterior cephalized structure [1,2]. These morphological features might explain why they are

considered as primitive animals. However the close relationship with chordates and their striking regenerative capabilities allows us to consider them as an attractive model for the study of such fundamental mechanism [6]. The regenerative capacity that is present in all echinoderms classes ensure the replacement of external and internal organs which are frequently lost after traumatic injury, predation, or even autotomy [21, 35]. In echinoderms, although predation is the often-cited cause of autotomy there are environmental stresses such as abiotic mechanical damage (excessive fluid-flow velocities caused by storms), rapid fluctuations in salinity or even in temperature that might induce autotomy [107]. Regeneration has been studied most thoroughly in crinoids, ophiuroids, and asteroids, both in terms of mechanisms at the tissue/cellular level and ecological significance [21,35,107-116]. Although echinoids present a reduced condition for regeneration because they do not have large exposed appendices susceptible to predation, it might occur at spines, pedicellariae and at the test [21,35,108,110,115,116].

Regeneration is not exclusive of echinoderms and is a phenomenon, which in most cases follows autotomy. Autotomy is the mechanism which involve the adaptive detachment of animal body structures acting as a defensive function, that is neutrally controlled and it is widely observed throughout Metazoa [21,35,117]. Echinoderms have extraordinary regeneration but also autotomy capacities; for instance, crinoids and holothurians regrowth autotomized viscera, some asteroids, holothurians and ophiuroids reproduce asexually through fission or autotomy with a complete rebuilding of missing body parts and the great number of echinoderms regrows lost appendages [21, 35,108].

Autotomy planes, that divide different anatomical components, comprise breakage zones with particular weakness. However, the autotomous detachment is not a result of such isolated fragility. It also depends on nervous system-controlled reduction in the mechanical resistance at the instant of autotomy [117]. There are permanent breakages zones that always present low levels of mechanical resistance (e.g. epidermis of ophiuroid) and potential zones of weakness, that just become weak at autotomy (e.g. intervertebral ligament) [21, 116].

Two mechanisms can be involved in the detachment of body parts: the muscular contraction that creates tension sufficient to split body parts as an example autotomy of salamander and lizard tails, bivalve siphons, and hydromedusan tentacles; and a endogenous change in the mechanical properties of the tissue present at the autotomy plane that result in a completely loss of tensile strength, the examples are all the autotomy processes of echinoderms [21, 117].

Undoubtedly it seems that there are structures at the autotomy planes that reduce trauma, endorse fast fluid compartment sealage, enhancing wound closure and healing, important requisites for efficient regeneration [21, 35,108,118]. For example, amputation at levels other than autotomy planes in crinoids results in anomalies, and sometimes on the complete absence of regeneration after wound repair [21,35,108,117]. Also, data comparing regeneration events after autotomy and amputation at different levels in asteroids reveal that the rate of arm regeneration is greater after autotomy than after amputation at distal levels [119]. However there is in echinoderms some detachment of body parts that occurs in a response to external stimuli at sites with absence of autotomy structures and with a different time scale (hours or days), the unique feature in common to these sites is the presence of mutable collagenous tissues (MCT) [21].

Some investigations have demonstrated that in all five echinoderm classes, the detachment mechanism of body parts depends on the rapid and nervously controlled disintegration of MCT that is present in the intact autotomy plane [21]. The MTCs associated with autotomy structures as the capacity to undergo an abrupt irreversible decrease in tensile strength, allowing the detachment of body parts under the influence of external factors. Nevertheless there are some structures that can be detached at sites without adaptations for autotomy due to the irreversible destiffening of MCT [21]. It is possible that MCT gives the adequate stimulation (destiffening enough) and time allowing the body part to be detached passively, examples are the crown by stalked crinoids, dropping the spines by asteroids, ophiuroids and echinoids and shedding of the arms by asteroids at levels distal of the autotomy plane. Also they are involved in fission in asteroids, ophiuroids and holothurians [21].

The role of JLCs on connective tissue disruption was also confirmed due to ultrastructural changes observed in JLCs during autotomy of the central disc of *Amphipholis kochii* [53]. The presence of sparsely cellular MCT at the autotomy planes results in a wound with an expectable topology and with minimal cell damage after detachment. The presence of MCTs in the breakages zones in echinoderms generates less cell debris and reducing also the need of a strong rearrangement leading to an accelerate wound closure and healing, minimizing the delay before the beginning of regeneration [21,35,108]. This is extremely interesting since the mechanism of fracture in other phyla, is thought to involve the application of muscular force [21,34,35].

Several evidences suggest that MCTs are probably one of the crucial elements of the outstanding regenerative capacities found in echinoderms, since they appear to participate in the regenerative process, providing a "plastic" and reversible microenvironment with growth-promoting characteristics favouring tissue repair and regeneration [15, 21,35,108]. In fact tissue microenvironment play a fundamental role in several processes involved in regeneration, such as wound-healing, inflammation, cicatrization, morphogenesis, tissue differentiation, cell fate determination as outlined in several regeneration studies [120,121,122] .

## 8. The requirement of reversible and dynamic structures in tissue regeneration

Regenerative medicine seeks to devise new therapies for patients with severe injuries or chronic diseases in which the body's own responses do not suffice to restore functional tissue [120,121,122]. It attempts to replace damaged tissues through the seeding of stem cells on synthetic/natural or semi-synthetic structures designed to mimic ECM, which will restore cell and tissue function [34,120,121,122]. Regeneration of tissue or even completele organs is not a simple phenomenon. It is a well-coordinated process that implicates several steps such as tissue remodelling, cell proliferation and differentiation to get a fully functional situation, where biophysical basis are considered as important signals to attract stem cells to the site of injury [120,121,122].

The complex microenvironment found in living tissues is a mixture of proteins such as collagen, non-collagen glycoproteins, growth factors, PGs, GAGs and other macromolecules that assemble forming a network that supports cells *in vivo* [8,9,12,123-128]. It compromises also essential biochemical and biomechanical cues inhomogeneously distributed that are required for tissue morphogenesis, differentiation and homeostasis. ECM composition, architecture and multi-functionality changes from tissue to tissue and is generated during biological development throughout a dynamic and reversible biochemical and biophysical communication between the cellular and protein microenvironment [9,125,126].

Far from being completely static, ECM is constantly being remodelled where cells build and reshape ECM from the compositional but also structural point of view by degrading and reassembling mechanisms. The mechanism of ECM assembly, often coincident with morphogenesis, is dependant on ECM molecules and cells and it is regulated by the 3D architecture and the cellular tension that is transmitted through integrins. On the contrary, proteolysis cascades control ECM degradation. All this processes occur during development, differentiation but also wound repair [12,125,126].

Both mature and tissues in development are subjected to a wide range of mechanical forces that are common in development processes and which have some impact on the physiology of their own cells. The dynamicity of the ECM result from processes that subject cells to force, require cells to generate force or both which is for example the cyclic stretch of pulsatile blood flow, muscle stretching, movements of joins. It has been evidenced that cells exhibit diverse behaviors depending on the elasticity of the substrate they are anchored to, or on the elasticity of the extracellular microenvironment. Examples of this arise from cancer cells that grow on soft agar rather than on a solid substrate, or the differentiation of different cell phenotypes modulated by substrate stiffness. It is also known that the biomechanical environment has been considered as a potential regulator of the stem cell niche, since stem cells exist in microenvironments of diverse stiffness both during development and into adulthood and in between different tissues. The elastic moduli of various tissues vary from less than 1 KPa for fat, brain and mammary tissue to aproximately 10 KPa for

skeletal muscle and 10 MPa to bone [9,129-132]. Various mechanical inputs are experienced by these cells through adulthood due to aging and diseases, which involve profound changes in ECM stiffness. Furthermore, cells also reside and experience cyclic strains of approximately 1 Hz, such as muscles, tendons, ligaments and in the heart, where several groups already demonstrate that cyclic stretch enhances tissue regeneration [9,129].

Natural ECM microenvironments of tissues and organs are undoubtedly dynamic with specific mechanical, architectural and biochemical characteristics that regulates cell fate [128-130]. These microenvironmental cues associated with molecular mechanisms (e.g. signalling pathways, structure, gene expression, protein localization) maintain cell homeostasis, induce differentiation, support self-renewal, or regulate assembly [9,129-132]. The rate of tissue regeneration is dependant on the tissue as well as on its microenvironment. An utmost important issue in the biomedical field is the development of biomaterials that mimic ECM microenvironment and that allow a dynamic and reversible dialogue between the microenvironment and the cells, but at the same time can degrade at a similar rate at which the new tissue is being formed.

## 9. Marine/echinoderm models and their molecules against mammalian diseases

Invertebrate animals have been used as animal models since the late 1800s, where their use led new findings (tested by billions of years of trials, successes and failures) in biology but aso in the medicine field [115,133-136]. Species range from terrestrial (nematode and insects) to fresh water and marine life (planarian, molluscs) however the most often used are the fruit fly (*Drosophila melanogaster*) and nematode (*Caenorhabditis elegans*) [136]. Superior biological diversity is found in the ocean, where from the 33 modern phyla, 28 occur in marine habitats. Some of the most interesting of them are the deuterostomes that includes vertebrates, echinoderms and the tunicates, that shares a common origin with mammals [2].

Marine animals can be very useful for biotechnological applications. New natural substances were already discovered with applications in agriculture (pesticides), engineering (antifouling) fields as well as biomedical research (adhesives, anti-tumour substances) [12-17]. Marine animals and plants are reservoirs of bioactive compounds where 14000 pharmacological substances have been already identified. Alkaloids bis and tris (indole) are the compounds most isolated with potent antimicrobial, anti-inflamatory, antiparicidal, anticancer, and antiproliferative activity [136]. In the past two decades pharmaceutics industry has been developing new products obtained from marine organisms and some of them are already in the market and other approved for commercialization [137-140].

Currently, there are marine natural products in various phases of preclinical and clinical development, in cancer therapeutics [6,136-138,140,141]. A component from sponge (C-nucleosides) has been used as a treatment for leukaemia and lymphoma and sulphated fucans and galactans has demonstrated antimetastatic properties [135,140]. Some natural molecules, echinoderm derived with anti-coagulant activity on human blood cells and with cytotoxicity against some tumour cell lines have been already identified and purified [29,136,141]. For example polysaccharides isolated from sea cucumbers have been reported to have several important actions such as anticoagulant (fucosylated chondroitin sulphate) and antithrombotic activity (heparin). Antifungal, cytotoxic and antiviral properties were also detected in holothurian saponins and carotenoids respectively. Another interesting molecule found was Frondoside A, a molecule derived also from sea cucumber that play a fundamental role modulating angiogenesis and inhibiting breast cancer metastasis [136,138,140]. Pro-cancer genes were also identified in visceral tissues of holothurians that regenerate, suggesting that they evolved a strong antitumor mechanism, opening perspectives for the development of new cancer therapeutic tools [136]. In asteroids, also molecules with antitumoral activity was detected, the gangliosides and purines (roscovitine) a kinase inhibitor, that blocks the proliferation of various tumor cells including neuronal cells [136,138,140].

However marine animals can be considered as a model for more fundamental studies regarding some molecular bases of cellular mechanisms and diseases.

Understanding the principles of osmoregulation in marine animals for example leads to an increase in understanding how human kidney maintains the blood with 300mOsm and also how the kidney cells stand the osmotic stress generated by the kidney, where cells are exposed to concentrations as high as 3000 mOsm [136,141]. Also, the understanding of the function of the human immune system, how the body fights against infection and disease, was obtained from studies with marine deuterostems such as echinoderms (with its innate immune system), tunicates (model for tissue transplantation – tissue recognition or rejection), but also sharks (model for studying both innate and acquired immunity) [140]. Sea urchin embryos for example are good models for development, fertilization and embryogenesis studies due to its relative simple organization and because of its optical transparency that allows visualization in vivo possible [141].

Investigations considering non-mammalian models have been widely used since most results are in many cases applicable to humans, offering new insights in the evolution of vertebrates and also mammals [29]. Although adult echinoderms are considered as primitive animals, their embryological development present strong similarities with advanced animals. Recently it was found that echinoderm (sea star *Marthasterias glacialis*) nervous transmission relies on chemical synapsis similar to the synaptic activity of mammals [142]. It is feasible that echinoderms still share many developmental processes and genetic processes with chordates [6,10,13,73,143].

Lately, the discovery of the genome of the sea urchin *Strongylocentrotus Purpuratus* provide the opportunity to answer questions related with developmental biology, cell cycle regulation but also pathways involved in some diseases. The sea urchin genome reveals 23300 genes with representatives of all vertebrate gene families, where orthologs of some human disease associated genes were also found. Many of these genes cover a wide range of systems such as the nervous, endocrine, blood, muscle and skeleton [7]. The potential use of the regenerative capacity of echinoderms as a model for tissue regeneration applications remains mostly unexploited. Their muscle regenerative capacities were recently studied with the viewpoint of demonstrating to the biomedical community that they are a suitable

model systems for pharmacological studies in muscle development but also in regeneration [6].

The study of MCTs might be very important not just to follow a biomimetic approach to develop a dynamic biomaterial allowing tissue regeneration, but also to give new insights in the study of certain pathologies associated with alterations of the viscoelastic properties of the connective tissues [61,144,145].

## 10. References


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# Chapter III

## New insights into mutable collagenous tissue: correlations between the microstructure and mechanical state of a sea-urchin ligament*

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## Abstract

The mutable collagenous tissue (MCT) of echinoderms has the ability to undergo rapid and reversible changes in passive mechanical properties that are initiated and modulated by the nervous system. Since the mechanism of MCT mutability is poorly understood, the aim of this work was to provide a detailed morphological analysis of a typical mutable collagenous structure in its different mechanical states. The model studied was the compass depressor ligament (CDL) of a sea urchin (*Paracentrotus lividus*), which was characterized in different functional states mimicking MCT mutability. Transmission electron microscopy, histochemistry, cryo-scanning electron microscopy, focused ion beam/scanning electron microscopy, and field emission gun-environmental scanning electron microscopy were used to visualize CDLs at the micro- and nano-scales. This investigation has revealed previously unreported differences in both extracellular and cellular constituents, expanding the current knowledge of the relationship between the organization of the CDL and its mechanical state. Scanning electron microscopies in particular provided a three-dimensional overview of CDL architecture at the micro- and nano-scales, and clarified the micro-organization of the ECM components that are involved in mutability. Further evidence that the juxtaligamental cells are the effectors of these changes in mechanical properties was provided by a correlation between their cytology and the tensile state of the CDLs.


## 1. Introduction

The 'mutable' collagenous tissue (MCT) of echinoderms (starfish, sea-urchins and their relations) has the capacity to undergo reversible changes in mechanical properties (viscosity, tensile strength, and stiffness) within timescales of around 1s that are under the control of the nervous system [1,2]. MCT is present in all living echinoderm classes, in the form of dermal connective tissue, interossicular ligaments and tendons [2]. In addition to fulfilling the mechanical functions associated with 'conventional' collagenous structures (i.e. energy storage, transmission and dissipation), MCT provides mechanisms for the detachment of appendages or body parts in response to disease, trauma or predator attack [2] and for the energy-sparing

maintenance of posture [3]. Most mutable collagenous structures consist largely of parallel aggregations of collagen fibrils to which proteoglycans are covalently and non-covalently attached, as in mammalian connective tissue [2-11]. An elastomeric network of microfibrils surrounds and separates collagen fibers (bundles of fibrils), maintaining their organization and providing a long-range restoring force [2,12,13]. One constant morphological feature appearing in all MCTs is the presence of juxtaligamental cells (JLCs), which contain large electron-dense granules and come into close contact with motor axons [2,14,15].

The mechanical adaptability of MCT depends on the modulation of interfibrillar cohesion, and there is good evidence that this is mediated by effector molecules secreted from the JLCs [2]. Candidate effector molecules that influence the mutability of sea-cucumber dermis have been isolated and partly characterized [16-19].

The aim of our investigation was to advance knowledge of the basic biology of MCT by (i) providing a detailed morphological analysis of a typical mutable collagenous structure - the compass depressor ligament (CDL) of a sea-urchin, and (ii) identifying changes in morphological aspects of its cellular and extracellular components that are correlated with different mechanical states (‘standard’, ‘stiff’ and ‘compliant’). Using histochemistry, transmission electron microscopy (TEM), cryo-scanning electron microscopy (CSEM), focused ion beam/scanning electron microscopy (FIB/SEM), and field emission gun-environmental scanning electron microscopy (FEG/ESEM), we detected differences in both extracellular and cellular constituents of CDLs in different mechanical states, which provide an insight into the micro-organizational basis and control of MCT mutability. Our results help to characterize the functional role of this specialized ECM, which has striking morphological similarities to mammalian collagenous ECM.

# 2. Materials and methods

## 2.1 Experimental animals and solutions

Sea-urchins (*Paracentrotus lividus*) for the TEM and FIB/SEM analyses were collected by scuba divers along the Ligurian coast of Italy and kept in tanks of aerated seawater at 16ºC in the University of Milan. Animals for the FEG/ESEM, CSEM and histochemical analyses were collected in Aguda on the northern Portuguese coast and kept in tanks of seawater at 16ºC in Estação Litoral da Aguda.

CDLs are components of the masticatory apparatus ('Aristotle's lantern') of the sea-urchin (Fig.1A). Each lantern contains ten CDLs (Fig. 1B) whose functions include stabilizing the position of the lantern [20, 21, 22].

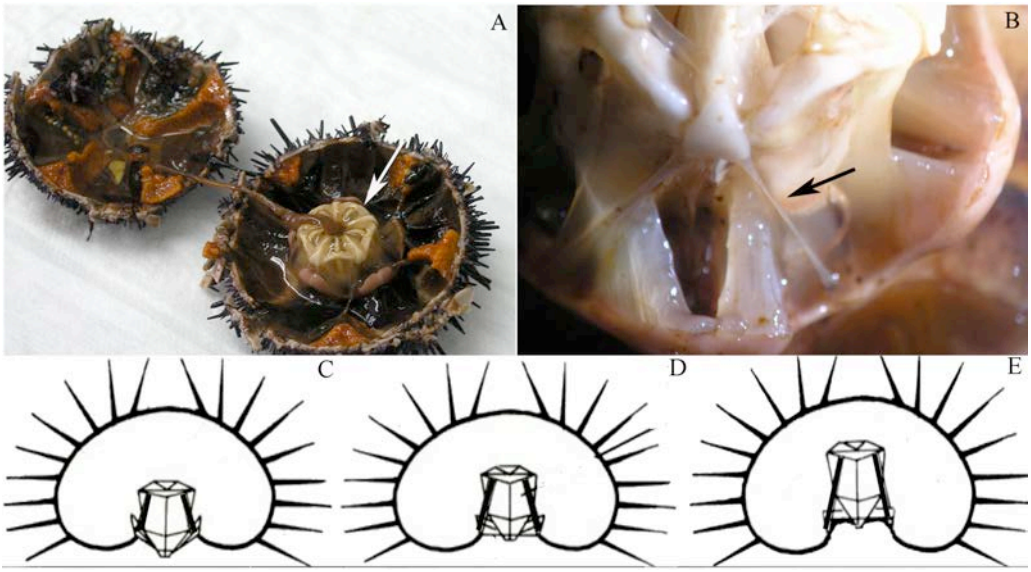


**Figure 1: Anatomical relations and behavior of CDLs.** (A, B) Specimen of P. lividus split into two halves: (A) The Aristotle's lantern (arrow) is observable in the oral half. (B) Enlargement of the Aristotle's lantern showing the anatomical location of the CDLs (arrow). (C-E) Diagrams of sea-urchins with lanterns in different positions; CDLs shown in black. (C) Protracted position; CDLs compliant; (D) Resting position; CDLs in standard state; (E) Retracted position; CDLs stiff.

In order to compare CDL morphology in different functional states, animals were subjected to three different treatments. To obtain the 'compliant' condition, the lower half of an animal, which includes the lantern, peristomial membrane and CDLs (Fig. 1A), was immersed in an anesthetic solution of 0.1% propylene phenoxetol

(Sigma Aldrich 484423) in seawater for 45 min [20, 23]; this treatment results in the protraction (lowering) of the lantern and slackening of the CDLs (Fig. 1C). To obtain the 'stiff' condition [21, 22], half animals were immersed in 1mM acetylcholine chloride (Sigma Aldrich 6625) in seawater for 15 min, which causes retraction (raising) of the lantern and stretching of the CDLs (Fig. 1E). Controls, which were in the ‘standard’ condition’ (Fig. 1D), were kept in seawater alone [21].

We chose to induce mechanical changes in intact CDLs left in situ in the lantern, rather than use excised CDLs held artificially at a constant length, in order to mirror as closely as possible in vivo conditions, where CDLs tend to undergo simultaneous changes in stiffness and length.

### 2.2 Ethical treatment of animals

This study was carried out where no specific permits were required for the described field studies since sea-urchins (*Paracentrotus Lividus*) are invertebrates. This work was performed with a species that is not endangered or protected. The location of the field studies is also not privately owned or protected in any way.

### 2.3 TEM and LM

After incubation in the respective solutions, half animals were fixed at 4 ºC for 2 h with 2% glutaraldehyde in 0.1 M cacodylate buffer. After an overnight wash in the same buffer, the specimens were post-fixed for 2 h with 1% osmic acid in 0.1 M cacodylate buffer. After fixation specimens were washed with distilled water. CDLs were removed carefully from the lantern, pre-stained with 2 % uranyl acetate in 25% ethanol for 2 h, dehydrated in a graded ethanol series and embedded in an Epon-Araldite resin mixture. Semi-thin (0.90-0.99 μm) and thin (0.1-0.075 μm) sections were cut with an LKB V Ultrotome using a diamond knife. Thin sections were stained with aqueous uranyl acetate and lead citrate, and observed in a JEOL 100SX TEM. Semi-thin sections were stained with basic fuchsin and crystal violet and observed in a Jenaval light microscope.

### 2.4 FIB/SEM

FIB/SEM samples were treated as above as far as the dehydration step, after which the ethanol was progressively substituted with hexamethyldisilazane (HMDS) and the samples allowed to dry at room temperature. They were then mounted on a stub, carbon coated and observed in a Dual Beam system for FIB/SEM operation (FEI Strata DB 235 M). Milling was performed in the form of a rectangle block with different dimensions (20 x 40µm or 20 x 15 µm), with ion currents ranging from 10 pA to 30 nA, and beam energy of 30-40 keV. Lower beam currents were used for the cleaning mill. SEM imaging was obtained by means of the electron column available. The system operated with column pressures in the $10^{-5}$ Pa range and the specimen chamber pressure between $10^{-4}$ and $10^{-3}$ Pa.

### 2.5 FEG/ESEM

Two sample procedures were followed. In the first the lantern complex was processed as for the FIB/SEM samples. In the second, samples were processed and embedded as for TEM and the internal surface of the resin-embedded CDLs was exposed by cutting a few longitudinal semithin sections. The resin blocks containing CDLs were placed in contact with a solution of 1% NaOH in absolute ethanol at room temperature for 30 minutes to dissolve the resin and expose the internal structure of the tissue for observation with FEG/ESEM. After resin dissolution, CDLs were washed in 100% ethanol and dried in HMDS. All CDLs were then observed in a FEI Quanta 400FEG ESEM/EDAX Genesis X4M microscope at low vacuum (70 Pa) without any coating.

### 2.6 CSEM

Unfixed and hydrated CDLs were examined by CSEM. Immediately after dissection, CDLs were immersed in liquid nitrogen and carefully mounted onto a cryo sample holder. By means of a vacuum cryo transfer system, CDLs were placed on the cryo-stage where they were kept under high vacuum at a minimum temperature of -210ºC. After appropriate positioning of the stage, a blade was used to create a transverse freeze fracture. Water was removed by gentle sublimation at -90ºC for 5

min. The CDLs were sputter-coated with gold palladium for 80 s and the fracture face was inspected in a JEOL JSM 6301F/ Oxford INCA Energy 350 / Gatan Alto 2500.

## 2.7 Proteoglycan histochemistry

### 2.7.1 Alcian blue

CDLs were fixed with 4% paraformaldehyde in PBS, dehydrated in an ethanol series, cleared in xylene and embedded in paraffin wax. Sections 7 μm thick were cut in a Reichert Jung microtome, deparaffinized with xylene and hydrated to distilled water. Sections were stained with alcian blue 8GX (Sigma-Aldrich 05500; staining solution: 1% alcian blue in 3% acetic acid, pH 2.5) for 30 min, washed in running tap water for 2 min, rinsed in distilled water, dehydrated in an ethanol series, and washed in xylene. The sections were observed in a Jenaval light microscope.

### 2.7.2 Cuprolinic blue

Half animals in the standard state were fixed overnight at room temperature in 0.1% cuprolinic blue containing 2.5% glutaraldehyde in sodium acetate 0.025 M and MgCl2 0.3M (pH 5.6). CDLs were washed twice in sodium acetate 0.025 M and MgCl2 0.3M (pH 5.6) followed by a wash in distilled water. After fixation, CDLs were removed carefully from the lantern under distilled water, washed in ethanol 25%, and in 0.5% sodium tungstate in ethanol 50%, dehydrated in a graded ethanol series and embedded in an Epon-Araldite resin. Sections (70-100 nm) were cut with an LKB V Ultrotome using a diamond knife, stained with aqueous 2% uranyl acetate in distilled water, and observed in a JEOL 100SX TEM.

## 2.8 Quantitative evaluation and statistical analysis

TEM micrographs at a magnification of x30,000 were acquired of one CDL from each of four different animals. Twenty-five measurements of the distance between adjacent collagen fibrils were obtained in each micrograph, using the program ImageJ 1.41o. Since the data were not normally distributed (D´Agostino and Pearson test), the Mann–Whitney U test was used to compare the distance between collagen fibrils in standard, stiff and compliant CDLs. Results were considered

statistically significant when $p<0.05$. ImageJ 1.41o was also used to measure the diameter and number of the 'dark' (i.e. completely electron-opaque) and 'light' (i.e. partly electron-lucent) granules inside JLCs in TEM images (x16, 000). These cytological data were subjected to statistical analysis, and results were considered statistically significant when $p<0.05$. Means are given ± standard deviation. Statistical differences between CDLs in different functional states were determined using Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's post-hoc test. All statistics were performed using GraphPad Prism 5 Demo software (version 5.02).

# 3. Results

## 3.1 Background: microstructural organization of the CDL

Each CDL was a strap-shaped band of tissue 9-10 mm long and 0.2-0.4 mm wide, and consisted mainly of a parallel aggregation of cross-striated collagen fibrils. The fibrils were enclosed within a coelomic epithelium, which became a myoepithelium on one side of the CDL (Fig. 2A). The parallel organization of the collagen fibrils with globular cells was shown well by FEG/ESEM and FIB/SEM (Fig. 2 B, C, D).

Fig. 3A,B, C shows that the fibril diameter was variable, and measurements of TEM images revealed that fibril diameters ranged from 26.52 nm to 179.42 nm (mode 45.5 ± 19.0 nm; N=1033). The nanometer resolution of TEM revealed that the D-spacing of the cylindrical collagen fibrils (Fig. 3 C, F) varied from 39.7 to 77.9 nm (mean 59.2 ± 6.2 nm; N=344).

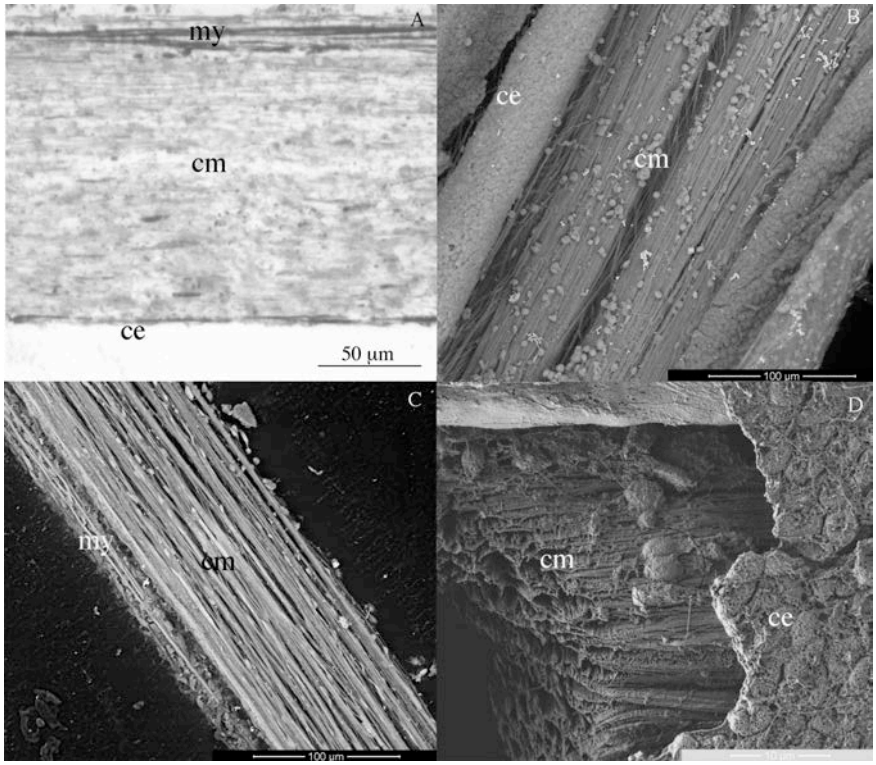


**Figure 2: General view of CDL internal structure:** (A) Semi-thin longitudinal section of CDL and (B, C) FEG/ESEM micrograph of resin-embedded CDL revealing the dense collagen array surrounded by a coelomic epithelium. Fig.2A shows clearly the coelomic myoepithelium. (D) FIB/SEM micrograph of milled CDL showing small globular cells between the collagen bundles covered by a ciliated coelomic epithelium. ce, coelomic epithelium; my, myoepithelium; cm, collagen matrix.

The collagen fibrils were aggregated into bundles (i.e. 'fibers') with a wide range of diameters (0.4-1.3 µm; mean 0.87 ± 0.46 nm; N=13). Between the collagen fibers there were beaded filaments of mean diameter 25.6 ± 10.3 nm (N=24), which formed a loose meshwork or compact bundles with mean diameter 245 ± 16.5 nm (N=14) (Fig. 3B, D-F). In addition to these elongated filaments, short filamentous structures of variable length (10-30 nm) were observed by FEG/ESEM to directly connect adjacent collagen fibrils, thus forming interfibrillar bridges (Fig. 3G, H).

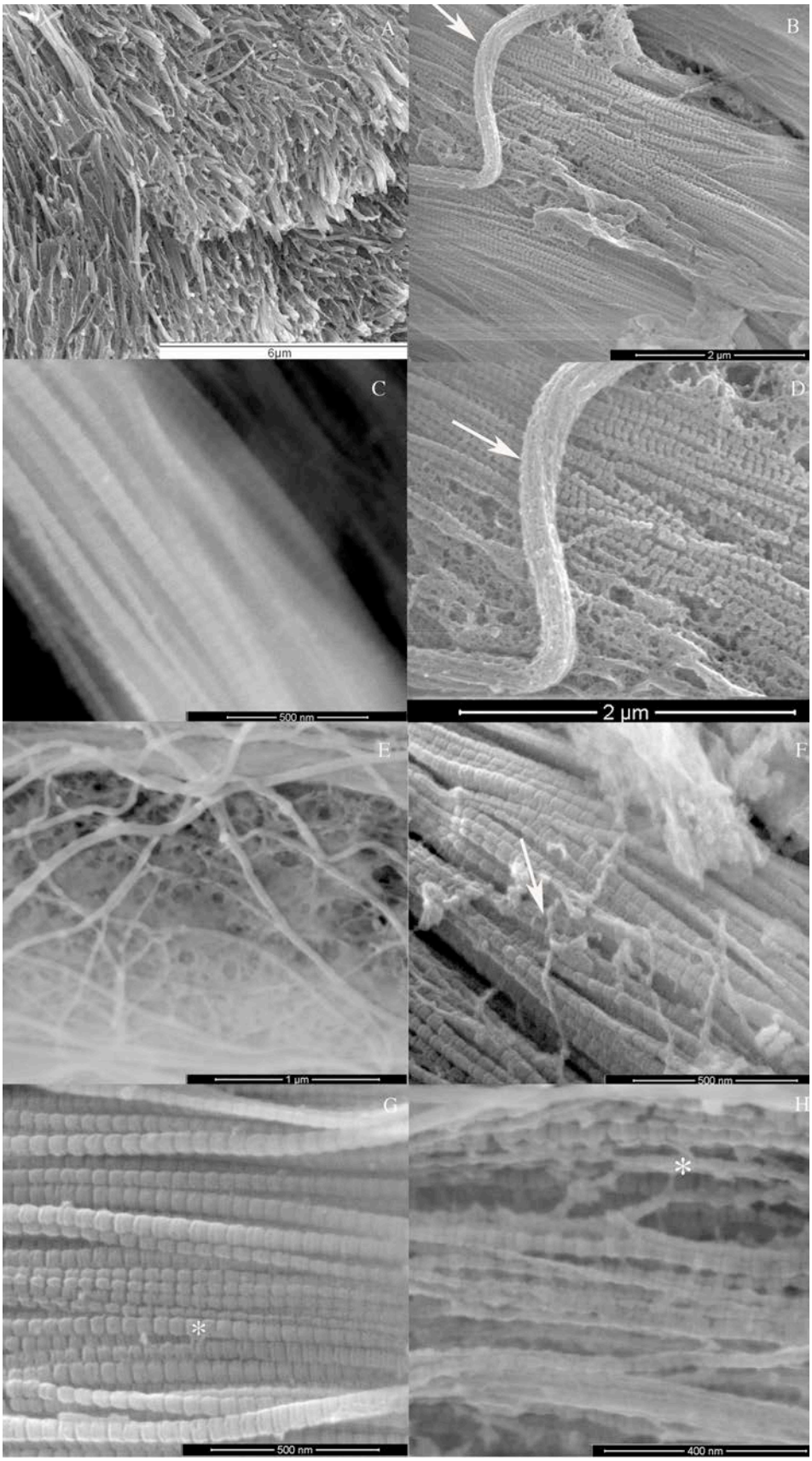


**Figure 3: CDL extracellular matrix:** (A) Cryo-SEM and (B-H) FEG/ESEM micrographs showing a (A) general view of CDL compliant matrix. (B) Detail of collagen fibrils and fibrillin (arrow). (C) Parallel array of collagen fibrils showing a clear D-banding pattern. (D) Enlargement of Fig. 3B

showing a fibrillin bundle (arrow). (E) Enlargement of Fig. 3B showing a fibrillin meshwork. (F) Loose fibrillin meshwork (arrow) on surface of collagen fibrils. (G, H) Interfibrillar bridges linking adjacent fibrils (*).

The fibres of the CDL were stained moderately and uniformly by alcian blue at pH 2.5, indicating the presence of GAGs (Fig. 4A).

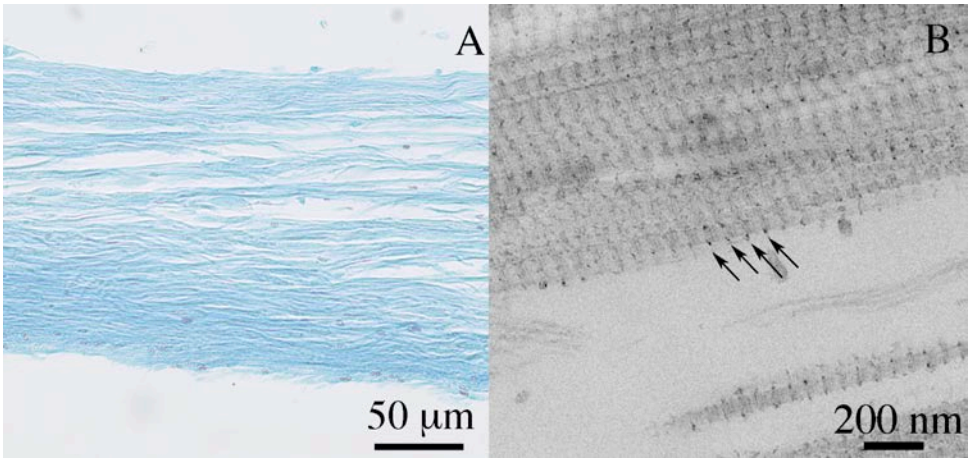


**Figure 4: Proteoglycans presence and distribution:** Longitudinal section of CDL stained with (A) alcian blue at pH 2.5 and cuprolinic blue (B) (arrows, cuprolinic blue stained precipitates).

Staining of CDLs with cuprolinic blue produced electron-dense globular or ellipsoid precipitates on the surface of the collagen fibrils. On each side of a particular collagen fibril, one such precipitate was located within each D-period and at the same location within each D-period, so that the precipitates were spaced very regularly (Fig. 4B).

The ligament also contained many granule-containing cell bodies and processes (Fig. 5), which will be described below.

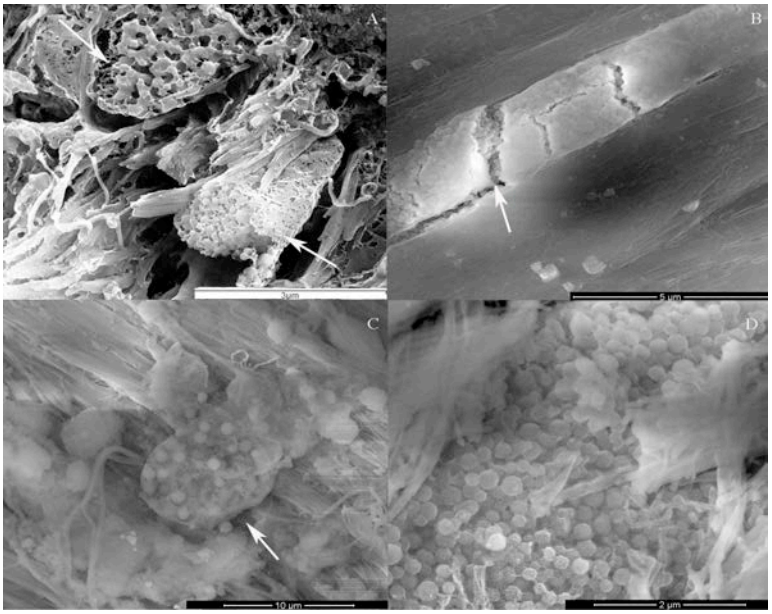


**Figure 5: Juxtaligamental cells:** (A) CSEM micrograph showing a transverse section of a JLC (arrow) within the collagen array. (B-D) FEG/ESEM micrographs of resin- embedded samples. The dissolution of resin allows the direct visualization of a fractured JLC process (arrow), showing (B) the internal granules, (C) granules inside the cell membrane (arrow), and (D) the variation in JLC granule size.

## 3.2 Microstructural organization of CDLs in the different mechanical states

### 3.2.1 Gross changes

Anesthetization reduces the stiffness of all MCT in the lantern, including that of the peristomial membrane to which the lantern is attached. As a result, the entire masticatory apparatus is protracted and the CDLs shorten and thicken (Fig. 1 C). In response to acetylcholine, the CDLs and peristomial membrane become stiff, the lantern is held in a retracted position, and the CDLs are longer and thinner than in the standard condition (Fig. 1 E).

### 3.2.2 Microstructural changes

Histological sections and FEG/ESEM micrographs showed that the organization of the collagen array and cells changes when CDLs shift from the standard to the stiff or compliant states (Fig. 6).

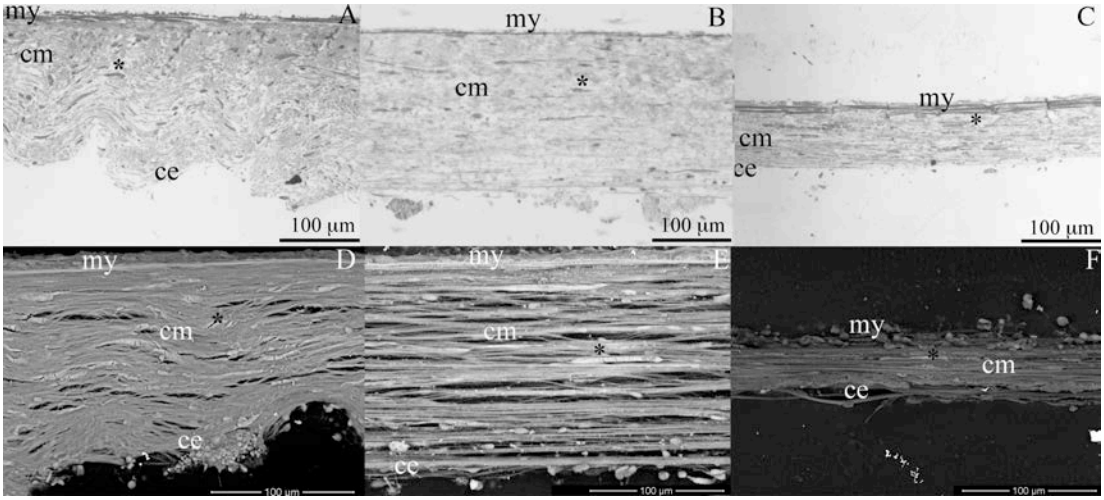


**Figure 6: Histology of CDLs in different functional states:** (A-C) Semi-thin longitudinal sections and (D-F) FEG/ESEM micrographs showing CDLs in the compliant state (A, D), standard state (B, E) and stiff state (C, F). Asterisks, cells; ce, coelomic epithelium; cm, collagen fibers; my, myoepithelium.

### 3.2.3 Extracellular components

Collagen fibrils were more tightly packed in stiff CDLs than in compliant or standard CDLs (Fig. 7).

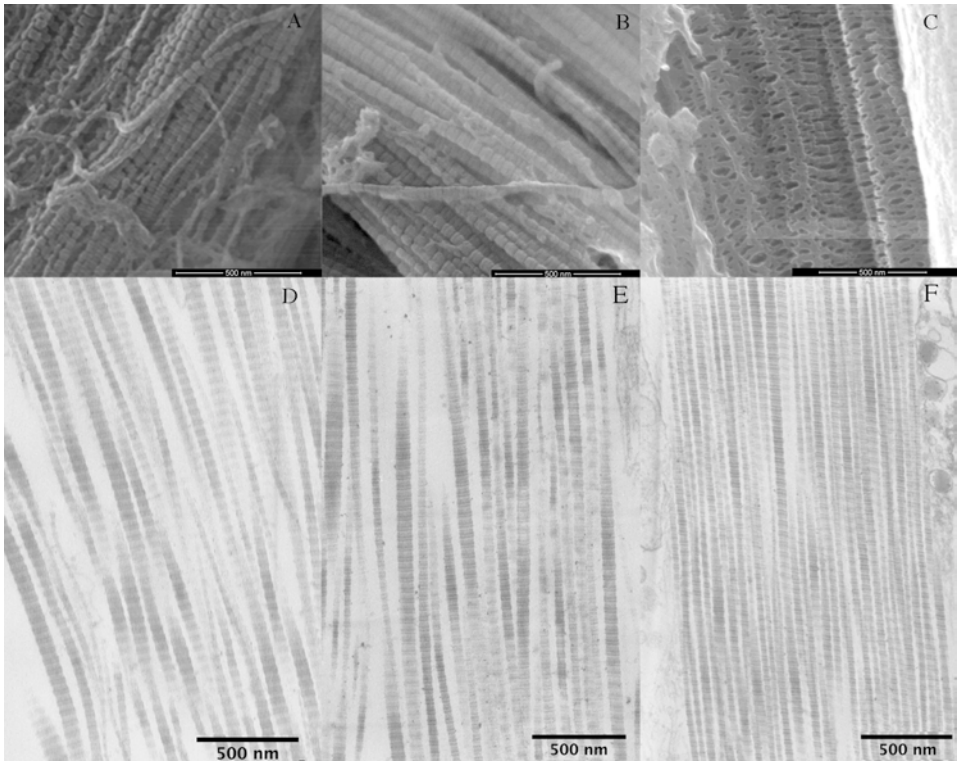


**Figure 7: Arrangement of collagen fibrils:** (A-C) FEG/ESEM and (D-F) TEM micrographs. (A, D) Compliant state. (B, E) Standard state. (C, F) Stiff state.

The mean interfibrillar distance was significantly lower in stiff CDLs than in compliant and standard CDLs (Mann Whitney U: 5446, P<0.0001 and U: 7617, P<0.0001 respectively), although there was no significant difference between compliant and standard CDLs (Fig. 8).

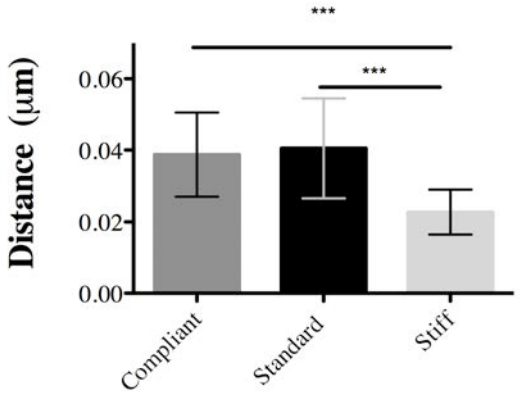


**Figure 8: Distance between collagen fibrils:** Mean interfibrillar distance of CDLs in the three mechanical states. ***P<0.0001. Data are expressed as means ± SD.

The only effect of mechanical state on the distribution of fibrillin microfibrils seemed to be a consequence of collagen fibril packing. In the stiff condition, the reduction in interfibrillar space resulted in the microfibrils forming more densely packed sheets (Fig. 9B). In the compliant and standard conditions they are more dispersed between the collagen fibrils (Fig. 9A).

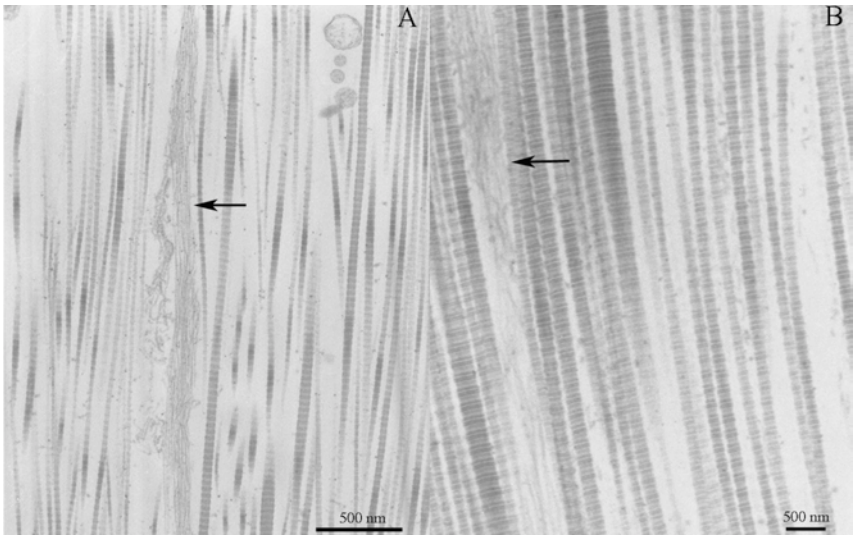


**Figure 9: Microfibrils:** TEM micrographs showing the distribution of microfibrils in CDLs in standard (A) and stiff (B) states.

### 3.2.4 Cellular components: JLCs and intracellular granules

The main cellular elements observed in TEM and FEG/ESEM contain many membrane-bounded, electron-dense granules, and thus resemble the juxtaligamental cells (JLCs) that are present in all previously examined mutable collagenous structures in echinoderms. The profile of the granules is usually circular. The presence of less frequent oval profiles suggests that at least some of the granules are ovoid rather than spheroidal in shape (Fig. 10A-C). Fig. 10D shows that in all three mechanical states, the mean diameter of 'dark' (completely electron-opaque) granules is lower than that of 'light' (partly electron-lucent) granules, although the difference is statistically significant only for stiff CDLs.

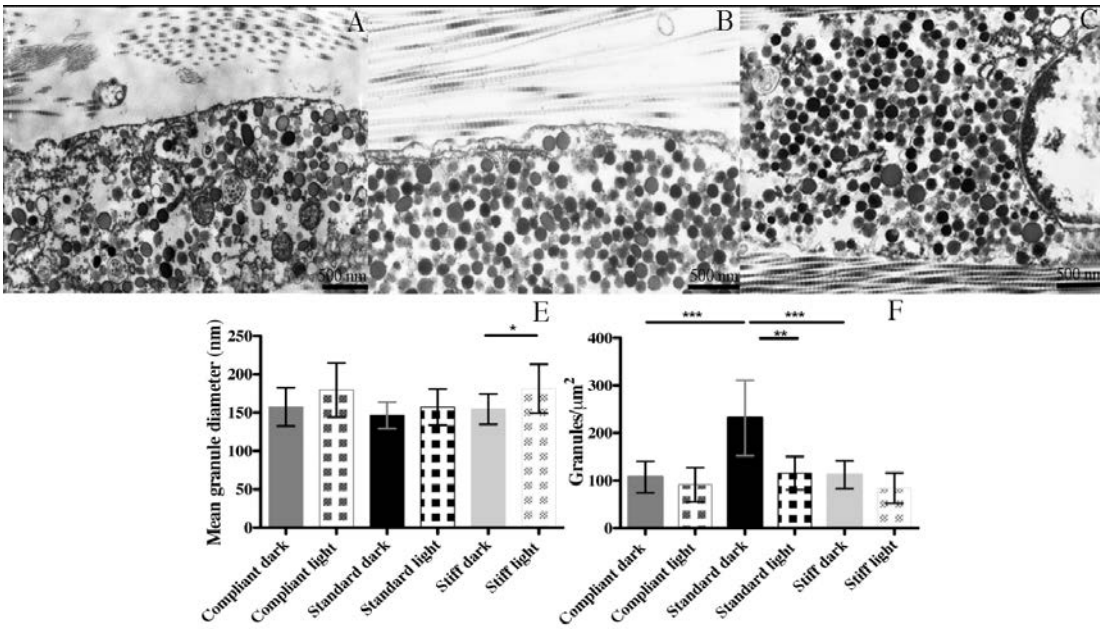


**Figure 10: JLCs in the different mechanical states:** (A-C) TEM micrographs of compliant (A), standard (B) and stiff (C) CDLs. (D) Size of JLC granules in different mechanical states. *P<0.05. (E) Number of JLC granules in different mechanical states. **P<0.01; ***P<0.001. Data are expressed as means ± SD.

The size of neither the dark granules nor the light granules differs significantly between the different mechanical states. Regarding granule abundance, dark granules are more numerous than light granules in all three mechanical states, although the difference is significant only in the standard condition. Dark granules are considerably less numerous in compliant and stiff CDLs than in standard CDLs, whereas there is

no significant difference between the numbers of light granules in the three mechanical states (Fig. 10E).

# 4. Discussion

## 4.1 Organization of CDL microenvironment

The CDL of *P. lividus* resembles most other echinoderm mutable collagenous structures in consisting mainly of collagen fibrils with a banding periodicity of ca. 60 nm [21]. Available data on the molecular biology of collagens from different MCTs indicate that they are evolutionarily close to those of vertebrate fibrillar collagens and share no features that can be correlated with the mechanical adaptability of the tissues [2,24-27]. In the CDL, the collagen fibrils are organized into parallel fibers that determine the tensile strength of the ligament and set a limit on the degree to which it can stretch during routine movements of the sea-urchin's lantern [20]. It is interesting to note that the general architecture of CDL collagen, such as fibril organization and bundle orientation, is identical to the structure and organization of collagen observed in several human tissues such as tendon, ligament, cornea, skin and blood vessels [28].

Beaded filaments like those forming a meshwork or bundles between the collagen fibers of the CDL are ubiquitous in MCT and are also present in echinoderm ligaments that show no evidence of mutability [2]. Those in the mutable dermis of a sea-cucumber are morphologically, biochemically and immunologically similar to mammalian fibrillin-containing microfibrils [13, 29], and a preliminary investigation has detected fibrillin-1-like immunoreactivity in the CDL of *P. lividus* (Barbaglio, unpublished results). In vertebrates and invertebrates, aggregations of fibrillin-rich microfibrils are thought to provide tissues with the capacity for strain energy storage and elastic recoil [29], and we envisage that shortening and thickening of CDLs, which we observed in intact preparations of the lantern treated with propylene phenoxetol, is due in part to microfibrillar mechanisms.

The CDLs were stained by alcian blue at pH 2.5, which is fairly selective for GAGs [5, 30]. GAGs, and the proteoglycans (PGs) into which they are incorporated as side-chains, are likely to play a significant role in the mechanical adaptability of MCT, since they are directly or indirectly involved in interfibrillar cohesion, the reversible modulation of which underpins the mutability phenomenon [2,31]. PGs were visualized by the polycationic dye cuprolinic blue, demonstrating that polyanions are periodically distributed along collagen fibrils surface, being attached to specific sites in each D-period of the collagen fibril. Scott [32] demonstrated that collagen fibrils in holothurian dermis are surrounded and interconnected by a PG lattice of orthogonal and axial filaments, and interfibrillar PGs have been identified in a sea-urchin spine ligament [7,31,33,34]. The short interfibrillar cross-bridges observed by FEG/ESEM in the CDL may have been PGs, although they were not periodically distributed.

### 4.2 Correlations between microstructure and mechanical state

This investigation has revealed previously unreported differences in both extracellular and cellular constituents of a representative mutable collagenous structure, the sea-urchin CDL, in different mechanical states that mimic the mutability of the tissue in vivo. We found that the mean interfibrillar distance of stiff CDLs was significantly lower than that of standard or compliant CDLs, and that there was no significant difference between those in the standard and compliant state. The denser fibril packing of the stiff CDLs obviously involves a reduction in interfibrillar space and displacement of materials previously occupying that space. This is probably a result of the stretching of the CDLs in acetylcholine-stimulated preparations and, by shortening the distance between adjacent fibrils, may facilitate the stiffening mechanism by making it easier for interfibrillar cohesion to be strengthened, for example by the attachment of crosslinking agents such as tensilin [16]. Such facilitation of the stiffening mechanism may occur in other mutable collagenous structures that consist of parallel fibril arrays and undergo simultaneous stretch and stiffening, such as sea-urchin spine ligaments ("catch apparatus") [35], crinoid arm ligaments [36] and ophiuroid arm ligaments [37].

Like all previously examined mutable collagenous structures, the CDL contains many juxtaligamental cell (JLC) bodies and their processes. These cells, characterized by their abundant intracellular granules, are probably the effector cells that directly bring about changes in the tensile properties of the extracellular matrix, because (1) they terminate within MCT, (2) they are in close contact with the motor nervous system, (3) immunological methods have demonstrated the presence in their granules of molecules that influence MCT tensility (such as tensilin and stiparin), and (4) there is no other candidate cell-type within or near MCT [2,17-19,38]. The electron density of the granules in the JLCs of the CDL is variable, as has been observed in all other MCTs, and for the purposes of this investigation the granules were categorized as being 'dark' or 'light'. We suspect that these represent only different stages in granule maturation, the dark being fully mature and the light various immature stages (because changes in only the dark granules could be correlated with mechanical state: see below). The size of the dark and light granules did not differ significantly between the three mechanical states, and there was no significant difference between the quantity of light granules. However, dark granules were much more numerous in standard than in compliant and stiff CDLs, implying that dark granules are involved in the standard→compliant and standard→stiff events. How can the dark granules have a role in both antagonistic processes? An unusual feature of the JLCs of the CDL is that only one cell-type has been identified [22]. Most other MCTs possess at least two JLC types distinguishable by the size and/or shape of their granules. It is believed that these are also functionally distinct, perhaps representing separate 'stiffener' and 'de-stiffener' cells [2].

To explain these results we therefore hypothesize that:

(1) There are two populations of CDL granules (each comprising mature and immature stages), which are functionally distinct (one is involved in the standard→stiff shift and the other in the standard→compliant shift), but not morphologically distinct. These may be present in separate, but morphologically indistinguishable, cell-types or in a single cell-type.

(2) Only mature (i.e. 'dark') granules are involved in their respective processes, and as a consequence of this involvement they are depleted by an as yet unknown mechanism. They do not, through a decrease in the electron density of their contents, become light granules, because there is no corresponding increase in the number of light granules. After examination of many electron micrographs, we have found no evidence that JLC granules undergo exocytosis and we can only surmise that the transport of their contents to the extracellular environment and the recycling of their membranes are achieved by particularly rapid and efficient processes.

Living systems in nature are frequently multifunctional and dynamic, providing a source of inspiration for the design and synthesis of new classes of materials that have potentially a wide range of medical and non-medical applications [39, 40]. A priority in the biomedical field is the development of biomaterials that mimic ECM microenvironments and that allow a dynamic and two-way dialogue between the microenvironment and the cells, whilst also degrading at a rate similar to that at which the new tissue is being formed. The use of echinoderms as an animal model creates a unique opportunity to find new concepts for the development of a biomaterial that favours tissue regeneration, since their ECM, which closely resembles that of mammals, and particularly MCT, is present at anatomical sites at which there is a strong regenerative capacity [41]. In the present work, the sea-urchin CDL was used as a model MCT that has the advantages of being easily accessible and having a typical MCT organization uncomplicated by the incorporation of skeletal components. Although the molecular mechanism of mutability is understood incompletely, the concept is sufficiently appealing to deserve further consideration by biomaterials scientists.

## 5. Acknowledgements

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# Chapter IV

## Correlations between the biochemistry and mechanical states of a sea-urchin ligament: a mutable collagenous structure*

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## Abstract

The mutable collagenous tissues (MCTs) of echinoderms can be regarded as intelligent biomaterials, due to their ability to reversibly change their mechanical properties in a short physiological time span. This mutability phenomenon is nervously mediated and may involve secreted factors of the specialized juxtaligamental cells (JLCs), which, when released into the extracellular matrix (ECM), change the cohesive forces between collagen fibrils. MCTs exist in nature in several forms including some associated with echinoderm autotomy mechanisms and provide a dynamic environment that may enhance tissue regeneration. Since the molecular mechanism of mutability is still incompletely understood, the aim of this work was to provide a detailed biochemical analysis of a typical mutable collagenous structure and to identify possible correlations between its biochemistry and mechanical state. The MCT model used was the compass depressor ligament (CDL) of a sea-urchin (*Paracentrotus lividus*), which was analyzed in different mechanical states that mimic the mutability phenomenon. Spectroscopic techniques, namely Fourier transform infrared (FT-IR) and confocal Raman, were used to identify the specific molecular components that contribute to the CDL biochemical microenvironment and to investigate the possibility that the remodelling/synthesis of new ECM components occurs during mutability phenomena, by analogy with events during pregnancy in the uterine cervix of mammals (which also consists mainly of mechanically adaptable connective tissue). The results demonstrate that CDL ECM includes collagen with biochemical similarities to mammalian type I collagen, as well as sulphated GAGs. CDL mutability seems to involve a molecular rearrangement of the ECM, without synthesis of new ECM components. Although there were no significant biochemical compositional differences between CDLs in the different mechanical states studied, adjustments of tissue hydration seemed to occur.


**Keywords:** Mutable collagenous tissue, echinoderm, sea-urchin, ECM rearrangement, water exudation.

## 1. Introduction

The mutable collagenous tissues (MCTs) are peculiar morpho-functional adaptations of echinoderms [1-3]. They are considered to be “smart”, or “intelligent”, connective tissues due to their ability to reversibly change their biomechanical properties in a short time-scale, under nervous control [1-4].

Recently published data from our group confirm that the compass depressor ligament (CDL) of the sea-urchin masticatory apparatus (Aristotle’s lantern) has a typical MCT structure consisting of a dense parallel array of collagen fibrils, which is permeated by glycosaminoglycans (GAGs), proteoglycans (PGs) and a network of fibrillin-containing microfibrils, as well as specialized cells called juxtaligamental cells (JLCs) [4,5]. The CDL exhibits in nature different mechanical states, which we called “standard”, “compliant” and “stiff” and correlations between both extracellular and cellular constituents and CDL mechanical state have been identified. Regarding the extracellular matrix, we also showed that stretching of the CDL reduced the interfibrillar distance, possibly facilitating the stiffening mechanism [5].

The fibrils that dominate the CDL ECM consist of collagen, the most abundant structural protein in the connective tissues of both invertebrates and vertebrates [6-8]. With regard to phylogenetic relationships, available data on the morphology and molecular biology of collagens from different MCTs indicate that they are evolutionarily close to those of vertebrate fibrillar collagens (type I) in terms of their structure, chain composition and gene organization [2-4,9-15].

The literature also suggests that the reversible modulation which underpins the mutability phenomenon does not result from effects on the mechanical properties of collagen fibrils but from the physiological control of interfibrillar cohesion, whereby PGs and GAGs are directly or indirectly involved, since they serve as binding sites for molecules responsible for interfibrillar cohesion [2,3]. As reported for mammalian connective tissues, some interfibrillar PG bridges were visualized in CDLs, which were also preferentially located at specific sites in each D-period of the collagen

fibrils [4,15-19]. GAGs were also identified through selective staining with alcian blue [5,16]. Although PGs identified biochemically in the spine ligament of another sea-urchin were shown to be sulphated, until now no attempts have been made to identify their presence in the CDL or their possible contribution to the mutability phenomenon.

In the present study, spectroscopy techniques such as FT-IR and confocal Raman were used in order to reveal the biochemical fingerprint of the CDL. As there is a strong similarity between mammalian and MCT collagen, ultrapure collagen type I of bovine origin and chondroitin sulphate (a GAG) from shark cartilage (GAG), were used as references. Also the possible correlation between CDL biochemistry and different mechanical states was investigated by spectroscopic techniques. The possibility that remodelling (degradation and synthesis) of the ECM occurs during mutability phenomena was also evaluated since this is one of the mechanisms responsible for the mechanical adaptability of mammalian connective tissues, like the uterine cervix during pregnancy [20-23].

Our results demonstrated that CDL contains collagen with some biochemical similarities to mammalian type I, and GAGs belonging to the chondroitin sulphate family. CDL mutability seems not to involve the synthesis of new ECM components. However, adjustments of tissue hydration seem to occur. This study contributes to the biochemical characterization of this specialized dynamic ECM, which has striking biochemical and structural similarities to mammalian connective tissues.

## 2. Materials and Methods

### 2.1 Animals and tissues collection

In this study, the model used was the compass depressor ligaments (CDL) obtained from specimens of the sea-urchin *Paracentrotus lividus* that were collected on the north of the Portuguese coast, and maintained in an aquarium as described elsewhere [5]. The top half of the test (“shell”) of an animal was removed and discarded in order to expose the masticatory apparatus, which contains ten CDLs. The half animal was then immersed in 0.1% propylene phenoxetol (Sigma 484423) in

seawater for 45 min, or in 1mM of acetylcholine (Sigma A 6625) in seawater for 15 min to obtain CDLs in the compliant and stiff state respectively. Standard CDLs were obtained from half animals immersed in seawater. During all analyses, tissues were always kept in the specific solutions in order to maintain their respective mechanical states. All experiments were performed using five different animals.

## 2.2 Spectroscopic characterization of CDL microenvironment

### 2.2.1 Pure components

Ultra-pure bovine collagen type I (Sigma - C4243) and chondroitin sulphate sodium salt from shark cartilage (Sigma - C4384) were used as reference for the identification of their corresponding components of the extracellular matrix of CDL. Collagen films were prepared by mixing chilled (99%) bovine collagen solution with 10xPBS. After pH adjustment to 7.2-7.6, 5mL of collagen suspension were poured into a petri dish (∅ 35 mm) and kept at 37ºC for 1 hour in order to form a reticulated 3D gel. The gel was further dried in a vacuum system at room temperature to form a film that was characterized by vibrational spectroscopies. Chondroitin sulphate powder was analyzed as received.

### 2.2.2 Fourier transform infrared spectroscopy (FT-IR)

Collagen films, chondroitin sulphate and hydrated CDLs in the different mechanical states were analyzed by FT-IR using a Perkin Elmer 2000 spectrometer. The specimens were analyzed with attenuated total reflectance (ATR-FTIR) using the SplitPeaTM accessory (Harrick Scientific), provided with a silicon internal reflection element and configured for external reflectance mode, where the spectra were acquired from a 200 μm diameter sampling area. At least five different areas from each sample were analyzed. All samples were run at a spectral resolution of $4cm^{-1}$ and two hundred scans were accumulated in order to obtain a high signal-to-noise level. A nitrogen purge of the sample compartment was performed to minimize artifacts that could arise from residual air bands ($CO_2$ and $H_2O$ vapour). All spectra were automatically smoothed, and normalized using Spectrum software, version 5.3. The heights of the peaks of amide I (1640 $cm^{-1}$, C=O stretch), amide II (1550$cm^{-1}$, C-N

stretch and N-H in-plane bend) 1450 $cm^{-1}$ (CH bending) and the one between 3100 $cm^{1}$-3600 $cm^{-1}$ (OH and NH) of CDLs and collagen spectra were measured using the software Spectrum version 5.3.

#### 2.2.2.1 Spectral curve fitting

In order to identify the secondary structure of collagen (the main protein present in CDL ECM), ultrapure collagen films (reference) and CDLs were analyzed by FT-IR spectroscopy. Samples were maintained at room temperature in a vacuum system for 24 hours to evaporate water. Spectral acquisitions were obtained as described before (section 2.2.2).

Derivative and curve fitting algorithms were performed with the software PeakFit from AISN Software. Initial peak positions were obtained from second derivative spectra of the raw data. The number and position of the peaks obtained were used as initial input parameters in the curve-fitting algorithm. A Lorentzian band-shape was used to fit the contours [24,25]. The curve-fitting algorithm creates Lorentzian bands that are added to produce a computed spectrum, which is compared with the experimental one. The process is iterated until a satisfactory fit between the computed and experimental bands is obtained by a least square regression analysis. The calculated area of each sub-band is reported as a percentage of the computed contour. Following methods described in the literature, once the adequate curve fitting was achieved for type I ultra-pure collagen, every parameter was fixed for the analysis of CDL spectra [24,25]. To obtain correct band area determinations, curve fitting was performed on larger spectral intervals than those used for peak assignment. Protein secondary structure parameters were further assessed using absorption bands assigned as α-helix, triple helix, β- sheets, β-turns, and unordered structure (% of total amide I absorption). The curve fitting procedure was applied to 1480-1720 $cm^{-1}$ (ν(C=O amide I absorption) interval that allows the determination of collagen secondary structure parameters. Furthermore, other spectral regions giving additional information that contributes to the classification of collagen types, namely the intervals 1350-1480 $cm^{-1}$ (δ($CH_2$) and δ($CH_3$) absorptions), 1180-1300 $cm^{-1}$ (amide III absorptions) and 1005-1100 $cm^{-1}$ (ν(C-N) and δ(N-H)), were also investigated.

### 2.2.3 Confocal Raman spectroscopy

A LabRAM HR 800 confocal Raman microscope system (Horiba-Jobin Yvon) comprising a spectrometer and a fully integrated confocal microscope Olympus BX41 was used to characterize hydrated CDLs in the different mechanical conditions, as well as collagen films and chondroitin sulphate powder. All analyses were conducted on samples placed on $Ca_2F$ discs. Raman spectra were generated using 785 nm laser diode as excitation source with an effective laser power of 6 mW on the sample, focused on the sample with a 100x or a 50x (LWD) objective for analysis in liquids. The scattered light was dispersed by a grating with 1800-lines/mm (Jobin-Yvon), and a thermoelectrically cooled charged-coupled detector (CCD) camera recorded the spectra. The CCD was connected to a computer for data collection and analysis using Labspec software (Horiba Jobin Yvon, Lille, France) and OriginPro data analysis and graphing software. Collagen and chondroitin sulphate spectral acquisitions were performed in 400 to 1800 $cm^{-1}$ wavenumber range using the following conditions: exposure time 100s, 5 accumulations, confocal pinhole 100 µm, spectral resolution 4$cm^{-1}$. In order to avoid CDL tissue dehydration, Raman analyses were performed in the conditioning media (propylene phenoxetol, acetylcholine and sea-water) with a LWD objective. The liquid environment also offers the advantage of dissipating the heat generated by the laser beam, thus preventing tissue damage. Spectral data were acquired in different areas of the sample. All spectra were baseline corrected and smoothed, using an automatic polynomial function and vector normalized on the whole spectra. Additional data manipulation included standard signal averaging and cosmic event (spike) removal.

#### 2.2.3.1 *Ex vivo* determination of water concentration profiles in CDL in the different mechanical states

The water content of tissues was determined from the ratio of Raman intensities of the OH stretch vibration of water at 3390$cm^{-1}$ and the $CH_3$ stretch of protein at 2935 $cm^{-1}$. Hydration measurements were carried out on CDLs in the different mechanical states and in 5 different animals. Samples were analyzed using a 785 nm laser as the excitation source. Water concentration profiles were obtained by

acquiring Raman spectra at different depths below CDL surface with 20 μm increments. For determination of water/protein ratios in CDL we used the ratio of the integrated intensities of water (3350 – 3550cm$^{-1}$) and protein (2910 – 2965 cm$^{-1}$) in order to maximize the signal-to-noise ratio and avoid overlap of the water signal with the NH vibration of protein at 3329cm$^{-1}$. The signal collection time per spectrum was 5 s.

### 2.3 Tissue Hydration assay

Tissue hydration of CDLs in different mechanical conditions was evaluated by subtracting the dry from the wet weight and deviding by the wet weight. Dry weight was measured after a thermal treatment performed in a Perkin Elmer Differential Scanning Calorimeter (DSC), where tissues were heated from 5°C to 180ºC in order to ensure complete dehydration.

### 2.4 Sulphated glycosaminoglycan quantification with alcian blue

The concentration of sulphated GAGs in CDLs in the different mechanical states was measured according to the procedure of Bjornsson *et al.* [26]. This protocol uses alcian blue, a cationic dye that is able to bind to sulphated GAGs. GAGs were extracted from CDLs in the different functional states after immersion in 4M guanidine–HCl for 15 minutes, at 4º C. GAGs were precipitated with alcian blue 8GX dye (Sigma-Aldrich A9186-10G) stock solution (alcian blue in 0.1% $H_2SO_4$/0.4 M guanidine-HCl) after overnight incubation at 4ºC and collected by centrifugation (15 min, 12000g g). The excess stain and contaminating proteins were removed by washing the pellet in DMSO-$MgCl_2$ for 15 minutes. The proteoglycan-alcian blue complexes were than immersed in guanidine-HCl/propanol until completely dissolved. After dissociation of the complex, the GAG concentration was determined photometrically using the corresponding calibration curve established using chondroitin sulphate sodium salt from shark cartilage (Sigma-Aldrich C-4384) as the standard, since the amount of GAG/PGs is directly proportional to alcian blue concentration. The absorbance was recorded on a microplate reader with a 605-nm filter, where a calibration curve was derived from the chondroitin-sulphate standard.

The sulphated GAG weight was normalized by tissue weight to calculate sulphated GAG concentration.

## 2.5 Proteins extraction and identification

### 2.5.1 Protein Extraction and Separation by 1D SDS-PAGE

Animals were incubated in specific solutions (see section 2.1) in order to obtain CDLs in the standard, stiff and compliant states. After incubation, tissues were cut in RIPA lysis buffer (200 mM Tris-HCl buffer pH 7.5 plus 1% Triton-X 100, 150mM NaCl and 1% of NP-40), homogenized, and sonicated during 30 min at 4ºC to allow the release of proteins. Protein concentration was determined using a DC Protein Assay kit from Bio-Rad (500-0112). For gel electrophoresis, 25 µl of XT sample buffer (1610791 XT sample buffer from Bio-Rad) in 5 µl of reducing agent (161-0792 XT Reducing Agent from Bio-Rad) were added to the 25 µg of CDLs extracts and the resulting solution was heated for 5 min at 95°C. Protein separation was achieved using Criterion™ XT Tris-Acetate Gel, 3-8% from Bio-Rad (345-0129-MSDS), and electrophoresis was carried out at a constant voltage of 150 V. Ten µl of protein standard (HiMark™ Pre-stained Protein Standard, ref. LC 5699 from Invitrogen) was used for molecular weight (MW) determination. The separated proteins were visualized by staining overnight with very sensitive Colloidal Coomassie Brilliant Blue G 250 (Sigma 27816).

### 2.5.2 Protein Identification by Mass Spectrometry: in-gel digestion

Protein bands were removed from the gels using a disposable scalpel and were digested as previously described [27,28]. Milli-Q $H_2O$ was used to wash the excised bands that were subsequently destained in 50% acetonitrile (ACN) and 100% ACN. Disulfide bonds were reduced with 10 mM DTT and alkylated with 50 mM iodoacetamide. The dried band pieces were allowed to swell in a digestion buffer (50-mM $NH_4HCO_3$ containing 6.7 ng/µL of trypsin (modified porcine trypsin, sequencing grade; Promega, Madison, WI, USA) on an ice bath. The supernatant was removed after 30 minutes and 20 µL of 50 mM $NH_4HCO_3$ was added to the gel pieces where

digestion was performed overnight at 37°C. After the complete digestion, the supernatant was stored at –20°C until used [27,28].

### 2.5.3 MALDI-MS/MS

The digested peptides were desalted and concentrated using home-made chromatographic microcolumns packed with different affinity materials, as described elsewhere [27-28]. Briefly, constricted GELoader tips (Eppendorf) were packed with POROS R2 chromatographic resin (PerSeptive Biosystems) or graphite powder (activated charcoal; Sigma-Aldrich) [27-29]. Then, each digested spot was subsequently passed through R2 and graphite microcolumns and eluted separately onto the MALDI plate using 0.5 µl of 5 mg/ml CHCA in 50% (v/v) ACN with 2.5% (v/v) formic acid.

Tandem mass spectrometry was performed using a MALDI-TOF/TOF 4800 plus mass spectrometer (Applied Biosystems). The mass spectrometer was externally calibrated using des-Arg-Bradykinin (904.468 Da), angiotensin 1 (1296.685 Da), Glu-Fibrinopeptide B (1570.677 Da), ACTH (1-17) (2093.087 Da), and ACTH (18-39) (2465.199) (4700 Calibration Mix, Applied Biosystems). Each reflector MS spectrum was collected in a result-independent acquisition mode, typically using 1000 laser shots per spectra and a fixed laser intensity of 3500V. The fifteen strongest precursors were selected for MS/MS, the weakest precursors being fragmented first. MS/MS analyses were performed using CID (Collision Induced Dissociation) assisted with air, with a collision energy of 1 kV and a gas pressure of 1 x 106 torr. Two thousand laser shots were collected for each MS/MS spectrum using a fixed laser intensity of 4500V. Raw data were generated by the 4000 Series Explorer Software v3.0 RC1 (AB Sciex) and contaminant m/z peaks resulting from trypsin autodigestion were excluded when generating the peptide mass list used for database search. The interpretation of the combined MS+MS/MS data was carried out using the algorithm, MOWSE (provided with MASCOT; version 2.2; Matrix Science) and two different databases (UniProt/Swiss-Prot joined together with the purple sea urchin *Strongylocentrotus purpuratus* genome predicted protein database and UniRef100). The search was performed using monoisotopic peptide masses and the following

criteria: one missed cleavage, p<0.05 significance threshold, 50 ppm peptide mass tolerance, 0.25 Da fragment mass tolerance, carbamidomethylation of cysteine as fixed modification, and methionine oxidation as variable modification. Significant hits were visually inspected to eliminate false positives [27,28,30].

### 2.6 Statistic analysis

At least five experiments were performed for each analysis. Statistical differences between CDLs in different functional states were determined using Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's post-hoc test. Data are given as mean ± standard deviation (SD). Results were considered statistically significant when $P < 0.05$.

## 3. Results and discussion

### 3.1 Biochemical characterization of CDL extracellular matrix

#### 3.1.1 *Ex vivo* FT-IR spectra of CDL

Mammalian collagen type I and chondroitin sulphate powder were analyzed by FT-IR (Figs 1 and 2) and Raman spectroscopy (Fig. 3) to be used as positive controls when evaluating their possible presence in the CDL extracellular matrix (ECM).

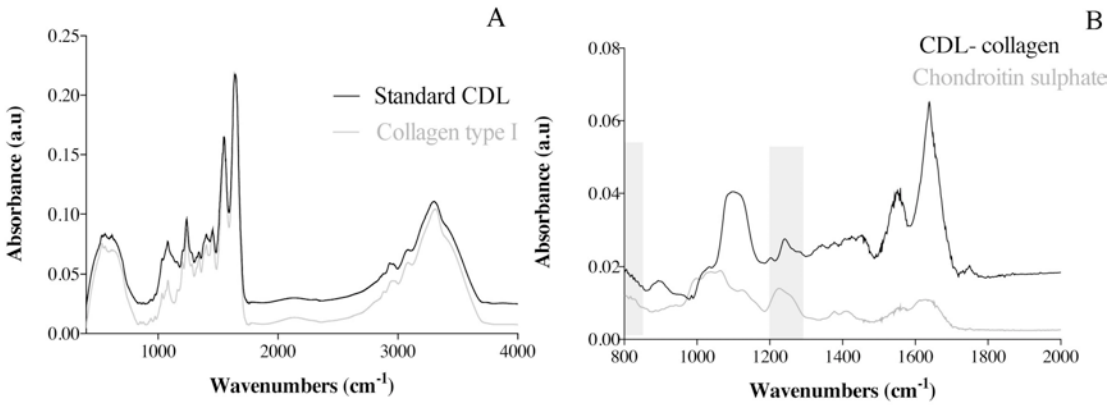


**Figure 1: Fourier transform infrared (FT-IR) spectra of:** (A) standard CDL and bovine collagen type I. (B) The difference spectra of CDL and collagen type I (CDL-collagen) shown together with the

spectrum of chondroitin sulphate from shark cartilage. The grey areas indicate characteristic wavenumber intervals for sulphate groups of chondroitin sulphate.

A comprehensive assignment of the major bands was derived from literature. The main bands of collagen (Fig. 1A) arose from the peptide bond vibrations: amide A (NH stretch), amide I (C=O stretch), II (C-N stretch and N-H in-plane bend) and III (C-N stretch, N-H bend and CO in-plane bend). The spectrum of chondroitin sulphate (Fig. 1B) exhibited features arising from the protein entities (amide I) but also from sulphate ($SO_4^{2-}$) groups and sugars.

FT-IR spectra of CDL exhibited bands assignable to collagen, since the striking resemblance between the spectra was immediately apparent (Fig. 1A), confirming that collagen was the dominant protein present in CDL ECM, as previously implied by morphological data [1-5].

The characteristic absorbance bands of CDL were amide I (1640 $cm^{-1}$), amide II (1550 $cm^{-1}$) and amide III (1239 $cm^{-1}$), arising from the peptide bonds vibrations of the main structural proteins, namely collagen. The spectral region between 1000-1500$cm^{-1}$ was the most crowded in the CDL spectrum, due to the absorptions from many biologically important functional groups (such as $CH_2$) $CH_3$ bending vibration arising from lipids, as well as $COO^-$ absorptions attributed to carbohydrate moieties, fatty acids and amino acid side chains.

As revealed in Fig. 1A, the major FT-IR bands of CDL and collagen overlapped markedly, making it difficult to identify the characteristic sulphate bands of chondroitin sulphate GAGs in CDL spectrum. In order to overcome this limitation, the collagen spectrum was subtracted from that of the CDL. The resultant spectrum showed absorptions at 1240-1250 $cm^{-1}$ ($\nu_{S=O}$) and 825-850$cm^{-1}$ ($\nu_{C-O-S}$) due to the presence of sulphates, apart from spectral features at 1630-1650 $cm^{-1}$ and 1552 $cm^{-1}$ (amide I and II, respectively).

### 3.1.2 Determination of protein secondary structure

FT-IR spectroscopy provides information on the secondary structure content of proteins. Different collagen types exhibit similar IR absorption bands but can be distinguished by their secondary structures [24,25]. The spectral region most sensitive to protein secondary structural components is the amide I band (1700-1600 $cm^{-1}$) which is due almost entirely to the stretch vibrations of the C=O peptide linkages. Figure 2 shows the deconvoluted spectra of collagen type I and CDL in the 1550-1700 $cm^{-1}$ interval.

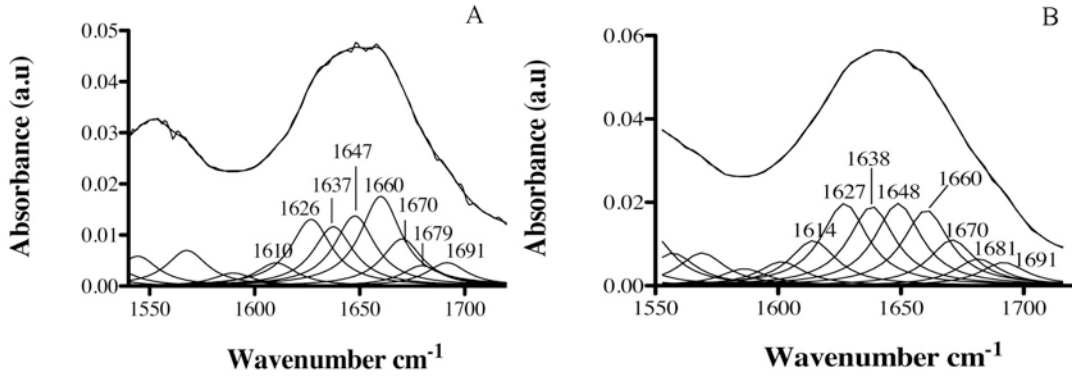


**Figure 2: FT-IR deconvoluted spectra of Amide I absorption band:** (A) ultrapure collagen type I; (B) standard CDL.

The decomposition of the collagen amide I band revealed several components (1626 $cm^{-1}$, 1647 $cm^{-1}$, 1660 $cm^{-1}$, 1670 $cm^{-1}$ and 1690 $cm^{-1}$) that were observed in both collagen type I and CDL spectra. The curve fitting procedure applied to the amide I band of ultrapure collagen type I and CDL spectra allowed determination of secondary structure parameters of collagen according to the band positions: α-helix (1660 $cm^{-1}$), β-sheets (1679 and 1626, 1691 $cm^{-1}$), β-turns (1608 and 1669 $cm^{-1}$), triple helix (1637 $cm^{-1}$) and random coil structure (1647 $cm^{-1}$). Spectral area per band was used for calculating the percentage of secondary structures (Table 1) [24,25].

**Table 1:** Secondary structure (in %) of collagen type I from bovine origin and CDL (N=5 for both collagen type I and CDL spectra).

| | **α-helix** | **β-sheets** | **β-turns** | **Triple helix** | **Unordered** |
|---|---|---|---|---|---|
| **Collagen type I** | 18 | 14 (±1) | 9 (±2) | 17 (±1) | 16 (±1) |
| **CDL** | 15 (±2) | 14 (±1) | 11 (±3) | 18 | 18 (±2) |

It was found that deconvoluted spectra of collagen type I and CDL ligament show a predominance of triple helix and α-helix structure, compared to β-sheets. This could be explained by the lack of globular domains in the protein. The presence of a triple helix structure on collagen type I and CDL was also confirmed by the mean absorption IR ratios between amide III at 1239 $cm^{-1}$ (CN stretch, NH bend and NH in-plane bend) and 1454 $cm^{-1}$ (CH bending) which were 1.19 (N=5) and 1.18 (N=5), respectively. Values higher than 1.0 indicated the presence of triple helices, in contrast to the 0.59 of gelatin (a protein devoid of a triple helix secondary structure) [31,32].

The curve fitting of collagen films and CDL spectra (results not shown) in the intervals 1350-1480 $cm^{-1}$ ($\delta(CH_2)$ and $\delta(CH_3)$ absorptions), 1180-1300 $cm^{-1}$ (amide III absorptions) and 1005-1100 $cm^{-1}$ ($\nu$(C-N) and $\delta$(N-H)), presented differences in the number and position of the bands suggesting the possibility that besides collagen type I, other collagen types contribute to the CDL matrix. Nevertheless, in the analysis of these data, it should be taken in consideration that intensity of spectral signal, as well as signal-to-noise ratio in those intervals, was significantly lower then in the amide I zone, a fact that could have influenced the quality of the curve fitting.

### 3.1.3 *Ex vivo* Raman spectra of CDL

Raman measurements were performed on regions where the ECM was visibly abundant. Several predominant bands at 855, 939, 1004, 1245, 1452 and 1664 $cm^{-1}$ were evident in Raman spectra of both collagen type I and CDL (Fig. 3). The peak in the 1655-1667 $cm^{-1}$ region was assigned to the amide I band of collagen, that at 1452 $cm^{-1}$ to the $CH_2/CH_3$ deformation of lipids and that at 1241-1272 $cm^{-1}$ to amide III. The presence of some amino acids, such as phenylalanine (1003 $cm^{-1}$) and tyrosine (646$cm^{-1}$), was also detected in both spectra.

The aromatic or saturated side chain rings of proline and hydroxyproline amino acids present in the collagen structure appeared in CDL spectra as two bands at 926 and 856 $cm^{-1}$ for proline and 878 $cm^{-1}$ for hydroxyproline. The peak at 939 $cm^{-1}$ is related to C-C vibrations of the collagen backbone.

As with FT-IR, the Raman spectrum of CDL revealed the presence of sulphate groups of chondroitin-sulphate with main sulphate vibrations of $OSO^{-}_{3}$ at 1067 $cm^{-1}$ and C-O-S vibration at 996$cm^{-1}$ (Fig. 3C).

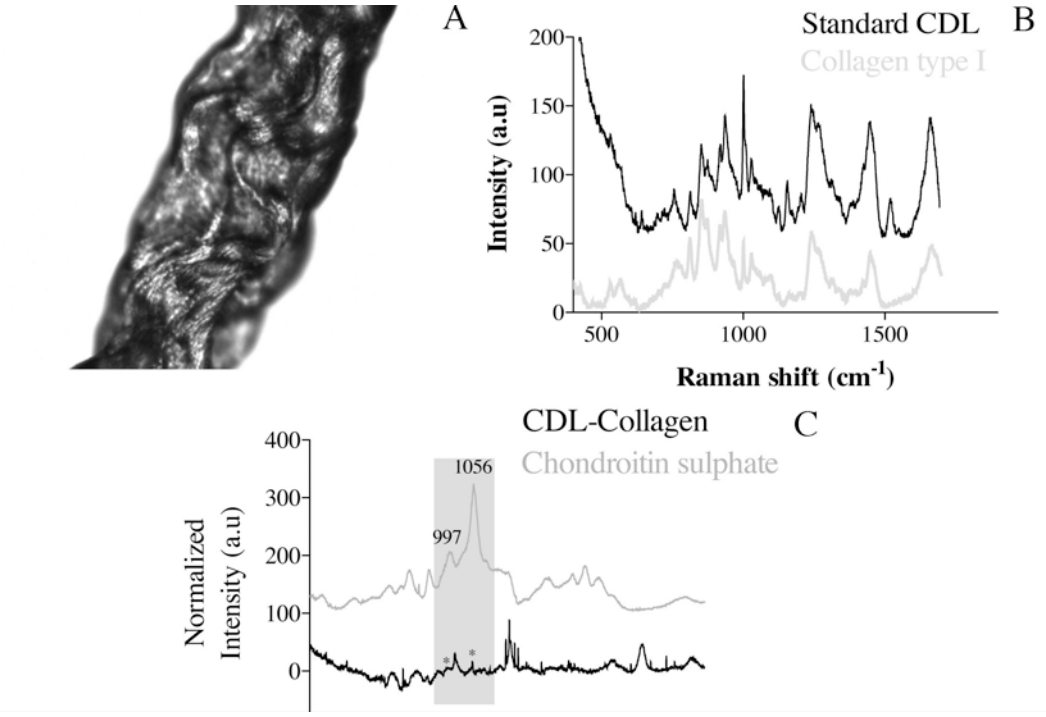


**Figure 3: Raman characterization of the CDL microenvironment:** (A) Video image of standard CDL (10x objective); (B) Raman spectra of collagen type I and standard CDL; (C) Difference between the spectra of CDL and collagen, together with the spectrum of chondroitin sulphate from shark cartilage. * Indicates the presence of sulphate groups of chondroitin sulphate.

Both in FT-IR and Raman spectra, pure collagen type I showed peaks with higher intensity than those of CDL, an observation that is strictly correlated with the amount of organic contents absorbing radiation. In the CDL tissue, other components besides collagen, like nucleic acids, lipids and sugars, give rise to absorptions in the vibrational spectra, contributing to the increase in intensity in most spectral regions. Although there was a strong similarity between the collagen and CDL Raman spectra,

a new band was observed in the CDL spectrum (between 1500-1600cm$^{-1}$), due to the presence of additional biochemical species in the ECM.

### 3.2 Biochemical characterization of CDLs in the different mechanical states

One-dimension gel electrophoresis showed a pattern of protein bands common to CDLs in the different mechanical states, with no significant differences being observed in terms of protein quantity. Numerous protein bands with apparent molecular masses ranging from 10 to 250 kDa, were observed (Fig. 4). However, only the most intense were excised and identified using MALDI-TOF/TOF.

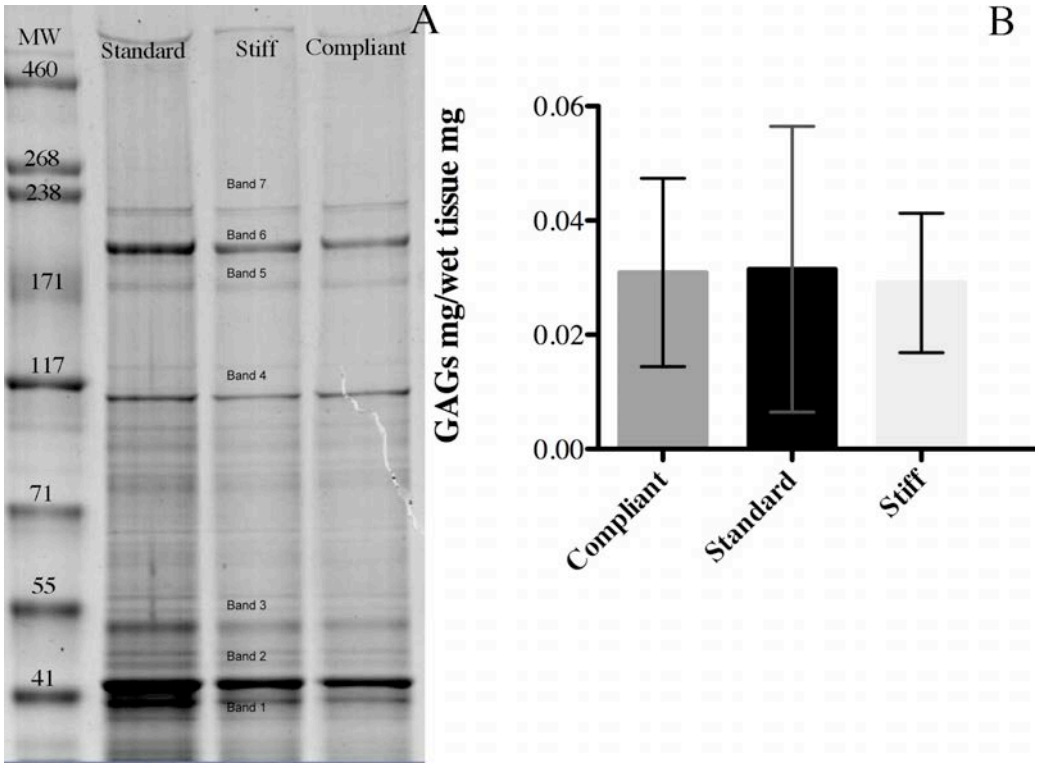


**Figure 4: Quantification of some ECM components of CDLs in the different mechanical states:** (A) Separation of proteins extracted from CDLs. Sections excised for in-gel digestion are indicated with a band number. (B) Sulphated GAG quantification with alcian blue staining (mean values). MW-molecular weight of marker proteins is shown in kDa in the left.

**Table 2** – Proteins identified in ECM of CLD from the sea urchin *Paracentrotus lividus* identified by MALDI-TOF/TOF-MS of in-gel tryptic digests of the bands indicated in Fig. 4A. Peptide mass (MS) and fragmentation (MS/MS) data were used to search against the UniProt/Swiss-Prot database joined together with the purple sea urchin *Strongylocentrotus purpuratus* genome predicted protein database and UniRef100.

| Gel band | Protein name | Species | Accession number [1] | Database | Protein | | Peptide | | Number of Distinct Peptides [4] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Score [2] | C.I. (%) [3] | Score [2] | C.I. (%) [3] | |
| **1** | Predicted similar to cytoskeletal actin | *Strongylocentrotus purpuratus* | gi\|72007954 | Uniprot-SwissProt/S.p. | 791 | 100 | 662 | 100 | 16 |
| **2** | Predicted similar to cytoskeletal actin | *Strongylocentrotus purpuratus* | gi\|72007954 | Uniprot-SwissProt/S.p. | 749 | 100 | 631 | 100 | 15 |
| **3** | Tubulin alpha-1 chain | *Paracentrotus lividus* | P18258 | Uniprot-SwissProt/S.p. | 569 | 100 | 496 | 100 | 11 |
| **4** | Predicted similar to myosin heavy chain | *Strongylocentrotus purpuratus* | gi\|115692122 | Uniprot-SwissProt/S.p. | 661 | 100 | 580 | 100 | 23 |
| **5** | Predicted similar to major yolk protein precursor | *Strongylocentrotus purpuratus* | gi\|115924727 | Uniprot-SwissProt/S.p. | 137 | 100 | 118 | 100 | 8 |
| | Toposome | *Paracentrotus lividus* | Q6WQT5 | UniRef100 | 265 | 100 | 151 | 100 | 17 |
| **6** | Major yolk protein | *Strongylocentrotus purpuratus* | gi\|47551123 | Uniprot-SwissProt/S.p. | 226 | 100 | 212 | 100 | 9 |
| | Toposome | *Paracentrotus lividus* | Q6WQT5 | UniRef100 | 873 | 100 | 673 | 100 | 32 |
| **7** | Predicted similar to myosin heavy chain | *Strongylocentrotus purpuratus* | gi\|115692122 | Uniprot-SwissProt/S.p | 900 | 100 | 766 | 100 | 31 |

[1] Accession numbers of the identified protein in UniProt-SwissProt/S.p. or Uniref100 databases

[2] *Mowse* scoring algorithm.

[3] Confidence index that the protein/peptide match was not random.

[4] Peptide whose sequence differs in at least 1 amino acid residue

The combination of mass spectrometry with homology-database search, allowed the identification of the following proteins: actin, tubulin, myosin and major yolk. Bands 1 and 2 were identified as either muscle (41 kDa) or as cytoskeletal actins (42 kDa), respectively. Gel band 3 was identified as tubulin, and band 4 and 7 as myosin bands. Gel band 5 and 6 were identified as toposome protein (173 and 180 kDa) (Fig. 4A, Table 2). The remaining bands could not be identified despite the quality of the obtained MS and MS/MS spectra.

Concerning sulphated GAGs, no significant differences were found between CDLs in the different mechanical states. Any real differences may have been masked by the strong variability that was always observed between animals and which was indicated by the large standard deviations (Fig. 4).

FT-IR spectra obtained for CDLs in the different mechanical states were similar (Fig. 5). The mean IR ratios between amide III (1239 $cm^{-1}$) and 1454 $cm^{-1}$ for CDL in the stiff and compliant condition were also higher than one, 1.8 (N=5), 1.9 (N=5) for stiff and compliant CDLs respectively. The most noticeable difference concerned the amide I/amide II intensity ratio. Stiff CDLs presented a significantly higher amide I/amide II intensity ratio than did compliant tissues. The characteristic bands in the infrared spectra of proteins that include the amide I and amide II arise from the amide bonds that link the aminoacids. The absorption associated with the amide I band leads to stretching vibrations of the C=O bond of the amide whereas the absorption associated with the amide II band leads primarily to bending vibrations of the N-H bond.

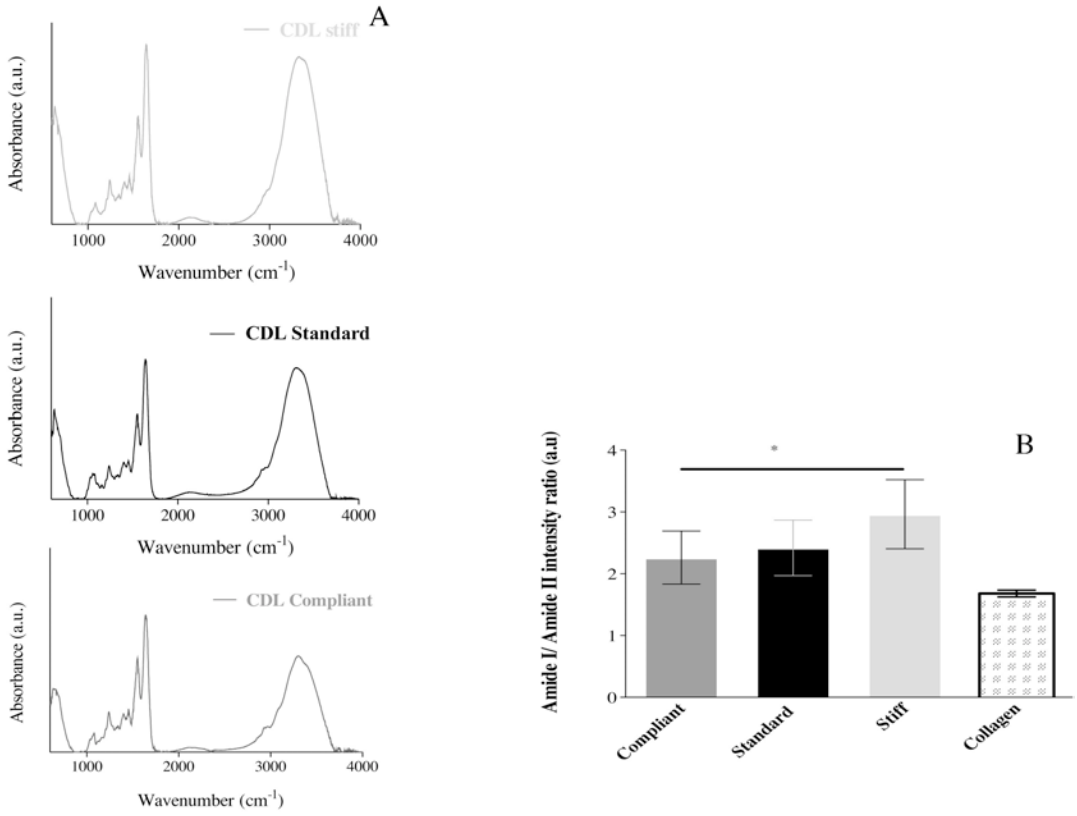


**Figure 5: FT-IR characterization of CDLs in the different mechanical conditions:** (A) FT-IR spectra of CDL and (B) mean amide I/amide II intensity ratio for CDL in the compliant, standard, and stiff mechanical conditions, as well as for collagen. * Asterisk represents statistically significant differences ($P<0.05$).

Non-protein components of tissues also contributed significantly to the amide I region of the spectrum, the most intense arising from the O-H vibration of water (a band around 1640 $cm^{-1}$). Consequently, the variation of the amide I/amide II intensity ratio can be related to both the protein and water content of the tissues. The collagen type I film also showed a lower amide I/amide II intensity ratio, compared with CDLs.

### 3.3 Contribution of water to CDL mutability phenomena

No statistically significant differences were observed between the amounts of water present in the three mechanical states of CDLs when water loss was estimated by heating (Fig. 6A).

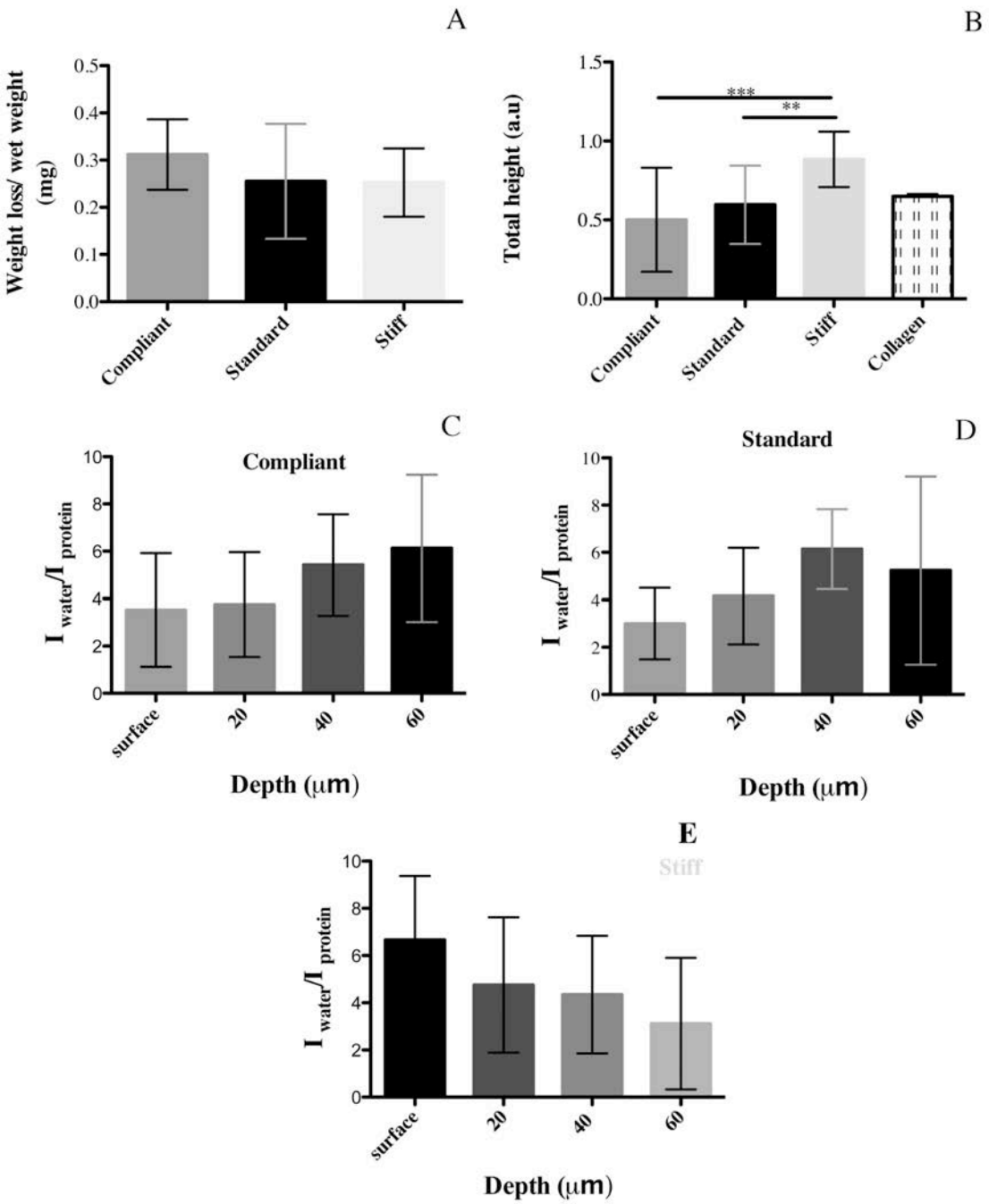


**Figure 6: Role of water in mutability phenomenon:** (A) the weight loss of CDL after thermal treatment; (B) Quantitative estimation of the mean intensity band in the frequency region 3100 $cm^{-1}$ - 3600 $cm^{-1}$ present in FTIR spectra; (C, D); Water content at depth increments calculated from the intensity ratio of OH band (3350 $cm^{-1}$) and protein bands (2910 – 2965 $cm^{-1}$) for (C) compliant and (D) standard and (E) stiff CDLs (Raman results).

However, the FT-IR spectra lead to a different conclusion. The intensity of the broad band in the 3100-3600 $cm^{-1}$ region (Fig. 6B), that arises from the overlapping of the absorption bands of O-H groups from water molecules, N-H groups from proteins

and $NH_2$ groups from nucleic acids, was higher in stiff tissues, thus suggesting a higher water content in those tissues compared to the compliant and control ones. We decided to investigate if the amount of water varied from the periphery to the centre of the tissues. For that, Raman spectra were collected at different depths. The data indicate that in the standard and compliant states the interior of the CDL is richer in water than the surface, whereas in the stiff state the reverse is the case (Fig.6 C-E).

## 4. Discussion

### 4.1 CDL biochemical microenvironment

As in mammalian connective tissues, the ECM of the CDL consists mainly of collagen fibrils with a structure (D-banding periodicity around 60 nm), chain composition and gene organization similar to that of vertebrate type I collagen [9-19]. About 30 collagen types have been identified, which vary in amount in each tissue. Collagen fibrils may contain types I, II, III, V, XI, XXIV, XXVII [6,7]. However, a sea urchin collagen has been found to have two $\alpha$1(I) chains and one $\alpha$2(I) chain, which is characteristic of collagen type I [6,10,12-15]. Taking this into consideration we used commercial collagen type I as a positive control in our investigation. Our FT-IR and Raman data revealed that collagen made a strong contribution to the CDL spectrum, and demonstrated the presence of amino acid side chains characteristic of collagen, such as proline and hydroxyproline, as well as other constituents like carbohydrates, fatty acids, nucleic acids and phospholipids [2-5,33-44].

FT-IR has also been used to differentiate between different collagen types on the basis of their structural parameters [24,25,40-44]. The amide groups (amide I, II, III and A) vibrations of protein backbones received particular attention, since they are present in all proteins and provide information on secondary conformation [40,44]. Using a similar approach to that of Petibois *et al.* [25], curve fitting was performed for different regions of the FT-IR spectra of collagen and CDLs, particular attention being given to the amide I zone, since its shape is sensitive to the type and amount of secondary structures and is not influenced by side chains [24,25,42]. The results confirmed that CDL collagen has a triple helical structure and has strong similarities to mammalian type I. However, differences were observed in other spectral regions,

suggesting the possible presence of collagen other than type I in the CDL. This is expected, because mature connective tissues of mammalians, such as cornea, skin, and cartilage, contain heterotypic collagen fibrils consisting of more than one collagen type. [6,6,45].

In addition to collagen, PGs were previously identified in the CDL by histochemical methods. It was shown that they are located at specific and periodic sites of collagen fibril surface and their GAG side-chains were labelled by alcian blue [5]. The spectroscopic characterization performed in this work identified sulphate spectral features (chondroitin sulphate) in CDLs; nevertheless the possible existence of non-sulphated GAGs cannot be excluded [46-53]. Sulphated GAGs have been identified in other mutable collagenous structures of echinoderms [1-3]. Sulphated GAGs appear to have been conserved in invertebrate evolution possibly because, apart from their role in MCT mutability, they contribute to protective reactions against foreign bodies [54-56].

### 4.2 Remodelling or reorganization of CDL during mutability phenomena?

Like all ECMs, that of the CDL is complex. Its morphology and organization resemble those of mammalian ECM of mammals, consisting of a dense array of collagen fibrils and fibrillin-containing microfibrils (which provide tensile strength and elasticity respectively) surrounded by proteoglycans and glycoproteins, which maintain hydration and link fibrillar components and cells.

Dynamic ECMs are not exclusive to echinoderms; mammals also have mechanically adaptable connective tissue in the uterine cervix, which is normally rigid, but which becomes very pliant during pregnancy, thereby permitting delivery [20-23,57-60]. This mutable connective tissue passes through different stages (softening, ripening and post partum) that are coordinated by the neuro-endocrine mechanisms and occur over a longer time scale (hours to days). In this case, the reversible changes in mechanical properties results from modification of the biochemical composition and structure of the cervix, with matrix metalloproteinases having an important role in ECM degradation. So far, no relationship between

collagen (type I and III) content and the variations in the tensile strength has been detected. The increase in tissue compliance is strongly correlated with the synthesis of a collagen with higher solubility and fewer cross-links, and also with changes in biochemical composition, collagen degradation, increase in tissue GAG content and tissue hydration [20-23,57-60]. Finally, after labour there is a significant increase in transcription of genes involved in matrix repair which achieves both ECM remodelling and the recovery of tissue integrity [20-23,57-60].

A novel objective of the present study was to look for evidence of ECM remodelling during the mutability phenomenon, as occurs in the human cervical stroma. One-dimension gel electrophoresis tests indicated that other proteins besides collagen are present in CDLs but that the protein pattern and quantity is the same in the ligaments in the different mechanical states. FT-IR data suggest that subtle adjustments of protein content may occur in stiff CDLs. However, it is possible that these are due to the release of protein effectors such as tensilin into the ECM. FT-IR and Raman results suggested that the water content of stiff CDLs is higher than of compliant CDLs.

No significant differences in the content of sulphated GAGs in CDLs in different mechanical states was observed, suggesting that sulphated GAGs act exclusively as binding sites for effector molecules (e.g. tensilin). Further work needs to be done to look for non-sulphated GAGs in the CDL [2,3,8,9,17].

It appears that the variable tensility of CDLs does not involve the degradation of collagen fibrils, as suggested by Ribeiro *et al.* [5], but is more a molecular rearrangement process. The IR ratios of Amide III and the peak positioned at 1450 $cm^{-1}$ of CDLs in the different mechanical conditions were higher than 1.0 demonstrating that variable tensility of CDLs does not involve changes in the molecular conformation of molecules of collagen [21,22,31-32,61-63]. Given that collagen is a very stable protein, and mutability phenomena occur in short physiological time-scales, it is highly unlikely that there is enough time for stiffening to result from the synthesis of new collagen.

A first trial to identify the proteins involved in CDL mutability by mass spectrometry conjugated with protein database search using the recently sequenced genome and predicted protein database of purple sea urchin, *S. purpuratus* [30] was also performed. Amongst the identified proteins are two structural proteins, tubulin and actin, whose presence is related to the cellular elements of the CDL. Myosin was also identified, which was expected since a myoepithelium occupies around 8% of CDL cross-sectional area [4]. Although bands 5 and 6 could represent major yolk protein, a very abundant glycoprotein of the coelomic fluid that surrounds the CDL, a higher score was observed with toposome from *P. lividus* [64]. Toposome is a protein essential for sea urchin cell adhesion and development [65]. Further work on protein extraction protocols, as well as other separation techniques, is being done in order to identify possible key-proteins involved in CDL mutability.

### 4.3 The contribution of water to the mutability phenomena

In the present work, Fourier Transform Infrared Spectroscopy and Raman Spectroscopy techniques were used to investigate the possible contribution of water to the mutability phenomenon, but revealed no significant differences between the three mechanical states. FT-IR data regarding the intensity ratio of amide I/amideII (Fig. 5B) and the height of the absorption band in the 3100-3600 cm-1 region (Fig. 6B) suggest that water exudation could be involved in the change from the standard to the stiff states, since these tissues have high content of O–H groups. However, we must keep in mind that the estimated penetration depth (0.15 $\mu$ m) to which the sample is probed means that we are at the surface of CDL. These results are in agreement with Raman data acquired in different depths profiles, which demonstrated that the water content of stiff CDLs increases, as the analysis was performed from the interior of the CDL to its surface. The opposite pattern was observed in standard and compliant CDLs. Although a trial of MRI analysis was performed in order to demonstrate the movement of water in CDL, its small size constitutes a limitation on the usefulness of this specific technique. However, water 'exudation', together with tissue shrinkage, is not exclusive to the CDL, since it has been observed in mammalian tendon, articular cartilage and intervertebral disc, and also in another MCT, i.e. excised holothurian dermis when it stiffens from the standard state [66-72]. Tamori et al. adduced evidence supporting the view that water exudation resulted from the release of

extracellular water molecules, which had been previously bound through electrostatic forces, and was caused by those changes in non-covalent intermolecular linkages that underpinned tissue stiffening. For example, water molecules masking fixed charges on GAG side-chains might be displaced by stronger interactions between these charges and putative effector macromolecules such as tensilin, resulting in water exudation [66,73,74]. Our observation that the pattern of water distribution was similar in standard and compliant CDLs corresponds to the similar finding of Tamori *et al.* regarding holothurian dermis, and provides more evidence that different molecular mechanisms underpin stiffening from the compliant to standard states and stiffening from the standard to stiff states [66, 73].

## 5. Conclusions

This investigation has revealed previously unreported biochemical differences between experimentally induced mechanical states of the sea urchin CDL, which simulate the mutability of the tissue in vivo.

The CDL ECM is a composite structure comprising a dense array of collagen fibrils, PGs, GAGs, water and other glycoproteins. We found that the fibrillar collagen has strong biochemical similarities to mammalian collagen type I as suggested by FTIR and Raman data. CDL GAGs were found to be sulphated. Although these may be involved in mutability, since they serve as binding sites for molecules responsible for interfibrillar cohesion, they are polyanionic molecules whose electrostatic properties render them osmotically active. When the CDL shifts from the standard to the stiff state, stronger interactions between GAGs and effector proteins (such as tensilin), may expel water molecules.

Our results also demonstrate that CDL mutability does not involve the synthesis of new ECM components, but that adjustments of tissue hydration may occur during shifts in mechanical state.

Although there are similarities between the mutability of the CDL ECM and the human cervical stroma during pregnancy, the time scale is completely different: 1s

to a few minutes in the CDL and hours to days in the cervix. It is highly unlikely that CDL collagen and GAGs are degraded and synthesized in such a short time. As has been inferred with regard to other echinoderm MCTs, the mechanism of CDL mutability seems to depend on molecular rearrangement rather than remodelling.

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# Chapter V

# Metalloproteinases in a sea-urchin ligament with adaptable mechanical properties*

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## Abstract

The mutable collagenous tissues (MCTs) of echinoderms show reversible changes in tensile properties (mutability) that are initiated and modulated by the nervous system via the activities of cells known as juxtaligamental cells. The molecular mechanism underpinning this mechanical adaptability has still to be elucidated. Adaptable connective tissues are also present in mammals, most notably in the uterine cervix, in which changes in stiffness result partly from changes in the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). There have been no attempts to assess the potential involvement of MMPs in the echinoderm mutability phenomenon, apart from studies dealing with a process whose relationship to the latter is uncertain. In this investigation we employed the compass depressor ligaments (CDLs) of the sea-urchin *Paracentrotus lividus*. The effect of a synthetic MMP inhibitor - galardin - on the biomechanical properties of CDLs in different mechanical states ("standard", "compliant" and "stiff") was evaluated by dynamic mechanical analysis, and the presence of MMPs in normal and galardin-treated CDLs was determined semi-quantitatively by gelatin zymography. Galardin reversibly increased the stiffness and storage modulus of CDLs in all three states, although its effect was significantly lower in stiff than in standard or compliant CDLs. Gelatin zymography revealed a progressive increase in total gelatinolytic activity between the compliant, standard and stiff states, which was possibly due primarily to higher molecular weight components resulting from the inhibition and degradation of MMPs. Galardin caused no change in the gelatinolytic activity of stiff CDLs, a pronounced and statistically significant reduction in that of standard CDLs, and a pronounced, but not statistically significant, reduction in that of compliant CDLs. Our results provide evidence that MMPs may contribute to the variable tensility of the CDL, in the light of which we provide an updated hypothesis for the regulatory mechanism controlling MCT mutability.

## 1. Introduction

Echinoderms (starfish, sea urchins and their relations) have connective tissues with the unique ability to change mechanical properties such as elasticity and viscosity in short physiological time scales (<1 s to minutes) under nervous control [1,2]. They are called either “mutable” collagenous tissues (MCTs) to reflect the reversibility of their tensile changes, or “catch” connective tissues due to their energy-sparing capacity to maintain body or appendage posture with low oxygen consumption [1-4]. Each shows one of three patterns of tensile change: (1) only reversible stiffening and destiffening (e.g. sea urchin peristomial membrane and compass depressor ligament) [5,6]; (2) only irreversible destabilization (always associated with autotomy (defensive self-detachment)); e.g. crinoid syzygial ligament) [7]; (3) irreversible destabilization as well as reversible stiffening and destiffening (e.g. ophiuroid intervertebral ligament) [8]. MCTs are present in all five living echinoderm classes in several anatomical forms (dermal connective tissue, interossicular ligaments and tendons). Most MCT structures resemble mammalian connective tissues in that they consist largely of collagen fibril arrays, proteoglycans (PGs), fibrillin-containing microfibrils and water [2,9-17].

MCTs are also characterized by the invariable presence of specialized neurosecretory-like cells known as “juxtaligamental cells” (JLCs) [2,18]. There is evidence that the intracellular granules of JLCs store molecules that directly affect the interfibrillar cohesion of MCTs [2]. So far, only one such potential effector molecule has been identified and fully characterized. This is tensilin, a glycoprotein present in the dermis of holothurians (sea cucumbers) that forms interfibrillar bridges between collagen fibrils, preventing interfibrillar slippage and increasing the resistance of the tissue to tensile forces [19,20]. Tensilin and another incompletely characterized molecule from holothurian dermis [21] may be regulatory stiffening agents. So far, no potential regulatory destiffening agents have been identified. It has been speculated that enzymes might have such a role. For example, since the C-terminus of tensilin, which contains a collagen-binding domain, is susceptible to proteolysis, it has been suggested that rapid destiffening of holothurian dermis could depend on the

inactivation of tensilin by a specific protease [19]. The fact that the amino acid sequence of tensilin indicates 21-36% homology with mammalian tissue inhibitors of metalloproteinases (TIMPs) [19], raises the intriguing possibility that metalloproteinases (MMPs) may be directly involved or that the regulatory mechanism has evolved from a MMP-TIMP system [2].

MMPs are a family of enzymes that can degrade all ECM components and are extensively involved in the ECM remodelling that accompanies morphogenesis and wound healing in mammals [22-25], and development and regeneration in echinoderms [26-28]. Furthermore, MMPs contribute to the destiffening of the mammalian uterine cervix, which precedes and facilitates the dilatation of the cervix during fetal delivery [29-35]. Whilst the uterine cervix can also be regarded as a mutable collagenous structure, its changes in mechanical properties differ from those of echinoderm MCTs in having a much longer time course (hours to weeks) and in being under primarily endocrine rather than neural control [34]. Another important difference is that cervical destiffening is achieved partly through the degradation of collagen fibrils [30,34,35], whereas there is no evidence that this accompanies the destiffening of echinoderm MCTs and indeed the capacity of most of these tissues to rapidly restiffen makes this highly unlikely a priori [2,36]. MMPs could, however, destabilize echinoderm MCTs by hydrolyzing non-collagenous components that contribute to interfibrillar cohesion. Such a mechanism may be responsible for the dermal liquefaction shown by some holothurians, which appears to result from the digestion of interfibrillar molecules (possibly proteoglycans) by a gelatinolytic enzyme that has no discernible effect on the collagen fibrils themselves [37].

Dermal liquefaction is an extreme (or even fatal) phenomenon whose relationship to reversible MCT mutability is unclear [37, 38]. We chose to explore the possible role of MMPs in the latter by using as a model a more conventional mutable collagenous structure – the compass depressor ligament (CDL) from the lantern (masticatory apparatus) of the sea urchin *Paracentrotus lividus* (Lam.). The sea urchin lantern contains ten CDLs, which, when stiff, help to stabilize the position of the lantern, and which destiffen to permit movement of the lantern by its intrinsic musculature [5]. We examined the effect of a broad-spectrum MMP inhibitor on the

mechanical properties of CDLs in different tensile states and we used gelatin zymography to quantify MMPs in such CDLs. Our results provide evidence that MMPs may contribute to the variable tensility of the CDL, in the light of which we provide an updated hypothesis for the regulatory mechanism underpinning MCT mutability. This proposes that changes in the tensile properties of MCTs result from the rapid activation and inactivation of MMPs and adjustments in the balance of these enzymes and their respective inhibitors.

## 2. Materials and Methods

### 2.1 Animal tissues and bathing solutions

Adult individuals of P. lividus of similar size were collected in Aguda (north Portuguese coast) and maintained in an aquarium as described previously [36]. Isolated preparations of compass depressor ligaments (CDLs) were obtained from the lantern and mutability was mimicked as described previously [36]. In brief, the ‘compliant’ state was reproduced in vitro by immersing isolated CDLs for 45 minutes in 0.1% propylene phenoxetol (Sigma Aldrich 484423) in seawater (PPSW), which is an effective anaesthetic for echinoderms. The 'stiff' state was obtained by immersion of CDLs in 1mM acetylcholine chloride (Sigma Aldrich 6625) in seawater (AChSW) for 15 min. Controls, which were in the ‘standard’ state, were kept in seawater (SW) alone. Although the CDL is partly delimited by a contractile myoepithelium, in P. lividus this occupies only around 8% of its total cross-sectional area, and we have shown previously that destiffening and stiffening due to PPSW and AChSW respectively result from changes in the passive mechanical properties of the collagenous component and not from effects on the myoepithelium (Wilkie, Fassini and Candia Carnevali, in preparation) [5].

### 2.2 Mechanical properties

#### 2.2.1 Dynamic mechanical tests

The CDLs, which are strap-shaped bands of soft tissue 9-10mm, 0.2-0.4mm wide and less than 0.1mm thick, were dissected intact together with a small portion of

the skeletal ossicles to which they were attached at either end (Fig.1) The presence of the ossicle portions enabled the CDLs to be held firmly in the dynamic mechanical apparatus with minimal damage to the soft tissue. Fresh CDLs were always used and, before being analysed, were stored in SW. All CDLs were tested at a constant temperature of 20ºC.

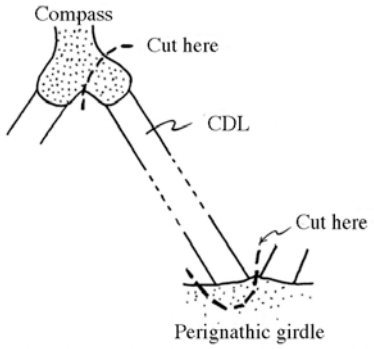


**Figure 1:** Schematic representation of the CDL dissection.

A dynamic mechanical analyser (DMA) (Tritec 2000; Triton Technology Ltd., Nottinghamshire, UK) with tension clamping geometry was used to determine the effect of chemical stimulation on the biomechanical properties of the CDL. Standard tension tests were chosen, because in the animal CDLs are continuously stretched due to routine movements of the "lantern". The ossicles at the ends of each CDL were glued to the clamps of the equipment with cyanoacrylate glue, which was found to be suitable because of its rapid polymerization time (a few seconds). Beeswax (Sigma-Fluka 14367) was used as a coating, in order to avoid contact between the cyanoacrylate glue and testing solutions. The mean thickness of each CDL was determined by several measurements using a digital micrometer, and it was assumed that sample cross-section was circular (average diameter between 0.2-0.4 mm). After mechanical fixation, and before starting the mechanical tests, tissues were allowed to equilibrate for 10 min in seawater. During the experiments sinusoidal force and displacement signals were measured simultaneously and this data set was resolved into complex modulus (E*), which is a measure of "stiffness", and Tan δ (damping), which is the ratio of the loss modulus (E´´) to the storage modulus (E´). The storage modulus represents the elastic modulus in phase with the stress, while the loss modulus represents the viscous contribution to stiffness because it is an out-of-phase

component. As the software provided E′, E´´ and Tan δ, E* can be calculated by the following formula:

$$E^* = SQRT (E´^2 + E´´^2)$$

### 2.2.2 Viscoelasticity of CDLs in different mechanical states

CDLs were clamped and left in seawater for 10 min before being treated with propylene phenoxetol or acetylcholine, as described above, to reproduce the compliant and stiff states, respectively. Five animals, and at least three CDLs from each animal were used for each mechanical state. To determine the optimal experimental parameters, i.e. those that provided maximum differentiation between the three mechanical states, CDLs in all three conditions were subjected to two types of experiment: (1) In constant frequency experiments, the frequency was kept at 1Hz and each CDL was subjected to a maximum strain which was changed stepwise in the following sequence: 5%, 10%, 15%, 10% and 5%, over the course of 5 sec (up to 15% strain leads to the rupture of some CDLs). (2) In constant strain tests, the maximum imposed strain was kept at 13% and each CDL was subjected to a frequency that changed stepwise as follows: 0.1 Hz, 0.3 Hz, 0.5 Hz, 0.7 Hz 1 Hz, 3 Hz, 5 Hz.

### 2.2.3 Effect of MMP inhibition on CDL viscoelasticity

The effect of the MMP inhibitor galardin (Calbiochem 364205) at 25 and 50 μM was investigated in CDLs using dynamic mechanical tests at 1 Hz and 13% maximum strain (the conditions that provide the maximum differentiation between the different mechanical states). Untreated CDLs were tested for 4 min before galardin was added. The duration of galardin inhibition was quantified as the time period between the point at which the E* maximized after addition of 50 μM galardin, and the point at which the E* returned to the value observed immediately before galardin addition. The effect of galardin on E* was quantified by normalizing the E* value when it started to decrease against the value just before the application of the chemical. The reversibility of the galardin effect was tested by immersing CDLs in the three mechanical states in SW, AChSW or PPSW containing 50 μM galardin, then

rinsing the CDLs with SW, AChSW or PPSW alone and treating them again with 50 μM galardin in the respective solutions.

### 2.3 Enzymatic activity

#### 2.3.1 Gelatinolytic activity in CDLs

Gelatinolytic activity was detected in more than five animals for each of the three mechanical states. Animals, from which the top half of the test (shell) had been removed to expose the intact lantern, were incubated in SW, AChSW and PPSW to obtain ligaments in the standard, stiff and compliant states, respectively. The CDLs were then quickly excised and placed in ice-cold RIPA lysis buffer (200 mMTris-HCl buffer pH 7.5 in 1% Triton-X 100, 150 mMNaCl and 1% of NP-40), homogenized, and sonicated for 30 min at 4ºC to release associated MMPs. After sonication, proteins were precipitated with acetone by overnight incubation at -20 ºC. Extracts were then centrifuged at 14000 rpm for 10 min at 4ºC and supernatants were collected. Protein precipitates were then diluted in PBS and the sample protein concentration was determined using a DC Protein Assay kit from Bio-Rad (500-0112). For each sample, 15 μg of protein was applied to non-reduced SDS-polyacrylamide gel electrophoresis using 10% gels containing 0.1 % gelatin (bovine skin, Type B, SIGMA, G9391). The gels were electrophoresed under 80 V with a maximum intensity current of 120mA, in a Mini-PROTEAN® Tetra Cell system from Bio-Rad. Following electrophoresis, the gel was washed twice with 2% v/v Triton X-100, to remove excess SDS, and incubated with MMP substrate buffer (50 mMTris–HCl, pH 7.5, 10 mM CaCl2) for 16 h. After incubation, the gel was washed with distilled water and stained with 0.1% w/v Coomassie Brilliant Blue solution (Sigma R-250). Areas of proteolysis appeared as clear bands against a blue background of gelatin substrate. Molecular mass determinations were made with reference to pre-stained protein standards. Stained gels were scanned and band densities were quantified by densitometric analysis (Quantity One Software, Bio-Rad). It should be noted that the gelatinolytic activity detected by this technique results from the presence of active MMPs, their inactive pro-enzymes and MMP-TIMP complexes (which are partly dissociated by SDS) [39].

### 2.3.2 Gelatinolytic activity in CDLs treated with galardin

To further assess the role of MMPs in CDL mutability, tissues in the different mechanical states were incubated with galardin. As there is considerable variability between individuals, we used two animals each for the standard and standard-inhibited conditions per gel. Per analysis, 10 CDLs (5 CDLs of each animal) were used for standard and standard-inhibited conditions. The same procedure was applied for the different mechanical states. More than five animals for each of the three mechanical states were used. For the standard state, samples were maintained in a solution of 50 μM galardin in SW; stiff CDLs were stimulated in a solution of 50 μM galardin in AChSW; and compliant tissues were incubated with 50 μM galardin in PPSW. After overnight incubation at 4ºC, tissues were prepared to measure their gelatinolytic activity as previously described in section 2.3.1.

## 2.4 Ethical treatment of animals

No specific permits were required for the described field studies since sea-urchins (*Paracentrotus lividus*) are invertebrates. This work was performed with a species that is not endangered or protected. The location of the field studies is also not privately owned or protected in any way.

## 2.5 Statistical analysis

All experiments were repeated at least five times. Statistical differences between CDLs in different functional states were determined using Kruskal-Wallis one-way analysis of variance with Dunn's post-hoc test. All statistics were performed using GraphPad Prism 5 Demo software (version 5.02). Data are given as mean ± standard deviation (SD). Results were considered statistically significant when $P < 0.05$.

# 3. Results

## 3.1 Mechanical properties

### 3.1.1 Viscoelasticy of CDLs in different mechanical states

Before evaluating the viscoelasticity of CDLs in the different mechanical states, the relationship between the maximum strain imposed on cyclically (constant frequency 1 Hz) loaded CDLs in different mechanical states and the resulting maximum stress was investigated (Fig.2A).

As is typical for collagenous tissues, the stress-strain curves were J-shaped, with a non-linear toe and heel region followed by a linear (constant stiffness) region, indicating that CDLs were more compliant at low strains and became stiffer as deformation progressed. This transition was abrupt and took place at 10% deformation.

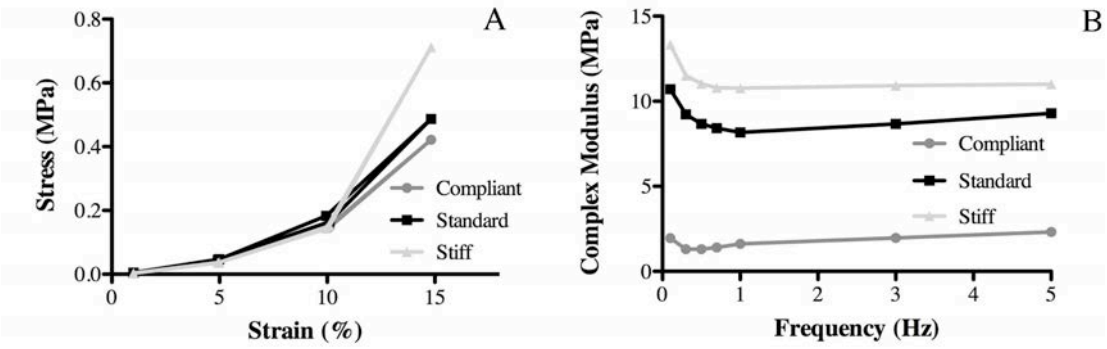


**Figure 2: Mechanical properties of CDL:** (A) Representative stress vs. strain curves of CDLs from one animal in the three mechanical states. (B) Effect of frequency on the complex modulus of CDLs from one animal in the three mechanical states tested at 13% strain.

Fig. 2B shows the relationship between the cyclical loading frequency at a constant maximum strain of 13% and the complex modulus (E*) of CDLs in the three mechanical states. On the basis of these results, it was decided that a maximum strain of 13% and a loading frequency of 1Hz should be employed in subsequent experiments.

In tests with CDLs in the standard state, the replacement of SW with PPSW resulted in a decrease in the complex modulus, which was partly reversible and repeatable (Fig. 3A).

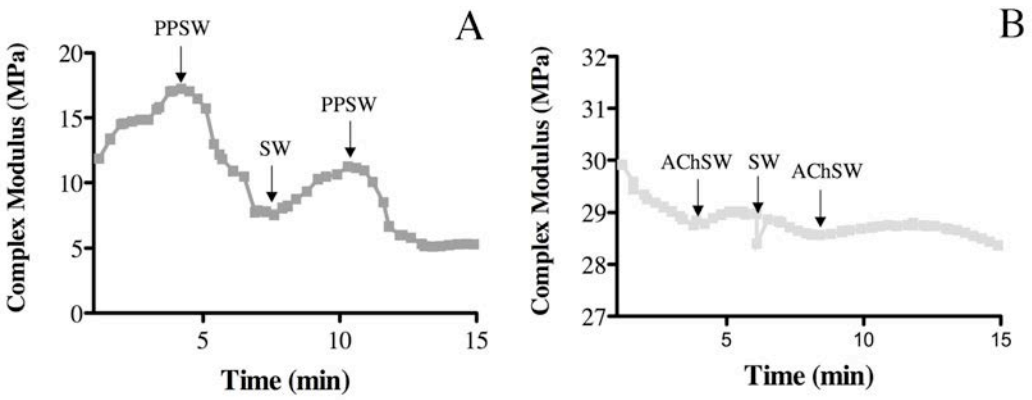


**Figure 3: Immediate effect of:** (A) PPSW (propylene phenoxetol in seawater - compliant state) and (B) AChSW (acetylcholine chloride in seawater – stiff state) on standard CDLs.

In equivalent tests in which SW was replaced with AChSW, there was a reversible and repeatable increase in the complex modulus (Fig. 3B).

### 3.1.2 Effect of MMP inhibition on CDL viscoelasticity

When galardin was applied to standard CDLs, there was some instability due to addition of the solution, and then the stiffness (E*), reached a plateau, and then decreased gradually, with E* returning to roughly the value observed at the beginning of the test (Fig. 4A, B).

The effect of MMP inhibition was concentration-dependent, the higher concentration of galardin (50 μM) being more effective on standard CDLs (Fig. 4A-D). Therefore this concentration was selected and used in further experiments with CDLs in the different mechanical states. As in standard CDLs, the MMP inhibitor enhanced rapidly the stiffness of compliant and stiff CDLs, which then decreased gradually to initial values (Fig. 5A).

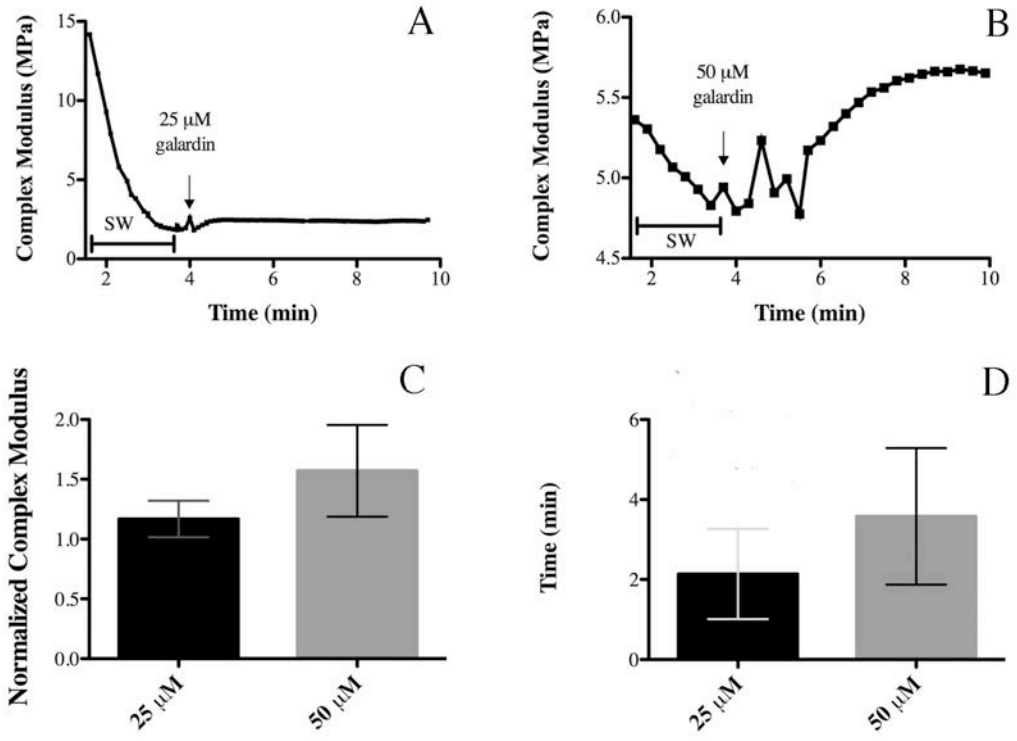


**Figure 4: Effect of MMP inhibition:** (A) Effect of 25µM galardin on a standard CDL. (B) Effect of 50 µM galardin on a standard CDL. (C) Comparison of the effects of different galardin concentrations. Values were normalized against E* values obtained before galardin addition. (D) Comparison of recovery times with different galardin concentrations: duration of the time period between the addition of galardin and the return of E* to the pre-treatment value. The action of galardin was quantified by normalizing the maximum E* reached after galardin addition against the value just before the application of the chemical.

The increase in E* caused by galardin was significantly greater in standard CDLs (mean fold of increase 1.57 ± 0.38; N=5) than in stiff CDLs (mean fold of increase 1.09 ± 0.06; N=5); the increase in E* of compliant CDLs (mean fold of increase1.5 ± 0.47; N= 5) did not differ significantly from the other two (Fig. 5A). Increases in the storage modulus showed exactly the same pattern, while increases in the loss modulus and tan δ did not vary significantly between the three mechanical states (Fig. 5B-D).

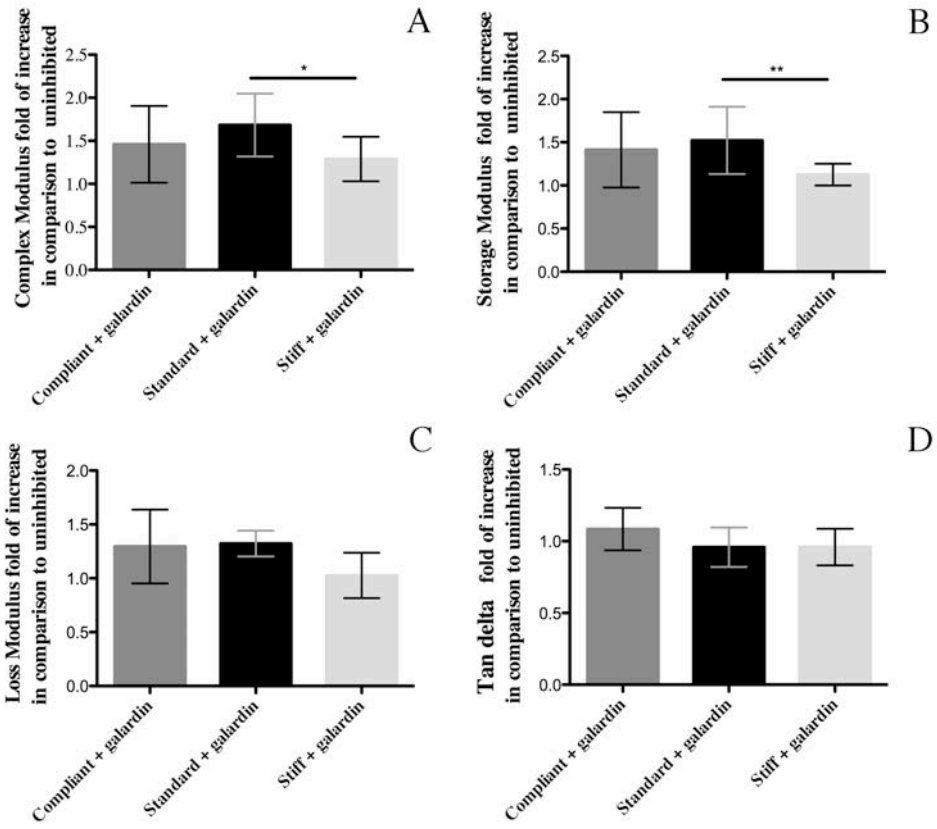


**Figure 5: Effect of MMP inhibition on CDL viscoelasticity:** (A) complex modulus, (B) storage modulus, (C) loss modulus and (D) tan delta of compliant, standard and stiff CDLs treated with 50 μM galardin in PPSW, SW, and AChSW respectively. The action of galardin was quantified by normalizing the maximum E* reached after galardin addition against the value just before the application of the chemical. The asterisk (*) represents statistically significant difference $P<0.05$ and the double asterisk (**) $P<0.01$.

The increase in the E* of standard CDLs caused by galardin was not significantly different from that observed when standard CDLs were stimulated with AChSW (mean fold of increase 2.19 ± 0.86; N=5).

As the mutability phenomenon is reversible, further tests were performed in order to determine if the effect of the MMP inhibitor was reversible. The reversibility of the inhibitory effect of 50 μM galardin on standard, stiff and compliant CDLs was demonstrated by rinsing CDLs with sea water (SW), acetylcholine chloride in sea water (AChSW) or propylene phenoxetol in sea water (PPSW), respectively, after exposure to galardin (Fig. 6A-C).

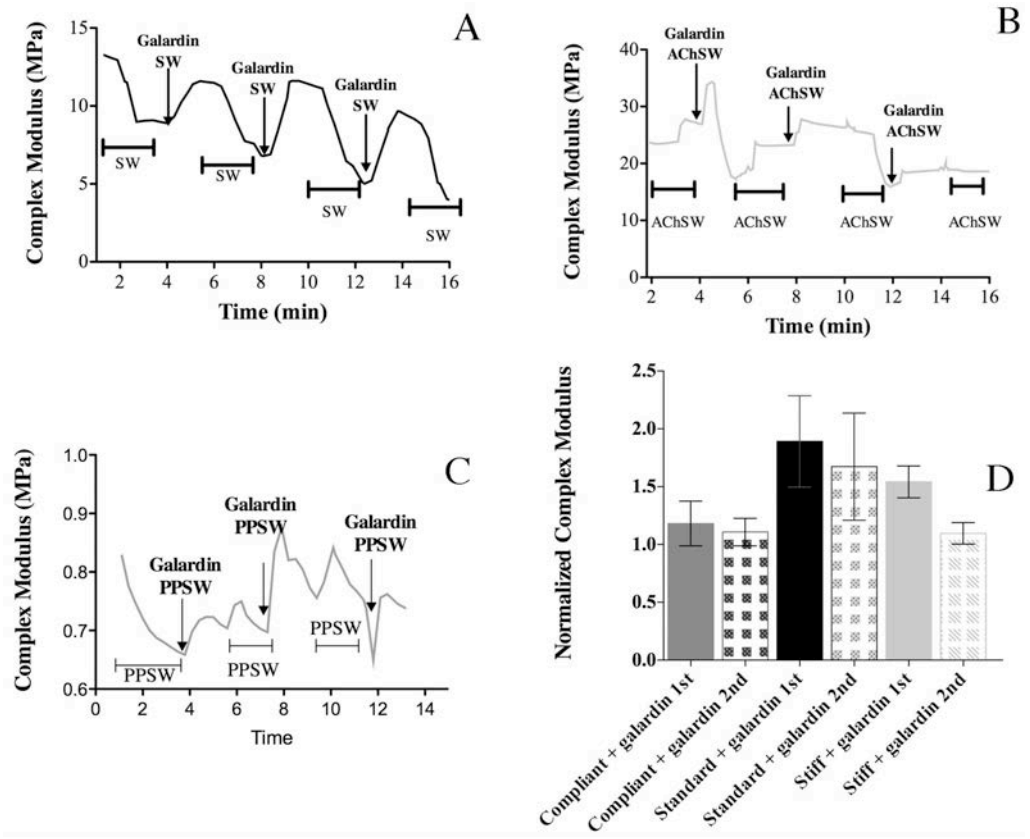


**Figure 6: Reversibility of MMP inhibition:** (A-C) Examples of recordings showing the reversibility of the galardin effect on (A) standard, (B) stiff and (C) compliant CDLs. (D) Normalized results. Tissues in compliant, standard and stiff states were stimulated with 50 μM galardin, washed and treated again with galardin. Data are expressed as means ± SD.

The inhibitory effect was reversible and could be subsequently repeated. Although in all three mechanical states the mean normalized stiffness of the second treatment was lower than that of the first, these differences were not significant (Fig. 6D).

### 3.1.3 Gelatinolytic activity of CDLs in different mechanical states and the effect of galardin

A similar pattern of gelatinolytic bands with molecular weights ranging from 35 to 250 kDa was obtained for standard, compliant and stiff states (identified in CDLs in the different mechanical states (Fig. 7A, D).

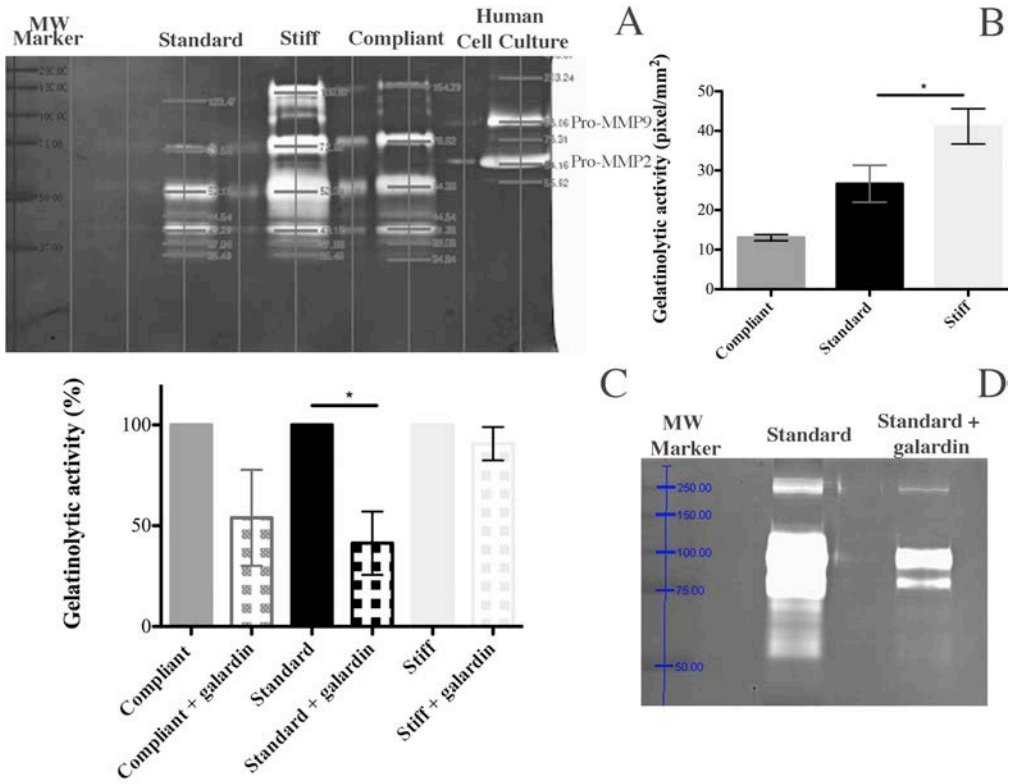


**Figure 7: MMPs of CDLs in compliant, standard and stiff conditions visualized by gelatin zymography:** (A) Zymogram showing pro-enzyme and active MMP band profile of CDLs in the three mechanical states, compared with human cell line showing MMP-2 and MMP-9 activity. (B) Optical density of MMPs activity in CDLs in the three mechanical states. (C) Comparative densitometric analysis of scanned gels of CDLs in the different mechanical states with and without 50 µM galardin. Data are expressed as means ± SD. The asterisk (*) represents statistically significant difference $P<0.05$. MMPs were detected in more than six animals for each of the three mechanical states. (D) Zymogram comparing standard CDLs with and without galardin treatment.

Stiff CDLs had clearly the highest level of gelatinolytic activity in all five zymograms (Fig. 7A), and densitometric analysis showed that there was a progressive increase in total gelatinolytic activity from the compliant to the stiff states (Fig. 7B).

Where the resolution of the bands was high enough (as in Fig. 7A), densitometric analysis indicated that the gelatinolytic activity of stiff CDLs was higher than that of standard and compliant CDLs at all molecular weights, the difference being particularly great at molecular weights above 53 kDa (up to six times higher in the example shown in Fig. 7A). This, however, could not be confirmed in all cases. Overnight treatment of CDLs with 50 µM galardin had little effect on the mean normalized gelatinolytic activity of stiff CDLs, caused a pronounced and statistically

significant reduction in that of standard CDLs, and caused a pronounced, but not statistically significant, reduction in that of compliant CDLs (Fig.7 C, D).

# 4. Discussion

## 4.1 Basic organization and mechanical properties of CDLs

The biomechanical behavior of the CDL is dependent on the composition and organization of its extracellular components. The CDL consists of parallel aggregates of collagen fibrils to which proteoglycans are attached, potentially serving as binding sites for molecules responsible for interfibrillar cohesion [2,5,19]. Available evidence suggests that interfibrillar cohesion is mediated by complexes of molecules, some constitutive and others regulatory [2,19-21,40]. The collagen fibril bundles ('fibers') provide the ECM with mechanical integrity and strength, and are delimited by a network of fibrillin-containing microfibrils, which is an elastic component that may provide resilience and help the CDL to re-shorten after elongation [2,5,11,12]. As for other mutable collagenous structures, the mechanical adaptability of the CDL depends on the modulation of interfibrillar cohesion and not on changes in the organization or mechanical properties of the collagen fibrils [5,36]. There is evidence that the juxtaligamental cells (JLCs) control this process in the CDLs and other MCTs [2,36].
In the present investigation, there was a high level of variation in the mechanical results generated by DMA, which is reminiscent of the inter-individual variability that has previously been reported in MCTs of P. lividus and other echinoderms [41,42]. To compensate for this effect, which could have masked the influence of chemical agents on CDL mechanical behavior, for each mechanical condition (compliant, standard and stiff) we used five animals, and a minimum of three CDLs from each animal, one for each mechanical condition.

## 4.2 Effect of MMP inhibition on CDL viscoelasticity

During the development, growth and remodelling of load-bearing connective tissues in mammals, fibrillar collagens are secreted and continuously degraded by MMPs [23-25]. It is notable that the sea urchin genome includes at least 26 MMP

genes with significant similarity to those of mammals [43,44]. If MMPs have a role in the variable tensility of MCTs, they are likely to contribute to MCT destiffening by hydrolyzing components of the interfibrillar crosslink complexes and therefore their inhibition should reverse and/or block destiffening.

The MMP inhibitor used in this investigation was galardin, or GM6001, which is highly potent against mammalian MMP-1, -2, -3, -8 and -9. It has a collagen-like backbone, to facilitate binding to the active site of MMPs, and a hydroxamate structure, which chelates the zinc ion located in the catalytic domain of MMPs [45-49]. Such chelation results in alteration of the attached MMP molecular conformation, blocking its proteolytic activity against extracellular matrix components and other substrates [45-49]. The involvement of MMPs in CDL mutability was supported by our finding that galardin (50 μM) increased the stiffness (E*) and storage modulus (E´) of CDLs in all three mechanical states, although it had a significantly lower effect on stiff CDLs. The preferential enhancement of the storage modulus and lack of effect on the loss modulus indicates that externally applied force was transferred more efficiently to the stiff and inextensible collagen fibrils, which would be a consequence of the strengthening of interfibrillar cohesion. The involvement of MMPs in CDL mutability was also suggested by the observations that (1) the increase in E* of standard CDLs following galardin treatment was not significantly different from that observed when standard CDLs were treated with AChSW and (2) that, as is the case for in vivo changes in mechanical properties, the action of the MMP inhibitor was reversible.

If MMPs contribute to the mutability of the CDL and of MCTs in general, their primary role could be reactive or constitutive: either it is only when the tissue is destiffening that activated MMPs are present in the extracellular environment and degrade their substrates, or activated MMPs are continuously present in the extracellular environment, and what varies is the extent to which the enzymes are inhibited, less inhibition resulting in destiffening and more inhibition resulting in stiffening. We found that galardin stiffened CDLs in all three mechanical states, implying that in all three states there is (1) ongoing MMP activity, which thus supports the constitutive model, and (2) ongoing production of crosslink components.

The significantly weaker effect of galardin on stiff CDLs than on standard or compliant CDLs would be expected, since in stiff CDLs MMP activity would already be greatly suppressed, together with the obvious fact that stiff CDLs must have much less capacity to stiffen further than standard and compliant CDLs. The constitutive model is illustrated in Fig. 8 (which is another "three-state" model: see Motokawa and Tsuchi, 2003) [50]. We hypothesize that the stiffness of the CDL is adjusted through the modulation of constitutive MMP activity. Since in the collagenous tissues of other animals, activated MMPs are constrained mainly by enzyme inhibitors, such as TIMPs [39,51] we further hypothesize that the control of CDL stiffness depends ultimately on the rate of release into the extracellular environment of endogenous MMP-inhibitors.

One aspect of our results that still requires explanation is the insignificant difference between the effects of galardin on CDLs in the compliant and standard states. At the moment we can only speculate that this relates to an asymmetry in the changes in MMP activity responsible for the compliant→standard and standard→stiff shifts, i.e. the increase in crosslink density associated with the compliant→standard shift, and the corresponding degree of MMP inhibition, may be much less (and perhaps too low to be detected by enzyme zymography) than the increase in crosslink density and degree of MMP inhibition that effect the standard→stiff shift. It is also possible that the compliant→standard shift relies mainly on a mechanism other than MMP inhibition. It appears that different mechanisms are responsible for the compliant→ standard and standard→stiff changes occurring in holothurian dermis [21].

### 4.3 Gelatinolytic activity of CDLs in different mechanical states and the effect of galardin

MMPs have a wide spectrum of activities: in particular, they degrade all extracellular matrix components, as well as growth factors, pro- and anti-inflammatory cytokines and chemokines, and they also modify apoptotic signals and regulate the immune response. As a consequence, they play a fundamental role in

tissue morphogenesis, wound healing, tissue repair and remodelling, and in the progression of some diseases such as cancer [22-25,31].

Included amongst the potential substrates of some MMPs is gelatin, i.e. collagen that has been denatured in the first stage of the degradation process [24, 39, 51]. We analyzed the gelatinolytic activity and distribution pattern of CDLs in different mechanical conditions using gelatin-zymography. This detects the presence of activated MMPs, inactive pro-MMPs and some MMPs previously bound to TIMPs [39]. No qualitative differences in the general pattern of gelatinolytic activity were observed between standard, stiff and compliant conditions. There were, however, significant quantitative differences, with a progressive increase in total gelatinolytic activity in the compliant, standard and stiff states (Fig. 7A, B). The absence of a P. lividus protein database and differences between the proteolytic patterns of the CDL and mammalian tissues prevented identification of the distinct enzymatic bands, although comparison with the gel of a human cell line suggested that mammalian MMP-2-like and MMP-9-like enzymes were not present.

Where there was good separation of the enzymatic bands, it appeared that the higher gelatinolytic activity of stiff CDLs was due to stronger activity at higher molecular weights, which probably resulted from the presence of MMP-MMP complexes and MMPs bound to endogenous inhibitors [39]. The presence of MMP-MMP and MMP-TIMP complexes is associated with the inactivation and removal of MMPs, the latter occurring at least partly by endocytosis [39,51] This positive correlation between degree of stiffness (compliant<standard<stiff) and increasing levels of higher molecular weight complexes (resulting from increasing inhibition of activated MMPs) is consistent with our model (Fig. 8). More difficult to explain is the apparent positive correlation between degree of stiffness and the total gelatinolytic activity (Fig. 7B), which seems to be the opposite of what would be expected if MMPs cause destiffening. However, it has to be stressed again that gelatinolytic activity results from the presence of not only active MMPs, but also of their inactive pro-enzymes and the molecular complexes associated with the removal and degradation of MMPs (i.e. MMP-TIMP and MMP-MMP complexes) [39]. It is possible that in the standard and stiff states, in which there is increasing inhibition of

MMPs, there is a progressive accumulation of MMP-TIMP and MMP-MMP complexes in the extracellular environment, perhaps because the rate of production of these "disposal complexes" exceeds their rate of removal and degradation (Fig. 8).

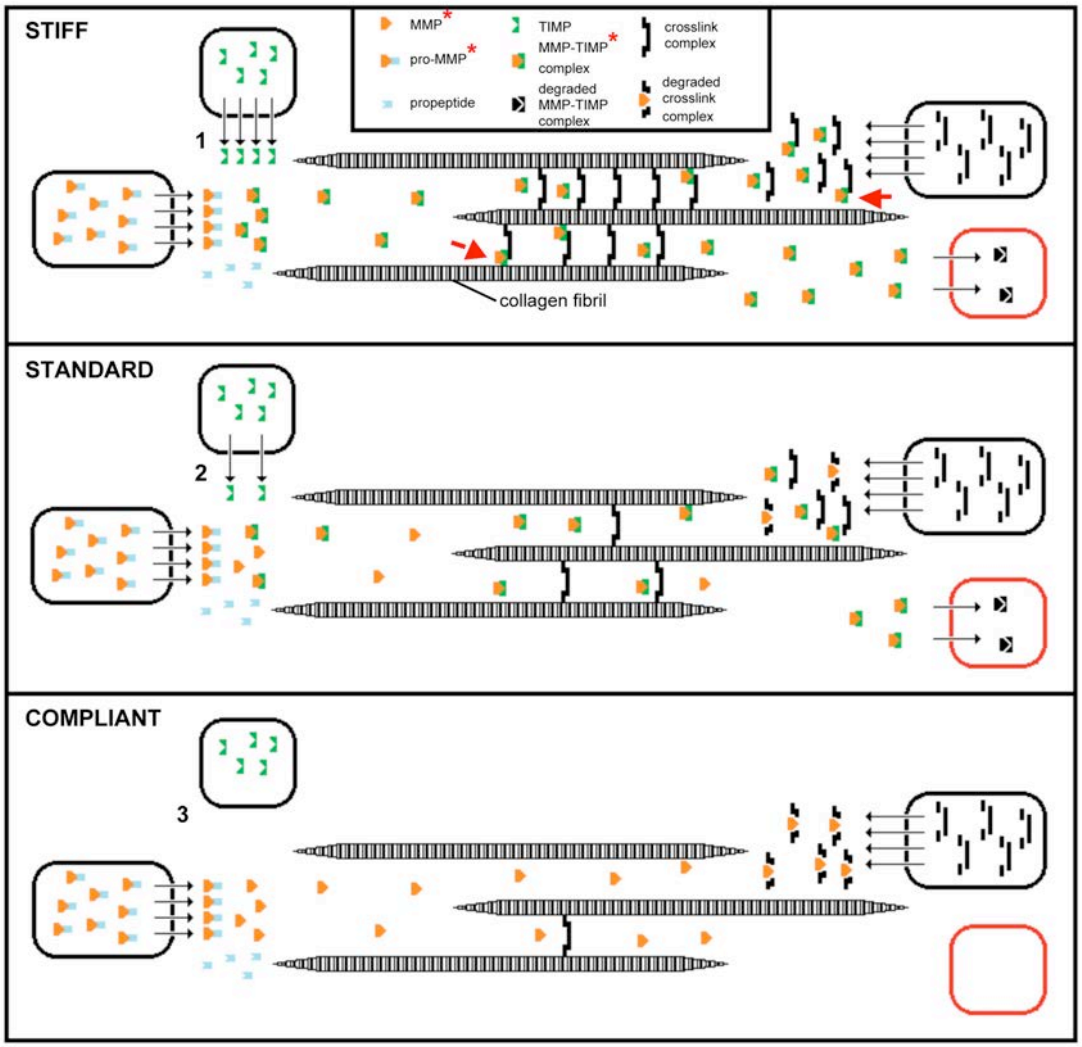


**Figure 8: Hypothetical model of the involvement of MMPs in MCT mutability.** It is known that MCTs consist of discontinuous collagen fibrils crosslinked by complexes of molecular components, and that changes in the mechanical properties of MCTs result from rapid changes in the strength of the interfibrillar cohesion that is mediated by these crosslink complexes. We found that the synthetic MMP inhibitor, galardin, increased the stiffness of CDLs in all three mechanical states, which suggests that in all three states there is ongoing MMP activity that has the potential to degrade components already incorporated into existing crosslink complexes and components that have been secreted but not yet incorporated, and ongoing synthesis and release of new crosslink components. The model acknowledges that MMPs are synthesized and secreted as inactive pro-enzymes, then activated extracellularly by proteolytic removal of the pro-peptide domain [39, 51, 52]. It is envisaged that crosslink components are synthesized and secreted separately, then assembled extracellularly to form functional complexes. The black boxes represent cells, although it should be noted that the three

processes do not necessarily occur in different cell-types. The red box represents the process by which MMP-TIMP complexes are removed and degraded. For the sake of simplicity, the model assumes that activated MMPs and new crosslink components reach the extracellular environment at a constant rate. It is hypothesized that interfibrillar cohesion is regulated only through changes in the rate at which an endogenous MMP inhibitor (which we assume is a TIMP-like molecule) is released into the extracellular environment. In the stiff state there are high levels of TIMP secretion (1), MMP inhibition and crosslinking. In the standard state there are intermediate levels of TIMP secretion (2), MMP inhibition and crosslinking. In the compliant state there are low levels of TIMP secretion (3), MMP inhibition and crosslinking. Also represented is the possibility that an endogenous inhibitor could function as a component of the crosslink complex (red arrowheads) and thus have a dual function (which may apply to TIMP-like tensilin). The model also assumes that the production of MMP-TIMP complexes exceeds the rate of removal and degradation of MMP-TIMP complexes, which would account for the positive correlation between degree of CDL stiffness and total gelatinolytic activity. The components marked with a red asterisk contribute to the gelatinolytic activity of CDLs as quantified by gelatin zymography.

The effects of galardin on the gelatinolytic activity of the CDLs provide further support for the model illustrated in Fig. 8. Galardin did not affect the gelatinolytic activity of stiff CDLs, which is expected, since, according to our model, MMP activity of stiff CDLs would already be maximally inhibited. Our model predicts that in both the standard and compliant states there should be significant MMP activity, and in accordance with this we found that galardin significantly reduced the total gelatinolytic activity of standard CDLs, although its effect on compliant CDLs was marked but not statistically significant.

As discussed in connection with the similar pattern of effects of galardin on the mechanical properties of the CDL, the lack of differentiation between the gelatinolytic activities of compliant and standard CDLs after galardin treatment may be due to an asymmetry in the changes in crosslink density and MMP inhibition associated with the compliant→standard and standard→stiff shifts, or because MMP inhibition is not involved in the compliant→standard shift.

### 4.4 Identity of the endogenous inhibitor

According to our hypothesis, the stiffness of the CDL and other echinoderm MCTs is determined by the rate of secretion of one or more endogenous MMP

inhibitors. It may of course be relevant that tensilin – a possible regulatory protein from MCT that has been fully sequenced – shows homologies to mammalian TIMPs. Holothurian tensilin aggregates collagen fibrils and stiffens samples of whole dermis. It is stored intracellularly and has been immunolocalized to the intracellular granules of juxtaligamental-like cells in holothurian dermis [19 ,Trotter et al., unpubl. data]. Whilst it is the role of tensilin as a potential regulatory stiffening agent that has been emphasized, it is notable that it cannot induce maximal stiffening (equivalent to the standard→stiff shift) of holothurian dermis, though another incompletely characterized protein can do this [20,21]. Furthermore, the homology of tensilin's deduced peptide sequence to mammalian TIMPs is stronger in its N-terminal domain [19], which in mammalian TIMPs forms the unit that inhibits MMPs [51,52]. It is therefore possible that tensilin retains the capacity to inhibit MMPs and even that this is its main function. Like mammalian TIMP-3, tensilin binds to the sulphated GAGs of ECM components through its C-terminal domain [19], which suggests that tensilin may be a TIMP that has evolved the additional capacity to act as an adjuvant stiffener. We therefore suggest in our model (Fig. 8) that the endogenous regulatory inhibitors are TIMPs that may also contribute to crosslink complexes.

### 4.5 Comparison with the uterine cervix

The mammalian uterine cervix can be regarded as another example of a mutable collagenous structure that demonstrates reversible changes in stiffness. Its mutability differs from that of echinoderm MCTs in, amongst other aspects, its much longer time course. It occurs in three stages: cervical 'ripening' begins about four weeks before birth and results in expansion of the cervical canal to 3-4 cm, followed by cervical dilatation to 10 cm during parturition itself, which occurs within hours, and then by the recovery of cervical stiffness, which takes days. These changes in mechanical properties result from modification of the biochemical composition and structure of the cervix and by MMP-dependent degradation of collagen fibrils and other ECM components. The MMP activity is modulated by endogenous inhibitors, including TIMPs, which become particularly important as a brake on the degradation process at the end of parturition [31, 34, 53]. The involvement of MMPs in cervical destiffening is reactive: the increase in MMP activity results from a dramatic rise in

MMP concentrations following increased synthesis by neutrophilic leukocytes, and is under mainly endocrine control [31, 53]. Thus regulation of MMPs at the transcriptional level is an important component of the cervical mutability phenomenon. In contrast to this, we postulate that in echinoderm MCTs the regulation of MMP activity occurs mainly at the extracellular level and is determined by the rate of inhibitor secretion. This provides a much faster responsiveness (within timescales of <1 s to minutes) than could a mechanism dependent on the adjustment of protein synthesis and is amenable to nervous control, which allows changes in the mechanical state of MCTs to be coordinated with the activity of contractile systems.

We believe that Fig. 8 illustrates the simplest model that can integrate our results with information derived from other MCTs. Aspects of it are admittedly speculative, but these are testable by further experimentation. The uniqueness of echinoderm MCT cannot be exaggerated: collagenous connective tissue that is directly innervated by the motor nervous system, and that can alter drastically and reversibly its mechanical properties within short physiological timescales, appears to have evolved only in the phylum Echinodermata (though a “pre-neural” version of the phenomenon occurs in the Porifera [54]). Despite this restricted taxonomic distribution, the investigation of MCT has the potential to provide information of widespread biomedical applicability [2, 55].

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# Chapter VI

## General discussion, summary and future perspectives

The central objective of this thesis was to understand the main basic mechanisms that govern CDLs hierarchical organization, biochemistry and biomechanical reversibility. The two focal questions that we tried to answer were:

- Which are the ECM components directly involved in CDL mutability?
- Which are the mechanisms that are behind tissue dynamicity? For that, we hypothesised the possibility of ECM remodelling and the potential involvement of MMPs in the mutability phenomenon.

We consider that a broader understanding of CDL physiological process within mutability could open new perspectives for the development of a dynamic and reversible biomaterial enhancing tissue regeneration.

## 1. The Compass Depressor Ligament Model

The mutable collagenous tissue used in this thesis was the compass depressor ligament (CDL) that *in vivo* shows reversible changes in its tensile properties [1]. We have mainly focused on the reversible stiffening and destiffening of CDLs, which means that CDL never loses completely its tensile strength such as the autotomy structures (irreversible destiffening) [1-5].

The different mechanical states that mimic the mutability of the tissue *in vivo* was achieved by using an effective echinoderm anaesthetic (propylene phenoxetol) and a neurotransmitter (acetylcholine) to obtain compliant and stiff CDLs respectively [1,6,7]. The standard condition was obtained by immersion of CDL in seawater. One problem regarding CDL and MCT variable tensility is the use of acetylcholine, which is an excitatory neuromuscular transmitter, and the presence of muscle cells in MCTs [8, 9]. However, it has been also confirmed that muscle cells are not always present in MCTs, and that acetylcholine induces changes in the passive mechanical properties of MCTs even on that tissues that lack myocytes (e.g. central spine ligament of echinoid, cirral ligament of crinoid), confirming that it acts in the extracellular matrix itself rather than in the muscle cells [9]. Also it has been argued that the contractile

myoepithelium that delimits the CDL occupies only 8% of CDL cross-sectional area. To be responsible for CDL variable tensility, the muscular component of the ligament would have to generate tensions several times higher than that observed in the strongest muscle known [1, 4, 9].

CDL was chosen as a model since one of the main objectives of this thesis was to understand the key mechanisms that govern the mutability phenomenon. Although these tissues are not present in anatomical sites with strong capacity to regenerate, they have the advantage of having a common constitution and a perfect parallel alignment without the presence of skeletal components [1, 4, 10,11].

## 2. Molecular Composition of CDL Extracellular Matrix

The composition and structure of CDL extracellular matrix regulates its ability to undergo variable tensility in a short timescale [1,4,12]. Collagen, the most abundant protein in animals, is mainly located in the extracellular matrix, and confers mechanical stability, promotes elastic energy storage but also regulates cell adhesion, supports chemotaxis and migration [13-19]. Although having mainly a structural role, collagen has the ability to interact with other extracellular molecules, playing an important role in tissue development and homeostasis [13,20-22]. Typical locations are the mesoglea of jellyfish, the cuticle of worms, basement membranes of flies and the connective tissues of mammals [13]. Many collagens are preserved from invertebrates to vertebrates, where their molecular hallmarks are the repetitions of Gly-X-Y sequences in which the amino acid residues in X- and Y- position are proline and hydroxyproline, and have a triple helical structure that is built by three polypeptide chains [13,14,22-24]. The long, rod-like collagen molecules, which are polypeptides with triple helical domains comprising 990 to 1020 amino acids per polypeptide chain, are arranged in a staggered configuration to form striated fibrils [13,14,23-25]. The molecules are deposited side by side and parallel but staggered with respect to each other creating a periodicity known as the D-band. Multiple fibrils assemble with the aid of cross-linking macromolecules (proteoglycans) resulting in fibers. The collagen fibrils aggregate into more complex supramolecular structures to became part of the structural connective tissues [13,14,20-27].

The morphological characterization presented in chapter III, revealed that CDL is a band of soft connective tissue 9-10 mm long and 0.2-0.4 mm wide that consists mainly of collagen fibrils with a variable diameter and organized into parallel fibers that determine the tensile strength of the ligament [1]. From literature it is known that fibrillar collagen is present in type I, II, III, V, XI, XXIV and XXVII, however only type I presents a two α1 (I) chains and one α2 (I) chain composition that were already identified in echinoderms, namely in sea-urchin embryos but also in adult tissues (i.e. *Paracentrotus lividus*) [13,14,22,28-36]. Molecular literature data on chain composition and gene organization as well as the morphological and spectroscopy results described in chapter III and IV suggest that the fibrillar structure observed in CDLs has similarities with collagen type I. The resemblances were found not only regarding its fibrillar structure, where a cross striation banding of 60 nm was found (mammalian d-banding - 67 nm) but also concerning the presence of some characteristic amino acids of collagen backbone like proline and hydroxyproline (detected by Raman) and protein conformation (FT-IR results revealed the prevalence of helical structure characteristic of type I collagen). These results are in agreement with the high homology that was obtained between some echinoderm and vertebrate proteins, namely the triple helix of collagen that was conserved in both invertebrates and vertebrates [29,37,38]. A clear phylogenetic proximity was found between echinoderm-striated fibrils and mammalian type I collagen fibrils. Wada *et al.* showed the equivalent phylogenetic tree for collagen genes where it is possible to observe the proximity of sea urchin genes to those coding mammalian type I collagen fibrils [22,38].

It is also known from literature that mature connective tissues are constituted by heterotypic collagen fibrils, meaning that tissues such as cornea, skin, tendon, cartilage, uterine cervix contain more than one types of collagen in their constitution [13,17,39-42]. Although we did not observe structural differences in collagen fibrils, we cannot exclude the possibility that other collagens types exist in CDL matrix.

The mechanical integrity and strength of the ECM is provided by collagen fibers. However, they are delimited by a network of fibrillin containing-microfibrils, which is the elastic component of MCTs (similar structures were already identified in

the sea cucumber dermis) providing resilience but also helping CDL to re-shorten after elongation [1,4, 43,44].

The extracellular matrix of CDL and of the main connective tissues of vertebrates is not exclusively composed of fibrous proteins, such as collagen and fibrillin; it also contains proteoglycans (PGs). PGs fill the interstitial space of the extracellular matrix in the form of a hydrogel, performing several functions such as buffering, binding and having force-resistance properties. The interfibrillar cohesion is maintained partly by the PGs-fibril relationship (PGs may be covalently and non-covalently attached to fibrils) that must hold fibrils against fluids flow and tissue stress history, preventing uncontrollable sliding, thus contributing to the stability of collagen fibers [45-48]. PGs in CDL were visualized at a specific position in the D-period of collagen fibrils. However, this is not exclusive of CDLs. Lattice and interfibrillar PGs were already observed in holothurian dermis and sea-urchin spine ligament, respectively [46-48]. PGs probably stabilize collagen fibrils acting as binding sites for specific proteins responsible for interfibrillar cohesion [4,45,48,49]. Proteoglycans are also composed of glycosaminoglycans side chains that are covalently linked to a specific protein core. Glycosaminoglycans chains are unbranched polysaccharides composed of repeating disaccharides units that can be divided into sulphated (chondroitin, dermatan, heparin and keratin sulfaphe) and non-sulphated (hyaluronic acid) [49-52]. Glycosaminoglycans were visualized distributed along CDL matrix, and identified by FTIR and Raman spectroscopy as belonging to the sulphate family. Sulphate polysaccharides were already identified in sea urchin (chondroitin/dermatan and heparin sulphate) embryos as well as in adult tissues (sulphated GAGs) but also in the body wall of sea cucumber (highly sulphated fucose branches of chondroitin sulphate) [4,53-55]. Oversulphated GAGs structures were identified in aquatic species (e.g. molluscs, sharks, crabs, squids, sea cucumbers), suggesting that they may play an important role in the protection of the organisms against foreign bodies [50,54,56]. As glycosaminoglycans are hydrophilic, they assume an extended conformation, inflating interfibrillar spaces, forming channels where water molecules move and enabling the matrices to resist high compressive forces [20,21,57]. Although they have an important role in maintaining the ECM hydrated, they also contribute to cell growth, differentiation, morphogenesis and

tissue homeostasis [20,21,49].

All ECMs are highly dynamic structures that are always under continuously remodelling, where tissue homeostasis is mediated by the coordinated balance between metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [20,58-64]. MMPs were thought to work mainly as enzymes that degrade structural components of the ECM, although it is known that MMPs proteolysis create space for cell migration, regulating tissue architecture and can/cannot trigger or modify the activity of signalling molecules. They play a fundamental role in morphogenesis, wound healing, in the progression of some diseases (cancer, cardiovascular, arthritis) in mammals, but also in echinoderm development and regeneration [58,61-70]. Analysis of the sea urchin genome of the *Strongylocentrotus purpuratus* has already revealed 240 metalloproteinase genes that represent the 23 families expressed in vertebrates [65-68].

In the present work, the possible involvement of MMPs in mutability was hypothesised. This is related to fact that tensilin (stiffening agent identified) presents in its C-terminus, a collagen-binding domain that is vulnerable to proteolysis and also because it has an homology (21-36%) with a TIMP [4,71]. It was proposed that the destiffening of holothurian MCT could involve a specific protease that inactivates tensilin. Also, apart from one study regarding the dermal liquefaction of holothurian body wall, whose relationship to mutability is not clear, till now, as far as we known, no efforts were done to understand the potential role of MMPs in CDL mutability phenomenon [72]. Taking this is consideration the main objective of the work described in chapter V was to evaluate the presence of MMPs and to explore their possible role in the mutability phenomenon. Regarding the presence of MMPs in CDLs, we were able to observe a distinct proteolytic pattern comparing to mammalians (MMP-2 and -9 expressed in humans were not expressed in CDLs). However, the absence of a *P. lividus* genomic database render problematic the identification of the enzymatic bands that could correspond to active MMPs and inactive pro-MMPs, as well as enzymatic complexes composed of MMPs bound to TIMPs [66].

As discussed above, strong similarities were observed between vertebrate and invertebrate connective tissues. However, an invariable difference in MCTs is the presence of neurosecretory cells called juxtaligamental cells (JLCs) [1,4,73-75]. They are considered as neurosecretory-like cells containing electron-dense granules, in which molecules are stored (in the sea cucumber model granules contain tensilin) that are able to interact with collagen fibrils, affecting interfibrillar cohesion and modifying MCT tensile state [4, 71,73,76,77]. Two populations of JLCs (distinguishable by size or shape of the granules) were identified in the holothurian model, suggesting that one cell type could release both stiffening and de-stiffening factors. In CDLs, the main cellular elements observed by TEM and FEG/ESEM were also the JLCs with a preferential circular profile. Only one population of JLC was observed, containing two different granules inside: the dark ones, which are electron-opaque, and the light ones that are partially electron-lucid. We think that the different granules represent different degrees of granule maturation, being the dark ones the fully mature comparing to the light ones (immature), since only differences in the dark ones could be correlated with the different mechanical conditions.

## 3. Molecular Mechanism underpinning CDL Mutability

The three different mechanical states that CDL can assume in *vivo* were mimicked *in vitro* by immersion of the animal in an anesthetic, in a neurotransmitter and in seawater to obtain the compliant, the stiff and the standard condition, respectively, as it was already described (chapters III, IV and V).

Biomechanical characterization of the three different mechanical states with a dynamic mechanical analyzer revealed that exposure of the CDLs to acetylcholine resulted in a reversible increase in stiffness due to action on Ach receptors in some kind of control pathway. This behavior has already been demonstrated in others sea-urchins MCTs structures such as the peristomial membrane and the ligaments of the spine-test joint (Candia Carnevali *et al.* 1990 and unpublished results) [1,4,6,78]. In response to acetylcholine, the Aristotle's lantern was held in a retraction position, making the CDLs longer and thinner than in the standard condition. On the basis of these results we can say that acetylcholine may lead to an increase in stability of the

linkages responsible for interfibrillar cohesion, thus inhibiting slippage between the collagen fibrils.

An opposite effect on CDLs is produced by propylene phenoxetol, which reduces CDL stiffness, since it blocks nervous conduction (as it has been demonstrated in ophiuroid and squid axons) to an extent that mimics tissue compliance in *vivo* [7]. Anaesthetization induces softening of all MCTs present in the lantern, provoking the change of the lantern to a protracted position and the shortening and thickening of CDLs due, in part, to microfibrillar retraction. It seems that softening is the result of the weakening/suppression of the proteoglycan-mediated binding of collagen fibrils, which allows interfibrillar slippage to occur [4,6,12].

### 3.1 Are the most abundant proteins of the ECM involved in CDL mutability?

It is known from literature that the molecular mechanism that underpins mutability is not a result of changes in the mechanical properties of collagen fibrils themselves. All the ultrastructural investigations performed have failed to demonstrate that MCT variable tensility is accompanied by modification of the banding pattern, shape or organization of collagen fibrils [4,79,80].

In chapter III, interesting correlations were achieved between the extracellular matrix of CDL and the distinct mechanical states. An ECM densely packed with a lower interfibrillar distance was found in stiff CDLs, compared to the standard and compliant conditions. This dense fibril packing seems to be the result of the stretching of CDLs due to the stimulation of particular lantern muscles (the compass elevators) by acetylcholine, and may possibly facilitate the stiffening mechanism. The reduction in the interfibrillar distance may facilitate the attachment of effector molecules, such as tensilin, strengthening the bonds between collagen fibrils, inhibiting slippage and increasing the resistance of the tissue to tensile forces. It is thus possible that a similar stiffening mechanism may occur in other MCTs with an analogous architecture and that are also simultaneously stretched and stiffened, such as the sea-urchin catch apparatus, arms ligaments of crinoids and ophiuroid [81-83].

As it occurs in other MCTs structures, CDL variable tensility depends on the modulation of the interfibrillar cohesion and not on the changes in the organization of collagen fibrils, as it occurs in the uterine cervix during pregnancy. The increase in cervix compliance is a result of biochemical changes and organization of ECM components, as well as degradation of collagen and other ECM components [42,84]. The destiffening of cervical stroma involves a disorganization of the matrix, since collagen with less degree of cross-links is synthesised, resulting in a matrix with high interfibrillar distance between fibrils [42,84-88].

As a consequence of the reduction in the interfibrillar distance in CDL in the stiff condition, all the others ECM constituents rearrange themselves to the new "space" available. In CDL, the consequence of the dense collagen packing has also a pronounced effect on fibrillin microfibrils. The reduced interfibrillar space results in the formation of more dense packed sheets of microfibrils compared to the compliant and standard condition. However, fibrillin-containing microfibrils do not seem to be actively involved in the mutability phenomenon since these structures also appear in tissues that are not mutable, such as lungs, ligaments, lenses, arteries, performing functions of elastic recoil. In CDL they just seem to adjust to the space available in the ECM helping the tissue to re-shorten after elongation [4,43,44,79,89-91].

This fibrillar packing associated with the discontinuous spindle shape of echinoderm collagen fibrils supports the hypothesis that CDL mechanical adaptability is a consequence of the interfibrillar sliding and not a result of changes in collagen structure itself (fibril shortening or elongation) [12]. As there were no significant differences in fibril packing when CDLs pass from the compliant to standard conditions, it seems that different mechanisms could be involved in the mutability phenomena. This is also observed in holothurian dermis and contributes to the conclusion that although exploiting common principles, more than one single molecular mechanism might govern MCTs mechanical reversibility [92,93].

### 3.2 What is the role of JLCs in variable tensility?

The role of JLCs in CDL mutability was studied in detail. Two different populations of granules were observed by TEM in JLCs in CDLs in the different mechanical conditions, where variations in their cytological appearance (namely the intracellular 'dark' granules) were correlated with the different CDL tensile states. The size of the light and dark granules, as well as the quantity of light granules did not change considerably in the different mechanical states. However, significant changes were observed in the quantity of dark granules. What we hypothesize is that the two populations of granules could comprise mature (dark granules) and immature (light granules) stages, since only dark granules present significant changes in number according to mechanical state. As they are more numerous in the standard rather than in the stiff and compliant state it is possible that they are involved in both the standard→compliant and in the standard→stiff transition.

The significant decrease in the number of 'dark' granules when CDLs shift from standard→compliant and from standard→stiff mechanical states, suggests that there are two populations of CDL granules, which could be functionally but not morphologically distinct.

Only mature (i.e. 'dark') granules seem to be involved in the reversible tensility being depleted by unknown mechanism. We did not find evidence of exocytosis. However, a study described in the literature indicated that JLCs granule disappear at the central disc (autotomy plane) of MCT ophiuroid *Amphipholis kochii*, implying the presence in the CDL of an adaptive cellular mechanism for dispersing granule contents rapidly [75].

### 3.3 ECM remodelling or reorganization during the mutability phenomenon?

Cervical remodelling during pregnancy is the main mechanism involved in the transformation of a closed cervix structure to one very compliant that opens sufficiently for birth [42,82-88,94,95]. A significant increase in collagen solubility

and disorganization, GAGs concentration and tissue hydration seems to occur with the progression of pregnancy, where the final phase after delivery ensure the completely recover of the initial tissue integrity by genes transcription involved in matrix repair [42,88,95]. The main function of the cervix is guaranty synthesis of proteins, glycoproteins and proteoglycans, as well as interactions between this components and the extracellular matrix. As this process share dynamicity with MCTs we decide to evaluate the possible CDL remodelling when the tissue change its mechanical properties (chapter IV).

Regarding changes in collagen concentration in the uterine cervix, apparent contradictions between studies seem to exist, but they are probably consequence of the result of the use of different methods [42,85,87,88]. The more recent studies do not report a decrease in collagen content, despite the hypothesis, the most consistent change in association with collagen remodelling with progression of pregnancy is an increase of collagen solubility, resulting in a collagen with less degree of cross-linking with subsequent increase in tissue compliance [42,84,88,95]. We did not evaluate the possible synthesis and change in content of collagen in CDLs in the different mechanical conditions since collagen is a protein with high stability that requires a specific kinetics to be synthetized in opposition to the reversible tensility which is a very fast phenomenon (takes less than one second to a few minutes to occur) [4,12,13,96]. By the contrary, the destiffening of uterine cervix present a higher time-scale of action. After softening (weeks), the cervical ripening is a more accelerated phase occurring in the hours preceding birth in mice and in the weeks or days preceding birth in women. The completely recovery of cervical stiffness after delivery can take days [84,88,94,95].

These changes in the mechanical properties of the uterine cervix are under endocrine control and another important difference is that cervical destiffening is achieved partly through degradation of collagen fibrils and other ECM components via MMPs [95,97]. Although CDL variable tensility involves MMP activity, the structure of collagen (morphological results reported in chapter III) as well as the maintenance of its conformation (biochemical characterization described in chapter IV) suggests that there is no evidence of degradation of collagen fibrils during

variable tensility. It seems that if MMPs are involved in the CDL they contribute to destiffening by working on the degradation of interfibrillar cross-link complexes responsible for interfibrillar cohesion rather than degrading collagen fibrils themselves. This is in agreement with the mechanism responsible for the dermal liquefaction of holothurian dermis that seems to be a result of the digestion of interfibrillar molecules (possibly proteoglycans) by an enzyme that has no apparent effect on the collagen fibrils [72].

Also, in mechanically adaptable connective tissues of mammals (uterine cervix during pregnancy), the alteration in collagen structure and packing is influenced by the composition of glycosaminoglycans (sulphated and non sulphated) attached to the proteoglycans in the ECM that absorb water providing compressive strength to the matrix. PGs have varied functions in signal factor binding and modulating collagen fibril size, spacing and access to proteases [45,94].

With progression of pregnancy, the total GAG content increases and changes its composition. As it was mentioned before, in CDLs GAGs were identified as belonging to the sulphate family. However, we could not observe a significant increase in sulphate GAGs content (compliant→standard and standard→stiff transitions) as normally occurs in mammals with pregnancy progression [42,94]. As we have discussed in the context of collagen, we do not believe that CDL mutability involves synthesis of GAGs due to the promptness of the phenomenon. It seems that they are exclusively involved in interfibrillar cohesion, perhaps serving mainly as binding sites for mutability effector molecules during changes in the tensile state. However, these data do not exclude the possible existence of others GAGs (non-sulphated) as well as its quality changes during mutability.

An outcome of the dense fibril packing of stiff CDLs that involves the reduction in the interfibrillar space is that all the molecules previously occupying that space can be displaced. One can envisage that CDL stretching causes a decrease in interfibrillar distance without necessarily also causing water loss since the spaces between the fibrils are narrower but they are also longer, so that the total volume of the interfibrillar space, and therefore interfibrillar water content, may not change. However, we were able to detect water exudation in CDL in the stiff condition by

FTIR and Raman spectroscopy, which seems to be not a result of stretching but the consequence of another mechanism. Water 'exudation' also occurs when holothurian dermis stiffen from its standard condition, but also in humans connective tissue such as tendon, cartilage and intervertebral discs [93,98-100]. It is possible that water molecules masking fixed charges on GAG side chains might be displaced by stronger interactions between these charges and intermolecular cross-linking agents (such as tensilin) that underpinned tissue stiffening. It is thus possible that the primary changes in macromolecular interactions responsible for CDL stiffening incidentally expel water molecules which itself may secondarily facilitate the development of interactions that strengthen interfibrillar cohesion.

Although we were not able to identify any key molecule responsible for CDL reversibility, and although CDL remodelling does not occur during mutability, FT-IR spectroscopy data revealed subtle adjustment of protein components in the compliant and stiff condition. The absence of CDL remodelling was also in agreement with extensive morphological characterization, where an evident reorganization of the ECM was observed.

### 3.4 Are MMPs involved in mutability phenomenon?

As it was mentioned previously, MMPs are involved in the degradation of collagen during development, growth and remodelling of the ECM of almost connective tissues [20,58,59, 64,101]. Their role in MCT mutability was suggested by Wilkie *et al.*, who surmised that enzymes might have a fundamental role on MCT destiffening, via the degradation of non-collagenous components (interfibrillar crosslink complexes) rather than collagen fibrils, which contributes to interfibrillar cohesion maintenance [4]. The destiffening should be reversed by enzymes inhibition. Taking this in consideration we decided to investigate the possible contribution of MMPs to CDL variable tensility. In chapter V we used the zymography technique (detects the presence of activated MMPs, inactive pro-MMPs and complexes such as MMP-TIMP and MMP-MMP) as well as dynamic mechanical tests to evaluate the presence of MMPs and the effect of an MMP inhibitor (galardin) on the biomechanical properties of CDLs in the different mechanical conditions. A simple

model that incorporates our results, as well as information regarding others MCTs, was described in chapter V.

CDLs in the different mechanical conditions revealed a pattern of MMP activity that seems to be related to the modulation of CDL variable tensility, since there was an increase in tissue stiffness upon addition of an exogenous MMP inhibitor (galardin a broad spectrum inhibitor), whereas it a significantly weaker effect on stiff CDLs was observed. In the context of the fundamental MCT model, the effect of the MMP inhibitor would be to alter the conformation of MMPs blocking its proteolytic capacity (the interfibrillar cohesion will be maintained) stopping the sliding of fibrils resulting in an increase of tissue stiffness. Also, the increase in stiffness of standard CDLs was not significantly different from the one observed when standard CDLs were stimulated with 1mM acetylcholine in seawater and, like the mutability phenomenon *in vivo*, the action of galardin was reversible.

The fact that galardin (MMP inhibitor) increase CDLs stiffness in the different mechanical conditions suggests that there is: (1) ongoing MMP activity that has the capacity to degrade components of ECM that can be already incorporated into the crosslinks but also components that have been already produced but not yet incorporated, (2) as well as ongoing synthesis and release of crosslink components. We hypothesised that CDL variable tensility is adjusted by the modulation of activated MMPs that are continuously present in the ECM environment and that what changes is the extent to which they are inhibited (less inhibition results in destiffening and more inhibition result in stiffening), which implicates the release of endogenous MMP-inhibitors to ECM. We hypothesised that CDL variable tensility depends on the rate of release of endogenous inhibitors (MMP-inhibitors) into the extracellular matrix.

The increase of tissue stiffness due to the action of a synthetic inhibitor (galardin) was higher in compliant and standard tissues than in stiff CDLs, suggesting that there is an upper limit on stiffness capacity together with the fact that MMP activity in stiff CDLs would be already significantly inhibited (by endogenous MMP-inhibitor). We hypothesised that CDL variable tensility is determined by the secretion

of one or more MMP inhibitors; however we cannot exclude the fact that tensilin, which has an important regulatory stiffening role on MCT mutability, has homologies with TIMPs. It is possible that tensilin has the capacity to inhibit MMPs as well as a supplementary stiffening capacity.

CDLs in the different mechanical conditions revealed a pattern of gelatinolytic MMPs that were different according to the degree of tensility. Stiff CDLs showed a remarkably higher MMP activity at higher molecular weights compared with the standard and the compliant conditions. The presence of higher activity at higher molecular weight suggests the presence of MMP-MMP complexes and MMPs bound to endogenous inhibitors (MMP-TIMP complexes), which is associated with inhibition and removal of activated MMPs. The possible correlation between the degree of stiffness and the total gelatinolytic activity is explained by the possible accumulation of MMP-TIMP and MMP-MMP complexes in the ECM where their production surpasses their degradation and removal rate.

From literature it is known that the destiffening of the human uterine cervix during pregnancy is characterized by modification of the biochemical composition and structure of the cervix with an increase in concentration and expression of MMPs that will degrade ECM components [97,101-104]. The action of MMPs are mainly concentrated in the rippening phase (begin in four weeks before birth), dilatation (during parturiation) and postpartum (after delivery). Although it is tempting to assume that a analogous mechanism is present in MCTs, it is possible that the MMPs involvement in mutability may be subtler and more diverse than the already known models [105]. The participation of MMPs in cervical destiffening is reactive (i.e. induced) and in contrast we postulate that MMP activity in MCTs is constitutive (i.e. always present in ECM) and the regulation of this activity is determined by the rate of inhibitor secretion. This is in agreement with fast time-course of mutability, since the production of proteins could take more time as it occurs in the uterine cervix.

## 4. Summary of CDL Mutability phenomenon

The results obtained in this thesis open new perspectives in biomedical research concerning the potentialities of echinoderms (sea-urchin) as an animal

model, whose particular structure, tissue organization but also biomechanical properties show strong similarities with mammalian connective tissues. As suggested by the results presented in this thesis (chapters III, IV and V), the ECM of CDL present microarchitecture and biochemistry similar to several human tissues, such as tendon, ligament, cornea, skin and blood vessels [13,14]. Furthermore, from the biomechanical point of view it is also known that the tensile strength of the mutable sea-urchin spine ligament can reach values analogous to those described for mammalian tendon [106,107].

Studying CDL variable tensility (see Fig.1) we were able to determine which are the contributions of the main ECM constituents to the phenomenon, as well as to understand that the entire matrix suffers a strong rearrangement and not a remodelling as commonly happens in the uterine cervix throughout pregnancy. Although strong similarities exist regarding ECM composition between both tissues we cannot forget that the timescale and the kind of control are completely different.

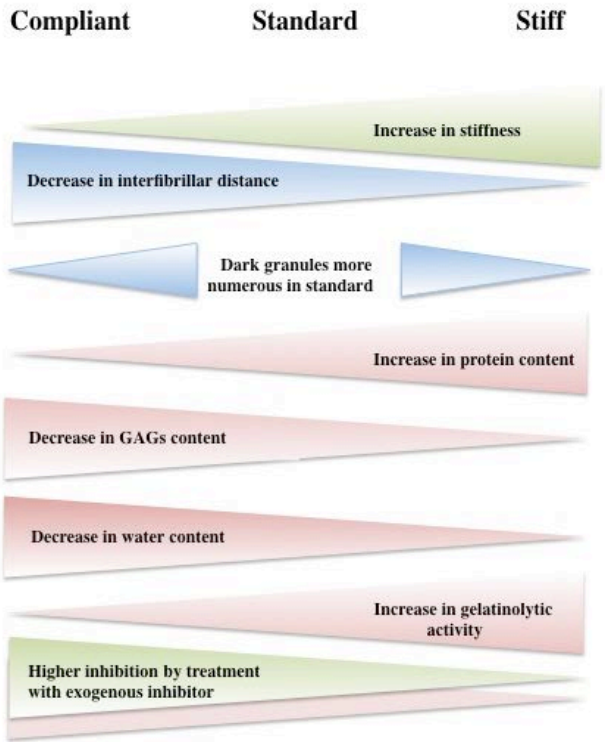


**Figure 1:** Summary of CDL mutability results: green represents biomechanics, blue morphology and red biochemistry.

Our results also suggest, that MMPs contribute to the mechanical adaptability of sea-urchin ligament. Mutability is an ancestral property of the ECM, that may have been lost during evolution. It is possible that what may be ancestral is the connective tissue consisting of collagen fibrils that are not connected by stable linkages, since such connective tissue occurs in Porifera, Cnidaria and Echinodermata. The absence of stable linkages between the fibrils could have facilitated the evolution of mutability mechanisms in Porifera and Echinodermata [4,108].

## 5. A promise to the future

The results reported in this thesis provide evidence that efforts should be performed in order to identify and characterize the protein effectors involved in CDL mutability. From literature, stiparin, stiparin inhibitor, plasticizer, tensilin and NSF (novel stiffening factor), have already been identified in the sea cucumber model, as the proteins involved in the mutability phenomenon. Although recent results (Tricarico *et al.,* unplublished) demonstrate that there is a sequence similar to tensilin in the peristomial membrane of the sea-urchin *Strogylocentrotus purpuratus* (the species whose genome has already been sequenced), we consider that other key proteins besides tensilin may be present and should be identified and characterized in the sea-urchin model. However, as the small size of the CDL was a limitation in this work, other sea urchin MCTs (namely the peristomial membrane (PM)) should be used to follow this approach. Preliminary identification of other proteins has been started (see chapter IV), but several extraction protocols, as well as chromatographic methods, need to be optimized.

Additionally, as JLCs may be reservoirs of proteins that influence the interfibrillar cohesion, efforts should be done in order to extract and isolate these specific cells. For example, laser capture micro-dissection (LCM), which has proven to be successful in tracing protein in cells and tissues, may be used to extract the cells, and a posterior proteomics analysis such as 2D-DIGE and mass spectrometry could be used in order to identify the key-proteins. Mechanical tests (namely DMA) should also be used to evaluate the possible contribution of the identified proteins to the mutability phenomenon since the experimental apparatus has already been optimized.

Even though this work has shown that MMPs activity plays a fundamental role in CDL mutability, its identification, localization and expression was not evaluated. As the pattern of MMPs is quite different from the human pattern it is possible that we could find new MMPs mainly involved in the reversibility properties that could be used in de development of the biomaterial.

Effectors should be also done in order to understand if the collagen fibrils present in CDLs are heterotypic as mammalian connective tissues, and what is its percentage in relation to fibrillin. Regarding to glicosaminoglycans, strengths should be done in order to identify non-sulphated GAGs, as well as its contribution to the mutability phenomenon.

A possibility of using the acquired knowledge resulting from the present work on the mechanical reversibility phenomenon would be to exploit CDL as a model to design a completely new tuneable biomaterial (incorporating or not CDL components) through mimicking the mechanisms responsible for MCTs reversibility. Since reproducing the complex natural structure of CDL is virtually impossible, a feasible approach would be to manipulate simpler ECM constituents, in order to obtain a scaffold with tuneable structural and mechanical properties for tissue regeneration applications.

Trotter and co-workers first foresaw a biotechnological potential in echinoderm MCTs proposing a theoretic model using collagen fibrils extracted from holothurians and a synthetic matrix that could reproduce MCT mutability [80]. Recently, Capadona *et al*. designed and developed a MCT-simulating nanocomposite with chemo-responsive adaptability inspired and mimicking holothurian architecture. The main goal of that investigation was to develop a stimulus-responsive biomaterial for intracortical microelectrodes applications (record brain unit activity) and not for a regenerative therapy since the nanofibres used (cellulose whiskers) are not degradable [109-112]. The same biomimetic approach was followed by Mendez *et al*. that inspired in the MCT concept, namely organization and water exudation, developed a stimuli-responsive nanocomposite of cellulose nanowhiskers incorporated into a

elastomeric polyurethane matrix that changes its tensility according to water exposition [113].

From our point of view, a novel composite structure with a site-specific microarchitecture and stiffness pre-set or continuously tunable adjusted by therapeutic or physiological manipulation could be achieved by the combination of components extracted from MCTs and other biological or even synthetic elements.

In order to mimic CDL ECM, commercially available type I collagen or alternatively sea urchin collagen (from CDL or peristomial membrane), could be used as a matrix, where the fibril structure and the parallel orientation of the polymer could be maintained using electron-spinning technique. The advantage of using collagen derived from MCTs is that intact collagen fibrils can be extracted by simple methods, retaining their intrinsic tensile strength and stiffness [4,80]. We believe that the immunological response of sea urchin collagen would not be the problem, since collagen of marine origin (e.g fish, sponges and jellyfish) has been already used in beauty and cosmetic products, mainly for soft tissues (e.g. skin) as well as cartilage and bone repair [114-116]. It is also considered as a good substrate for cell adhesion, with good capacities to interact with host tissues, owing to good chemical versatility and chemotactic properties [115-117]. Due to the outbreak of bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE) the use of collagen and products derived from bovine origin became a concern. As a consequence, collagen derived from aquatic animals, namely fish and molluscs, received much more attention [116-119].

GAGs or suitable analogues could also be grafted onto the collagen substrates, in order to produce a suitable interface for the mutability-effectors proteins. If necessary, other proteins (e.g. fibrillin or elastin) could be added to achieve the required biomechanical properties of the matrix.

As mimicking reversibility would certainly be the most challenging goal, a different approach based on chemical or/and electrical stimulation would be possible routes to be explored. However, much more interesting would be if the scaffold could

modulate its mechanical properties according to the *in situ* physiological requirements of the regenerating tissue.

The requirement of tuneable scaffolds for tissue regeneration is of extreme importance, since the ECM of tissues and organs are not completely static; they ensure a perfect microarchitecture and microenvironment with biochemical and biomechanical cues required for tissue homeostasis. Possible applications of these dynamic biomaterials could be in the regeneration of soft connective tissues. A better target might be the therapeutic destiffening of scar tissue, such as that resulting from deep burns to the skin, which tends to contract (so-called “burn contractures”).

Another important application could be for pregnant women who show cervical insufficiency (incompetence) [120,121]. This pathology is associated to a preterm birth and it is assumed to be due to a deficiency of ECM components such as collagen, elastin, or other structural component of cervical connective tissue that normally resists softening, and dilatation triggered by the loading effect of the fetus and amniotic fluid [120,121]. The treatment for cervical insufficiency is a suture around the uterine cervix (cervical cerclage) in order to avoid premature dilatation. However, no satisfactory solution has been found. Maybe this could be a completely new field to explore, where a MCT-inspired scaffold could provide the necessary mechanical support and with progression of pregnancy could soften, allowing delivery [122].

Another application could be in the cosmetic industry, as an anti-aging treatment particularly developed to encourage the physiological de-stiffening of the skin. However, it could be applied for other connective tissues pathologies since with aging there is a combination of elevated and inappropriate collagen crosslinking, becoming the aged tissue mechanically weaker and less elastic but also more rigid than younger. This aberrant tensility of the ECM can compromise its function and organization endorsing age-related diseases such as cancer [20]. Alternatively, it could find applications in the pharmaceutical industry as medical therapies to treat connective tissue pathologies characterized by alteration of the viscoelastic properties, such as Ehlers-Danlos Syndrome (EDS). In this case the study of MCTs could

enhance the understanding of this pathology that is characterized by a hyperplasticity of connective tissues, that are associated with collagen fibrils that are weaker than normal [123-126]. Recently, efforts have been made to associate specific gene abnormalities with certain types of EDS. However, little is know about how collagen fibrils behave in this syndrome: are they unable to transmit stress? Or do they slide past each other as it occurs in MCTs?

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