Metabolic diseases and tendinopathies

the missing link

IBSA FOUNDATION
for scientific research

Metabolic diseases and tendinopathies
the missing link

IV Forum

21 June 2014, Lugano

Index

SESSION 2

Presentation

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The IV Forum "Metabolic diseases and tendinopathies: the missing link" took place in Lugano, Switzerland, on June 21, 2014, at the Auditorium of USI (Università della Svizzera Italiana). The purpose of the meeting, organized by IBSA Foundation in collaboration with I.S.Mu.L.T. (Italian Society of Muscles, Ligaments and Tendons – www.ismult.com), was to stimulate further research into the relationship between metabolic conditions – that are often subclinical, hence not easily diagnosed – and alterations of the extracellular matrix in tendon diseases.

Over 200 participants attended the Forum, that brought together prominent experts of the international scientific community to discuss about the latest scientific results on this topic. It was a real translational medicine meeting. Many interesting theories have been proposed in order to clarify the influences of hormones on tendon homeostasis and health, which are all reported and discuss into this paper.

The scientific sessions were chaired by Professor Michael Hirschmann (Department of Trauma & Orthopaedics, Kantonsspital Baselland, Bruderholz, Switzerland) and Dr. Christian Candrian (Canton Hospital System, Lugano, Switzerland) together with Professor Maffulli and Dr. Oliva, experts around the world as a tangible contribution to scientific progress in the area.

We wish to thank all the speakers and attendees for the enthusiastic participation. We hope this meeting is the first step to improve studies on this topic and to understand the complex relationship between hormones and tendon injuries.

Introduction

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The Forum "Metabolic diseases and tendinopathies: the missing link", is the ultimate goal of the collaboration between IBSA Foundation and I.S.Mu.L.T.

We have to say that joining two medical branches such as Endocrinology and Orthopedics has not been easy, but the research and the last four years of hard work has allowed us to find much in common.

Thanks to the open mind of the IBSA Foundation all of this has been done, indeed the Forum brought together some 200 members of the international scientific community, including physicians and surgeons, basic scientists, physiotherapist, sports scientists and a pre- and doctoral students to discuss this topic: a real translational medicine meeting.

Tendon conditions adversely impact the quality of life of millions of people, yet their causes and healing mechanisms are still unknown. Despite the array of hypotheses made, there is still a large number of factors affecting tendon health that remains unknown.

Humans and animals develops and grow under the physiological control of hormones; many soft tissues and bone diseases are associated to hormones diseases during the organisms development and this is clearly known by many centuries. Despite this postulate, soft tissue diseases, as tendinopathies, have been poorly investigated from this point of view.

This Forum is the first orchestrated scientific consensus to focus on this aetiopathogenetic hypohesis. We advocate that clinical and basic scientists involved in the study of tendons and tendinopathy explore this novel hypothesis. This is but the beginning.

SESSION 1

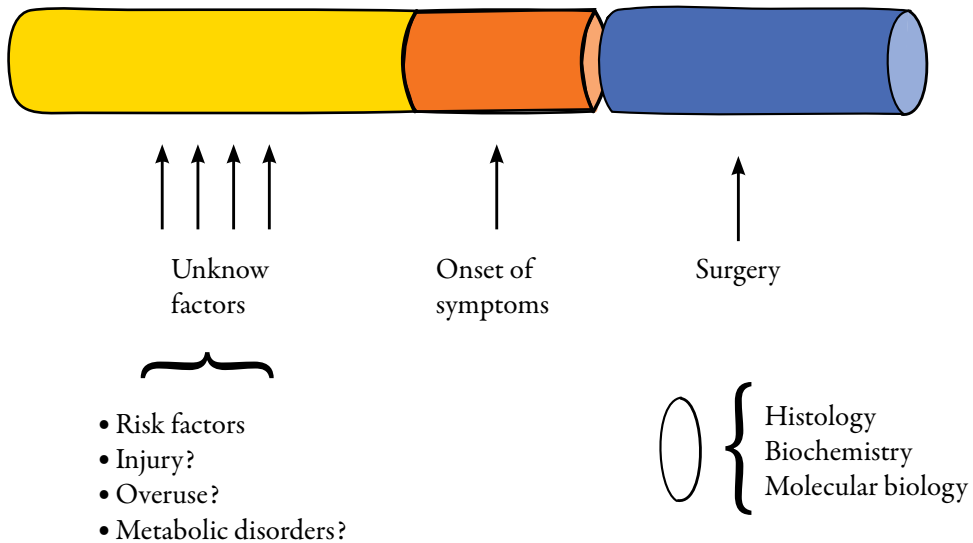# Metabolic diseases and tendinopathies. The missing link

Nicola Maffulli

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Tendinopathy is a common injury which affect both young and active patients, and older sedentary people. Many studies are published in literature trying to explain its pathogenesis, natural course and to develop new and effective treatments. Many human clinical studies, animal and histological models have been used to better understand the pathogenesis. But pain, which is the most important symptom that drive patient to the doctor, appear only after several months or years from the onset of the failed healing response typical of tendinopathy. So, for many years our studies were focused on the final stage of tendinopathy and little is known about the process which precede the onset of symptoms. These is still a gap of knowledge between the beginning of disease and clinical presentation (● **Figure 1**).

The etiopathogenesis of tendinopathy still remains unclear. It has probably a multifactorial origin, and it has been attributed to a variety of intrinsic and extrinsic factors [1]. An hypoperfusion theory has been proposed. Ischemia occurs when a tendon is under maximal tensile load. On relaxation, reperfusion occurs, generating oxygen free radicals; this may cause tendon damage, resulting in tendinopathy [2]. Peroxiredoxin 5 is an antioxidant enzyme that protects cells against damage from such reactive oxygen species. Peroxiredoxin 5 is found in human tenocytes. Its expression is increased in tendinopathy, supporting the view that oxidative stress may play a role. Hypoxia alone may also result in degeneration, as tendons rely on oxidative energy metabolism to maintain cellular ATP levels [3]. During vigorous exercise, localized hypoxia may occur in tendons, with tenocyte death. Tenocyte apoptosis has been implicated in rotator cuff tendinopathy [4]. Application of strain to tenocytes produces stress-activated protein kinases, which in turn trigger apoptosis. Oxidative stress may play a role in inducing apoptosis, but the precise details remain to be elucidated. There are more apoptotic cells in ruptured supraspinatus tendons than in normal subscapularis tendons [5].

● Figure 1. Temporary gap between the onset of tendinopathy and symptoms

Unknow
factors

Onset of
symptoms

Surgery

- Risk factors
- Injury?
- Overuse?
- Metabolic disorders?

Histology
Biochemistry
Molecular biology

The term "tendinosis" has been in use for nearly three decades to describe the pathological features of the extracellular matrix network in tendinopathy. Despite that, most clinicians still use the term "tendinitis" or "tendonitis," thus implying that the fundamental problem is inflammatory. Histological examination of tendinopathy shows disordered, haphazard healing with an absence of inflammatory cells, a poor healing response, noninflammatory intratendinous collagen degeneration, fiber disorientation and thinning, hypercellularity, scattered vascular ingrowth, and increased interfibrillar glycosaminoglycans [1]. Histopathological studies revealed thinning and disorientation of collagen fibers, myxoid degeneration, hyaline degeneration, chondroid metaplasia, calcification, vascular proliferation, and fatty infiltration [6]. Frank inflammatory lesions and granulation tissue are infrequent and are mostly associated with tendon ruptures [7]. For these reasons we advocate the use of the term "tendinopathy" as a generic descriptor of the clinical conditions in and around tendons arising from overuse.

Many studies advocate the importance of extra cellular matrix (ECM) for the homeostasis of connective tissue. Physiological and pathological modifications of the ECM seem the most important intrinsic factors involved in tendinopathy and tendons ruptures. Transglutaminase (TGs) have been implicated in the formation of hard tissue development, matrix maturation and mineralization. TG2 is widely

distributed within many connective tissues. Injured supraspinatus tendons showed reduction of TG2 protein expression, both at mRNA and protein level [8]. TG are important in maintaining the structural integrity of tendons thanks to its mechanical or crosslinking function in normal condition, and the fall of TG2 may mean the exhaustion of the reparative tendon's capabilities. The turnover of ECM in normal tendon is also mediated by matrix metalloproteinases (MMPs), such as collagenases and stromelysins [9]. They are able to denature collagen type I. After tendon rupture, the activity of MMP-1 increases, with a reduction of MMP-2 and MMP-3 [10]. An increase in MMP-1 activity and degradation of the collagen fibril network is a potential cause of the weakening of the tendon matrix and may contribute to a mechanically less stable tendon that is susceptible to rupture. These findings may represent a failure of the normal matrix remodelling process.

Pain is the cardinal symptom tendinopathy, but the source of pain has not been clarified yet [4]. Studies on Achilles tendon showed that chronic painful tendinopathy often present neovascularisation outside and inside the ventral part of the tendinopathic area. However, neovascularity in absence of pain is not necessarily pathological, and, in athletes, it can just indicate a physiological response to physical training. The ingrowth of sensory and sympathetic nerves from the paratenon accompanies the neovessel in chronic painful Achilles tendinopathy. These sensory and sympathetic nerves can release nociceptive substances, and may be the primary source of pain.

Much progress has been made in the last decades in diagnosis and treatment, but are we sure we truly understand the pathology? Many risk factors and etiopathogenetic process still remain unknown. An association between tendinopathy and metabolic disorders is emerging. Several studies showed that tendinopathies are more frequent in patients with hyperglycemia, diabetes, obesity, or metabolic syndrome. Diabetes mellitus has been considered a risk factor for rotator cuff tears by some authors. In a study on asymptomatic subjects Abate *et al.* [11] found that age-related rotator cuff (RC) tendinopathy is more common in diabetic paients, who showed a restricted shoulder range of motion, higher incidence in retears after a surgical repair, and higher rate of complications and infections are reported both after open and arthroscopic repair of RC tendons [12]. An association between calcific tendinopathy and diabetes and thyroid disorders has been shown, but the precise mechanism is still unknown [13]. More than 30% of patients with insulin-dependent diabetes have tendon calcifications. The exposure of proteins to high levels of sugar moieties causes the glycosylation of several extra-cellular matrix proteins, which can modify the extracellular matrix by cross-linking proteins [14]. In an animal study, tenocytes obtained from porcine patellar tendon have been incubated with glycated type I collagen, which increased transglutaminase (Tg) activity. This may represent an additional pathway mediating pathological changes, and could contribute to calcific tendinopathy in diabetes [15].

Some authors focused their attention on the correlation between serum levels of lipids and RC tears. The interest in this relationship arises from the potential role of high serum lipid concentration in complete rupture of the Achilles tendon. Fatty degeneration, or tendolipomatosis, was found in the histopathological examination of specimens harvested during surgery for tendinopathy in the lower limb [16]. However, similar results were not obtained from tendon samples of the RC and the long head of the biceps. Abboud and Kim [17] observed higher levels of total cholesterol, triglycerides, and low-density lipoprotein cholesterol, and lower levels of high-density lipoprotein cholesterol in patients with RC tears compared to patients with shoulder pain but without RC tears. Nevertheless, this data was not confirmed by histological/pathological evidence of cholesterol deposition. On the other hand, in a recent study, no statistically significant difference in serum triglyceride and total cholesterol concentrations between patients undergoing arthroscopic RC repair and patients of a similar age undergoing arthroscopic meniscectomy has been reported [16]. Consequently, no definitive conclusion on the role of serum cholesterol and triglyceride concentration in the pathogenesis of RC tears can be formulated.

Obesity can be considered another important risk factor for the development of tendinopathy [18]. Overweight patients have elevated cholesterol, atherosclerosis, diabetes, hypertension, metabolic syndrome, and decreased physical activity. Since vascular supply is essential for the metabolic processes of the tendons, all these conditions associated with obesity or with increased body mass index (BMI) may represent a cause of decreased vascularity, interplaying in the onset and progression of RC tears. In a cross-sectional study, abdominal obesity was associated with chronic RC tendinopathy [19]. Furthermore, both obesity and metabolic syndrome are associated with increased concentration of proinflammatory cytokines including IL-1, IL-6, and TNFα, as well as reactive oxygen species (ROS). Proinflammatory cytokines have been proved to be upregulated in rat and human models of RC tendinopathy. Prolonged systemic, low-grade inflammation and impaired insulin sensitivity act as a risk factor for a failed healing response after an acute tendon insult, and predispose to the development of chronic overuse tendinopathies [20]. Morover proinflammatory cytokines play a crucial role in the apoptosis process, particularly in apoptosis induced by oxidative stress, leading to a failed healing response in tendons.

The role of hormones in the pathogenesis of tendinopathy is not well recognised, even though the use of anabolic steroids is correlated with a higher incidence of spontaneous tendon ruptures. A recent study investigated the effects of dihydrotestosterone (DHT) on human tenocyte cultures from the intact supraspinatus tendon of male subjects, showing a possible correlation between testosterone abuse and shoulder tendinopathy [21]. Cultured human tenocytes were incubated for 24 h, then DHT was added to the culture plate wells. Cell morphology assessment and cell proliferation tests were performed 48, 72 and 96 h after DHT treatment (● Figure 2). DHT-treated tenocytes showed an increased proliferation rate at DHT concentration higher

• Figure 2. The effect of dihydrotestosterone (DHT) on tenocytes proliferation
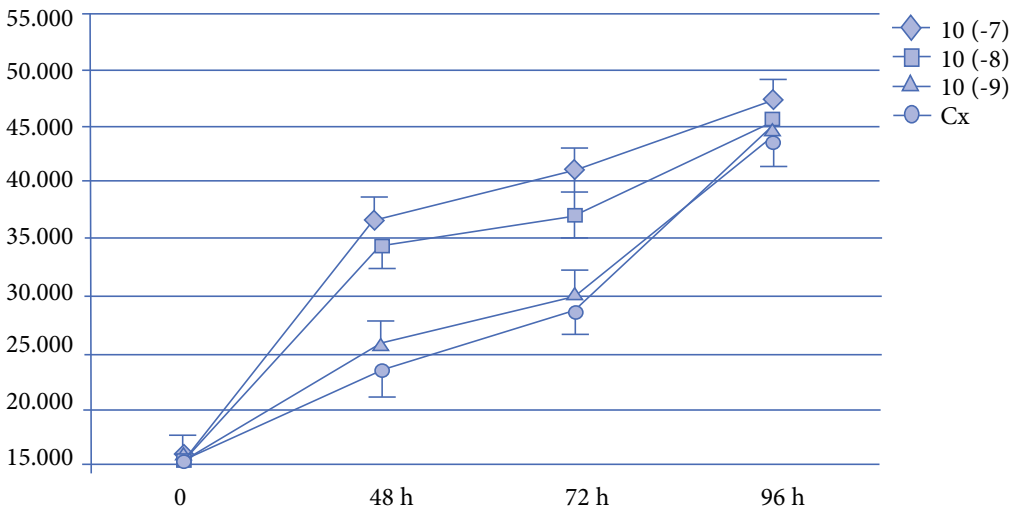
than 10^{-8} M. Differences in cell numbers between control and DHT-treated cells were statistically significant. The tenocytes treated with DHT were more flattened and polygonal compared to control cells that maintained their fibroblast-like appearance during the experiment at each observation time (• **Figure 3**). Progressively increasing concentration of DHT had direct effects on male human tenocytes, increasing cell number after 48 and 72 h, and leading to a dedifferentiated phenotype after 48 h of treatment. This effect can be important during tendon-healing and repair, when active proliferation is required.

Genetics could also play a role in tendinopathy. In the last two decades, several evidences have been provided to support the relationship between single nucleotide polymorphisms and the susceptibility to develop tendon injuries. Brothers and sisters diagnosed with full thickness RCTs had more than twice the relative risk for developing a lesion and nearly five times the risk of experiencing symptoms than spousal controls. A familiar predisposition and inherited genetic components have also been postulated as a cause of calcific tendinopathy in some circumstances [13]. Recently, the genes associated with tendon injuries and the onset of musculoskeletal injuries, have been identified. Variants within the COL5A1 (• **Figure 4**), tenascin C and matrix metalloproteinase 3 (MMP3) genes are associated with increased risk of Achilles tendon injuries [22]. A recent review concluded that the genes currently associated with tendon injuries include gene encoding for collagen, matrix metallopeptidase, tenascin and growth factors [23]. However, tendon and ligament injuries seems not to have a single

● **Figure 3.** Cells morphology assessment of tenocytes cultured with the addition of dihydrotestosterone (DHT) at 48 and 72 h
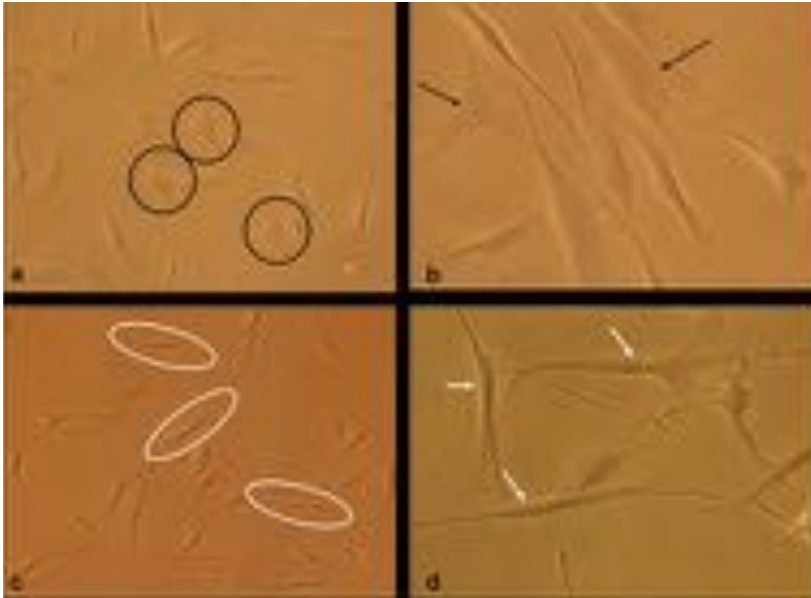
genetic cause. They are associated with the effects of multiple genes in combination with lifestyle and environmental factors. Although complex disorders often cluster in families, they do not have a clear-cut pattern of inheritance [24]. This makes it difficult to determine a person's risk of inheriting or passing on these disorders.

In conclusion, tendinopathy can be viewed as a failure of the cell matrix to adapt to a variety of stresses as a result of an imbalance between matrix degeneration and synthesis. The pathogenesis is multifactorial. Some components of the mechanical environment seem to contribute to the manifestation of tendinopathies, but recent evidence underline the importance of intrinsic factors. Tendon injuries arising from overuse are a difficult clinical problem. Lack of information about their etiology makes the pursuit of effective treatments almost a random process. Metabolic disorders are a new frontier and a novel field of research, and, if these associations are confirmed, assessment and treatment of patients with tendon conditions may have to be revisited. Researchers continue to look for major contributing genes for many common complex disorders. Several genes have been related to musculo-skeletal disorder, in particular tendinopathy. However, the identification of the genetic background related to susceptibility to injuries is challenging yet and further studies must be performed to establish the specific role of each gene and the potential effect of their interaction.

● **Figure 4.** Variant within the COL5A1 gene
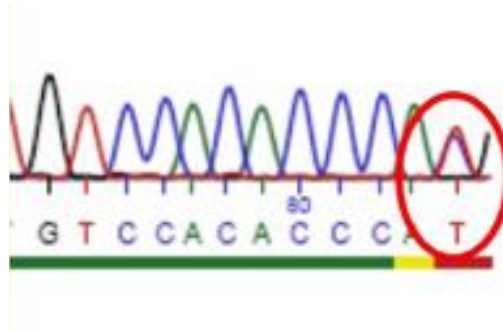
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How tendons modify during diabetes. Glycation related crosslinks in mechanics and connective tissue disease
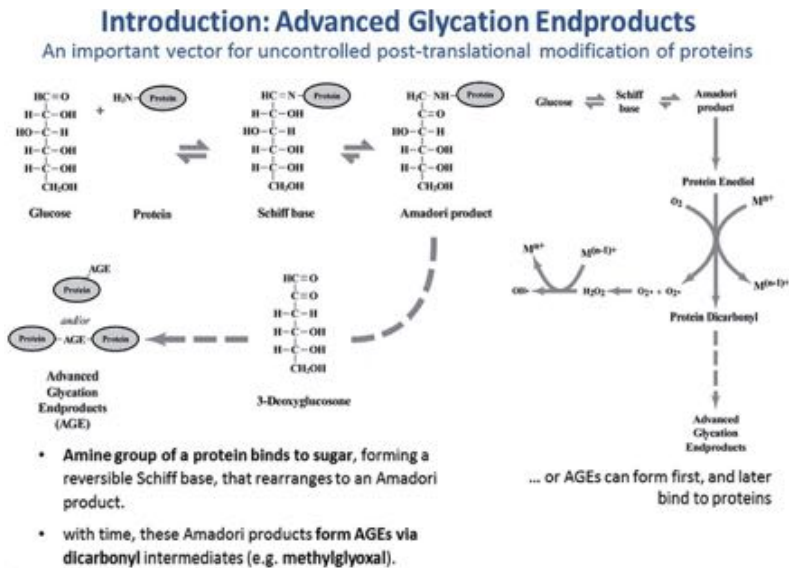
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Diabetes mellitus is a disorder characterized by hyperglycaemia due to an absolute or relative deficiency of insulin and or insulin resistance. It affects 1-2% of the population worldwide. Diabetic patients are prone to long-term complications which drastically reduce life quality, such as cardiovascular disease, nephropathy, renal failure, retinopathy, cataract, and poor wound healing.

Connective tissue aging and diabetes related comorbidity are associated with compromised tissue function, increased susceptibility to injury, and reduced healing capacity. This has been partly attributed to collagen crosslinking by Advanced Glycation End-products (AGEs) that accumulate with both age and diabetes. While such crosslinks are believed to alter the physical properties of collagen structures and tissue behavior, existing data relating AGEs to tendon mechanics is contradictory.

Diabetic complications appear to be multifactorial in origin, but advanced glycation has been postulated to play a central role in its pathogenesis. Protein glycation is a spontaneous reaction depending on the degree and duration of hyperglycaemia, the half-life of the protein and permeability of the tissue to free glucose. Glycated proteins can undergo further reactions, involving dicarbonyl intermediates, such as 3-deoxyglucosones (3-DG), giving rise to poorly characterized structures called advanced glycation endproducts (AGEs) (● **Figure** 1) [1]. AGEs are complex, heterogenous molecules that can cause protein crosslinking. Not all AGEs have been identified and the mechanisms underlying their formation remain unclear. Given their slow formation, it was believed that only long-lived extracellular proteins accumulate AGEs. Increased glycation and build-up of tissue AGEs have been implicated in diabetic complications because they can alter enzymic activity, decrease ligand binding, modify protein half-life and alter immunogenicity [2]. But their role in the pathogenesis of diabetic complications is still unclear. A recent study has reported the presence of autoanti-

Figure 1. Protein glycation by glucose and the formation of AGEs

Introduction: Advanced Glycation Endproducts
An important vector for uncontrolled post-translational modification of proteins

- Amine group of a protein binds to sugar, forming a reversible Schiff base, that rearranges to an Amadori product.

- with time, these Amadori products form AGEs via dicarbonyl intermediates (e.g. methylglyoxal).

… or AGEs can form first, and later bind to proteins

Source: Ahmed, 2005 [1], adapted.

bodies against serum AGEs capable of forming AGE-immune complexes in diabetic patients that may play a role in atherogenesis [3].

Among proteins collagens are particularly susceptible to AGE formation, because of their long half-life, and this process may be involved in physiopathology of tendinopathy. Extracellular matrix protein glycation have been also related to the pathogenesis of calcific tendinopathy by some authors [4]. The collagen molecule is synthesized as a trimeric molecule containing two α1, and one α2 chains, which assemble into fibrils and are enzymatically crosslinked into the extracellular matrix (ECM). In aging, type I collagen becomes less flexible and more acid insoluble, which correlates with the accumulation of AGEs. The low biological turnover of collagen makes it therefore susceptible to interaction with metabolites, primarily glucose. Besides the elderly, people who suffer with type II diabetes are particularly badly affected by AGE crosslinking [5]. AGE related collagen crosslinks alter physical characteristics of collagen fibers, for instance increasing denaturing temperature and resistance to enzymatic break-down, while decreasing solubility in water was shown in tendons. In tendons, AGE formation has been shown to affect protein interactions within the matrix as well as between cells and their matrix [6]. These changes have been associated both with reduced healing capacity [7] and altered mechanical properties of connective tissues [8].

• **Figure 2.** Mechanical property changes in AGE crosslinked tendons
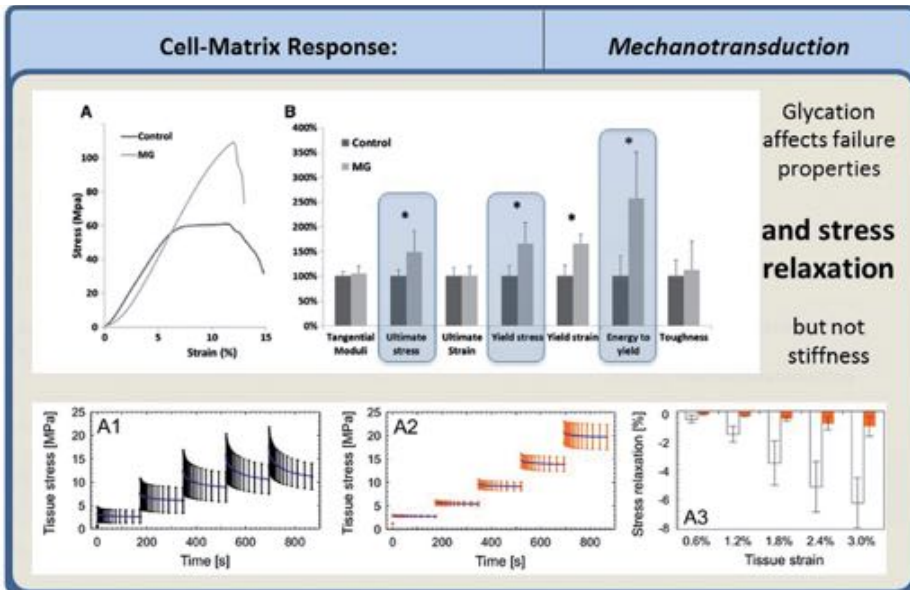
Source: Li *et al.*, 2013 [9], adapted.

In order to better understand how AGEs may adversely affect the mechanical properties of tendon collagen fibers, we recently studied their effects in a rat model [9]. To better isolate the functional effects of AGEs on tendon collagen fiber mechanics, methylglyoxal (MGO) to induce AGEs in rat tail tendon fascicles (RTTFs) were used. Mechanical testing of tendon fascicles with induced AGE crosslinks showed nearly complete removal of stress relaxation behavior, significantly altered failure stress, significantly altered yield behavior, but only a slight, non-significant increase in tissue elastic modulus (• **Figure 2**). Then the fiber mechanics were studied. Tendons were incrementally stretched under a multiphoton microscope while force-time curves were recorded. A 60 s relaxation period after each stretch was not sufficient for the samples to reach equilibrium, but was sufficient to permit the majority of the stress relaxation response. Analysis of cell nuclei permitted quantification of individual fiber kinematics. In control samples, fiber-fiber sliding dominated the tissue response compared to fiber stretch, while AGE laden tendons demonstrated a dominant fiber stretch relative to fiber sliding (• **Figure 3**). An important finding of this study was that the formation of AGEs alter the manner in which tendon reacts to loading at the fiber level, in particular significantly reducing collagen fiber sliding. On the other side tendons try to compensate this loss of function by increasing collagen fiber stretch,

● Figure 3. Fibers mechanics of normal and AGEs tendons
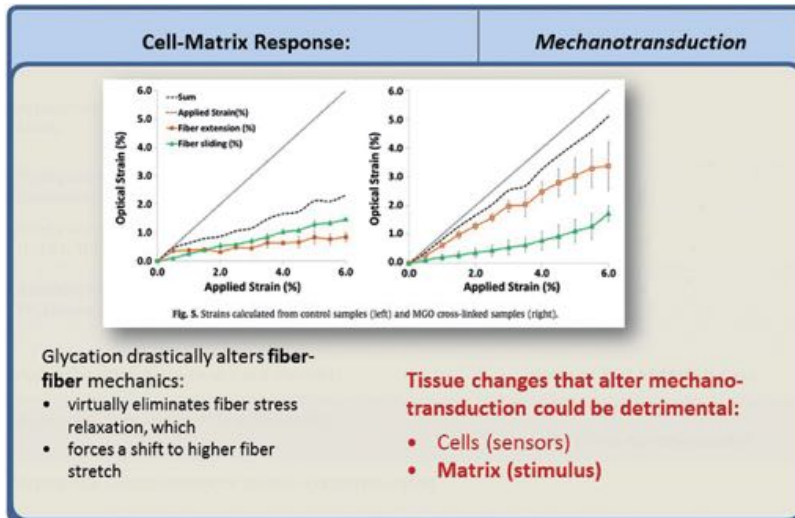
The tendon collagen fibers showed a loss of sliding which is compensated by an increase in collagen fiber stretch.
Source: Li *et al.*, 2013 [9], adapted.

which may have potentially important implications for predisposing collagen fibrils to damage during everyday use. The tissue stiffness does not appear to be significantly affected. Therefore physiological loads in aged and diabetic tendons could involve fiber "over-stretching" that leads to accelerated accumulation of damage.

Despite the recognized importance of AGEs, there are still several important open questions about their role in the onset of pathological conditions. Where AGEs and AGE related crosslinks form is still not well known, as well as how they act to affect mechanical properties of collagen structures. AGE crosslinks are probably formed between triple-helical regions of collagen molecules, potentially altering the transfer of mechanical force between the bridged molecules within a collagen fibril [6, 10]. A recent study has identified potential lysine-arginine protein cross-linking sites for glucosepane, a key non-enzymatic collagen crosslinker [5]. The authors identified 14 specific lysine-arginine pairs that, due to their relative position and configuration, are predisposed to form glucosepane. The residues predicted to be involved in AGE crosslinks were determined to lie within key collagen domains, such as binding sites for integrins, proteoglycans and collagenase, hence providing molecular-level explanations of previous experimental results showing decreased collagen affinity for key molecules, affecting the biological properties of collagen tissues.

In conclusion advanced glycation end-products (AGEs) accumulate with age and are associated with chronic, age-related diseases, in particular diabetes mellitus. AGEs comprise a broad group of post-translational protein adducts and crosslinks that can alter protein physical properties and adversely affect their function. Tendon collagen has long high half-life, and therefore is particularly susceptible to AGE formation.

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Cushing, acromegaly, GH deficiency and tendinons

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This communication aimed at reporting any possible correlation between high level of glucocorticoids, Cushing's syndrome, acromegaly and GH deficiency and tendinopathy, showing the effect of cortisol, GH/IGF-I on tenocytes and extracellular matrix (ECM) proteins, in particular Collagen type I and III.

Cushing's syndrome (CS) is induced by prolonged exposure to endogenous or exogenous cortisol excess [1, 2], and can be caused by excessive treatment with synthetic steroids that have glucocorticoid activity (*i.e.* iatrogenic CS), or by endogenous ACTH dependent (mainly Cushing's disease due to an ACTH-secreting pituitary tumor, or more rarely ectopic Cushing's syndrome) or independent (due to spontaneous adrenal glucocorticoid hypersecretion from adrenal tumors) forms [1, 2]. Clinical features include rapid weight gain, particularly of the trunk and face with sparing of the limbs (central obesity) [1, 2]. Common signs include the growth of fat pads along the collarbone, on the back of the neck or "buffalo hump", and on the face – "moon face", dilation of capillaries, thinning of the skin which causes easy bruising and dryness, purple or red striae and many others symptoms which are well described in literature [1, 2]. Literature also provided many information about the effects of long exposure to high level of cortisol deriving from *in vitro* and animal studies. However, in these studies the authors did not investigated the effect of prolonged exposure to endogenous cortisol excess but the prolonged effects of treatment with corticosteroids, which is a different disease, named iatrogenic Cushing's syndrome. Finally the results of these studies are quite controversial because some of them demonstrated that corticosteroids have no effects on tendons, and in facts they are commonly used to manage tendinopaties [3]. On the contrary, many others studies shows that corticosteroids, such as dexamethasone [3] e, inhibit type I collagen mediated upregulation of MMP-2 and -9 by canine flexor digitorum profundus tendon tenocytes, rat

● **Figure 1.** The effects of dexamethasone on collagen synthesis and gene expression of tendons in an animal study. Dexamethasone inhibits collagen synthesis and gene expression by embryonic chick tendon
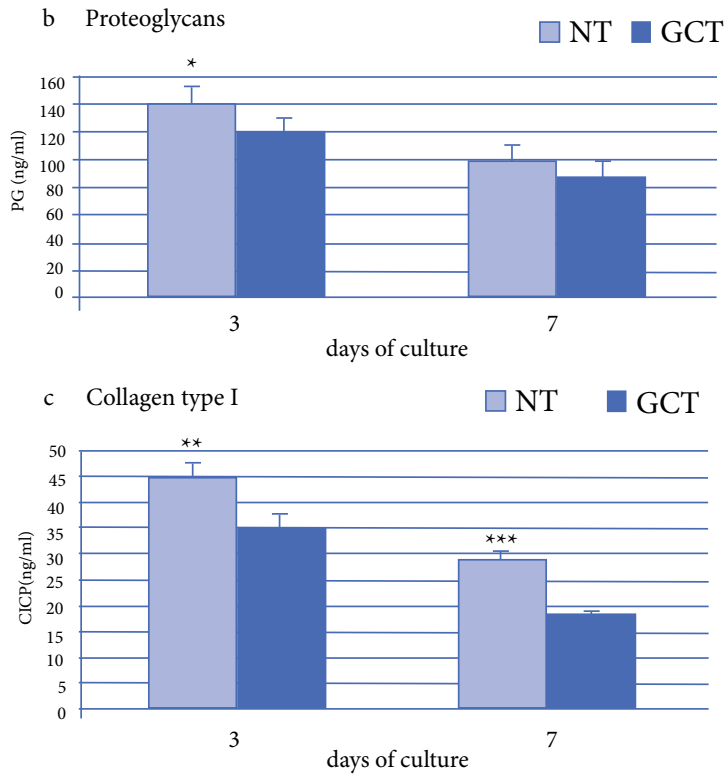
30 adult rats treated for 8 weeks with 4 mg/kg methylprednisolone (n=15) or sterile saline injection (n=15).

Source: Torricelli *et al.*, 2006 [6].

Achilles tendon tenocytes migration, the proliferation of rat Achilles tendon and patellar tendon tenocytes [4, 5].

Torricelli *et al.* found that dexamethasone inhibits collagen synthesis and gene expression by embryonic chick tendon [6] (● **Figure 1**). Similar results were found in another study that showed a reduce Achilles tendon collagen synthesis and reduced tenocytes proliferation in rats treated with glucocorticoids [7]. The authors also found that this effect was not only time dependent, but also dose dependent (● **Figure 2**). Corticosteroids are widely used for many types of tendinopaties, in particular subacromial corticosteroid injections are commonly used in the nonoperative

● **Figure 2.** Glucocorticoids inhibit tenocytes proliferation and tendon progenitor cell recruitment in a dose and time dependent manner
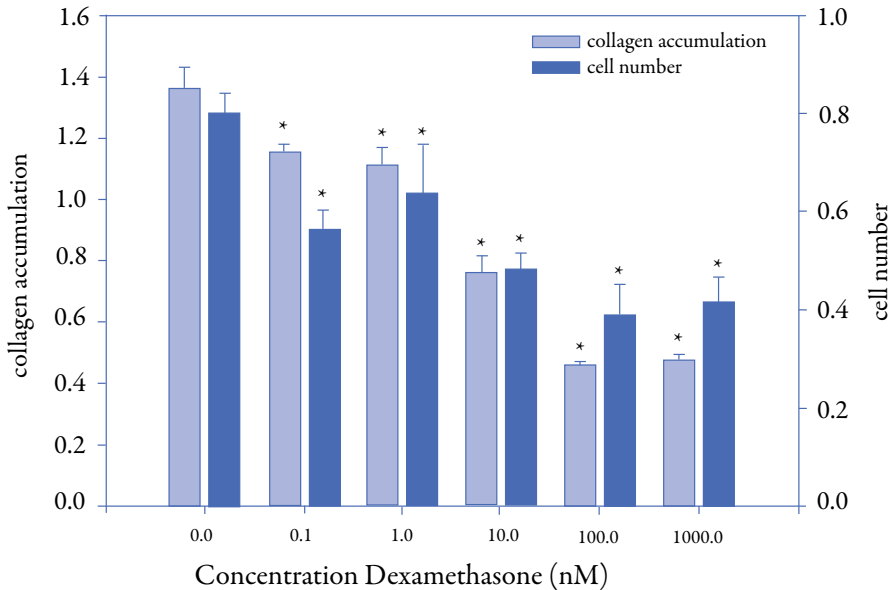
Source: Scutt *et al.*, 2006 [7].

management of rotator cuff (RC) disease. In their study, Wei *et al.* tried to characterize the acute response of RC tendons to injury through the analysis of the type-III to type-I collagen expression ratio, a tendon injury marker, and to examine the effects of a single injection of corticosteroid on this response in an animal model [8]. They found that a single dose of corticosteroid did not alter the acute phase response of RC tear in the rat. However, the same steroid dose in uninjured tendons initiates a short-term response equivalent to that of structural injury. These findings suggest that while a single corticosteroid dose may have no long-term effects on tendon collagen gene expression, collagen composition may be acutely altered by the injection. The authors concluded that therapy and activity recommendations following subacromial corticosteroid exposure should be made with the awareness of possible compromised rotator cuff tendon properties.

In patients with Cushing's syndrome, high cortisol levels induce an increased protein catabolism and myopathy. The clinical presentation is due to the physiological effects of cortisol, which reduces the utilization of amino acids for the formation of protein everywhere except the liver. Extrahepatic protein stores are reduced and amino acid levels in the blood increase. Extrahepatic utilization is decreased thereby

reducing protein synthesis. By searching in Pubmed and Medline for any possible correlation between Cushing's syndrome and tendinopathy, only 4 case reports are reported in literature [9, 12]. However all these studies reported the spontaneous tendons rupture in this selected patients.

Acromegaly and GH deficiency

Many animal studies in literature have shown a direct correlation between the growth hormone/insulin-like growth factor-I (GH/IGF-I) and collagen synthesis. The administration of IGF-I (and IGF-II) has been found to accelerate protein synthesis and recovery after injury in tendons in rabbits [13]. The administration of GH for 3 weeks has been shown to increases circulating levels of procollagen propeptides and to increase the expression of both collagen type I and III in intramuscular fibroblasts in rats. IGF-I is present in human Achilles tendon linked directly to fibroblasts, and a detectable interstitial concentration has been demonstrated in human tendon [14]. Finally, several growth factors, such as IGF-I, stimulate collagen synthesis and are expressed in response to mechanical loading [15].

In a recent study 20 male rats were randomly assigned to recombinant human GH (rhGH) (1.25 mg rhGH/kg body weight twice daily for 14 days) or vehicle to evaluate the impact of rhGH on collagen growth and maturational changes of tendons and ligaments [16]. Recombinant human GH administration was able to stimulate dense fibrous connective tissue growth, suggesting that a short course rhGH treatment can affect the rate of new collagen production. However, the maturation of the tendon and ligament tissues decreased 18-25% during the rapid accumulation of *de novo* collagen. Thus, acute rhGH administration in a dwarf rat can up-regulate new collagen accretion in dense fibrous connective tissues, while causing a reduction in collagen maturation. The expression of IGF-I was studied in rat muscle and tendon after training. Levels of IGF-I after training was higher than before training, therefore a possible role for IGF-IEa in adaptation of tendon to training has been hypothesized [17]. Changes in IGF-IEa expression could explain the important effect of eccentric actions for muscle hypertrophy.

The effect of chronically altered GH/IGF-I levels on connective tissue of the muscle-tendon unit is not known. Nielsen *et al.* recently studied the effect of GH deficiency on the Achilles tendon in a rat model [18]. They studied three groups of mice, giant transgenic mice that expressed bovine GH (bGH) and had high circulating levels of GH and IGF-I, dwarf mice with a disrupted GH receptor gene (GHR$^{-/-}$) leading to GH resistance and low circulating IGF-I, and a wild-type control group (CTRL). The authors created an animal model of Laron syndrome, which is an autosomal recessive disorder characterized by an insensitivity to growth hormone (GH), caused by a variant of the growth hormone receptor. It is characterized by a very high circulating levels of GH and low levels of IGF-I. The number and size of collagen fibrils in Laron mice were significantly reduced as compare to controls, while the tre-

● **Figure 3.** mRNA expression of IGF-I isoforms and collagens in a mouse model. The mRNA expression of IGF-I isoforms was generally higher in both tendon and muscle collagen in transgenic mice with GH and IGF-I excess compared to controls
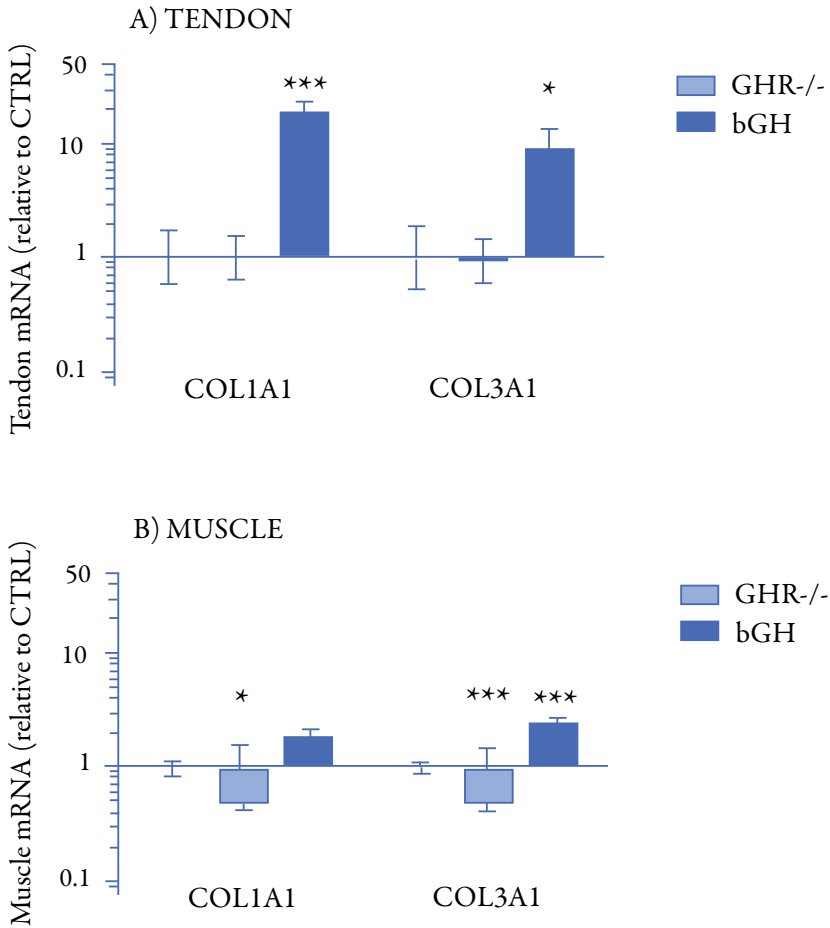
Source: Nielsen *et al.*, 2014 [18].

atment with rhGH determined a significant increase in size and numbers of fibrils. A decreased mRNA expression of IGF-I isoforms and collagen types I and III in muscle was also found in lice as compared to controls. In contrast, the mRNA expression of IGF-I isoforms and collagens in bGH mice was generally high in both tendon and muscle compared to controls (● **Figure 3**). Chronic manipulation of the GH/IGF-I

axis influenced both morphology and mRNA levels of selected genes in the muscle-tendon unit of mice. Whereas only moderate structural changes were observed with up-regulation of GH/IGF-I axis, disruption of the GH receptor had pronounced effects upon tendon ultra-structure.

The reason is related to physiological effects of GH, which is an anabolic hormone, acting directly or throughout stimulation of IGF-I, insulin, and free fatty acids. In a normal nutritional state the effects of GH on protein metabolism are modest, but in fasting conditions the effects on protein metabolism are more pronounced. Lack of GH during fasting increases protein loss and urea production rates by approximately 50%, with a similar increase in muscle protein breakdown.

Axial and peripheral arthropathy affects the majority of patients with acromegaly, being a leading cause of morbidity and functional disability [19]. Joint cartilage and tendons thickness was investigated in patients with acromegaly. This open prospective study was designed to evaluate the effect of a long term treatment with octreotide (OCT) on acromegalic arthropathy assessed by ultrasonography examination [19]. Joint cartilage thicknesses of shoulder, wrist, and knee and the thickness of Achilles tendons were measured in 30 acromegalic patients. The thicknesses of articular cartilages as well as the thickness of Achilles tendon were significantly increased in patients with active acromegaly compared to healthy subjects [19]. No significant differences was found in tendon thickness between patient who were affected by acromegaly for more or less than 10 years, meaning that in these patients the circulating levels of GH and IGF-I excess rather than the duration of symptoms may have an influence on tendon structure [19]. The long term OCT treatment induced a slight decrease on tendon size. Treatment with OCT has been found to improve symptoms and signs of acromegalic arthropathy, but objective detection of structural changes in bone and cartilage has not been reported to date [19]. Few years later, in 12 newly diagnosed patients suppression of circulating GH and IGF-I levels by LAN treatment was followed by a significant decrease in thickness of Achilles tendons [20]. In another study 9 acromegalic patients and 9 GH deficient treated patients were compared to healthy controls [21]. The authors found that the synthesis of collagen and the number of collagen fibres were higher in patients with acromegaly compare to patients with GH deficiency, even if the difference was not statistically different.

In conclusion the results of these studies suggest that glucocorticoid overexposure may impact tendon ultra-structure by inhibiting collagen synthesis, particularly in uninjured tendons. Cortisol excess is associated to spontaneous tendon rupture in patients with Cushing's syndrome, in particular with Achilles tendon rupture. Acromegaly and GH deficiency are associated to an altered tendon thickness and type I and type III collagen synthesis. In acromegaly treatment with somatostatin analogs seems to reduce Achilles tendon size, with octreotide and lanreotide displaying different effectiveness. In GHD treatment with rhGH has been shown to increase type I and type III collagen synthesis and to induce moderate structural changes in tendons.

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How thyroid hormones modify tendons

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The etiology of tendinopathy is currently considered to be multifactorial. Many studies have been published on rotator cuff (RC) pathologies and tears (RCTs) and several anatomic and surgical investigations with histologic sections have been performed to clarify its pathogenesis. Recent evidence strongly suggests that most of RCTs are caused by primary intrinsic degeneration [1]. Emerging studies have elucidated the complex process of RC degeneration and the attempts to unify intrinsic and extrinsic theories can be made to explain the natural history of RCTs. However the relative contributions of each factor still have to be determined. Resent researches suggest an association between RC tendinopathy and diabetes and thyroid disorders, but the precise mechanism is still unknown [2]. Patients with associated endocrine disorders present earlier onset of symptoms, longer natural history, and they undergo surgery more frequently compared to a control population [1, 3, 4].

Thyroid hormones (THs) T3 (triiodothyronine) and T4 (thyroxine) play an essential role in the development and metabolism of many tissues and organs, both in early and adult life, including changes in oxygen consumption, protein, carbohydrate, lipid and vitamin metabolism [5]. The effects of THs are mediated mainly through T3, which regulates gene expression by binding to the TH receptors (TRs)-a and -b. T4 is important for both collagen synthesis and extra cellular matrix (ECM) metabolism. Hypothyroidism causes accumulation of glycosaminoglycans (GAGs) in the ECM, which may predispose to tendon calcification. GAGs are also involved in others pathologies during hypothyroidism, like carpal tunnel syndrome.

The relationship between thyroid disorders and shoulder pain has been suspected since the late 1920s [6]. More recently, such association has been more formally hypothesized [7], and thyroid diseases have been linked to idiopathic tendinopathies. But, despite RC tendinopathies and tears are the most frequent diseases of the shoulder joint, no systematic

Figure 1. Western Blot analysis of TR receptor isoforms express on tenocytes membrane
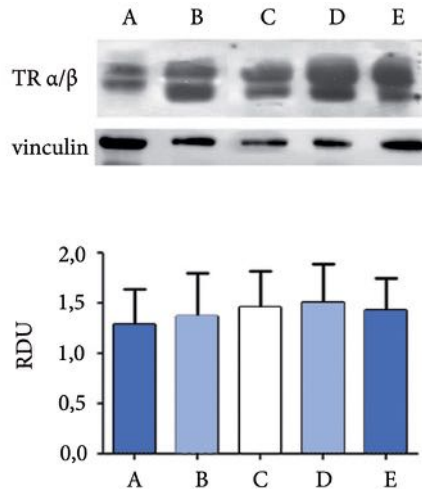
A: patients with healthy rotator cuff tendons. **B-C**: patients with rotator cuff tears without thyroid disease. **D-E**: patients with rotator cuff tears and thyroid disease. The polyclonal antibodies against TRs α/β recognize two specific bands at 47 and 55 kDa, respectively.

Source: Oliva *et al.*, 2013 [8].

study on the thyroid disease as a risk factor has been performed. The first part of the study is an epidemiplogical study. More than 1000 patients have been operated in the last 5 years for RCT by our surgical team. Of them, 441 patients have been enrolled in the study. Each patient was investigated with a telephone survey and the presence of thyroid disease, thyroidectomy, diabetes and hypertension were recorded. Body mass index (BMI), glucose, total, and HDL cholesterol were also evaluated. 63% of patients submitted to rotator cuff surgical repairs were female, and more than 58% of these patients referred a thyroid disease. Male were 37% of patients, and 19% of them suffered of a thyroid disorder.

These clinical data let us think that thyroid disease could be another important factor in the etiopathogenesis of the RCTs. To solve this problem we try to answer some questions: are thyroid hormones receptors expressed on tenocytes? What are the roles of thyroid hormones in the homeostasis of tendons? Do thyroid hormones induce collagen production? Do thyroid hormones induce biglycan and fibromodulin expression? Do thyroid hormones induce Cartilage Oligomeric Matrix Protein (COMP) expression? Do thyroid hormones play direct or indirect roles on tendon extracellular matrix?

Although TRs are ubiquitous, their presence on tendons has not been previously investigated.

● **Figure 2.** The addition of THs in tenocytes culture induce an increase of cell's proliferation
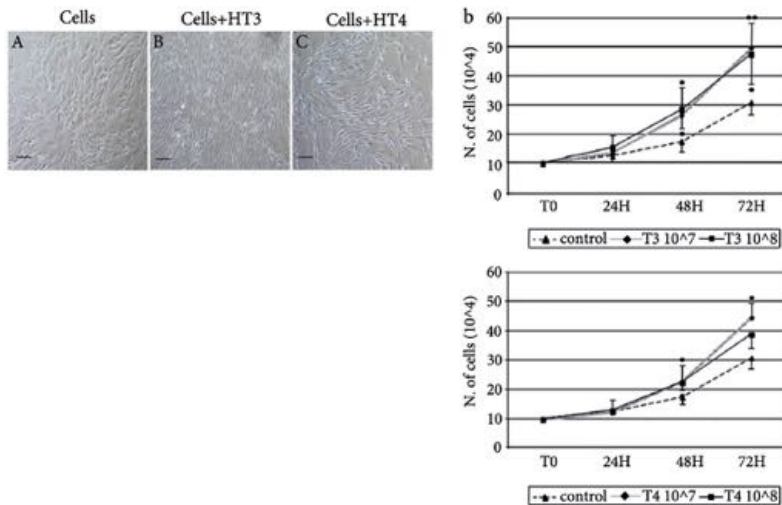
Cell growth: primary tenocyte-like cells were cultured and exposed to different thyroid hormone concentrations. At 72 h, T3 at concentration of 10-7M in vitro given for 3 days stimulates, more than T4 (19% for T3 and 10% for T4), cells cycle and growth of tenocytes of healthy rotator cuff tendons.
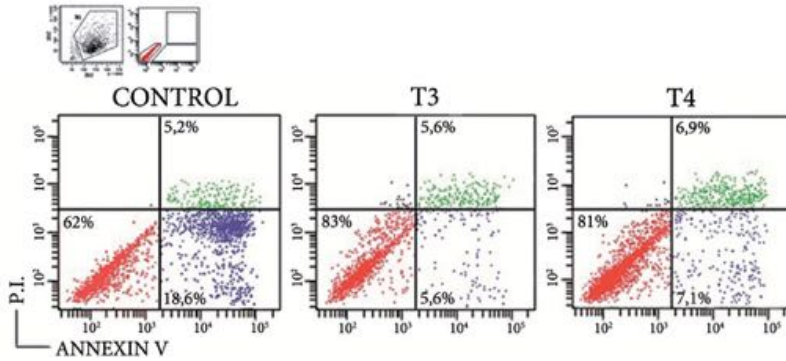*Source:* Oliva *et al.*, 2013 [8].

In an histological study the expression pattern of TR isoforms have been studied in three groups of patients: one with RCTs and thyroid diseases, one with RCTs without thyroid diseases, and one with healthy RC tendons [8]. The TRα and TRβ protein expression level were characterized by western blot analysis. All healthy and pathologic RC tendons analyzed expressed high levels of TRα/β nuclear receptor isoforms, indicating that TRs are present on tenocytes and that TRα/β expression pattern is not influenced by thyroid diseases (● **Figure 1**).

To further investigate the role played by THs in the homeostasis of tendons, in vitro condition that may allow THs to induce proliferation of tenocytes has been designed. As expected, both T3 and T4 induced cell growth. The higher increase was obtained by 72 h of hormone treatment, being 19% for T3 and 10% for T4 (● **Figure 2**). Tenocytes grew with a doubling time of approximately 49 h. The addition of the THs in the culture medium led to stimulation of cell growth with a reduction of the doubling time. In particular, T3 induced a reduction in doubling time of 27% (36 h) and T4 of 19%, with the 10^{-7}M dose.

Was also noted that THs were able to counteract apoptosis in human tenocytes primary cells after 48 hr serum deprivation. T3 and T4 caused an increase in vital

● **Figure 3.** THs are able to counteract apoptosis of human tenocytes

THs were able to counteract apoptosis in human tenocyte primary cells after 48 hr serum deprivation.
Source: Oliva *et al., 2013* [8].

cells (83, 81 *vs* 62%) and a reduction of apoptotic (5.6, 7.1 *vs* 18.6%) cells after 48 h compared with the control cells (● **Figure 3**).

The relationship between thyroids pathologies and collagens disorders has been described by some authors [9]. In particular hyperthyroidism seems to be accompanied by increased rates of catabolism of both soluble and insoluble collagen, while hypothyroidism seems accompanied by decreased rates of catabolism of collagen. After 14 days of in vitro culture with THs and the addition of Ascorbic Acid, an increased expression of collagen type III and a significantly increase of synthesis of Collagen type I have been found.

Decorin, biglycan, fibromodulin, are prostaglandings with collagen-binding proprieties and they interact with the collagen fibers and other matrix molecules, regulating the ECM assembly, including fibrillogenesis. After 14 days of culture with THs and Acid Ascorbic the production of byglican was increased, while no expression of fibromodulin with or without T3 or T4 has been found.

Cartilage Oligomeric Matrix Protein (COMP) or trombospondin 5, first identified in cartilage, is a glycoprotein particularly present in tendon exposed to compressive load. It belongs to the thrombospodin gene family with the ability to bind to type I, II, and IX collagen molecules as well as fibronectin. COMP modulates the organization of collagen fibrils. A significant increase of COMP synthesis has been found with the addition of THs.

In conclusion, these results show that the TRα/β nuclear receptor isoforms are present in healthy and pathologic rotator cuff tendons. They also reinforce the concept of a physiological action of THs in the homeostasis of tendons. THs seems to enhance, in vitro, tenocytes growth, and counteract apoptosis in healthy tenocytes isolated from

tendon in abdose- and time-dependent manner. They also seem to increase the synthesis of Collagen type I and III, and the synthesis of other ECM proteins, in particular COMP and byglican. For these reasons we think that THs may have a role also in the failed healing response during tendinopathies [10]. Much research remains to be performed to clarify the exact role of THs in tendon tissues and their implications in tendon ruptures, tendinopathies and tendon healing. If this association is confirmed, assessment and treatment of patients with tendon conditions may have to be revisited.

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Exploring the role of hypercholesterolemia in tendon health and repair*

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High cholesterol remains a significant healthcare problem, as more than 13% of adults in the U.S. are affected by hypercholesterolemia [1]. The detrimental effects the disease has on cardiovascular health are well-documented, but the effects on the musculoskeletal system, and more specifically on tendons, have not been thoroughly examined. Few studies have been reported in the literature about the relationship between hypercholesterolemia and tendon disease. Some clinical studies have demonstrated an association between familial hypercholesterolemia and Achilles tendon xanthomas and subsequent ruptures [2, 3] and others have suggested that there is a link between rotator cuff tears and high cholesterol in shoulder patients [4]. The aim of our work is to determine how high cholesterol impacts the mechanical properties of otherwise healthy tendons, and whether the disease impairs the tendon's ability to heal after acute injury.

The first animal study utilized a porcine model and consisted of seven male Yorkshire pigs with a high cholesterol group (n = 4) that received a high cholesterol diet for 5 months and a control group (n = 3) [5]. The animals were sacrificed after five month. Mean cholesterol levels at the time of sacrifice were 290 mg/dL for the hypercholesterolemic (HC) group and under 100 mg/dL for the control (CTL) group. No differences were noted in tendon size as measured by cross-sectional area, but biomechanical testing revealed significantly reduced stiffness ($p<0.002$) and Young's modulus ($p<0.0001$) in the HC group compared to CTL tendons. HLB tendon mechanical properties were substantially reduced and this supported clinical observations relating high cholesterol and the incidence of rotator cuff tendon tears [4].

To examine the cumulative effects of hypercholesterolemia murine knock out models have been used. Forty male C57BL/6 CTL mice and 40 male C57BL/6 mice deficient for Apolipoprotein E (APOE) representing a hypercholesterolemic group [6]. The aim was to investigate the effects of an accumulation of exposure to high cholesterol on mouse patellar tendon compared to controls. Half of the animals were sacrificed at 14 weeks while the other animals were sacrificed at 10 months. Tensile testing of patellar tendons from 14-week-old APOE mice receiving a unilateral full-thickness central defect resulted in normalized (comparing injured to contralateral sham) cross-sectional areas was closer to baseline compared to controls. But after 10 months, APOE mice showed a decrease in elastic modulus, indicating a detrimental cumulative effect of hypercholesterolemia on tendon properties in this model.

A second study was designed to evaluate patellar tendon healing in normal and hypercholesterolemic knockout mice [7]. It was hypothesized that tendons from aging hypercholesterolemic mice would exhibit inferior baseline mechanical properties and tendon healing compared to normal controls. Uninjured patellar tendons from APOE mice showed a significant decrease in elastic modulus but a trend toward increased cross-sectional area compared to control. Normalized maximum stress was significantly lower in the APOE group than in the controls and there were no differences in normalized area or modulus. As hypothesized, APOE tendons exhibited reduced healing strength and baseline elastic modulus compared to controls. It should be noted that the mice here were older than in the previous study. The reduction in tendon healing in aging hypercholesterolemic tendons may be linked to the cumulative effects of intratendinous cholesterol deposition or relative tissue ischemia due to vascular compromise, as seen clinically in older patients. This knockout mouse model is more comparable to the less-common condition of familial hypercholesterolemia Therefore, further work was performed to address the translational potential of this research direction.

The effects of diet-induced hypercholesterolemia on rotator cuff tendon mechanics have also been study in a rat model [8, 9]. Two high cholesterol diets have been designed. It was hypothesized that both would induce hypercholesterolemia in the rats and that supraspinatus tendons from hypercholesterolemic rats would exhibit reduced mechanical properties compared to rats fed a normal diet. Thirty male Sprague-Dawley rats (400-450 grams) were used in this study, with ten rats receiving a high-cholesterol diet consisting of 4% cholesterol and 1% sodium cholate (HC1 group) another group of ten rats receiving a different diet formulation consisting of 2% cholesterol (HC2 group), and the remaining ten rats receiving standard chow to serve as controls (CTL group). Lipid analysis confirmed that both high-cholesterol diets produced increased TC levels as well as TC:HDL ratios. Rats in the HC2 group also demonstrated a decline in HDL levels. Triglycerides were decreased in the HC1 rats. Biomechanical testing of supraspinatus tendons showed consistent *increases* in stiffness and elastic modulus in both high cholesterol rat groups. The findings of

increased stiffness and modulus in hypercholesterolemic rats were in direct contrast to the previous results found in the previously discussed porcine (biceps) and murine (patellar) experiments. This may be due to the differences in type, location, and function of the different tendons and how these relate to various intrinsic and extrinsic factors. While both three-month diet courses did produce marked increases in cholesterol as measured in the blood, this time frame may not have been long enough for the deleterious cumulative effects of hypercholesterolemia seen in previous work.

In the second study on a HC diet, the time course of healing of supraspinatus tendons in the rat rotator cuff injury model was evaluated [9]. All animals were subjected to a unilateral supraspinatus detachment and repair surgery, with contralateral limbs serving as within-animal comparative data. Animals continued their respective diet courses, and their supraspinatus tendons were biomechanically and/or histologically evaluated at 2, 4, and 8 weeks postoperatively. Biomechanical testing revealed a significant reduction in normalized stiffness in hypercholesterolemic rats compared with controls at 4 weeks after injury, whereas histologic analyses showed no significant differences in collagen organization, cellularity, or cell shape between groups.

Given the conflicting nature of the results, the utility of small versus large animal model systems for translational studies by exploring the effect of hypercholesterolemia on supraspinatus tendon elastic mechanical properties in mice, rats, and monkeys were assessed [10]. Supraspinatus tendons from normal and HC mice, rats, and monkeys were used. Cholesterol levels were manipulated for the mice through the previously described knockout model, and hypercholesterolemia for the rat and monkey models was investigated through changes in diet. HC animals had significantly altered plasma lipid profiles. Biomechanical testing showed a significant increase in stiffness compared with control in HC mice and rats, as well as a trend for HC monkeys. Elastic modulus was also significantly increased in HC mice and monkeys, with HC rats showing a trend. There was a strong consistency across species and between small and large animals which is important for future research. Interestingly, the aged mice were exposed to lifelong hypercholesterolemia while the rats and nonhuman primates only experienced increased cholesterol levels once they began their high-cholesterol diets. This suggests that these increased mechanical properties may be inherent to the effect of hypercholesterolemia on supraspinatus tendon rather than due to an effect of cumulative exposure time to the effects of HC.

In conclusion, although tendon disease is multifactorial, relevant factors must be evaluated in a systematic manner with the goal of reducing the impact of such costly musculoskeletal problems. These studies provided a better understanding of the implications of mixed hyperlipidemia on the long term structure and function of tendons. If a mechanistic cause of hypercholesterolemia induced tendon disease is identified, then potential therapeutic interventions such as the use of pharmacotherapy may be targeted to help combat this problem.

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How obesity modifies tendons. Implications for athletic activities

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Obesity is a world epidemic and one of the major public health problems in western countries. It is associated to an increased risk for diabetes, hypertension, and other cardiovascular diseases, as well as for musculo-skeletal disorders.

Several studies show that tendons frequently undergo to degeneration in obese subjects, which can progress to a symptomatic tendinopathy. Emerging studies suggest that tendinopathy is frequent in obese patients and that patients affected by tendinopathy or tendon rupture have significantly higher adiposity levels than controls [1]. Adiposity has been recognized as a risk factor for rotator cuff tears[2], and poorer outcomes have been reported after arthroscopic rotator cuff repair in obese patients than controls [3, 4].

Anatomic studies shows that Achilles tendon thickness is significantly higher in obese individuals than control group [5]. The average stress (force per unit area) experienced by the Achilles tendon is similar in both groups. After strenuous exercise (a series of 90-100 repetitions of standing calf raise) tendon thickness is reduced, due to the loss of interstitial water, associated with load-induced alignment of collagen fibers. However, the transverse strain in the tendon, calculated as the natural log of the ratio of post to pre-exercise tendon thickness, in obese subjects is almost half of that of normal weight counterpart. This finding suggests that obesity is associated with structural tendon changes that impair interstitial fluid movement in response to tensile load, and are responsible of a greater transverse stiffness [5]. Ultrasound evaluation shows thicker and hypoechoic tendons in obese subjects compare to normal people [6].

Histological changes have been observed in animals studies. Healthy tendons are formed by large and small fibrils, following a bimodal pattern distribution. Large fibrils are essential for the tendon to withstand tension forces, whereas remodeling of

the tendon results in the occurrence of fibrils with a smaller diameter [7]. In obese rats the fibril diameter shows an unimodal distribution, because of the relative prevalence of large fibrils, expression of an impaired remodeling process. Because thin fibers give greater elasticity to tendons, their relative paucity in obese animals could be responsible for increased stiffness and microruptures as a consequence of excessive loads [7]. Selective staining procedures show lipid droplets in the extracellular matrix, which could be expression of an early stage of tendolipomatosis, and it could progress to severe changes in tendon architecture and function [8]. At ultrastructural analysis by transmission electron microscopy, disorganized and tangled collagen fibrils can be observed in the tension region of tendons in obese animals [9]. Biochemical abnormalities are characterized by low levels of glycosaminoglycans (chondroitin and dermatan sulfate), which play an important role in the regulation of the extracellular matrix and collagen fibrillogenesis [10]. Their reduced concentration might be responsible for the inadequate deposition and organization of collagen fibrils [11]. On the contrary, obese rats showed an higher hydroxyproline content, probably secondary to the increased mechanical requirements.

Physiopathology of tendinopathy in obese patients has yet to be understood. Two theories have been proposed. The increased yield on the load-bearing tendons and the biochemical alterations attributed to systemic dysmetabolic factors. Weight-bearing tendons are exposed to higher loads with increasing adiposity, and the higher loads lead to overuse tendinopathy. The systemic hypothesis is based on studies showing that the association with adiposity is equally strong for non load-bearing and load-bearing tendons [12]. Many bioactive peptides and hormones are released by adipocytes, and adipose tissue is now considered as a major endocrine and signaling organ. These hormones called adipokinome, include chemerin, lipocalin 2, serum amyloid A3, leptin and adiponectin [13]. They are released by visceral fat and fat that surrounds blood vessels, during adipocity lipolysis, and they are able to influence mesenchymal stem cells activities, which may directly modify tendon structure (• Figure 1). In particular, adipokines are able to modulate cytokines, prostanoids, and metalloproteinases production [14, 15]. The persistently raised serum levels of PGE2, TNF-α, and LTB4, observed in obesity produce a systemic state of low-grade inflammation and may act as a prolonged disruptor of tendon homeostasis [16] (• Figure 2). TGF-β is also reduced, and this may have a detrimental effect on tendon healing, especially if the production of type I and III collagen is also reduced [17].

Obesity is frequently associated with other pathologies, such as diabetes mellitus and insulin resistance, which may also play a role in tendon pathology. High glucose levels determine the formation of Advanced Glycation Endproducts (AGEs). A key characteristic of reactive AGEs is the formation of covalent cross-links within collagen fibers, which alter tendon structure and function. The AGEs proteins, the cross-linking in collagen fibers and the up-regulation of pro-inflammatory mediators could impair the properties of tendon's Extra Cellular Matrix (ECM) [18, 19]. Dysli-

● **Figure 1.** Hormones called adipokinome are released by visceral fat and fat that surrounds blood vessels
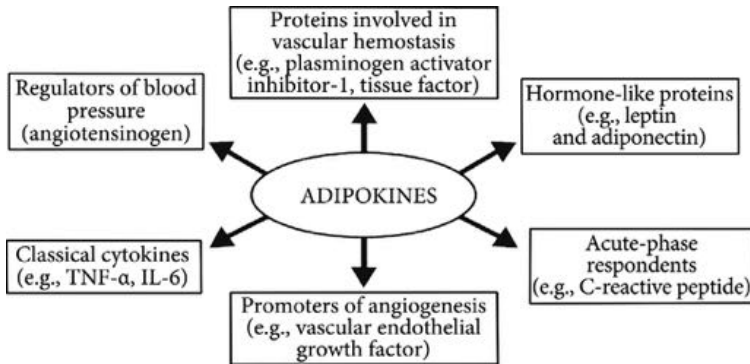
Source: Gaida *et al.*, 2009 [1]; Abate *et al.*, 2012 [6]; Del Buono *et al.*, 2011 [16].

● **Figure 2.** Systemic state of low-grade inflammation in obesity

How does obesity modify tendons?

● State of subclinical, chronic, low-grade inflammation acts as prolonged disruptor of tendon healing

● **Consistently raised levels of cytokines, prostanoids and metalloproteinases** (IL-1, IL-6 (and CRP), COX-2, PGE1, PGE2, TNF-α, TGF-β, MCP-1, LTB4, NOS type II) induce pain hypersensitivity, maintain inflammation and affect the synthesis of adipokines

● **They actively induce tendon changes** (thickening, decreased fibroblast proliferation and collagen type I and III synthesis, disorganization and degeneration of the ECM)

Source: Gaida *et al.*, 2009 [1]; Abate *et al.*, 2012 [6]; Del Buono *et al.*, 2011 [16].

pidemia is another consequence of insulin resistance associated to visceral adiposity. However, the deleterious effects of dyslipidemia on tendons are debated.

Another important topic is sport activities in obese patients. Physical activities and active lifestyle are commonly suggested to lose weight and to reduce cardiovascular risks. Exercise has beneficial effects on tendon morphology and function, because

mechanical loading is important to maintain tendon homeostasis. In obese patients adiposity may change tendon mechanical properties, and exercise could have a negative influence on tendon response to loading. Studies performed on Achilles tendon in runners support this idea [7]. During normal running, the tendon is highly solicited and the load can be as high as eight times body weight, so that modest increases in weight are amplified within the tendon. Leisure sport activity is useful in overweight or obese subjects. However, excessive overload can determine pathologic changes, and therefore some caution is necessary. Frequency and intensity of the sport performance should be increased gradually, in accordance with the progression of weight loss, avoiding agonistic activity and contrast sports, which are more likely to expose to acute injury. Non-weight bearing sports such as swimming and cycling should be preferred.

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SESSION 2

Calcification of the rotator cuff tendons and its relationship to endocrine disorders

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Calcific tendinopathy (CT) of the tendons of the rotator cuff (RC) is a common problem, with a reported prevalence varying from 2.7% to 22%, mostly affecting women between 30 and 50 years [1]. Although CT shows a strong tendency toward self-healing by spontaneous resorption of the deposits, it does not always follow this typical pattern.

The aetiopathogenesis of calcific tendinopathy is still unknown, especially because it remains difficult to clarify the primitive step which permit to the crystals to deposits in the RC. Many pathogenetic theories have been proposed, which are summarized in a recent review [2]. Uhthoff and coworkers hypothesized that a favorable environment permits an active process of cell-mediated calcification, usually followed by spontaneous phagocytic resorption. Benjamin and coworkers believe that calcifications are formed by a process resembling endochondral ossification, with bone formation and remodeling mediated by population of osteoblasts and osteoclasts. Other authors thought that ectopic bone derives from metaplasia of tendon cells into osteogenic cells. An association between CT and diabetes and thyroid disorders has been reported, but the precise mechanism is still unknown [3]. Patients with associated endocrine disorders present earlier onset of symptoms, longer natural history, and they undergo surgery more frequently compared to a control population [4]. More than 30% of patients with insulin-dependent diabetes have tendon calcification [5].

Different authors reported the prevalence of rotator cuff CT range between 2-20% of asymptomatic shoulders, and between 7-17% of symptomatic shoulders [1]. Specimens of RCTs calcific deposits consist of a gritty mass of sandy material or a toothpaste-like fluid, and the deposits were described as a white amorphous mass composed of many small round or ovoid bodies. Later, X-ray diffraction and infrared spectrom-

etry and other techniques identified the material of calcific deposits as calcium carbonate apatite [2].

An histological study described tissue changes and patterns of cell in relation to the stage of the RCTs and compared it with healthy RC tendons [6]. The study group consisted of 40 specimens from 40 patients. Eight specimens resulted from a small RC tear, 13 form a medium tear, 15 from large, 4 from massive, and 4 healthy tendon were used as control. All specimens in the study group showed oedema within the extracellular matrix and degenerative changes with fragmentation and disorientation of collagen fibrils. The fibroblast population, which are important to form granulation tissue and for normal healing processes of connective tissues, decreases as the size of the tear in the rotator cuff increases. Larger fibroblast population were seen in the smaller tears, which were actively proliferating taking part of an active reparative process. In contrast, fewer fibroblasts were present in the larger sized tears, with no evidence of cell proliferation. Contrary to many previous studies, the specimens examined showed changes of chronic inflammation and repair, which was mainly found in the smaller tears within the rotator cuff tendon. Macrophages were seen to decrease significantly in number from small to medium tears and from medium to large and massive tears. This trend was also noted for mast cells and leucocyte numbers. These observations indicate that the inflammatory process diminishes as the tear size increases and the potential for the tendon tear to heal by means of resolution, regeneration and repair diminishes as the tear size increases. The extracellular matrix also showed many differences. Fibrocartilaginous metaplasia was identified in 73% of specimens. Increased numbers of chondrocytes were observed in the proximal stump of bursal-side, partial thickness rotator cuff tears, in areas of relative avascularity and diminished numbers of fibroblasts. Chondroid metaplasia was evident in areas of low fibroblast cellularity and medium, large and massive tears had significantly more evidence of chondroid metaplasia than those from small tears. No association between the presence of chondroid metaplasia and either chronicity of the symptoms or the age of the patient was found. Amyloid deposition also appeared to be more prevalent in the larger sized tears and again was not associated with the chronicity of the symptoms, or the age of the patient. This study showed that there is a significant inflammatory component in smaller tears when compared with larger ones and therefore, these smaller tears show a greater potential to heal.

Another interesting morphological phenomenon that occurs in RC tendon tears is the modification of tenocytes' shape. Tenocytes become round cell and they changed behaviors. They seems to produce crystals, amyloid substance, and determine chondroid metaplasia. This phenomenon occurs mainly in the advanced stages of the pathology rather than in the earlier stages.

The belief of a possible association between CT and metabolic diseases, like diabetes and thyroid disorders, is now spreading among authors. So, we investigated the natural history of RC calcific tendinopathy with an emphasis on the association

with endocrine and connective tissue disorders. Given the prevalence of endocrine disease found in our study [3], we believe that these may have an important role in the etiology and pathogenesis of calcific tendinitis, although the mechanism of this effect is unknown. Computerized hospital records identified 149 patients diagnosed with calcific tendinitis. The study cohort of 102 patients (125 shoulders) comprised 73 women (71.6%) and 29 men (28.4%) (● Figure 1).

Calcific tendinopathy of RC was associated with endocrine disease in more than 64% of patients in our study, in particular with hypothyroidism, rheumatoid arthritis and diabetes mellitus. A high prevalence of both autoimmune and hormone-related gynecologic diseases was found. Compared with normal population, estimates for the prevalence of these diseases the prevalence of such diseases is significantly higher in this study. Than it is important to note that patients with associated endocrine disease have symptoms develop at a younger age (● Figure 2), have a significantly more protracted natural history, and more frequently undergo surgical treatment than patients with no associated endocrine disease.

Than, even if pain related to rotator cuff tendinopathy is a common problem little is known about the origin of pain from the tendon substance. We found a significant increase in the expression of glutamate and glutamate receptors in tendon tears [7]. Glutamate and the glutaminergic system play a key role in painful human tendon tears, even if the exact role is still uncertain, as glutamate is highly involved in both pain and metabolic pathways.

● **Figure 1.** Distribution of ages of symptom onset and variation with gender
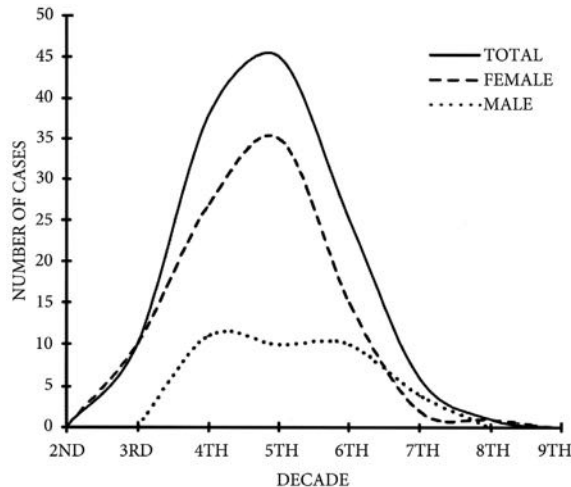
Source: Harvie *et al.*, 2007 [3].

● Figure 2. Distribution of ages of symptom onset for endocrine and non-endocrine cohorts
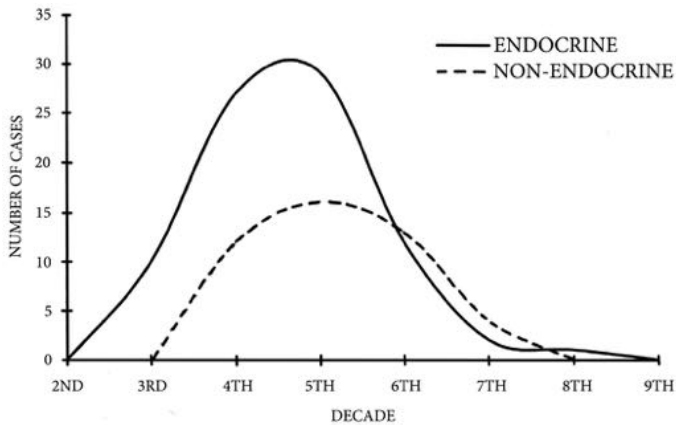
Source: Harvie *et al.,* 2007 [3].

Treatment of CT is controversial. There is currently no uniformity in the way shoulder disorders are labeled or defined. Measurement of outcome varies widely between clinical trials and, in general, the reliability, validity, and responsiveness of these outcome measures are not established. More recent studies supported the use of high-energy extracorporeal shock-wave (ESWT) and needle lavage for improving pain and shoulder function in chronic calcific shoulder tendinitis, which can result in complete resolution of calcifications [8, 9].

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Potential ways that estrogens and androgens may modify tendons

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Compared to male, women seem to have a greater risk of tendon injuries, but there is no monofactorial explanation for this connective tissue sex disparity. Many factors have been considered to be involved in this phenomenon, like anatomical differences and sex differences in hormonal levels.

Some studies show that there are differences in tendons physical and mechanical properties among genders. A morphological study compared Achilles and patellar tendons in trained and untrained women and men [1]. The authors found that the ability of Achilles and patellar tendons to adapt to loading was different among genders. In fact the Achilles and patellar tendon cross sectional area was greater in trained people compared to untrained people in response to mechanical loading, but in trained men the increase was greater than in trained women (● Figure 1). Compared to men, women have an attenuated tendon response to training, a lower tendon collagen synthesis rate following acute exercise, and a rate of tendon collagen synthesis which is further attenuated with elevated estradiol levels.

A histological study showed that the collagen synthesis in response to exercise was lower in women [2]. Tendon synthesis was still elevated in men 72 h after exercise, whereas in women no difference in tendon synthesis was observed between the leg which had performed exercise and the control resting leg. Therefore, these studies suggest that tendons have a lower rate of new connective tissue formation in women, their response to mechanical loading is reduced, and that the mechanical strength is lower compared to men.

Many authors studied the role of anabolic steroids in order to understand if they may influence tendons homeostasis. Anabolic steroids are strong stimulators of tendons and muscles, but there is little evidence about tendons. The influence of aging and sex hormones on connective tissue was previously investigated in an animal study

● **Figure 1.** In a cross-sectional study a greater tendon cross sectional area was found in trained men compared to untrained men, but also compared to untrained and trained women
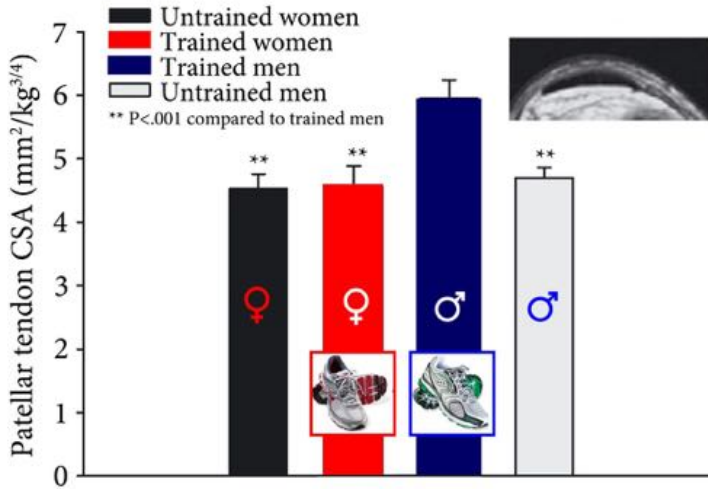
Source: Magnusson *et al.*, 2007 [1], adapted.

in the late '70 [3]. Collagen content was significantly greater in males than in females after sexual maturation. The collagen content and fibril diameter were considerably increased by ovariectomy and significantly decreased by the administration of estrogen. Furthermore collagen synthesis was significantly increased by the administration of testosterone in orchiectomized male rats, indicating that testosterone may have an effect on collagen synthesis. A more recent study reported that mechanical stimulus induced by sport is able to elicit adaptive changes in patellar in elderly subjects. However, lower collagen metabolic responsiveness in women was found [4]. Denaro *et al.* [5] investigated the effects of dihydrotestosterone (DHT) on human tenocytes cultures from the intact supraspinatus tendon. The role of hormones in the pathogenesis of tendinopathy is not well recognized, even though the use of anabolic steroids is correlated with a higher incidence of spontaneous tendon ruptures. In vitro, progressive increasing concentration of DHT had direct effects on male human tenocytes, increasing cell number after 48 h and 72 h of treatment, and leading to a dedifferentiated phenotype after 48 h of treatment. This study showed a preliminary evidence for a possible correlation between testosterone abuse and shoulder tendinopathy.

It seems that oral contraceptives affect directly or indirectly the tendon's collagen synthesis.

Women who are chronically exposed to high levels of estrogen, for example users of oral contraceptives (OC), may have an altered collagen content of tendons and ligaments, which may change the biomechanical properties [6]. In fact lower collagen synthesis has been observed in exercising women vs. men, and in users of oral contraceptives vs. nonusers [7] (• Figure 2). Endogenous or exogenous estrogen may influence the risk of injuries by changing the structural composition of ligaments and tendons. But equivocal data exist in the literature. Animal studies have reported no effect of estrogen on the mechanical properties of sheep knee ligaments [8], whereas a study on rabbits reported a lower failure load of ACL after 30 days of estrogen administration [9]. The effects of steroids hormones on tendons seem to be different between young compared to older post-menopausal women. While in younger women estrogens stimulation seems to have detrimental effects on tendons, in older post-menopausal women they seem to have stimulating effects. In fact, tendon collagen fractional synthesis rate is reduced in young oral contraceptives users compared with controls [6]. A clinical study was designed to test mechanical properties of the patellar tendon in oral contraceptives users and non-users at two different time points during the menstrual cycle and pill cycle, respectively [7]. No differences in patellar tendon structural composition, collagen cross-linking, and biomechanical properties

• **Figure 2.** Oral contraceptives seem to inhibits the exercise-induced increase in the initial synthesis of collagen either directly or indirectly
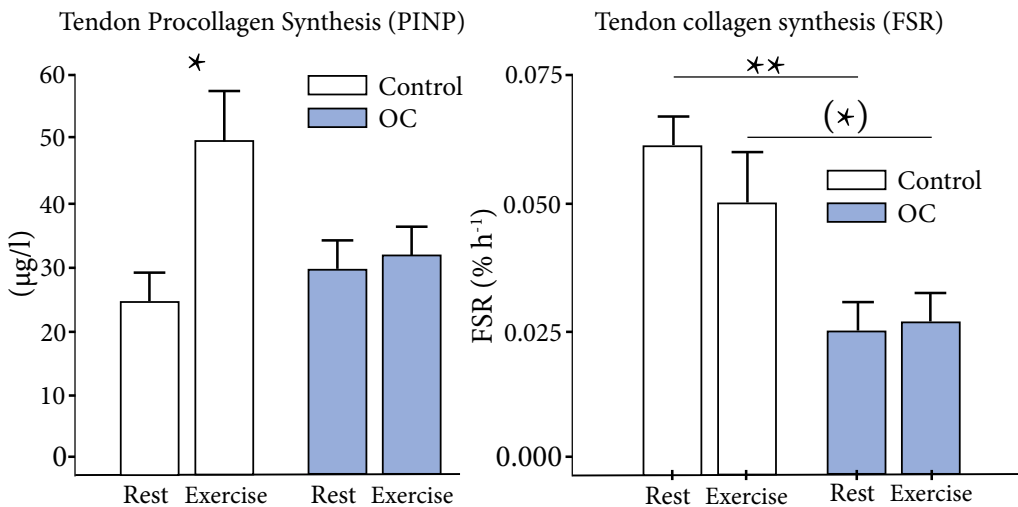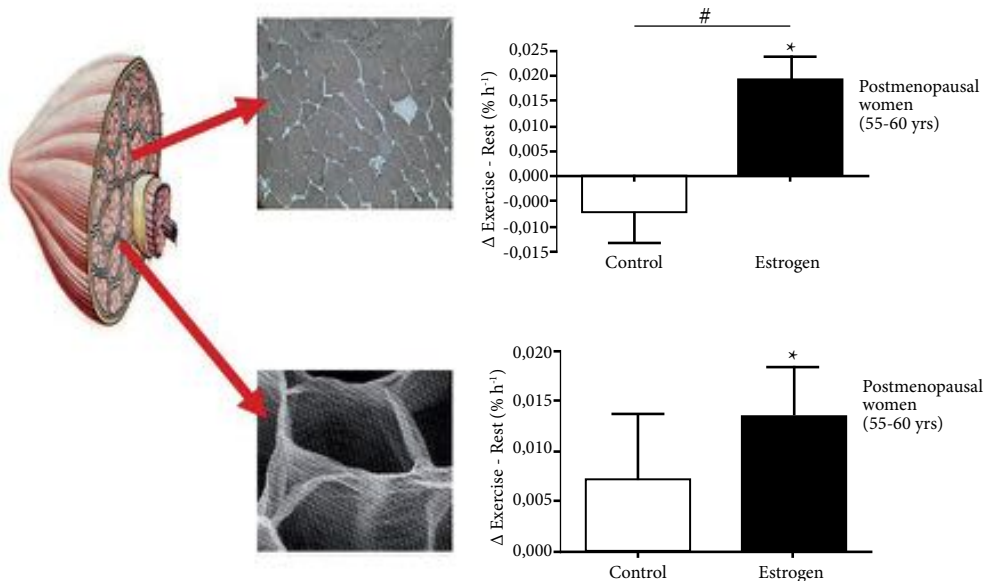
Source: Hansen *et al.*, 2009 [6]; Hansen *et al.*, 2013 [7].

were observed, but the study showed that high exposure to estrogen in young women to increased knee laxity and thus an elevated risk of an ACL injury. Different results have been found in older postmenopausal women. In a study comparing synthesis rate of myofibrillar and collagen proteins in 20 postmenopausal women, who were users of estrogen replacement therapy (ERT) after hysterectomy/oophorectomy or not (controls), the authors found that myofibrillar and collagen proteins were lower in hysterectomized/oophorectomized women using ERT compared with healthy postmenopausal women (● Figure 3) [10]. Nevertheless, resistance exercise in combination with ERT seems to have a counteracting effect on myofibrillar FSR in hysterectomized/oophorectomized women. The effects of transdermal ERT on type I collagen synthesis in tendon and skeletal muscle was investigated in 11 postmenopausal women. ERT was associated with enhanced synthesis of type I collagen in the skeletal muscle in response to acute exercise, indicating that the availability of estrogen in postmenopausal women is important for repair of muscle damage or remodeling of the connective tissue after exercise [11].
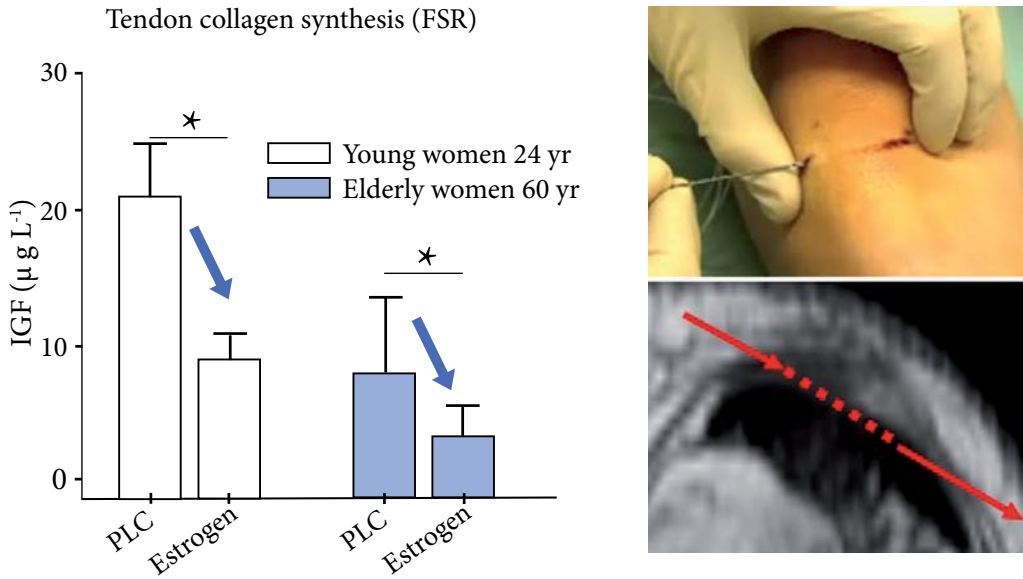
The growth hormone (GH) and insulin-like growth factor-I (IGF-I) are impor-

● **Figure 3.** The anabolic effect of estradiol in post-menopausal women: the increase in muscle and matrix proteins induced by exercise is more pronounced in patients with estrogen treatment

Source: Hansen *et al.*, 2012 [10].

● **Figure 4.** The decrease of IGF-1 in young and post-menopausal women

tant hormones which stimulate of collagen synthesis in connective tissue. An animal study showed that IGF-1is involved in mediating the effects of estrogen on tendons and muscle [12]. IGF-1 have in fat an important role in modulating the synthesis of tendons collagen protein. Studies in humans with pathologically high levels of GH/IGF-I, and in healthy humans who receive either weeks of GH administration or acute injection of IGF-I into connective tissue, demonstrate increased expression and synthesis of collagen in muscle and tendon [13]. Than the GH/IGF-I axis is able to influence both morphology and mRNA levels of selected genes in the muscle-tendon unit of mice [14]. A decrease of IGF-1 levels has been found both in young and post-menopausal women, but in older women the levels of IGF-1 are lower than in younger women and as well as the decrease (● **Figure 4**). So, the reason of the poor effects of estrogen on tendons in young women could be explained with the high suppression of IGF-1 levels.

A recent human study showed that injections of IGF-1 in animals is able to increase collagen synthesis in tendons and ligaments and to improve structural tissue healing, and that a local IGF-I administration can directly enhance tendon collagen synthesis both within and around the human tendon tissue [15]. In this study, two injections of either human recombinant IGF-I or saline into each patellar tendon were performed 24-h apart. The authors found that tendon collagen fractional synthesis rate was significantly higher in the IGF-I leg compared with the control leg.

In conclusion, the effects of testosterone seem to be very limited, but there are also limited data and more studies on the effects of testosterone are needed. The effect of estrogen seems to have small anabolic effects. They produce more effects in post-menopausal women, while in younger women these effects seem to be mediated by other mechanisms. GH and IGF-1 seems to stimulate the synthesis and collagen cross-link formation.

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# In vivo tenocyte metabolism in aging and oestrogen deficiency

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Little is known about tendons and tenocyte biological behaviour during aging and oestrogen deficiency. Tenocytes play a central role in maintaining the homeostasis of tendon extracellular matrix (ECM) and transmitting signals through their environment with proteins. The function, mechanics and homeostasis of tendon tissue depend on the orchestrated synthetic and degradative processes of tenocytes. In middle age, people used to playing sports regularly do not change their habits, even if changes begin to occur in the physiology and function of connective tissues. This leads to more frequent injury. Few studies focused on the effect of aging on tendons. Oestrogen levels might play an important role as observed in women that showed a lower risk of tendinopathies during pre-menopausal years, whereas, after menopause, the risk is increased [1]. Postmenopausal oestrogen deficiency seems to downregulate collagen turnover and to decrease elasticity in tendon [2]. Some differences in Achilles tendon structure in post-menopausal in comparison to young women have been found, and authors thought that hormone replacement therapy (HRT) with exogenous oestrogen may improve tendon structure by preserving collagen fibre diameter rather than collagen production and thus preventing tendon ruptures. Furthermore, oestrogen positively influences tendon morphology and biomechanical properties in postmenopausal women [3, 4]. The combination of HRT and physical activity has positive effects on Achilles tendon properties [5]. An animal study showed poorer Achilles tendon healing in oestrogen-deficient rats compared to controls [2]. However, others authors found that collagen synthesis was negatively related to estradiol concentration during a one-legged resistance exercise [4]. However few studies in literature have focused on the in vitro behaviour of tenocytes during aging and on oestrogen deficiency, and few data are available on in vitro ECM production and tenocyte metabolism.

● **Figure 1.** Tenocytes viability of young, old and ovariectomised rats
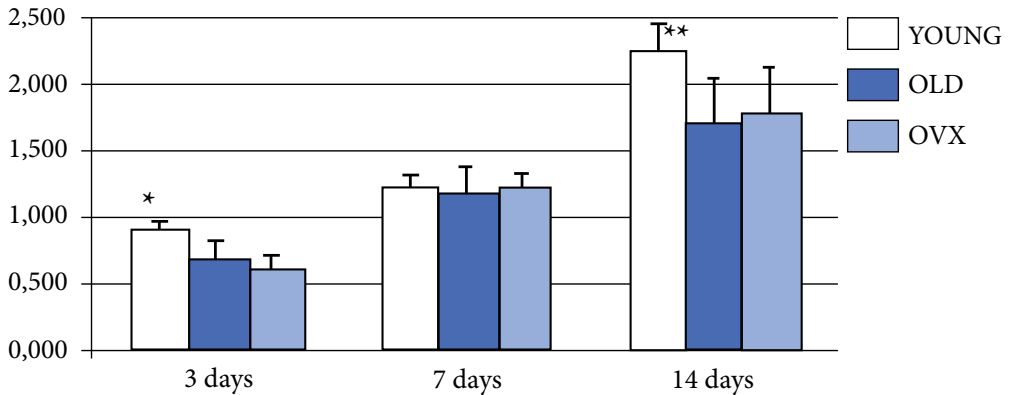
Source: by courtesy of Torricelli *et al.*, 2013 [6].

The aim of our study was to evaluate the in vitro the proliferation and metabolism of tenocytes isolated from the Achilles tendons of ovariectomised (OVX), middle-aged (OLD) and young (YOUNG) rats.

Three groups of Sprague Dawley rats have been used for the study, young rats (5 months old), middle-aged (13 months old) and ovariectomised rats (10 months at ovariectomy and euthanized 3 months later at the age of 13 months). Two different models have been used, a standard culture and an in vitro model of micro-wound healing. This model was used to assess age and oestrogen deficiency differences in tendon healing. In standard culture, tenocyte viability and the synthesis of ECM components, catabolic enzymes, growth factors and nitric oxide were evaluated at 3, 7 and 14 days of culture. In the in vitro tendon micro-wound healing model [7], micro-wound recovery rate, tenocyte migration and the synthesis of ECM-components, catabolic enzymes, growth factors and nitric oxide have been studied.

In standard culture condition, OLD and OVX tenocytes showed a significantly lower proliferation rate. No differences have been noticed at 7 days, but the YOUNG group proliferation was significantly higher than that of the OLD and OVX groups at 3 days (p<0.05) and at 14 days (p<0.005) (● **Figure 1**). Then, OLD and OVX tenocytes showed a significantly lower collagen I, aggrecan and elastin synthesis than YOUNG ones. In OVX group, fibronectin and elastin significantly decreased in comparison to YOUNG and OLD groups, respectively, whereas vascular endothelial growth factor and metalloproteinases-13 increased than those of both YOUNG and OLD groups (● **Figure 2**).

In the microwound healing model, tenocytes from both OVX and OLD showed a significantly lower healing rate (● **Figure 3**), proliferation rate, collagen I and nitrix

RESULTS

AGEING *Versus* **YOUNG**

CELL VIABILITY (at 3 and 14 days)
COLL I (at 3 days)
ELASTIN (at 3 days)

COLL III
FBN
NO
MMP-13
VEGF

ESTROGEN DEFICIENCY *Versus* **YOUNG**

CELL VIABILITY (at 3 and 14 days)
COLL I (at 3 days)
ELASTIN (at 3 days)
FBN (at 14 days)

MMP-13 (at 3 days)
VEGF (at 3 and 14 days)

COLL III, NO

ESTROGEN DEFICIENCY *Versus* **AGEING**

ELASTIN (at 3 days)

MMP-13 (at 3 days)
VEGF (at 14 days)
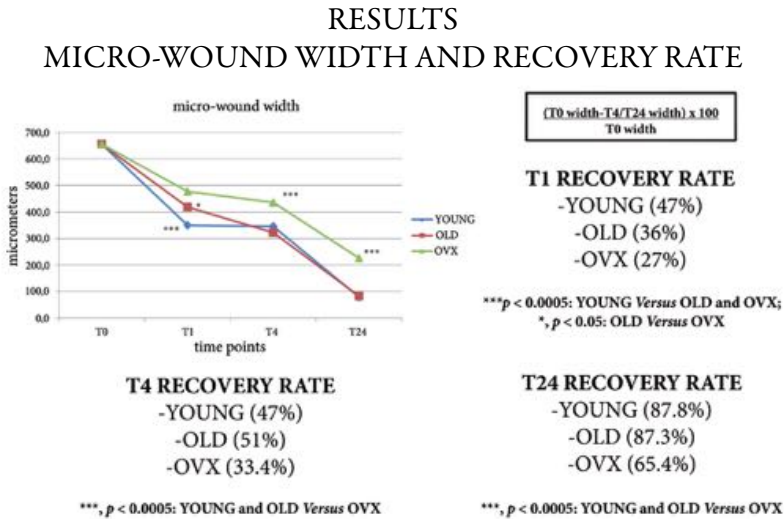
CELL VIABILITY
COLL I
COLL III
FBN
NO

Source: by courtesy of Torricelli *et al.,* 2013 [6].

oxide in comparison to YOUNG. OVX elastin value was significantly lower than YOUNG one and OVX healing rate and cell migration speed, proliferation rate and fibronectin results were lower, whereas collagen III and metalloproteinase-13 higher in comparison to both YOUNG and OLD groups (● **Figure 4**).

Aging is linked to a decline inmusclemass, strength and physical function, but little is known about what happens to tendons and tenocytes during aging and oestrogen deficiency. Since tendons are considered to have a pivotal role in the transfer of muscle force to produce movement it is important to investigate the changes in tendon features and behavior during aging. This study showed that proliferation and tenocyte biosynthesis are negatively and at least partially independently affected by aging and oestrogen deficiency, even though oestrogen deficiency exerts a greater negative effect than aging in culture. In micro-wound healing, the OLD group was able to recover the injury, although at a slower rate, whereas oestrogen deficiency showed higher negative effects on tendon healing because it was accompanied by lower cell proliferation, cell speed migration and an altered metabolism, by combining ECM protein loss and MMP overexpression. These results therefore highlighted how aging and, more significantly, oestrogen levels affect tendon metabolism and healing. However, further studies are needed to find solution for the prevention of tendon injuries in ageing and menopause.
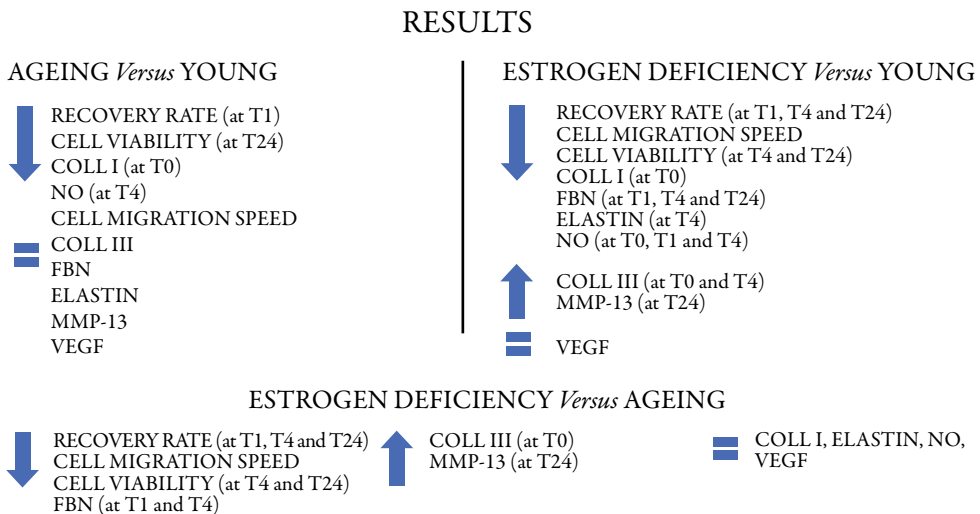
● **Figure 3.** In the microwound healing model, width and recovery rate

RESULTS
MICRO-WOUND WIDTH AND RECOVERY RATE

Source: by courtesy of Torricelli *et al.*, 2013 [6].

● **Figure 4.** Table comparing tenocytes proliferation, cells viability and ECM proteins synthesis in the three study groups

RESULTS

AGEING *Versus* YOUNG

↓
RECOVERY RATE (at T1)
CELL VIABILITY (at T24)
COLL I (at T0)
NO (at T4)
CELL MIGRATION SPEED

=
COLL III
FBN
ELASTIN
MMP-13
VEGF

ESTROGEN DEFICIENCY *Versus* YOUNG

↓
RECOVERY RATE (at T1, T4 and T24)
CELL MIGRATION SPEED
CELL VIABILITY (at T4 and T24)
COLL I (at T0)
FBN (at T1, T4 and T24)
ELASTIN (at T4)
NO (at T0, T1 and T4)

↑
COLL III (at T0 and T4)
MMP-13 (at T24)

=
VEGF

ESTROGEN DEFICIENCY *Versus* AGEING

↓
RECOVERY RATE (at T1, T4 and T24)
CELL MIGRATION SPEED
CELL VIABILITY (at T4 and T24)
FBN (at T1 and T4)

↑
COLL III (at T0)
MMP-13 (at T24)

=
COLL I, ELASTIN, NO, VEGF

Source: by courtesy of Torricelli *et al.*, 2013 [6].

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How endogenous factors can modify the efficacy of platelet rich plasma

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The aim of this work is to explain what platelet rich plasma (PRP) science and PRP therapies are, and current results in the conservative management of tendinopathy. Then an experimental study about the use of PRP in the context of hyperuricemia is showed.

PRP was defined by MeSH (Medical Subject Headings) in 2007 as a preparation consisting of platelets concentrated in a limited volume of plasma. This is used for various surgical tissue regeneration procedures where the growth factors (GFs) in the platelets enhance wound healing and regeneration [1]. Behind PRP is the concept of PRP therapies that is the ability to manipulate tissue healing using the molecular pool released from PRP. PRP science aims to develop a body of knowledge about PRP interactions in different conditions, here, for example, we have focused on PRP interactions with tenocytes in the context of hyperuricemia.

PRP therapies are a sensitive and controversial topic in Orthopedics and Sports Medicine. The use of PRPs has expanded to meet multiple medical problems where current treatment options were judged to be suboptimal. This rapid expansion has been possible given their safety profile, i.e. autologous source, and minimal manipulation.

In fact, PRP widespread use was not driven by the principles of the scientific methods instead patient demand has been boosted by sports news and propaganda reporting that outstanding elite athletes had been successfully treated with PRP. The need is clear, to investigate and describe main PRP targets and action mechanisms underlying their clinical effects.

● **Figure 1** shows the main milestones in the development of PRP therapies. In late '80 the first studies on the results of PRP therapy in the treatment of chronic ulcers have been reported. In the late 90s, maxillofacial surgeons stated that PRP regenerate bone around dental implants, while at the beginning of the new century PRP was

introduced in orthopedics and sports medicine. For the first time PRP was used for treatment of tendon diseases.

Due to the biosafety of these products, i.e. advantageous balance risk-benefit, clinical applications have preceded the basic research. Actually, in its very beginnings PRPs have been used with a vague idea of the biological mechanisms they were influencing. The use of PRP therapies started in the clinics rather than in the laboratories and physicians used PRP therapies without knowledge of its mechanism of function. Thereafter, most studies were directed to examining clinical outcomes rather than identifying the precise biochemical mechanisms underlying PRP effects, which remain to be elucidated in the most part.

PRPs differ from conventionally synthesized drugs in that they are products derived from living sources. Indeed platelets are lively cells and they may experiment several temporary transformations from preparation to local tissue delivery. The process is known as platelet activation and involves changes in platelet morphology, aggregation, centralization of granules, and secretion of their content to the extracellular milieu.

Another peculiarity is that PRP products are complex multi-molecular mixtures that cannot be readily characterized and reproducibility in the composition is influenced by biological inter-individual variability. Not only GFs are present in PRP. New proteomic analyses of platelet secretome list several hundreds of proteins including cytokines and chemokines, adhesive proteins, and enzymes: GF are in reality just a small subset [2, 3] (● Figure 2). This molecular pool is involved in mechanism governing healing neo-angiogenesis, inflammation, cells proliferation and tissue anabolism. PRP is currently considered as a tissue itself which is extract of the blood circulating tissue, and not a pharmaceutical preparation. All PRP components are the key active actors of the natural healing process (● Figure 3).

● **Figure 1.** Clinical milestones in the development of PRP therapies
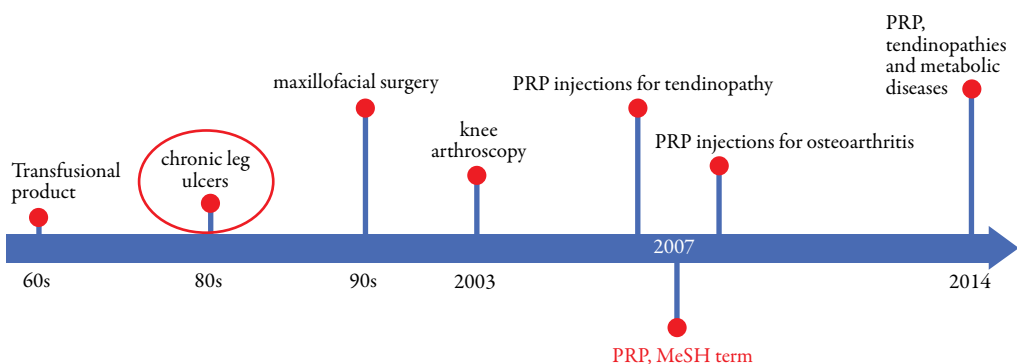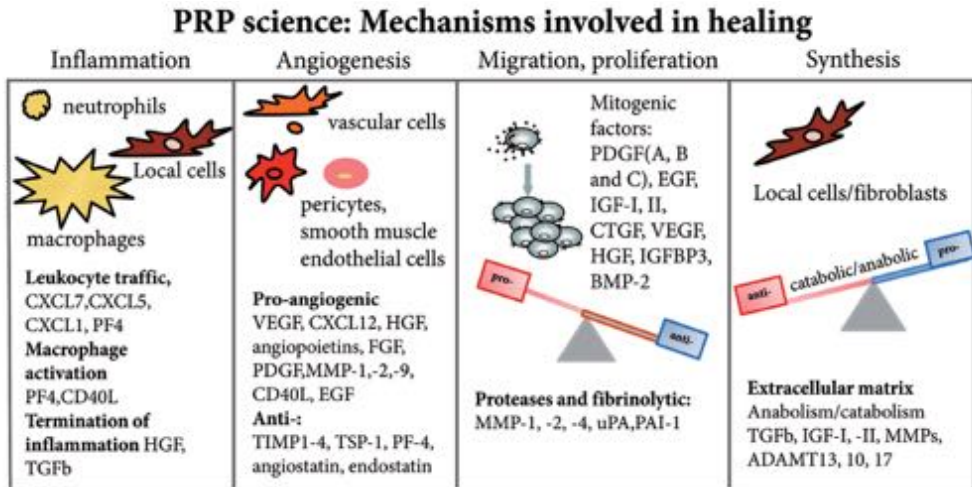

Source: Sánchez *et al.*, 2012 [4], adapted.

• **Figure 2.** Platelet secretome as part of PRP formulations
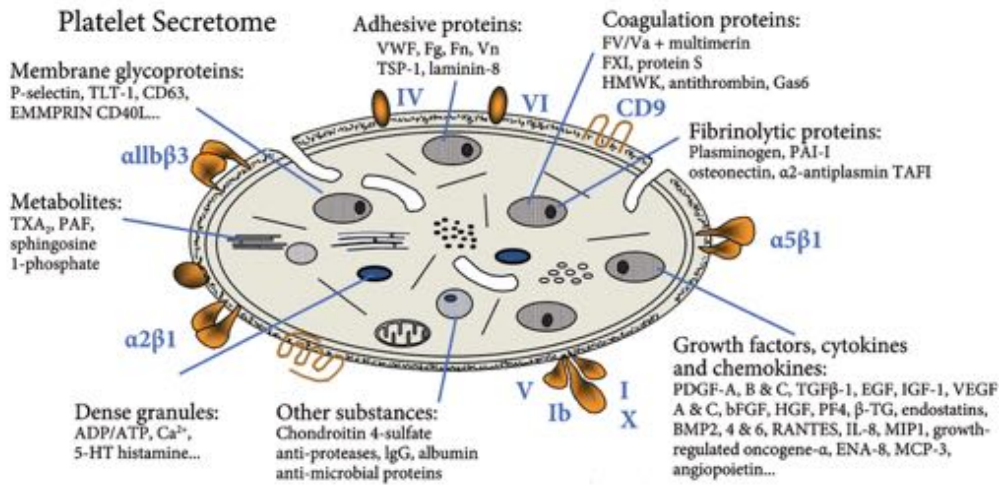
Source: Anitua *et al.*, 2004 [5], adapted.

• **Figure 3.** Signaling proteins released from PRP and their participation in mechanism involved in tissue healing

Source: Andia *et al.*, 2012 [6], adapted.

Combined together are forming a kind of engineered tissue extracted from the blood circulating tissue [7]. The need for better consideration of the cell population included in PRP formulations was advocated by different authors, and it is now one of the most important sources of debates in the field, particularly in sports medicine. Broadly speaking injectable PRPs were categorized as pure PRP and leukocyte and platelet rich plasmas [8] (● Figure 4). The presence of leukocytes in the PRP formulation adds further complexity. Considering fibrin architecture and platelet counts we can differentiate further PRP subsets [8]. Different PRP devices and harvest yield in terms of platelet and leukocyte count lead to the proposal of classification systems for PRP.

Leukocytes contained in PRP products play an important function in the healing process, regulating healing and inflammatory processes. Leukocytes are not only inflammatory cells, as they also present anti-nociceptive effects through different chemokines, anti-inflammatory cytokines (IL-4, IL-10 and IL-13) and opioid peptides (b-endorphin, metenkephalin, and dynorphin-A), and can therefore promote a clinically relevant inhibition of pathological pain. Recent studies showed the presence of several angiogenic proteins like VEGF, HGF, angiogenin, angiopoietin, IL-8, MCP-1 and RANTES, which are released both by platelets and by surrounding tissues. Some authors suggested that the balance between angiogenic and inflammatory modulators and their interaction with the host tissue may play an important role for tendon healing [2, 3].

However, the efficacy of PRP in the treatment tendinopathy is a controversial issue. Many clinical studies have been published in the last decade about PRP therapy in

● **Figure 4.** Different types of PRPs formulation, used in the conservative management of tendinopathies
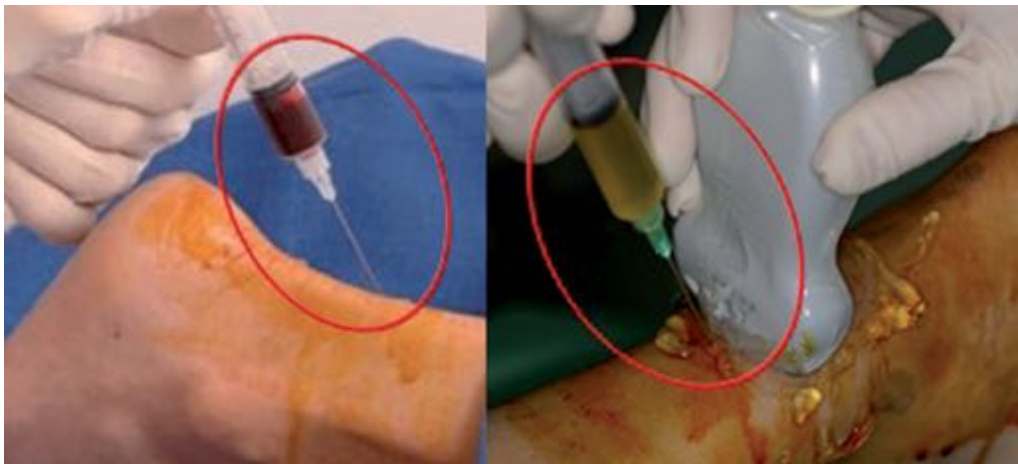
Source: Sánchez *et al.,* 2012 [9], adapted.

sports medicine with contrasting results. PRP is commonly used for the treatment of many tendon diseases, in particular when conservative treatment failed, in order to enhance tendon healing. In vitro studies on the use of PRP injections in tendinopathy showed that they can increase cell number, stimulate precursor cell differentiation and collagen fiber density, and restore extracellular matrix architecture. However, there is still not clear evidence that PRP improves in vivo tendon healing and function.

A recent systematic review [10] of literature reviewed 13 prospective controlled studies assessing PRP efficacy, comprising 886 patients and covering different tendinopaties (epicondylitis, rotator cuff tears, patellar tendinopathy and Achilles tendinopathy). 53.8% of studies used identical L-PRP protocol. This review showed that PRP has some beneficial effects on pain remission at the mid-term follow-up. But, even if the PRP formulation was the same in most studies, i.e. L-PRP, and the quality of studies was moderate, the great heterogeneity among studies hindered conclusive results. Mains sources of heterogeneity included different comparators, varied outcome scales and follow-up period, number of injections and protocols. Overcoming these methodological limitations will help to advance PRP therapies.

PRP and metabolic diseases

Most patients with metabolic diseases are excluded from clinical trials, because of the belief that metabolic diseases are a contraindication for PRP use. So, an important question we try to solve is if metabolic diseases are counter-indication for PRP application.

The application of autologous PRP to treat the diabetic foot illustrates this hypothesis. Actually, PRP has been used to treat non-healing wounds for more than 2 decades. In fact, the topical management of chronic leg ulcers was the first clinical application of platelets outside the blood stream with healing purposes. The goal of PRP is to re-activate healing in the non-healing wounds via the rapid formation of granulation tissue that prevents further deep tissue involvement and associated co-morbidities.

● **Figure 5.** PRP therapy for the treatment of an eight-month chronic diabetic foot ulcer showed excellent results after 5 week (5 injections)

Source: unpublished, original.

It has been used for diabetic foot ulcer, neuropathic ulceration, osteomyelitis with good results (● **Figure** 5). In general, concomitant pathologies such as diabetes do not hinder the therapeutic effects of PRP, and autologous PRP is effective in the diabetic foot [11] or systemic sclerosis [12]. Significant clinical outcomes indicated many previously nonresponsive wounds began actively healing in response to PRP therapy. Cost effectiveness analysis comparing the potential economic benefit of PRP to alternative therapies in treating non-healing diabetic foot ulcers, using an economic model based on peer-reviewed data showed that PRP resulted in improved quality of life and lower cost of care over 5-year period than other treatment modalities for non-healing diabetic ulcers [13].

Hyperuricemic PRP in tendons

The aim of our work is to study if tenocytes are influenced by hyperuricemia, thus we explored the response of tendon cells to the PRP-released molecular pool in terms of the synthesis of angiogenic and inflammatory modulatory proteins. We aim to assess whether hyperuricemia can be sensed as stressor by tenocytes, and whether this stressor can modify the angiogenic/inflammatory response to PRP.

Hyperuricemia, high levels of uric acid in body fluids, is becoming a critical medical problem. Its prevalence and its related comorbidities have increased dramatically in the last decades. The possibility that high levels of uric acid in biological fluids play a role in the development and progression of tendinopathy is based on recent knowledge on the influence of monosodium urate (MSU) crystals in tendon biology [14]. This is supported by the notion of uric acid acts as a death cell associated stressor, a danger signal (DAMP) that may stimulate macrophages to produce pro-inflammatory mediators such as IL-1b [15]. Recently, we have been shown that high concentrations of uric acid in the tendon cell microenvironment involve a mild alteration in extracellular matrix homeostasis in the context of platelet rich plasma [16].

Tendon cells were obtained through explant culture from the semitendinosus tendon of three healthy young donors undergoing ACL surgery. Cells were treated with PRP and hyperuricemic, and PRP, hyperuricemic and MSU. Cells treated with PRP were used as control. Pure PRP without leukocytes have been used. The concentration of uric acid in hyperuricemic cultures was 20 mg/dL. Cell proliferation, gene expression and protein synthesis have been assessed. The gene expression of SCX, DCN, ACAN, COL1A1, COL3A1, HAS2, TGF-b1, COX2, IL1b, IL6, IL8 genes have been evaluated with real time RT-PCR.

The proliferative effect induced by PRP was not affected by hyperuricemia (● **Figure** 6). When we analyzed gene expression we found a moderate increase of collagen type I and an increase of COMP induced by hyperuricemia (● **Figure** 7). They synthesis of collagen type I and COMP increased when MSU crystals were present in the culture (● **Figure** 8).

Abundant proteins present in cell culture supernatants were angiogenin, angiosta-

tin, Growth Regulated Oncogene (GRO-a/CXCL1), Regulated upon Activation Normally T cells Expressed and Secreted (RANTES/CCL5), IL-6/CXCL6, IL-8/CXCL8, and Monocyte Chemoattractant Protein (MCP-1/CCL2). Relevant levels of tissue inhibitors of metalloproteinases, TIMP-1, TIMP-2, MCP-3, angiopoietin, Vascular Endothelial Growth Factor (VEGF), and uPAR were also evidenced. Because inflammation and angiogenesis often occur in parallel several angiogenic proteins are also included in the inflammatory array. Therefore elevated levels of MCP-1, RANTES, IL-6 and IL-8 in a different array have been found.

These data show that tendon cells constitutively secrete low levels of VEGF, and IL8 but moderate levels of HGF, MCP-1 and GRO-a. The secretion of chemokines (RANTES, MCP-1, IL-6 and IL-8) and VEGF and HGF is boosted by PRP treatment. There were no differences between PRP or hyperuricemic PRP, except for IL-8 that showed a significant decrease in the presence of hyperuricemia. VEGF and GRO-a showed an increase in the presence of hyperuricemia, although not statistically significant. PRP induces a para-inflammatory response marked by the absence of relevant pro-inflammatory cytokines such as IL-1beta but characterized by elevated synthesis of chemokines inducing leukocyte migration. Tendon cells also produce a relevant amount of HGF, a crucial antiinflammatory protein in the context of PRP.

What emerges from these semi-quantitative results is that tenocytes are a source of numerous chemokines, extracellular signaling factors of the response to different stressors. We also found that, in this in vitro model, hyperuricemia is a minor stressor for tendon cells that does not modify significantly the angiogenic or para-inflammatory responses induced by PRP. In fact, major inflammatory triggers such as IL-1beta are not induced by PRP or hyperuricemic PRP.

● **Figure 6.** Hyperuricemia does not influence the proliferative effect induced by PRP on tenocytes
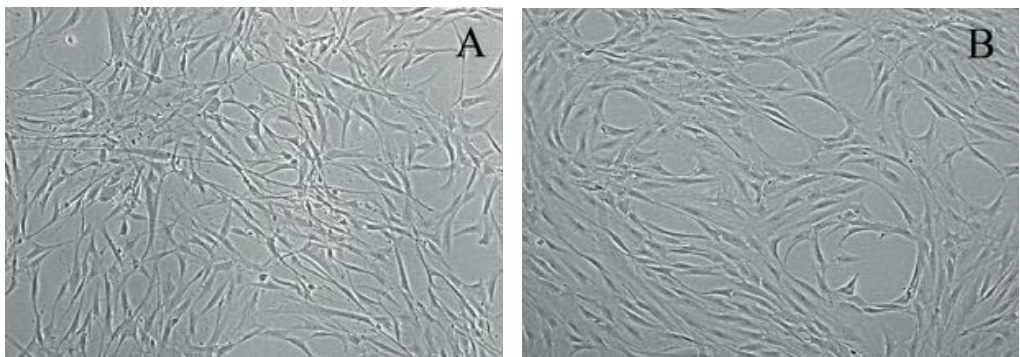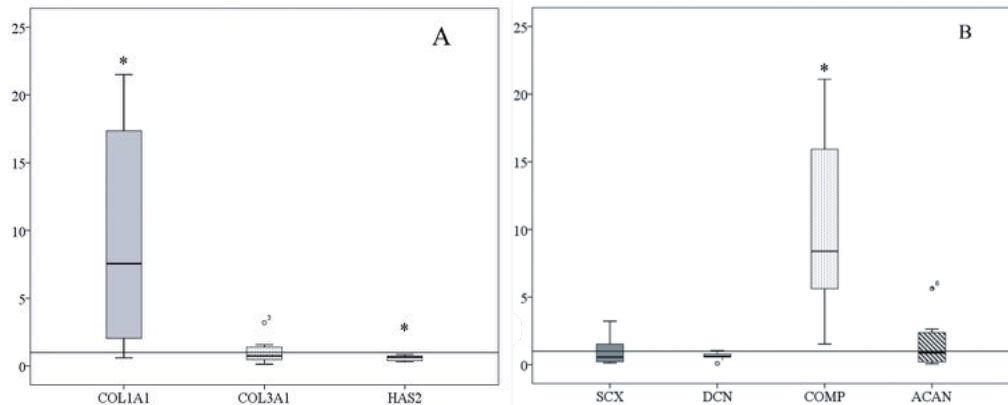

Source: Andia *et al.,* 2014 [16].

Relevant synthesis of chemokines, chemotactic cytokines with the ability to guide the migration of immune cells have been found. Tenocytes synthesize relevant amounts of monocyte chemoattractant protein (MCP-1/CCL2) and RANTES/CCL5. Both MCP-1 and RANTES mediate migration of monocyte/macrophages and are involved in inflammatory and angiogenetic mechanisms. These chemokines are typically induced during an innate immune response, and may also have a role as homeostatic chemokines involved in normal processes of tissue maintenance.

Although our results need in vivo confirmation it becomes apparent that a possible mechanism behind PRP is the enhancement and acceleration of the activation of the innate immune response by local tendon cells. Thus PRP therapies may be especially relevant in tendinopathic conditions marked by a failed healing response.

The production of these chemokines is similar in the presence of hyperuricemia. hyperuricemia is a minor stressor for tenocytes that does not induce changes in MCP-1 but in the synthesis of IL-8 confirming a previous study [14]. Of note, hyperuricemia elevates circulating CCL2 (MCP1) levels and primes monocyte trafficking in subjects with intercritical gout and serum MCP-1 is also elevated in patients with hyperuricemia compared to normouricemic controls. However, we cannot rule out that hyperuricemia could modify the polarization of infiltrating monocytes/macrophages.

Regarding inflammation, we did not detect production of IL-1b. Nevertheless, we detected a minimal intensity for IL-1alpha and TNF-alpha indicating that tenocytes might be marginally inflamed, but the presence of IL-1alpha and TNF-alpha coexists with slight levels of IL-10, which reduce inflammation. Even so, the presence of IL-6, IL-8 and GRO-a may indicate a parainflammatory state.

● **Figure 7.** A moderate increase of collagen type I and an increase of COMP induced by hyperuricemia have been found

Source: Andia *et al.*, 2014 [16].

● **Figure 8.** A higher increase of collagen type I and an increase of COMP has been found with the addition of MSU crystals to the culture
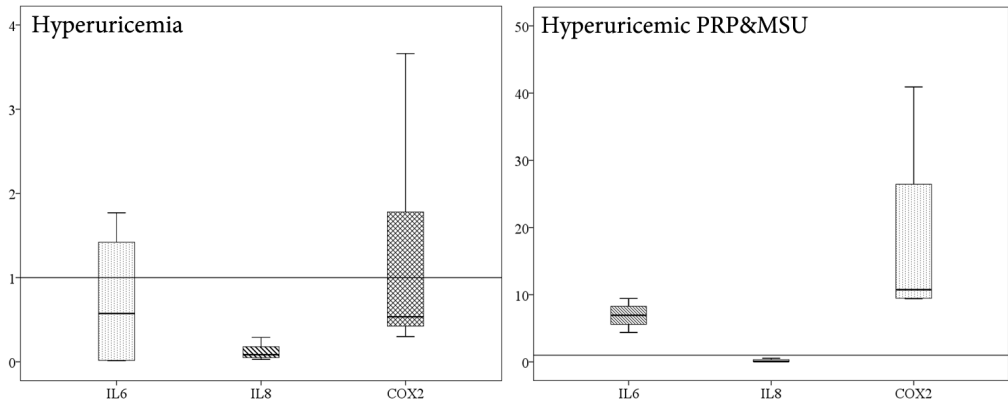
Source: unpublished, original.

The synthesis of several angiogenic proteins (VEGF, HGF, angiogenin, angiopoietin, IL-8, MCP-1 and RANTES) have been found in this in-vitro model. VEGF is a well-known angiogenic factor targeting endothelial cells and stimulating their proliferation. HGF is a potent stimulator of new blood vessel formation. Angiogenin is also involved in degradation of the basement membrane allowing endothelial cells penetration into the tendon. The latter mediates reciprocal interactions between the endothelium, surrounding matrix and mesenchyme. These proteins are released by tenocytes as a rapid response to PRP treatment. It is important to point out that signal transduction of PRP released signaling cytokines is reflected in an increase of pro-angiogenic proteins and a para-inflammatory response mainly involving monocyte/macrophage chemotaxis.

In conclusion, PRP science is in its very beginning, and PRPs are used with limited mechanistic understanding of their molecular and cellular properties. PRP science is now so complex that devising a successful formulation requires further attention to the host tissue context. Our efforts shall be focus on bridging the gap between PRP knowledge and identifying the molecular switch(es) for driving tendon homeostasis and the healing response.

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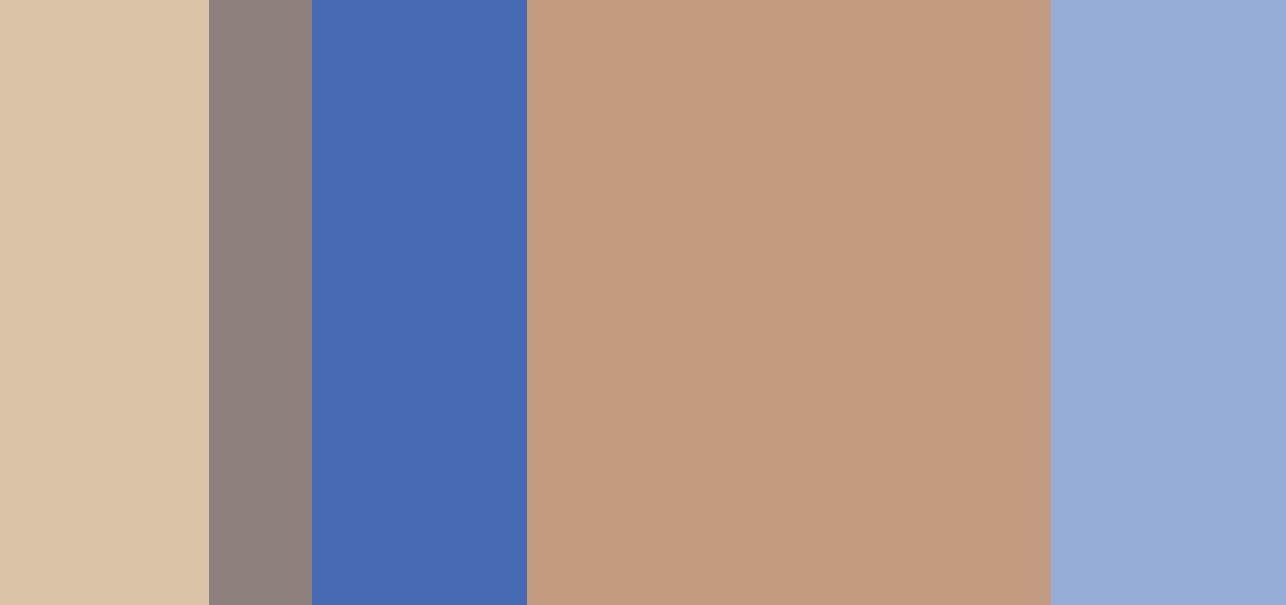
Conclusions

Tendinopathy is a common injury which adversely affect quality of life of millions of people all over the world, both young and active patients, and older sedentary people. Despite many hypothesis made about their pathogenesis and healing mechanisms, a large number of factors affecting tendon health are still unknown. The pathogenesis is multifactorial and it has been attributed to a many different intrinsic and extrinsic factors. In particular many studies advocate the importance of extra cellular matrix (ECM) for the homeostasis of connective tissue, and tendinopathic process can be viewed as a failure of the cell matrix to adapt to a variety of stresses as a result of an imbalance between matrix degeneration and synthesis.

Metabolic disorders are a new frontier and a field of research. If these associations are confirmed, assessment and treatment of patients with tendon conditions may have to be changed. With this work we tried to explain and understand how hormones modify tenocytes and tendon ECM, influencing its homeostasis. Clinical studies showed the significant relationship between hormones levels and tendinopathy and tendon ruptures, like diabetes mellitus, thyroid diseases, estrogens hormones, while interesting laboratory studies try to explain the physiopathology of hormones on tendons. Than many ideas could arise for further researches. We advocate that physicians and basic scientists involved in the study of tendons and tendinopathy explore this novel hypothesis.

"Metabolic diseases and tendinopathies: the missing link" is the theme of the IV Forum organized by Fondazione IBSA in collaboration with I.S.Mu.L.T. (Italian Society of Muscles, Ligaments and Tendons). Disorders of tendon or tendinopathies affect daily lives of millions of people: both young, active people and older sedentary people. However, the exact causes and healing mechanisms are still obscure.

The purpose of the meeting – attended by prominent experts of the international scientific community – was to stimulate further research and improve studies to understand the complex relationship between metabolic conditions and alterations of the extracellular matrix in tendon diseases.