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USE OF PLATELET RICH PLASMA IN TENDONS' AND LIGAMENTS' INJURIES IN SPORT HORSES

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USE OF PLATELET RICH PLASMA IN TENDONS' AND LIGAMENTS' INJURIES IN SPORT HORSES

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INTRODUCTION

Tendons and ligament injuries in horses are a major problem for any kind of discipline and attitude, often demanding long periods of rest, introduction in lower classes of competition and sometimes retirement from the sport activity (Smith, 2008). The site of the lesion, the affected limb and the use of the horse are the main concerns in assessing the possibility of recovery and the prognosis (Ross & Dyson, 2006). Throughout the decades a number of possible therapies has been experimented yet none of them has ever turned out to be able to ensure a complete restoration of the anatomical and functional integrity of the injured tissue. In the past, effort has been put to improve the quality of the healing process of these tissues and the aim was to obtain a scar tissue enough strong and elastic to allow somehow the reintroduction to activity. Therapies, from stall rest to shock wave treatments, basically were trying to improve the process of reparation. Nowadays scientific research has brought to light the possibility to work on healing processes not in the sense of reparation but of regeneration. Regenerative medicine principles are based on the belief that healing processes cannot just be improved in a quality or timing side, yet they can be led to

produce matrix tissue other than scar tissue with the help of autologous blood derivatives and MSCs (Fortier & Smith, 2008). The regenerative properties of tissues are studied everyday more to understand to what point the regeneration can be led either with the implantation of cells to differentiate in the desired way or with the application of sources of growth factors to boost the tissue's own capability to regenerate. In this sense, platelets concentrates have been in the last decades addressed as possible proper solutions to the problem, being a substantial source of growth factors potentially boosting the reparative process and improving the quality of the heal (Borzini & Mazzucco, 2005). Nevertheless there is a general lack of knowledge about the precise indications, timing, number of applications and minimum number of platelets for the treatment and its effectiveness is yet to be clinically investigated. Too many are in fact the possible interactions of platelets' growth factors in the site of lesion and each one can affect the outcome of the healing process: each tissue has different healing features regarding the behaviour of cells after the injury in the sense of time for healing, environment, biochemical and physical interactions, not forgetting that the age, the characteristic of the lesion itself and the concomitant local and systemic situations play a role too (Weibich *et al.*, 2002). All of these variables are hard to investigate for importance and far to be completely decoded at the moment for some tissues (as tendon, ligament and cartilage) more than for others (skin and bone).

All over the world researchers have been working both in vitro and in vivo to gain as much knowledge as possible as the difference between a scar and a regenerated tissue is a fundamental topic in human medicine. In veterinary medicine preliminary studies have gained advantage from these researches and have later on faced the problem of having to deal with many different species. Skin and bone have been widely investigated from veterinary researchers for the importance of their lesions in dogs and much is the interest in understanding how these tissues can benefit from the development of regenerative medicine. Unfortunately for a number of reasons for horses it is quite different and the

tissues target of interest are necessarily tendons and ligaments; too little can in fact be done for bone major lesions in horses and, on the other hand, too little is usually the importance of skin lesions compared to the possible dramatic effect of an injured tendon or ligament on the activity of the horse.

Laboratory research can point out some fundamentals to refine the treatments but in horses, more than in other species, the final assessment for use must pass by in vivo trials. In many countries, such as Italy, in vivo research on horses is nearby impossible to be performed, thus the closest way to approach the meaning of the leading of a biological process in one sense or the other is the clinical trial. Clinical trials are of great support to research but definitely lacking of some cornerstones of research. The application of treatments on large numbers of subjects could help to outline the guidelines for the effective and actual use of blood derivatives and stem cells.

The present study was outlined as an answer to the need to create some guidelines for the application of autologous blood derivatives (i.e. PRP) in tendons and ligaments lesions in sport horses referred for lameness at the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine of Alma Mater Studiorum – University of Bologna. The first step of the research focused on the preparation protocol to obtain a sort of internal “standard of quality” of the product, whose aimed characteristics were pictured on the basis of updated literature. The second step consisted on the application of PRP obtained with the standardized protocol in those horses that had been assessed to have an injury either in the SDFT, or the DDFT, or the ICL or the SL both in front or hind limbs. Horses were followed for one year after treatment and the outcome was considered for results. Thus the aims of this study were to standardize a protocol of preparation for PRP and to verify the effectiveness of a PRP application in tendon and ligament spontaneous lesions in sport horses.

SECTION 1

Understanding bio-mechanical and functional features, injuring and healing processes of tendons and ligaments in the equine distal limb

Chapter 1

Histology, morphology and functional anatomy of superficial digital flexor tendon, deep digital flexor tendon, inferior check ligament and suspensory ligament

The distinction between tendons and ligaments is substantially anatomical: tendons connect muscles to bones while ligament tie two bones to each other. Connecting a muscle to the bone, tendons are often erroneously considered inert structures involved in the movement of joints. It is though to be considered that, even if the function of maintenance of position is important, tendons have the fundamental role of storing kinetic energy and allow locomotion. This is especially true in horses where tendons of the palmar/plantar surface of the metacarpal/tarsal region (SDFT, DDFT) bear the stress of the hyper-extension of the fetlock joint in the stepping phase and release the energy during elevation.

One of the differences between these structures that behave like springs and other tendons whose role is limited to maintenance of position (i.e. extensor tendon) is the stiffness of the structure, which is almost doubled in these last ones (Smith and Goodship, 2004).

HYSTOLOGY AND MORPHOLOGY OF TENDONS

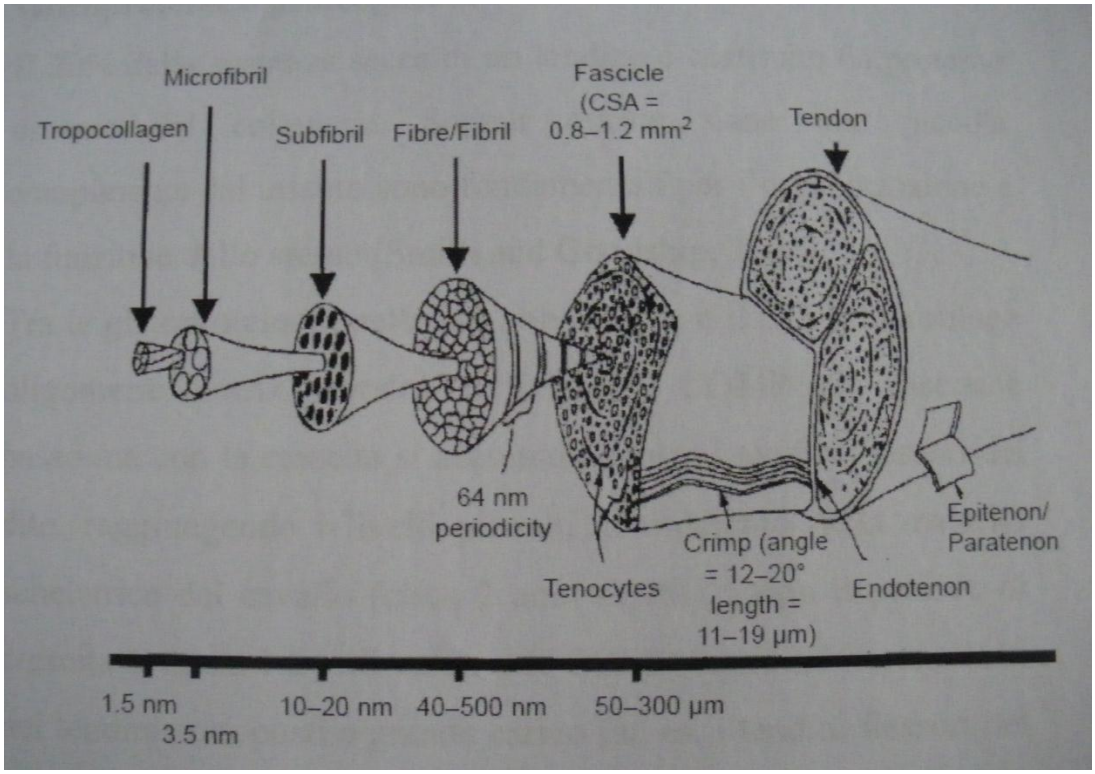
Tendons are tough strands of fibrous connective tissue having a role of "intermediate items" between a muscle and a bone. The dense connective tissue is organized in a regular pattern, with a peculiar spatial disposition responding to the bio-mechanical requirements of the tendon. Tendons are mainly composed of water (70%). The remaining 30% of dry mass includes mainly collagen and a "non-collagen" matrix (Dowling *et al.*, 2000).

Water is fundamental to ensure the elastic properties to the tissue. Density of tendons increases when dehydration occurs causing a loss of elastic properties (Smith and Goodship, 2004).

In the past the fibrils of collagen were considered the major responsible for the strength of the tendon; the intra-and inter-fibrillar covalent bonds of collagen though and the electrostatic bonds of non-collagen proteins have recently been demonstrated to have greater importance in giving the tendons their bio-mechanical features (Dowling *et al.*, 2000).

Tenocytes are displayed in long parallel rows in the spaces among collagen fibers. On the basis of the nuclear morphology three different types of cells (I, II, III) have been identified in the tendinous fascicles. A fourth type of cells has been recognized instead inside the endotenon (Smith and Goodship, 2004). The distribution of the cellular types varies among tendons and within the same tendon depending on the age. The specific features of these different cells is not yet well known even though it is clear that type I and III cells have a higher metabolic activity as their nuclei are larger and richer in nucleoli. These cells may be primarily involved in the synthesis of the extra-cellular matrix (Dowling *et al.*, 2000).

Tenocytes have numerous cytoplasmic ramifications connecting close cells through gap junctions. The deriving syncytium creates a transduction system similar to that created by osteocytes in bones (Smith and Goodship, 2004).



Tendon Structure (Dowling et al., 2000)

Type I collagen is the prevalent type of collagen in normal tendons. Every molecule of type I collagen is assembled inside the endo-plasmatic net of the cell starting from a procollagenic molecule. This last one is made of two alpha-1 chains and one alpha-2 chain that create a triple helix along with the terminal –N and –C domains. The pro-collagen molecule is cleaved by specific proteases cutting the terminal peptides and producing a molecule of tropocollagen. These molecules are then rearranged in a regular pattern to obtain insoluble collagen fibrils. Each collagen fibril overlaps with the next one for one fourth of their length. The fibrils are tight together by covalent bonds between the residues of lysine/hydroxyl-lysine of adjacent fibrils (Smith and Goodship, 2004). The fibrils then assemble further to form fascicles , and finally into a tendon fiber.

Groups of fascicles are bounded by the epitendon and peritendon to form the tendon organ.

Type II, III, IV and V collagens are minor components confined in specific sites. Type II collagen is allocated at insertions and in the regions where the tendon changes direction around a bony spike. In these areas the fibro-cartilaginous nature of the matrix is enhanced with the aim to withhold the forces of compression and tension that the tendon undergoes. Type III, IV and V collagens are confined close to the basal membranes and to the endotenon (Dowling *et al.,* 2000).

Collagen is organized in tough parallel arrays closely packed together and disposed longitudinally. Collagen fibrils and fibroblasts are the basic structure of a tendon, representing the primary unit of a tendinous fascicle. Primary fascicles are assembled in secondary fascicle, furthermore arranged in tertiary fascicles. Collagen fibrils within the primary fascicle are parallel yet are disposed in a helix figure for the whole length of the tendon. The fasces of tendon fibrils are held together in an waved display (crimp pattern) on the surface. The crimping is only histologically visible in longitudinal sections of the tendon.

The complex structure of the collagen fasces leads to a profound lateral cohesion within the tendon that obstacles, together with the inter-fibrillar fundamental matrix, fibers to skim among them. The crimp pattern plays a fundamental role for the elastic features of the tendon during the first phase of weight charge. The stress strain curve correlates the shrinking of the tendon with the stress to which it undergoes. Moderate forces cause the shrinking of the tendon with the flattening of the crimps while stronger pulling forces may cause the rupture of the fibers (Smith and Goodship, 2004).

In young animals the angle within the crimps is of about 19°-20°and each crimp is extended for 17-19 mm in the central region of the metacarpus in the SDFT. In older subjects the angle is reduced to 12°-17° and the crimp to 11-15 mm. The reduction of the crimps contributes to the increase of stiffness proper of the aging process (Dowling *et al.*, 2000).

The 20% of dry mass of a tendon is made of protein different from collagen. Despite the small presence they are fundamental for the structure and the function of the tendon itself (Smith and Goodship, 2004).

Among glycoproteins the COMP (cartilage oligomeric matrix protein) is the most represented. The level of COMP at birth is low and it increases with age cumulating within the flexor tendons and reaching its highest level with the skeletal maturity of the horse (about 2 years of age). After the developing period its levels start to decrease again. COMP is mainly stored in those tendons which bear a lot of weight and harsh pulling forces (i.e. digital flexor tendons) other than in tendons which are less stressed (i.e. digital extensor tendons). The COMP storing process during growth makes the tendon stronger. The function of the COMP is not yet completely determined yet it is known that it binds the fibrillar collagens (including type I) with a Zinc-related mechanism. This interaction with collagen takes place thanks to the C globular terminal domains of the COMP with 4 analogue places displayed along the collagen molecule. The COMP cannot occupy two spaces with only one domain, yet it can bind 5 molecules of collagen, sorting them to form a fibril during the initial phases of formation of collagen (Smith and Goodship, 2004). If the COMP does not bind the collagen fibrils it is taken away. Recent studies have shown how the COMP accelerates the genesis of collagen fibrils in vitro and it is possible that this protein acted more as a building manager other than having a structural role. This would explain the higher concentration of COMP during growth that is when the synthesis of the matrix takes place (Smith and Goodship, 2004).

Proteoglycans are made of two lateral chains of glycosaminoglycan connected to a central protein. Proteoglycans are divided in two different classes: large and small. Large proteoglycans have numerous lateral sulfate chains binding water. These molecules are majorly present in those sites where the tendon undergoes compressive forces. Small proteoglycans are thought to take part in the regulation of the diameter of collagen fibrils (Smith and Goodship, 2004).

Among horse SDFT's proteoglycans condroitin-sulfate, dermatan-sulfate, cheratan-sulfate, heparin and sulfate heparin, and ialuronic acid have been identified (Dowling *et al.*, 2000).

Specific structural differences and compositional features among tendons

Among tendons there are differences in the content of water, collagen and other proteins. These compositional features reflect the diverse functions each tendon is aimed to (Smith and Goodship, 2004).

Tendons can be divided in weight bearing structures, which act like springs, and in steady positioning structures, responsible for the maintenance of position of the skeleton. The first ones are less rigid and more elastic compared to the second ones which must be very stiff (Davis and Smith, 2006).

Digital flexor tendons, which are weight bearing, have a higher percentage of water and less collagen compared to the extensor tendons, which are positioning tendons. Another compositional difference regards the COMP: at birth its level is low in all tendons increasing with aging ten times in flexor tendons, whereas in the digital extensor tendons its level does not change (Smith and Goodship, 2004). The DDFT has lower levels of COMP compared to the SDFT and its collagen fibrils are large (in SDFT the fibrils are both large and small) (Davis and Smith, 2006).

ANATOMY

In this study PRP has been applied in some tendons and ligaments of the distal part of the limb in horses. To understand the characteristics of the injection site it is necessary to provide a brief description of histology, morphology, anatomy and biomechanics of the structures where PRP was applied. Digital extensor tendons are mentioned whereas they have not been submitted to any application: the aim of the description is to highlight the peculiar functional and structural features of flexor tendons.

In horses the muscular part of flexors in fore and hind limbs is well represented until the carpus and the tarsus respectively; distally to these only the tendon part can be found. The anatomy of the tendinous portion of flexors in hand and foot is nearly the same.

The superficial digital flexor tendon (SDFT), the deep digital flexor tendon (DDFT) and the suspensory ligament (SL) are the three major structures counteracting the hyperextension forces stressing the fetlock joint on the palmar/plantar aspect of the distal part of equine limbs. At the dorsal aspect of the limb in the same regions there are the digital extensor tendons only responsible for the extension of the limb at walk. The proper functioning of a tendon requires its possibility to skim smoothly among surrounding tissues. In those areas where the tendon direction is suddenly changed, which mainly is nearby joints, the tendon itself is wrapped by a tendon sheath. This structure reduces the frictions between tendons and other structures like retinacula (McIlwraith, 2002).

The tendon sheath is a compound of connective tissue layers both parietal and visceral, covered by synovial cells and forming a closed environment filled with synovial fluid.

The palmar aspect of the synovial sheath (called great sesamoidean sheath) presents two annular ligaments: the palmar annular ligament (PAL) and the digital proximal annular ligament (DPAL). The PAL is attached to the abaxial-palmar aspect of proximal sesamoid bones. The PAL is a strong transverse ligament embracing the sheath and the flexor tendons, forming a canal along with the proximal scutum (proximal sesamoid bones and palmar ligament). The DPAL is a squared thin layer of connective tissue laying between the skin and the SDFT. Distally it is attached, along with the SDFT, to the first phalanx. The dorsal aspect of the sheath is formed by the palmar aspect of the inter-sesamoidean ligament, the distal sesamoid ligaments, the scutum medium (thin fibro-cartilage layer attached to the second phalanx) and the second phalanx itself.

The sheath is extended for 4-7 cm proximally to sesamoid bones and distally to the mid second phalanx. The sheath has many pouches: proximal, collaterals and distal.

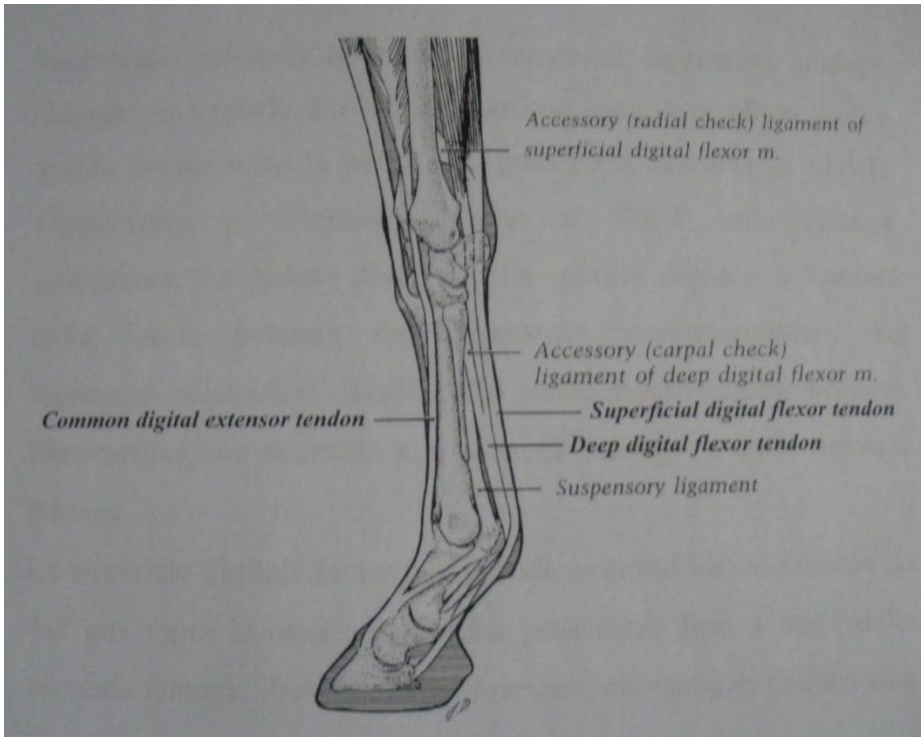
The proximal pouch of the sheath is in the distal part of the metacarpus, just proximally to the manica flexoria and the PAL. The collaterals are on the lateral and medial aspects of the first phalanx, between the flexors and the sesamoidean distal ligaments. The distal pouch is between the second phalanx and the dorsal aspect of the DDFT, separated from the proximal pouches of the podothrochlear bursa and the pouches of the distal inter-phalangeal joint by a thin layer of connective tissue.

The DPAL adheres to the palmar aspect of the distal part of the DDFT and wraps the terminal expansion of the tendon. The ligament is formed of a fibrous layer tightened to a strong fascia which covers the distal tract of the SDFT (Denoix, 1994).

A synovial bursa is similar to a tendon sheath yet it covers only a part of the circumference of the tendon. It is usually found where tendons pass on bony spikes. Along the straight course of the tendons, where a tendon sheath is not

necessary, surrounding tissue is composed by soft connective tissue and called peritendon, which is elastic and full of blood vessels.

Despite the role of protection from traumas the sheaths have toward tendons, they also slow the healing process of tendons themselves. This action is due to the synovial fluid and to the absence of the peritendon. The peritendon is in fact considered the source of fibroblasts which are essential to the healing of tissues (McIlwraith, 2002).



Anatomy of the distal part of the limb (Smith *et al*., 2002)

Superficial digital flexor tendon (SDFT)

The superficial digital flexor muscle has a small muscular portion with numerous and strong tendon attachments. Its origin is on the upper part of medial epicondyle of the humerus, either directly or by a tiny tendon, often merging with one of the near muscles (medial ulnar muscle) (Denoix, 1994).

The tendon takes shape in the distal part of the radius and receives, nearby its origin, a stiff fibrous structure called superior check ligament (SCL or SDFT

accessory ligament), which origins from the palmar aspect of the radius. In this area the tendon has circular or prismatic shape; distally the tendon enters the carpal canal and going through it is on the medial-palmar aspect of the DDFT. At the proximal part of the metacarpus the tendon flattens to assume the shape of half moon, embracing in its concavity the DDFT. Just above the proximal sesamoid bones it forms a ring (manica flexoria) through which the DDFT passes. This ring though doesn't extend more than to the median part of sesamoid bones. The SDFT is afterwards the most superficial structure until the distal part of the first phalanx where is splits into two stiff and short branches attaching to the extremity of the glenoid cartilage of the second phalanx.

In hind limbs the superficial digital flexor muscle's carny portion is small and merged with abundant fibrous tissue, having the shape of a rope and extending from the femur (supracondylar fossa) to the second phalanx. The tendon flattens in the calcaneal region where its shield skims over a large synovial bursa, reassuming its ropy shape at the palmar aspect of the metatarsus. Its stiff, fibrotic structure and reduced elastic properties allow the tendon to strongly contribute to the keeping of correct angulation of the hock and fetlock joints.

Deep digital flexor tendon (DDFT) and inferior check ligament (ICL)

The deep digital flexor muscle has three heads. One originates from the humerus, one on the palmar aspect of the proximal side of the radius, one on the proximal side of the ulna (medial aspect of the olecranon). The heads are quite different in dimension, being the humeral one the strongest, thickest and prismatic. It has a large tendinous component which can be easily spread in many fasces, which collect distally to form a bulky tendon. The ulnar head has a pyramidal short carny part and its tendon runs distally in vertical direction between the two ulnar muscles. The radial head is the shortest and thinnest,

becoming distally a small, flat tendon merging to the superior check ligament and reaching later on the humeral head, in the radius-carpal joint area.

The tendon common to the three heads (DDFT) seems to be the direct prosecution of the humeral head; it runs through the carpal canal along with the SDFT in a deeper position. Half the way along the metacarpus it receives the inferior check ligament. The inferior check ligament (ICL) is a stiff and strong fibrous strand which prolongs the common palmar ligament of the carpus. Its proximal insertion is on the most proximal palmar aspect of the metacarpus and it is wide and thick, with rectangular shape. Running distally the ICL becomes thinner and narrower, merging to the DDFT on its dorsal aspect (Denoix, 1994).

Behind the metacarpus-phalangeal joint the DDFT runs through the manica flexoria being hidden by the distal part of the SDFT until the pastern joint. In correspondence of the proximal scutum it becomes larger, elliptic and fibro-cartilaginous. At the proximal half of the proximal pastern the DDFT is divided into two symmetric cylindrical parts and its fibers assume a spring-like disposition. Both parts decrease in width and thickness running distally. The smallest cross sectional area (CSA) of the DDFT is found at the level of the half of the proximal phalanx where the tendon passes through the two terminal branches of the SDFT becoming superficial. At the distal half of the first phalanx the CSA of the DDFT increases. In correspondence of the upper part of the second phalanx the dorsal aspect of the tendon presents a fibro-cartilage thickening. Running distally, passed the palmar aspect of the navicular bone, the DDFT widens in a fan shape of cartilaginous nature; the palmar aponeurosis occupies the whole space between the lateral and medial palmar processes of the coffin bone. It is attached to the cresta semilunaris of the third phalanx and it is kept in site by a strong reinforcement fascia (Denoix, 1994).

In hind limbs the deep digital flexor muscle derives by the merging of the lateral digital flexor muscle and the medial digital flexor muscle. The lateral flexor is quite strong, its carny portion is thick and first prismatic, then cone-shaped in its distal part. It presents many fibrous laminas and is wrapped by a solid

aponeurosis. The tendon has an oval section, becoming independent at the foremost distal part of the tibia and receives the tendon of the tibial caudal muscle in the tarsal area. It continues in the tarsal sheath where it is wrapped by a large synovial bursa with a proximal pouch projected upward along the plantar aspect of the tibia, while the distal pouch goes down until the mid metatarsus.

The medial digital flexor muscle has a thin fusiform carny portion. The tendon is of cylindroid shape and origins around the distal third of the tibia, passes through the malleolar canal and afterwards through a sheath formed by the tarsal medial collateral ligament and the plantar fascia. The tendon crosses caudally the surface of the proximal extremity of the medial metatarsal bone and reaches, forming an acute angle, the tendon of the lateral flexor at the mid metatarsus. Starting from this point the DDFT receives the superior check ligament, which arrives from the distal plantar ligament of the tarsus. Distally to this the anatomy is similar to the forelimb.

Suspensory ligament (SL)

The suspensory ligament (SL) originally derives from a muscle and it still contains some muscular fibers both in the proximal portion and the mid body. The muscular portion is well represented in the fetus and in young animals, whereas in adults the fibrous component takes over. The SL can be considered as a structure where the tendinous component is substituted by muscular fibers, it is yet classified as a ligament because of its cell morphology and the lack of a muscular body properly called.

The SL is a long, tough and strong strand extending from the upper part of the palmar aspect of the metacarpus to the sesamoid bones, between the metacarpus and the flexor tendons.

In the distal third of the metacarpus it branches in two, forming an acute angle. Both branches are tightly inserted on the apex and abaxial aspect of the

corresponding sesamoid bone (medial/lateral), and extending further distally with a stiff fibrotic branch which reaches the digital dorsal extensor, embracing the proximal pastern, and attaching with the extensor at the coffin bone (Dyson *et al.*, 1995).

In clinical practice the SL is divided in three parts: proximal, mid-body and branches (Dyson and Genovese, 2003).

Dorsal digital extensor tendon

The muscular part of the dorsal digital extensor muscle has its proximal insertion on the distal edge of the epicondyle crest and at the basis of the lateral condyle of the humerus. It becomes a tendon in the distal third of the radius after dividing in two unequal parts. The strongest of the two represents the digital extensor properly called and its distal attachment is provided by a long and stiff tendon running on the dorsal aspect of the carpus and provided with its own synovial membrane. The tendon spreads in the fetlock region and is there provided with a sub-tendinous bursa for correct skimming. Further distally it spreads and flattens even more becoming thicker in correspondence of the dorsal aspect of the phalanxes. By the distal edge of the first phalanx it receives the branches of the SL. Its distal insertion is on the pyramidal eminence of the third phalanx. The second part of the muscle is much smaller and it is lateral to the other part. It continues with a thin tendon which passes in the same bursa on the dorsal aspect of the carpus with the large tendon and bends afterwards with an acute angle to reach the lateral digital extensor. This thin tendon is although often found on its own running distally to the proximal pastern forming with the tiny muscular part to which it belongs the Philips muscle (Barone, 2004).

In hind limbs the dorsal digital extensor muscle's proximal insertion is in the homonymous femoral fossa, between the trochlear lateral ridge and the lateral condyle, through a small tendon. It continues with a muscular fusiform body,

flattened in dorso-plantar direction and covered by an aponeurotic membrane which becomes thinner running distally. The tendon that afterwards originates begins in the distal fourth of the tibia with a cylindrical shape, flattening as it passes in front of the tarsus, the metatarsus and the phalanxes. In the tarsal region it runs under the three extensors' retinacula (proximal, mid and distal) being wrapped in the same area by a long and narrow synovial membrane. It is reached by the lateral digital extensor tendon as it runs by the dorsal aspect of the fetlock. The distal anatomy is comparable to the fore limb (Barone, 2004).

Lateral digital extensor tendon

The lateral digital extensor muscle has a thin muscular body that originates from the proximal lateral tuberosity of the radius and from the edge between radius and ulna. Its tendon runs on the dorsal aspect of the radius becoming independent by the distal third of the forearm and, passed the carpus, it folds toward the dorsal lateral aspect of the third metacarpal bone. It receives a tough branch from the pisiformis bone and the ending of the tendon of Philips. It extends then on the dorsal aspect of the fetlock and ends on the proximal edge of the proximal pastern (Barone, 2004).

VASCULARIZATION OF TENDONS

Tendons receive blood supply from four sources: from the muscle, from the bone it attaches to, from the mesotendon layer (vinculum) inside the synovial sheath and from the peritendon where there is no sheath (McIlwrhaith, 2002). The tendon matrix, inside the sheaths, can be moreover nourished by the synovial fluid for diffusion (Davis and Smith, 2006).

The blood supply deriving from these sources varies in its extent depending on the tendon and the side of the tendon. In the metacarpal region, for example, the SDFT mainly receives blood supply by the proximal muscle-tendon junction (Smith and Goodship, 2004).

It has been demonstrated that the muscle and the bone supply blood only to the proximal and distal 25% of a tendon thus it can be assumed the paratendon layer to play an important role. In a few studies about equine forelimb have shown that proximally and distally the SDFT and the DDFT receive blood vessels from their respective muscle of derivation and from their insertions on the bones. Within the carpal sheath the principal branches of the median artery give vascularization to the surface of these tendons through the mesotendon; other branches run parallel to the flexor tendons along the metacarpal bone (McIlwraith, 2002).

Between the carpal sheath and the digital sheath tendons are wrapped by the peritendon layer through which blood vessels enter the tendons themselves.

The SDFT receives blood supply by various arteries. One artery derives from the median artery and reaches the tendon in correspondence of the muscle-tendon junction. Close to the proximal edge of the PAL the tendon receives an artery from each side (lateral/medial metacarpal distal branch) which originates from the corresponding digital artery. Nearby the distal edge of the PAL the

tendon receives again a vessel branch (digital proximal) deriving from the corresponding digital artery. All of these branches give supply to a wide intra-tendinous vascular net made of arterioles placed longitudinally along and among the fasces of fibers and that generate numerous anastomoses among themselves with perpendicular branches (Denoix, 1994).

The DDFT is supplied by three sources of blood. Proximally to the fetlock region the tendon receives a branch either from the second common palmar digital artery or the third common palmar digital artery. This branch enters the synovial sheath in correspondence of the proximal pouch and runs distally on the palmar surface of the tendon. Distally to the fetlock, by the mid first phalanx, the palmar branches of the proximal phalanx (which originate from the lateral and medial digital artery) give origin to one or two branches which reach the dorsal aspect of the tendon. The foremost end of the tendon is nourished by two small arteries originating from the lateral and medial digital arteries.

These blood vessels build an intra-tendinous vascular net within the DDFT other than in the region of the fetlock: in this area the vessels are confined to the palmar surface of the tendon (Denoix, 1994; Hay Kraus *et al.*, 1995). Some nourishment is guaranteed by the synovial fluid of the digital sheath, and it reaches the tendon for diffusion.

The lack of vascularization of some portions of tendons has been related to a predisposition to the development of degenerative diseases and to tendinitis (McIlwraith, 2002), yet the DDFT presents some histologic adaptation features to this closely ischemic environment: it presents in fact, in the fetlock area, a little number of cells and a high quantity of fibro-cartilaginous matrix. The proteoglycans of the cartilage allow the movement of the synovial fluid during the cyclic compressions favoring the passage of nutritive substances to the less vascularized parts. Recent studies have moreover shown the greater resistance of tenocytes compared to other types of cells to survive in situations of relative hypoxia, where they are able to keep their proliferative activity. Furthermore

studies on other species have excluded an alteration of the vascularization as possible primary cause of tendinitis (Smith, 2003).

FUNCTIONAL ANATOMY OF TENDONS AND LIGAMENTS

Tendons and ligaments have similar histological and biochemical features yet have different biomechanical importance. Tendons actively transfer the weight charge from the muscle to the bone, being thus "power transducers." They own a great resistance to tension and have relatively scarce extensibility. Ligaments instead resist passively to the charge.

Flexor and extensor tendons may act either independently to bend or extend digital joints or together to give stability to joints. In horses during locomotion flexor tendons are responsible for bending the digit when the limb is lifted and for supporting the fetlock in the stepping phase. In this phase elastic energy is stored by flexors to be released in the following step, thus reducing the energy required and increasing the efficiency of the locomotion system. When galloping the fetlock is hyperextended and flexor tendons and the suspensory ligament undergo huge pulling forces that stretch them to the limit of rupture. In the majority of homogeneous substances there is a linear correlation between applied force and deformation that can be expressed by an elastic constant; tendons instead respond with a non-linear deformation to the charge. At the beginning there is a great stretching for small increments in the charge which is followed by a phase of linear correlation between increasing of charge and stretching (Goodship and Birch, 1996).

In a mono-directional extension the tendon appears yielding to the pulling forces; with an increase of the pulling although there is a stiffer response. In this transitional phase the crimp pattern disappears. To see the straitening though the tendon must be extended at least of the 3% of its length.

Riemersma *et al.* ran a study on 5 ponies and demonstrated that the lengthening of the SDFT, the DDFT and the SL increases a lot along with the speed of gait. At walk the lengthening is of 2.19%, 1.15%, and 3.36% respectively, while at trot they are of 4.15%, 1.70%, and 5.78% (Riesmersma *et al.*, 1996).

When the pulling force stops the crimp pattern returns. After this phase of initial elastic response the mechanic characteristics of the tendon change and become viscoelastic. When submitted to a constant charge the tendon extends progressively in time. The biomechanical behavior of a tendon submitted to a force is represented by a stress-strain curve (Smith and Webbon, 1996).

Stress-strain curves

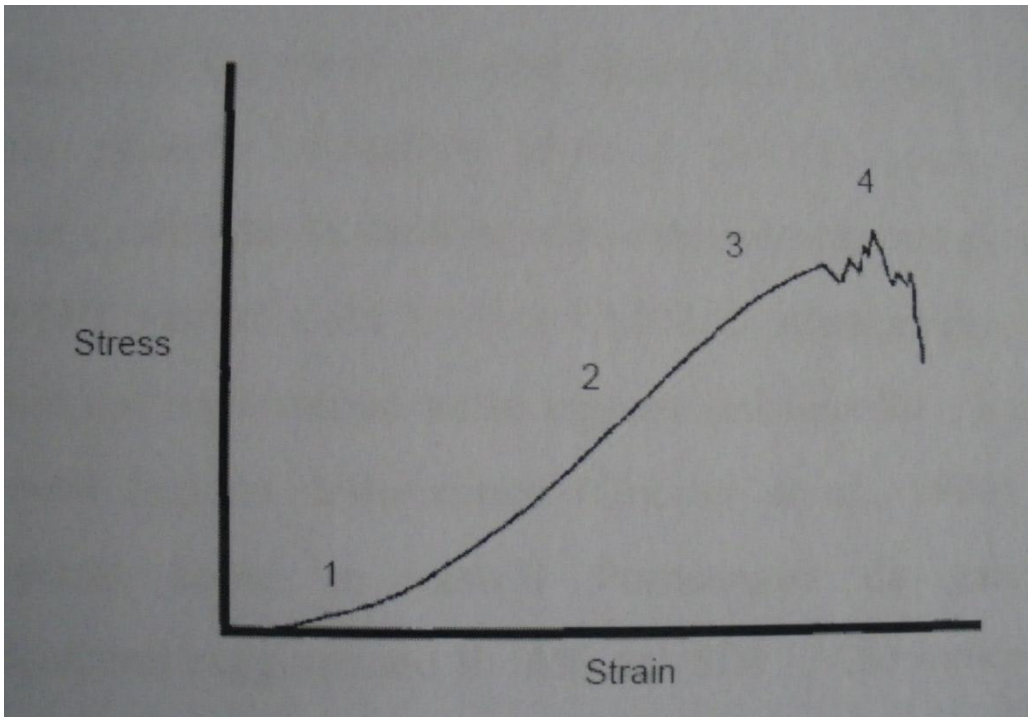
In the stress-strain curve the percentage of lengthening (strain) is put in relation with the applied force per space unit (stress). The obtained curves are different for each tendon and part of the tendon.

The curve can be divided in three regions (phases):

1-initial part (elastic phase): there is a non-linear lengthening of the tendon; this condition is related to the flattening of the crimp pattern;

2-intermediate part (viscoelastic phase): there is a linear deformation of the tendon; in this part of the curve the elasticity of the tendon itself is determined

3-final part (rupture phase): the curve slumps to the 0 point; this phase corresponds to the rupture of the bonds among collagen fasces or to the rupture of whole fibrils (Smith and Webbon, 1996).



Stress-strain curve (Dowling et al., 2000)

Biomechanical parameters

From the stress-strain curve some biomechanical parameters can be drawn:

- Final straining power (% of extension of the tendon at the rupture point). Abrahams states that the limit for the lengthening of a tendon is about 5%. Herrich *et al.* performed in vitro studies applying cyclic compressions on normal SDFT specimen observing that equine flexor tendons can stretch up to the 10-12% without rupturing. Crevier *et al.*, also with in vitro studies, obtained quite inconstant responses for SDFT rupture point, varying between 8.1% and 12.5% of stretching. The lowest values were recorded for the fetlock region and the highest for the metacarpal portions (Crevier *et al.*, 1996). In Warmblood gallopers the possible stretching of the SDFT has been demonstrated to be up to the 16%. This datum shows how the tendon can be functional to the limit of its straining power.

- Final tension power (force/unit of space where the tendon ruptures).
- Elasticity module

Conditioning and hysteresis

The visco-elastic properties of a tendon are represented by the shifting of the stress-strain curve from left to right. Repeated charges cause a shifting of the curve to the left, thus an increase in the stiffness of the tendon: this process is called "conditioning."

Another known property of the tendon is called "hysteresis." This one is represented by the difference between the charge curve and the discharge curve. The difference between the area under the curves represents the energy "lost" by the tendon (that in horses is about 5%) during the charging cycle. Part of this energy is responsible for the increase of temperature of the tendon. This phenomenon is associated to repeated stresses and time, and is thought to be a cause for the development of tendonitis in the SDFT. The tendon is quite resistant though to this physical process and tenocytes are not damaged by high temperatures. Thus the tendon shows much more complex biomechanical properties than a normal elastic substance (Smith and Webbon, 1996).

In flexor tendons the cross sectional area changes along its length. There is obviously also a great variability among horses as it is related to height, weight, and metacarpal circumference of the horse. Riemersma *et al.* have demonstrated that a tendon under charge stretches homogeneously. They also showed that the elasticity module is inversely proportional to the content of collagen. They deduced that the final stretching strength is inversely proportional to the cross sectional area while elasticity is quite constant along the whole length of the tendon (Crevier *et al.*, 1996). The SDFT is a quite homogeneous tendon, yet in the metacarpal region its proportional content of collagen (responsible for a greater elasticity) is higher than in other parts of the tendon. In any way this

stronger structure of the tendon is not capable to compensate the reduced cross sectional area owned at this level. In the metacarpal region infact the SDFT is more often injured both in vivo and in vitro (Crevier *et al.*, 1996).

The age of the animal also affects the biomechanical properties of tendons and ligaments. Wilmink *et al.* demonstrated how the angles of the crimps in the mid portion of tendons of adults were reduced compared to those of the younger. Thus adult subjects resulted more likely to develop tendon straining (Smith and Webbon, 1996).

Chapter 2

Tendons' and ligaments' injuries in the equine distal limb

In horses the tendons of the distal limb are subjected to exogenous and endogenous traumas. Exogenous traumas are usually wounds and contusions. Wounds caused by foreign bodies often sever completely the tendon and are accompanied by septic contamination. In contusions instead the physical injury to the local vascular net provokes a blood effusion which causes the edema and triggers the inflammation process.

Endogenous traumas are much more frequent and various: they are roughly called strains (stretching of the tendon beyond its physiologic length range) and can vary a lot in their seriousness. Minor strains are often subclinical, featured by a partial rupture of a portion of the tendon or of only a part of the fibrils. In severe strains the amount of ruptured fibrils increases up to get to the complete rupture in the cross sectional area. The fibrillar architecture is at this point completely destroyed.

Also SL stress injuries are classified as strains, as this structure is the relic of the III interosseous muscle and not a ligament properly called (McIlwraith, 2002). The following inflammation is called tendonitis. In horses the word tendonitis is correctly used to refer to the inflammation following a strain and involving the portion of tendon surrounded by the peritendon and not by the tendon sheath. The word tendo-synovitis refers instead to the inflammation of the part of tendon wrapped by the sheath, while the tenosynovitis is the inflammation of the only tendon sheath. The inflammation of a ligament is called desmitis.

There can be a thickening close to the lesion as a result of a fibrosis process of the tendon, of the surrounding peritendon and eventually of the tendon sheath. This thickening is usually visible as a palmar/plantar swelling of the SDFT. The tendinitis of flexor tendons has also been classified on the basis of the site (Watkins *et al.*, 1985 A):

-proximal: right under the carpus or the tarsus

-mid: in the mid third of the metacarpus or the metatarsus

-distal: in the distal third of the metacarpus or metatarsus and in the region of the PAL. The DDFT can also be involved distally to the fetlock joint. This lesion is described as low tendonitis.

TYPES OF LESION

Straining

A tendon strain can occur when the tendon is suddenly loaded with a charge greater than its tensile resistance. Any change in the structural features of the tendon can't be considered positively because tendons already always work to their limit of resistance. Thus when it is overloaded a mechanic disruption of tendon matrix occurs predisposing the tendon itself to develop tendonitis.

This probably is the mechanism that most frequently turns out in ligament injuries and some DDFT injuries (Davis and Smith, 2006). The most common lesions caused by straining involve the structures of the palmar aspect of the metacarpal region and the clinically evident lesion is probably often preceded by a phase of molecular degeneration.

Degeneration is usually the first phase of a tendinopathy. A pure degenerative process does not induce either a clinically evident inflammatory response or a healing process, yet it does likewise contribute to the weakening of tendon structure (Davis and Smith, 2006). Running speed during sport activity and some predisposing factors play a fundamental role in the development of a strain.

Many causes have been hypothesized to predispose to such injuries like impair training, muscular fatigue and rapidly repeated stresses (Dowling *et al.*, 2000). An abnormal conformation or angulation of the fetlock joint , caused either by muscular flaccidity or developmental defects, also increases the stresses on the tendon; likewise do an excessive sloping angle of the pastern, incorrect shoeing, and untrimmed hoof tips. Other concurrent factors may be: incoordination and disproportion between body weight and tendon resistance, training on irregular

or slippery soil, training on short turns courses where the tendon is solicited more on one side than on the other (Davis and Smith, 2006).

Tendon ruptures

The mechanism through which tendon and ligament ruptures happen within a synovial sheath is nowadays not completely clear. The structures that are more often damaged are the DDFT (especially in forelimbs) and the manica flexoria (especially in hind limbs). DDFT lesions are mainly caused by excessive stretching and usually occur by the its lateral edge, where the tendon folds cranially downwards, beyond the fetlock. Many ruptures of the manica flexoria are found instead after long lasting tenosynovitis, thus it is possible that the hypertrophy of the synovial layers, reducing the space for the skimming of the tendon, increases the chances for the ring to break up (Davis and Smith, 2006).

Transcutaneous tendon lesions

In horses, in the distal part of limbs, tendons are easily subjected to traumas as they are only covered by a thin layer of skin. The worst consequences possible occur when the palmar/plantar aspect is involved. A tendon can be severed for more than 50% of its cross sectional area and still be functioning at walk. Complete severing of the extensor tendon on the dorsal aspect of the limb seldom causes long term functional impairment (Davis and Smith, 2006).

SDFT TENDONITIS

SDFT lesions seriously compromise a horse's sport performances, to the point of having to retire the horse from the activity. SDFT in horses is a charge tendon, quite similar in its function and structure to Achilles's tendon in humans. It is very often subject of injuries, up to the point that 93% of tendon and ligament lesions in horses have been estimated to affect the SDFT (Ely *et al.*, 2004).

In trotting horses the incidence of SDFT injuries ranges between the 7% and 43% depending on the studies and forelimbs are mainly affected. The problem is often bilateral with a prevalence in the left limb.

Racehorses are considered the ones with the highest risk of SDFT damage as they either run at high speed or run and jump (steeplechase) at the same time. SDFT is loaded with charge especially in the first phase of the step and the major stress is put in the landing phase after jumping (McIlwraith, 2002). SDFT lesions occur instead less frequently in dressage horses, Arabians, Quarter Horses and fox hunting horses. Nevertheless spontaneous SDFT lesions are found also in sedentary and untrained horses older than 15 years old. These lesions are often very severe and mainly involve the proximal portion of the tendon, in the proximal metacarpal, the carpal or the musculo-tendinous junction area (Jorgensen and Genovese, 2003).

Sport activity related SDFT lesions occur most commonly in the mid third of the metacarpus, yet they may also affect any other portion of the tendon. In hind limbs the most affected part of the tendon is by the plantar aspect of the hock, and occasionally the lesion may involve the mid metatarsal portion (Jorgensen and Genovese, 2003).

Some authors advocate the development of degenerative changes in the tendon structure as preceding and predisposing to the rupture. This theory is confirmed by the fact that histologic evaluations of tendons of clinically normal racehorses showed the presence of numerous structural alterations. These changes are generally limited to the portion of tendon covered by the peritendon layer (mid metacarpal region) where the tendon has the thinnest diameter and the intratendinous vascularization is believed to be relatively lacking. Lesions generally develop symmetrically and bilaterally. They are initially identified in the center of the tendon and include a reduced formation of fibrous tendon fasces, degeneration and death of tenocytes (Smith, 2003).

DDFT TENDONITIS

DDFT tendonitis are less frequent than SDFT's. An explanation for this to happen may be that the loading of the charge on this structure increases slowly and progressively in the gait, while the SDFT receives the load basically all at once (Smith and Goodship, 2004).

In the metacarpal region, proximally to the tendon sheath, DDFT tendonitis are found in association with ICL desmitis, while in the carpal sheath area they are often secondary to the presence of an osteo-chondroma (Dyson, 2003).

Unlike the SDFT, the DDFT is more often damaged within the digital sheath. It is not yet known whether if these lesions are preceded by degeneration. The pathology seldom develops bilaterally (Smith and Goodship, 2004).

DDFT tendonitis can be claimed as cause of digital sheath swelling in the fetlock, even though the causes can be numerous. On the other hand without a swelling of the sheath it is hard to identify DDFT's lesions (Dyson, 2003).

The types of DDFT tendonitis are two: the first one is proper of and within the tendinous substance (even though it may extend to the edges of the tendon) and is quite similar to the classic forms of tendonitis; the second type originates from the borders of the tendon, medially or laterally, and usually in the metacarpus-phalangeal joint area, involving or not the inner part of the tendon. These tendon tears are thought to originate from suddenly hitting pressures on the tendon when the fetlock joint is hyperextended (Smith and Goodship, 2004).

ICL AND SL DESMITIS

ICL desmitis may occur alone or in association with SDFT tendonitis. Thus the pathogenesis is related more to the SDFT than to the DDFT where the ICL attaches with its distal insertion. These desmites are frequent in ponies, which are instead less like to develop tendinopathies caused by straining (Smith and Goodship, 2004).

The SL is divided in three areas that may be injured: the proximal part, the mid body and the branches (Dyson and Genovese, 2003).

The SL proximal desmitis often occurs in forelimb of sport horses and can be unilateral or bilateral. In hind limbs this desmitis is of great importance for dressage horses competing at high level. Horses with straight or hyperextended fetlock and pastern seem to be more likely to develop this pathology (Dyson and Genovese, 2003).

The mid-body SL desmitis is most commonly found in forelimbs than in hind limbs. It is quite frequent both in Standardbreds and Thoroughbreds (Dyson and Genovese, 2003). Trotting horses seem to tolerate better serious damages to the SL compared to Thoroughbreds (Dyson *et al.*, 1995).

The desmitis of SL branches (medial and lateral) often occur in any type of sport horse, both in fore and in hind limbs. The lesion usually involves only one branch in one single limb, even though in hind limbs both branches may be affected (Dyson and Genovese, 2003).

SYMPTOMS AND DIAGNOSIS OF TENDONS AND LIGAMENTS INJURIES

The diagnosis of a tendon lesion is usually based on the anamnesis (usually lesions are related to traumas occurred during strenuous training) and on the relieving of signs of inflammation with the clinical examination (swelling, heat, pain, lameness). Thus the clinical symptoms of tendinitis vary depending on the poignancy of the inflammatory response and the degree of mechanical compression.

The cases of mild acute tendinitis present little increase of the volume of the tendon, secondary to the inflammatory edema. By palpation limited heat and pain are relieved. For the most subtle cases the palpation must be very accurate both in charge and with lifted limb.

when the tendinitis is more severe the inflammatory response is intense and swelling, heat, pain and lameness become much more evident. Increased pain can be shown either by bad laming or with high sensibility at touch on the interested area. In the worst cases the alteration of the tendon substance can be palpated (Smith and Goodship, 2004).

Lameness is not always evident and is related to the degree of inflammation rather than to the severity of the lesion. In the majority of cases lameness is severe at first when tendinitis is developed. The position of the limb while standing can be modified depending on the structural damage and the gravity of the injury. When there is a bad SDFT tendinitis the angle of the fetlock joint at rest can be normal thanks to the other means of sustenance of the joint (SL and DDFT). When the charge on the limb increases (i.e. when the contralateral limb is lifted) the injured limb shows a hyperextension of the fetlock (Smith and Goodship, 2004).

The chronic stage of a tendonitis manifests trough fibrosis and a swelling hard and stiff appears on the palmar/plantar aspect of the distal part of the limb. When the bulk is remarkable the posterior aspect of the metacarpus/metatarsus assumes a typical humpy appearance. When the lesion happens to be right under the fetlock the hump appears spheroidal. During chronicization there may still be hotbeds of acute inflammation within the lesion testifying the healing process is in progress or, sometimes, that a new trauma is interjecting.
In normal tendons the edges are clearly identified by palpation while in course of a chronic process it is not as easy because of the fibrosis and the thickening. The horse may seem normal at walk and at trot and present lameness at faster gaits (McIlwraith, 2002).

Diagnosing a **SDFT lesion** in its acute phase is easy because of the swelling of the involved area due to the exudative process. The local heat and pain contribute to the identification of the site of lesion. Very often it is recognized that an injury has occurred because of the lameness that appears, and that can be quite variable in intensity (McIlwraith, 2002). It must be remembered that in mild lesions the edema doesn't develop right away.

Diagnosing a **DDFT injury** is quite easy too, yet in this case the palpation must be much more accurate as the tendon is covered by the SDFT. Walking the horse can help as the lameness will increase with exercise and reduce with stall rest. As the inflammatory process goes on it becomes possible to identify a localized swelling going from the distal part of the palmar aspect of the carpus to the whole metacarpus, and which usually is more evident on the medial side and hot and painful at palpation. Ambulation is compromised and steps appear short and faltering (McIlwraith, 2002).

The clinical signs of **a SL desmitis** are quite similar to those of flexor tendons'. In the acute phase heat, edema and swelling can be appreciated by palpation in

the injured area, even though they are not always present. Slight differences are shown in the characteristics of the lameness depending on the affected portion.

Proximal desmitis

Proximal SL desmitis start with sudden lameness that can be transient and disappear in only 24 hours in forelimbs; in hind limbs the lameness is usually more severe and lasting. Often in trotting horses the lameness can only be seen with a high speed gait. Very frequently the single lesion passes unnoticed and when the desmites develops in the contralateral limb the horse begins to be reluctant to move other than showing a clear lameness.

Acute desmitis may present with localized heat in the proximal region and occasionally a slight edematous swelling on the palmar lateral aspect of the metacarpus or seldom on the palmar medial side. Pain can be evoked by palpation. Sometimes the edges of the ligament may feel rounded at touch. In chronic desmitis palpable anomalies are usually absent (Dyson *et al.*, 1995).

Mid-body desmitis

SL mid-body desmitis compromise horse's performances other than induce severe lameness. The clinical signs (localized swelling, heat and pain) are more evident compared to those seen with proximal desmitis; lameness is instead milder, indeed often undetectable. When lameness is present it usually improves or disappears after a few days of stall rest.

The appearance of pain referable to SL desmitis must be carefully evaluated and eventually related to recent strenuous work or training: pain is usually manifested right after an intense activity with no detectable alteration of the ligament. On the contrary the absence of pain cannot exclude the desmitis, as chronic pathologies mainly run by without pain manifestations. The degree of swelling is quite variable: there may be a bulk localized to the ligament or involving all of the surrounding structures.

In many horses when clinical signs disappear it is still possible to recognize the site of lesion by ultrasonography. These horses may still be capable to return back to sport activity. There is though a relatively high percentage of recurrent SL desmitis despite long periods of stall rest (over 12 months) which might be due to incomplete healing of the ligament (Dyson *et al.*, 1995).

Desmitis of the branches

The degree of lameness ranges enormously depending on the extent of the lesion but may not be present at all. Exception is made by old dressage horses often presenting both medial and lateral branch desmitis and being very lame (Dyson and Genovese, 2003).

A swelling of the involved branch and the surrounding soft tissues can usually be palpated along with localized heat. In course of acute desmitis pain may be evoked by palpation of the involved branch, its edges and the corresponding sesamoid bone. Palpating the branch may be difficult when the tendon sheath is swollen too.

SL branch's acute lesions in forelimbs may present in association a distension of the metacarpo-phalangeal joint capsule and pain after passive forced flexion. Also the injuries of the branches can be detected by ultrasound examination also long after the trauma (Dyson *et al.*, 1995).

COLLATERAL EXAMS

Ultrasound is the most important mean of collateral examination for tendon and ligament injuries. Tendonography has been used for years to define thoroughly the lesions within the tendon sheaths but has been now completely substituted by ultrasonography which is less invasive (Dyson and Denoix, 1995).
To perform tendonography air is initially injected in the digital flexor tendon sheath and afterwards in the surrounding subcutaneous tissue, among tendons, to outline the tendons. Afterwards radiographies are performed and compared between them. In case of alterations within the tendon sheath though, tenoscopy is the first choice technique to be used as goes far beyond the diagnostic sharpness of ultrasound.

Ultrasonography

The use of ultrasonography allows to determine thoroughly the site and the extent of the lesion. It plays a fundamental role also in the follow-up of clinical cases allowing to plan at the best the rehabilitation protocol for the horse. The management of the rehabilitation period should ideally plan ultrasound controls every 2-3 months (Smith, 2008). To achieve diagnosis the injured area should be evaluated within 4-7 days after injury, as many lesions tend to expand in a few days following the primary lesion (Davis and Smith, 2006).
Dyson and Denoix assessed that good ultrasonographic images can be obtained by using either a convex or a linear probe which must be as large as to be able to give visualization of the whole section of the DDFT. On the other hand the probe should have perfect contact with the limb also for longitudinal scans and

for this reason should not be too big (Dyson and Denoix, 1995). The structures found in the palmar region of the metacarpus can suitably be evaluated by placing the probe on the palmar aspect of the limb. To investigate the SL branches medial and lateral scans must be performed in the proper areas. Limbs must be completely scanned both in cross and longitudinal section and the tendons and the damaged areas should be measured.

The first structure the probe meets is the skin. The second structure to be visualized is the SDFT whose echo structure is quite homogenous. The tendon presents a rounded shape in the very proximal metacarpal region and flattens as it runs distally. Between the palmar surface of the tendon and the skin there is a thin anechoic line which is the synovial fluid within the tendon sheath.

The DDFT is slightly more echogenic and oval compared to the SDFT: its proximal and mid portions are thicker on the lateral than on the medial side, while the distal part is generically wider. Medially to the DDFT a consistent blood vessel (median artery) can be visualized: it can be differentiated from a lesion both for the presence of blood flow and the localization beyond the tendon edges.

The ICL can be observed in the proximal third of the metacarpus; it is separated from the DDFT by an anechoic layer, which is the synovial fluid within the carpal sheath. In normal conditions the ICL is the most echogenic among these structures especially in the distal third, where it becomes thinner and merges with the DDFT. Between the ICL and the SL the medial and lateral palmar veins of the fetlock can be visualized (Genovese and Rantanen, 1998).

With ultrasound all of the features of a lesion can be evaluated: the increase of volume, the modification of the shape, the alterations of echogenicity, the pattern of fibers, and the eventual surrounding inflammatory reaction.

The loss of the normal echogenicity can be due either to the damage to the structure of fibrils or to the inflammatory process (with hemorrhage, edema and cellular infiltration of the first stages). In general the increase in the content of water in the tissue causes a decrease of echogenicity.

At the ultrasound examination a tendon undergoing an acute process presents increased volume, decreased echogenicity (either generally or focally, i.e. core lesion), reduced longitudinal strand pattern, and modified shape, position, edges. There may even be completely anechoic areas.

Chronic tendinopathies are presented instead with a widely ranging increase of volume of the tendon which will appear with uneven echogenicity and pattern.

While investigating tendinopathies it is quite useful to evaluate dimension, shape and appearance of both the injured tendon and the contralateral, as many pathologies usually are bilateral (Davis and Smith, 2006).

The palmar aspect of the metacarpus is divided in six zones, each more or less 4 cm long (1A, 1B, 2A, 2B, 3A, 3B). Zone 1 includes the beginning of the SL and the exit of flexor tendons from the carpal sheath. Zone 2 covers the mid third of the metacarpus and includes the structures distal to the SL branching. Zone 3 corresponds to the distal third of the metacarpus and includes the fetlock. The metatarsus is longer and it is thus divided in eight zones (adding zone 4A and 4B) (Sande *et al.*, 1998).

The dimension of the lesion must be evaluated both in longitudinal and transverse scans. Placing the probe in longitudinal section the proximal-distal extension of the lesion and the degree of misalignment of fibers are evaluated. The transverse scan instead allows to measure the percentage of damaged cross sectional area (CSA) as a ratio on the whole diameter of the tendon. This scan allows to understand whether if the lesion is completely within the tendon matrix (central lesion) or it extends to the margins of the tendon (marginal lesion) (Rantanen *et al.*, 2003).

To characterize tendons and ligaments injuries all of the following parameters must be evaluated (Craychee, 1995):

- Region or site of lesion
- Length of lesion
- Modification of the echogenicity

- Pattern of the modified echogenicity (i.e. homogenous or not, focal or diffused)
- Longitudinal alteration of fibers
- CSA of lesion
- Changes of the aspect of the lesion over time

The classification of the lesion can also be made by type scoring (Rantanen *et al.*, 2003) based on the echogenicity pattern:

- Score 0: isoechoic
- Score 1: slightly hypoechogenic
- Score 2: mixed echogenicity (50% isoechoic and 50% anaechogenic)
- Score 3: totally (or almost) anaechogenic

The evaluation of the cross-sectional area (CSA) of the lesion gives an important parameter for the quantification of the damage and the severity of the injury. It must be measured in the point of maximum extent of the damaged area, in transverse scanning, and related to the total sectional area of the tendon/ligament (Smith, AAEP 2008):

- < 10% → mild lesion
- 10% - 40% → moderate lesion
- >40% → severe lesion

There also is a scoring system for fiber alignment in longitudinal scanning which refers to the amount of fibrils with destroyed crimp pattern (Genovese and Rantanen, 1998):

- Score 0: >75% of fibers are parallel
- Score 1: 50% - 75% of fibers are parallel
- Score 2: 25% - 50% of fibers are parallel
- Score 3: <25% of fibers are parallel

Chapter 3

Tendons' and ligaments' healing process

THE HEALING PROCESS

Tendons and ligaments are different in many ways, as for morphologic, biochemical, and tensile features. Even within the same joint, ligaments have their own peculiar features which include the healing response.

Studies about tendon and ligament healing processes have highlighted how low the intrinsic capability to heal of some ligaments can be, and how they would not recover after a rupture, while others would be fixed even without a complete restoration of morphologic and biomechanical properties of the original tissue (Woo *et al.*, 2000).

As for any other tissue, after an injury the tendon starts to heal with an inflammatory reaction. The severity and the extension of the lesion determine the proportion for the production of fibrin and of inflammatory cells (Davis and Smith, 2006).

In the whole process both intrinsic and extrinsic factors are involved. The extrinsic component is the main and most important, and it is represented by fibroblasts and capillaries produces by the peritendinous tissue. The intrinsic component is supported by the endotenon whose cells act as active fibroblasts.

This recent discovery has overcome the previous belief for which the tendon was considered an inert structure, almost without vessels and reparative properties (McIlwraith, 2002).

The pathologic process developing a tendinitis is divided in four phases; the initial, subclinical phase though is hard to be identified both by simple palpation and by an ultrasound examination. Thus the pathological conditions clinically detectable reduce to three phases: inflammatory acute phase, reparative sub-acute phase, and remodeling chronic phase.

Acute phase: it starts when a clinically evident problem onsets and lasts for about 1-2 weeks. Its duration is nevertheless quite variable depending on the type of lesion and the promptness of the therapeutic intervention. This phase is characterized by intra-tendinous hemorrhage, increased blood supply, development of edema and leucocytes infiltration (firstly neutrophils, then macrophages and monocytes) (Smith, 2003).

Sub-acute phase: it starts a few days after the lesion onset, it overlaps with the acute phase and culminates after 3 weeks. It is characterized by a marked angiogenic activity and storing of fibroblasts within the injured tissue. Fibroblasts derive by the resident tenocytes, by the cells of the endotenon and the peritendon and by blood monocytes. To repair a tendon (it is a properly called reparation and not regeneration) fibroblasts produce scar tissue mainly composed by type III collagen fasces randomly disposed. Type III collagen represents the 10% of total collagen in a normal tendon and increases up to the 50% in this process. GAG levels increase and COMP levels decrease. The scar initially produced is much weaker than the normal tendon and this makes the tendon more likely to incur in a relapse of the pathology in the same area. Relapses drag on over time these phases and the lesion worsens (Smith, 2003).

Chronic phase: it begins a few months after the injury. Type III collagen is converted in type I and the fibrils rearrange longitudinally along the tension lines (Smith, 2003). In association to these histologic changes the tensile resistance of the tendon increases of 3 times from the eighth to the twelfth week.

After twenty-four weeks the reparation tissue has been transformed in a properly called scar. Nevertheless even a small quantity of immature collagen tissue can predispose the scarred area to incur in a relapse (Davis and Smith, 2006).
The scar tissue is usually made of weaker substance compared to the normal tendon, but the large amount of scar tissue produced may even make the tendon stronger (Smith and Goodship, 2004). The final outcome of the tendon healing is a scar of connective tissue and not of tendinous tissue. This scar may restore the anatomic integrity of the tendon but not the tensile and elastic properties of the normal tissue (Davis and Smith, 2006).

The prevalence of the intrinsic healing mechanism other than the extrinsic potentially can lead to less problems due to the formation of peritendinous adhesions (McIlwraith, 2002). In the healing process of many tendon injuries though, the peritendinous tissues are quite involved, with the result that the fibroplasia binds all of the structures exposed in a mass of cartilaginous scar tissue. The resulting adhesion between the tendon and the surrounding tissues may have the negative effect to limit or bar the skimming of the healed tendon, grave consequence that can only be prevented with a cautious and early rehabilitation therapy (Davis and Smith, 2006).
The adhesions that form inside the sheath, and that progressively limit the movement of the tendon, although guarantee some supply for blood and cells, thus they must be considered as the physiologic response spurring the tendon to heal. In fact the portions of tendon within the tendon sheath have poor healing responses probably due to the absence of the blood supply of the peritendon (Smith, 2003).
Relapses are quite recurrent also after a complete recovery. They can occur in the same tendon, in the contralateral or in other structures supporting the joint. A completely recovered tendon (15-18 months after the initial lesion) is often stronger than before but less elastic. The result is that in the adjacent regions there is a higher concentration of tensile forces. When the relapse involves the

same tendon of a previous lesion it occurs in the same point or in the immediately adjacent portions. Contralateral tendon lesions are considered properly called relapses because bilateral are the degenerative processes and the clinical tendonitis as previously described (Smith, 2003).

THE PROGRESSING OF LESIONS: ACUTE VS CHRONIC, TWO DIFFERENT ENVIRONMENTS

Once a lesion is produced in any biological structure an inflammatory response is established: the progression of the acute inflammation must be considered as the first phase of the healing process.

The healing process of a lesion is a biological mechanism characterized by countless molecular interactions nowadays only partially understood. A fault in the harmonic succeeding of these interactions leads to the chronicization of the wound. From decades it has been demonstrated how important are the differences between the microenvironments of acute and chronic lesions from a biochemical point of view.

In chronic wounds the exudate obstacles the cell proliferation which is instead one of the basis of the success of the healing process and is stimulated by the acute wound exudate.

In vitro studies on endothelial cells and fibroblasts have showed that interleukine-1 and the tumor necrosis factor-α, respectively produced by neutrophils and lymphocytes, directly stimulate the synthesis of metallo-proteinasic matrix (MMPs) and inhibit the synthesis of the tissue inhibitors of metalloproteinases (TIMPs). From this it is easy to understand that after prolonged exposition to these pro-inflammatory cytokines the level of MMPs greatly increase compared to the TIMPs (Trengrove *et al.*, 1999).

The MMPs comprise a number of zinc-dependent enzymes (collagenase, gelatinase, and stromelysin) responsible for the degradation of the extracellular matrix like fibronectin, collagen, laminin, proteoglycans, and elastin. These enzymes have an homologous structure and are secreted in their proenzyme form (Wysocki *et al.*, 1993).

Both in vitro and in vivo studies have demonstrated the importance of proteases' role in the physiologic healing process, and how the levels and the time of expression of these enzymes must be limited and strictly controlled either by the activation of plasmin or by the presence of the TIMPs. An overproduction of MMPs fosters an excessive disruption of the matrix undermining the healing of the tissue. Studies about chronic wounds in humans have shown that a wound environment where proteases are widely expressed leads to the development of pathologic healing and chronicization. This finding is supported by the high concentration of MMPs retrieved in the exudate of such chronic lesions (Wysocki *et al.*, 1993; Trengove *et al.*, 1999).

It is possible that the imbalance between MMPs and TIMPs in a wound during the healing interjects not only with the disruption of the extracellular matrix but also with the degradation of growth factors and their respective receptors (Trengrove *et al.*, 1999).

In chronic processes growth factors are out of balance and their title decreases progressively. There are three possible explanations for this to happen; growth factors are in fact thought to be either entrapped in tissues, or bond by macromolecules, or hampered in their action by the presence of fibrin. Another mechanism inducing the decrease of growth factors is the increase of proteases. It is also true that, besides the imbalance between anabolic and catabolic factors, the capability of cells to properly feedback to growth factors is generally lacking. In chronic wounds there are large amounts of old cells that have poor or no capacity to respond to anabolic signals; these aged cells may also be in a resting phase of their cellular cycle, thus only temporarily inactive. The deficit of cell responses is confirmed by the decrease of fibroblast proliferation stimulated by TGF-β1 in human patients with venous ulcers. In the same studies a reduced expression of receptors and a number of dysfunctions in the transduction of biochemical signals have been recorded (Whitney, 2005).

THERAPY

Tendons and ligaments lesions are very common in sport horses and have a dramatic impact on the economy of the racing field.
The incidence of this sort of injuries is nowadays still high, even if in the last years the knowledge about the clinical aspects of the problem has greatly increased and the diagnostic and therapeutic means much improved.
Traditional therapeutic treatments include both a medical and a surgical approach aimed to reduce inflammation and prevent further damages (Dahlgren, 2009). The various therapeutic options can be classified in pharmacological, physical and surgical.

Cold: the application of ice or cold water (hydrotherapy) has been the cornerstone of treatment for acute tendonitis and desmitis and is even nowadays still considered of valuable help. It is aimed to the reduction of inflammation, thus of the exudate and the chemotactic agents responsible, over time, for damaging the tendon matrix originally healthy (Dowling *et al.*, 2000). Cold should be applied several times per day, for 20-30 minutes each time, until the acute phase of inflammation passes. Prolonged application should be avoided as it may cause a feedback vasodilatation (Henninger, 1994). Nowadays there are new commercial products that give the possibility to apply pressure cyclically along with cold water. Cyclic compressions stimulates lymphatic drainage and transmits the cold also to deeper layers (Dahlgren, 2009).

Bandage: in the acute phase of the lesion controlling the swelling and supporting the injured limb is essential. Bandages increase the pressure of the interstitial fluid contrasting Starling's forces that would otherwise induce licking

of fluid out of blood vessels (Henninger, 1994). A simple soft bandage is usually recommended even with habitual leg wraps. The bandage can be changed every day or even more often in case of repeated application of ice/cold water (Dahlgren, 2009).

Stable confinement: a period of stable rest is fundamental for the healing of tendinitis and desmitis. The length of the period ranges widely from a minimum of two months up to one year. For SL desmitis the minimum period of rest should be 4-6 months (Arthur, 1995).

Physiotherapy: physiotherapy, intended as a series of controlled exercises, could be started during the healing process, after the acute inflammatory phase. A good rehabilitation protocol should be applied regardless of the type of therapy chosen to treat the lesion. Rehabilitation schedules usually foresee progressively increasing physical activity and workload, from walk to gallop, and last often more than 12 months. An ultrasound control should be performed every 3 months to adjust the rehabilitative program to the case. The duration of the rehabilitation is quite important: a number of studies show how the possibility to get back to the activity without incurring in a relapse is very low for resting and rehabilitative period shorter than 6 months (Dowling *et al.*, 2000).

PHARMACOLOGIC THERAPY

In the past the only therapeutic forms in use were represented by thermo-genic treatments, in the form of creams or lotions. These treatments stimulate blood circulation and the production of abundant leukocytosis through which the decomposition of the products of inflammation was achieved. Their efficacy was to be retained valuable only in presence of an hyper-acute case of tendinitis; they would in fact not have any therapeutic effect in those cases where necrosis was already present. In later days the use of corticosteroids has taken over and more recently have the non-steroidal anti-inflammatory drugs, that unlike steroids have met unanimous favor.

Anti-inflammatory drugs: the use of short-action corticosteroids in the hyper-acute phase of the lesion (less than 24 hours after injury) seems to reduce the inflammatory reaction but there is no evidence of better final outcome with their use. The use of corticosteroids in the sub-acute/chronic phase instead inhibits the fibroblastic feedback which is essential for the tendon healing. The intra-lesion use of corticosteroids causes damages such as necrosis of collagen and hyalinization.

Dymethilsulfoxide (DMSO) has been widely used for its anti-inflammatory activity. It has good capacity to enter cells' membranes and inactivate free radicals which are quite harmful for the undamaged surrounding tissue. DMSO is commonly believed to have scarce toxicity, yet studies on rats have demonstrated how the local application of DMSO can weaken the tendons of the tail of such animals (Henninger, 1994).

The use of non-steroidal anti-inflammatory drugs (NSAIDs), like phenilbutazone and flunixine meglumine, has given controversial results in the

treatment of inflammation. They are useful to reduce inflammation, swelling and pain when used in the first two weeks after injury, even if they should be considered effective only for their analgesic power. In fact it is quite problematic to demonstrate in horses a significant action in vivo. The prolonged used of these drugs causes collateral effects such renal damages, gastric ulcers and colic syndrome (Henninger, 1994; Dahlgren, 2009).

Sodium hyaluronate: hyaluronic acid is a natural substance present in high concentration in synovial fluid of joints and tendon sheaths. It also has a structural role in connective tissues. Its use for intra-lesional or peritendinous injection as therapy for acute tendonitis presents conflicting results.

Preliminary studies on lab animals show that the high viscosity and molecular weight of the sodium hyaluronate gel, injected between the tendon and its sheath (peritendinous use) may favor scarring of the tendon and limit the adhesions. Gaughan promoted a study on horses and the tendon that were such treated did not develop significant adhesions with surrounding tissues. Good results were obtained in regards of speed and quality of the healing (Gaughan, 1994). Spurlock and colleagues also obtained good result treating 63 horses with SDFT tendonitis with Hilartil V: 60% of the treated horses showed echographic healing of the lesion in a lapse of time of 6 months at the most (Spurlock *et al.*, 1999).

Intra-lesional injection of sodium hyaluronate doesn't seem to reduce the incidence of relapses on the basis of the clinical outcome, when comparing cases of treated and untreated horses (Dahlgren, 2009). Hyaluronic acid's use isn't common because of its high cost and the difficulties of acquisition.

Polysulphated glycosaminoglycans (PSGAGs): glycosaminoglycans (GAGs) are an important component of cartilage. PSGAGs simply are GAG added with sulfates. The drug acts by inhibiting cartilage-destruction-linked enzymes and by reducing inflammation. PSGAGs have been used to treat joint degenerative diseases for several years. In the treatment of tendonitis they are used in the

initial inflammatory phase and reduce swelling and pain (Dahlgren, 2009). A study on 8 horses with a collagenase-induced SDFT lesion highlighted how the treatment with PSGAGs speeded up the tendon healing compared to control horses. The results were evaluated both with ultrasound and histopathology (Redding *et al.*, 1992).

Another study on a small number of horses with SDFT lesions and treated with PSGAGs showed how this therapy allowed horses to get back to sport activity much more than other conservative therapies or laser therapy. Data revealed though that less than 50% of the horses were able to compete again and the incidence of relapses was very high (Henninger, 1994). In 1996 PSGAGs were applied on 150 horses with SDFT acute tendonitis. On the basis of a clinical evaluation in 80% of the cases the treatments led to a favorable outcome. The medium rate of return to activity was 72% with a 72% of it with total recovery (Dow *et al.*, 1996).

Beta-aminopropionitrile Fumarate (BAPN-F; Bapten): the molecule acts binding irreversibly the lysil oxidase, an enzyme that is responsible for the formation of cross-links among collagen fibers during the initial reparation of the tendon. Crosslinks are soluble and easily denaturized with heat. When collagen maturates bonds stabilize to impart the characteristic biomechanical features to the fixed tissue. The BAPN decreases the formation of scar tissue encouraging the alignment of fibers (Henninger, 1994) and reduces the incidence of relapses but does not accelerate the healing process. The ideal moment to perform the intra-lesional injection of BAPN is between 30 and 60 days after injury, which is the lapse of time when the majority of crosslinks are built (Dahlgren, 2009). In 1998 Virginia Reef reported a study on Bapten's application to tendon lesions in horses. The 70% of treated horses returned to races despite the severity of the injury. The ultrasonographic appearance of the lesions showed substantial improvements too. In later days the enthusiasm about

this treatment faded because of some cases of spontaneous lacerations of treated tendons.

PHYSICAL THERAPY

Physical therapy includes low intensity ultrasound, laser therapy with low frequency infrared rays and electromagnetic therapy (Watkins *et al.*, 1985). In many cases the results obtained with these therapies aren't any better than those obtained with other preservative therapies (Dowling *et al.*, 2000).

Laser therapy: the biological effect of laser depends on the characteristics of the laser light (frequency, wave length and energy), of the type of treated tissue, and on the duration and the frequency of the application. Laser therapy is especially useful for the treatment of superficial wounds where the healing process is consistently sped up (Henninger, 1994). In Kaneps's study about horse tendons laser therapy's application results weren't any better than normal scarring of the lesion. The failure of the treatment is probably to be ascribed to the use of a power lower than the needed to influence the scarring of a wound (Kaneps *et al.*, 1984). More recent studies have ascribed to laser therapy the capability to stimulate collagen production in fibroblasts, to facilitate the remodeling of the newly formed tissue, but not to give back the tendon completely its mechanical properties (Kostoulas *et al.*, 1997).

Ultrasound: the biological effect produced by ultrasound depends on the frequency, the intensity and the type of wave produced. Ultrasounds generate heat in the tissue they hit modifying the blood flow, the metabolism of proteins, the extensibility of collagen, and the permeability of membranes (Henninger, 1994). Studies in humans have reported consistent therapeutic effects among which the increase in the production of collagen, the decrease in scar tissue, and the general improvement in the quality of the healing of lesions. In one study on rabbit tendons severed and treated with low density ultrasounds and increase of the tensile strength of tendons was reported after only ten days. Therapies with

high density ultrasound, on the other hand, seem to slow down the healing process causing blood stasis damaging the endothelium and decreasing the synthesis of collagen (Henninger, 1994). The application of ultrasounds on surgically damaged equine tendons revealed quite positive outcome at histology. After only 4 weeks from treatment no degenerated areas or signs of inflammatory reaction was visible. After 6 weeks the tendon seemed completely healed showing only a small scar (Morcos and Aswad, 1978).

Ionizing radiation: in 1979 Franks performed a study in which 25 of 48 horses with teno-desmic lesions got back to sport activity after a therapy with irradiation. The worst problems related to this kind of therapy regard the dosage of radioactive isotopes for which a proper equipment is strictly required (Franks, 1979).

Magnetic fields and electricity: the electric stimulation induces changes in the cellular environment, affecting the function and the differentiation of cells. Pain decreases, blood flow increases, the edema reduces and the migration of cells is stimulated. The biological effects vary on the basis of the properties of the electric power used, the schedule of the treatment and the kind of treated tissue. Experimental studies have demonstrated how various kinds of cells are able to respond to the electric stimulation. Many are the studies on equine soft tissues. The direct stimulation with low-power electricity does not bring any improvement to the histologic appearance of surgically created skin wounds. Watkins *et al.* demonstrated how a daily electro-magnetic therapy would increase vascularization of surgically created lesion in SDFT yet the maturation of the reparation tissue and the transformation of collagen were delayed. The observations were performed after 8 and 12 weeks from the creation of the lesion (Henninger, 1994).

Extracorporeal shock wave therapy: the mechanism of action of shock waves is unknown. Through experimental studies consistent effects have been relieved in the treatment of SL desmitis. The most frequent use of shock waves is

reported in the treatment of proximal SL desmitis where the average prognosis is much more favorable compared to those obtained with other conventional therapies. The 41% of horses affected by proximal SL desmitis in hind limbs returns to full activity within 6 months compared to the 13% of the horses treated with other conventional therapies. This treatment though cannot be considered appropriate in case of acute tendonitis (Smith, 2008).

SURGICAL THERAPY

Tendon surgery is used to treat either chronic tendonitis or the cases of serious lacerations and complete severing of the tendon.

Tenorraphy: a tendon suture is mandatory in case of wide lacerations or sharp cuts of the tendon. Some consider this procedure ineffective as the suture would offer little resistance to traction. Tenorraphy though is quite useful especially when the severed tendon is within the tendon sheath, as the two parts would tend to retract, creating a distance hardly fillable by granulation tissue. The utility of Tenorraphy is doubted when the loss of tendon substance is massive.

Splitting: this technique has been used since 1931 to treat chronic tendonitis. It consists in the longitudinal cutting of the tendon to create lesions in the fibrotic mass of the scar. These lesions are performed a couple of centimeters apart from one another on one or more than one parallel planes. The procedure was believed to lead to an improvement of the vascularization but recent studies have shown how it increases instead the trauma and the production of granulation tissue; furthermore there is no alteration on the production of collagen and the lameness persists. The surgical splitting of SL hasn't produced any favorable results in Warmbloods while in Standardbreds there seems to be an improvement (Arthur, 1995).

Splitting has recently been proposed to treat acute lesions other than chronic ones: the splitting would in fact allow the drainage of hematomas and intra-tendinous edema, reducing the dimension of the lesion and improving the alignment of collagen fibrils (Dowling *et al.*, 2000). A clinical study demonstrated how the splitting of tendons affected by acute inflammation with a core lesion would produce a significant reduction of the dimension of the lesion,

of the diameter of the tendon and thus of the grading of the lesion within the first 8-10 days after surgery. Later on a lower deposition of fibrous tissue was registered too. The 81% of horse got back to training and the 68% to activity, at the same level where they competed before surgery. These result were quite significant considering that the lesions were all graded of 3rd or 4th degree and involved an average of the 80% of the CSA of the tendon (Henninger, 1994).

Synthetic implants: the use of implants of fibers of carbonyls in tendons and ligaments was initially experimented by Jenkins (1976) in animals other than horses. The first results encouraged the use of carbonyl fibers to treat tendonitis and flexors' lacerations. Edwards (1978), Valdez (1979), and Langlois (1980) were the first ones to consider carbonyl fibers application in equine tendons; many other authors followed. In Italy the first one to use carbonyl fibers was De Gresti who treated 15 horses with chronic tendonitis (De Gresti, 1990). It was then shown that such treatment induces persistent anomalies in the newly formed collagen and the outcome intended as the return to competition was similar to those obtained with other sorts of treatments. Carbonyl fibers are inextensible thus resisting forces apply on the surface of the implant; these are probably responsible for the continuous tendon pain relieved in many horses undergoing the treatment. The implants cannot be indicated in case of stretch-induced tendonitis, where the peritendon is usually intact providing itself to the healing (Dowling *et al.*, 2000).

Cauterization: it consists in the application of heat on the lesion by using caustic substances (phenols and red mercury) or other burning instruments (thermo-cautery). This treatment is used on chronic muscle-skeletal lesions in horses with the aim to increase vascularization and exudation of inflammatory cells. It has been shown though how this treatment doesn't have any positive effect on tendon healing, on the type I collagen content and the athletic performances; healing is often slowed down and the incidence of peritendon adhesions increases. Heat treatment leads to the formation of a scar and the

treatment is very painful for the horse. There still are many fans of such therapy, either used alone or in association with other treatments, yet the obtained result are not straightforward positive (Dowling *et al.*, 2000).

Other surgical solutions

SCL desmotomy: this technique was proposed in 1986 to treat SDFT tendonitis. By severing the SDFT accessory ligament the peak of charge applied on the SDFT is reduced and the charge on the muscle increased. The results obtained after the desmotomy are wide-ranging. A percentage varying between the 52% and the 82% of the treated horses gets back to competition at the same or higher level than before (Dowling *et al.*, 2000). In a study on 62 Thoroughbred horses 92% of patients returned to training and 66% ran 5 times after surgery at the most. Forty-seven % of the horses returned to competition maintained or increased the gaining per start and 86% got back to its own competition level. Only 19% of the horses had a recurrence of the pathology. The results were quite encouraging compared to those obtained using conservative treatments (Henninger, 1994). Through a retrospective study it has been otherwise shown that the exercise following the desmotomy causes a worsening of the ultra-sonographic appearance of the acute tendonitis (Dowling *et al.*, 2000).

PAL desmotomy: this technique was proposed to treat lesions of SDFT and DDFT in the fetlock area. The procedure is indicated when the annular ligament forbids a normal skimming of flexor tendons. The surgery is performed in general anesthesia, better if with tenoscopy than with an open surgery (Davis and Smith, 2006).

Fasciotomy: this a surgical technique used to treat proximal SL desmitis in hind limbs that do not improve with conservative therapy. A fasciotomy of the fascia wrapping the SL is performed to reduce the compression in the proximal tract of this ligament (Davis and Smith, 2006).

REGENERATIVE MEDICINE

The aim of regenerative medicine is to restore the original architecture and biomechanical function to damaged tissues.

After a tendon lesion the scar tissue that forms induces a decrease on the performances and the lesion is likely to relapse. It is thus necessary to substitute the damaged tissue with a matrix more similar to the tendon and less to scar tissue. Knowing the molecular and mechanic processes involved in the development of a tendon or ligament lesion and the pathologic conditions of the lesion itself (acute/chronic) allows to a better choice in the most appropriate therapy to use (Fortier and Smith, 2008).

There are 3 fundamental instruments to obtain a good regeneration of the damaged tendon (Dahlgren, 2009):

1. An implant compatible with the attachment and the migration of cells able to lead the reconstruction of the damaged area in a three-dimensional fashion;
2. A source of cellular precursors able to recruit endogenous stem cells possibly differentiating in tenocites;
3. A physiologic combination of growth factors and cytokines to make the tendon matrix reorganize on the given implant.

Implant: there are implants obtained from porcine urinary bladder (UBM). Through mechanical and chemical procedures the basal membrane and the lamina propria of the bladder wall are isolated and then processed to powder. The obtained powder is reconstituted with saline solution and injected in the

lesion of the tendon/ligament. In the United States this treatment has been applied to hundreds of horses both with tendonitis and desmitis of SL. About the 85% of the horses returned to the previous athletic level (Fortier and Smith, 2008).

Stem cells: they are undifferentiated cells able to undergo differentiation in a specific cellular line and renovate. Mesenchymal stem cells (MSCs) are adult stem cells that can be isolated from bone marrow, muscle, tendon, adipose tissue, and can differentiate in cells such as osteoblasts, myocytes, chondrocytes, adipocytes, and tenocytes, depending on the tissue they have been implanted in. Adult stem cells can thus conduct a regenerative response; furthermore they contribute to healing producing bioactive proteins (growth factors and cytokines) sending local signals through the recruitment of endogenous stem cells; also they induce anabolic effects in the recruited cells and in those already present in the tissue (Dahlgren, 2009).

MSCs are used in tissue engineering, in genic therapy or they are directly injected into the lesion. Among these possibilities only the direct injection is applicable in clinical practice. Nowadays stem cells are applied in the form of a suspension inside the lesions of tendons, ligaments and joints (Dahlgren, 2009).

Bone marrow aspirate derived stem cells (BM-MSCs). The use of these cells has been experimented in laboratories on animals previously submitted to surgery to create a lesion. These studies have reported positive effects on the organization of the tissue, on the composition and the mechanic properties of treated tendons and ligaments.

The treatment can either consist in the injection of the entire marrow aspirate, of a concentrate of stem cells obtained with centrifugation or of a population of stem cells cultured in vitro (Fortier and Smith, 2008).

The injection of MSCs derived from bone marrow in teno-desmic lesions offers two great advantages: 1) MSCs can differentiate in mature fibroblasts able to

produce new matrix; 2) bone marrow is rich in blood thus it is a powerful source of growth factors that favor the healing of the tissue.

The intra-lesional injection of the whole aspirate is a simple technique, immediate (the application can follow right after the diagnosis) and relatively cheap. A negative effect, which is quite rare though, is the possibility to have a mineralization of the product after the injection (Fortier and Smith, 2008).

MSCs are nevertheless scarcely concentrated in bone marrow: to obtain a more effective concentration in the site of lesion with a marrow aspirate a large quantity of product should be injected, eventually provoking negative compressions on the healing process (Dahlgren, 2009).

The use of a centrifuge allows to concentrate the stem cells collected by aspiration. This technique offers all of the advantages of both the PRP (Platelet Rich Plasma) and of the bone marrow aspirate. The product is autologous and can be prepared right after the diagnosis. It also contains all of the three component important for regeneration previously described: the implant, the growth factors and the stem cells (Fortier and Smith, 2008).

Also the implant of MSCs cultured in a laboratory and re-suspended in a mean allows to overcome the problem of cells concentration, thus giving an effective number of stem cells in a small volume. The positive effects of this treatment must still be investigated much more thoroughly, yet no negative effects have been relieved. Ad advantage is for sure due to the better purity and availability of stem cells. The greatest disadvantage of this technique is due to the time necessary to cultivate the cells (2-4 weeks), thus the injection cannot happen in the acute phase of the lesion (Dahlgren, 2009). The lapse of time between the diagnosis and the treatment is fundamental for the positive outcome of the therapy. It has been demonstrated that the fibrosis of the sub-acute and chronic phases can obstacle the action of the injected product (Fortier and Smith, 2008). To be able to intervene in the acute phase a ready-to-the-use product should be available before the lesion is created, and this solution is far away to be

applicable as the product is autologous. The other disadvantage is the high cost of the therapy (Dahlgren, 2009).

Adipose tissue derived stem cells. The use of adipose tissue as source of adult stem cells offers numerous advantages compared to bone marrow: 1) the collection technique is easier and less invasive; 2) stem cells are more concentrated and the amplification in vitro is not necessary; 3) these stem cells show a higher proliferative rate in vitro.

The population of stem cells isolated from adipose tissue is heterogeneous and a physiologic combination of fibroblasts, endothelial precursors, pericytes, macrophages, B and T lymphocytes, pre-adipocytes and smooth muscle cells (Dahlgren, 2009).

At the moment the proves of the biosecurity of stem cells are only on the short-run; so far none of the treated cases has shown any collateral effect, such as the development of a tumor, in a time over 5 years from the treatment (Fortier and Smith, 2008).

Growth Factors. The therapies implying the use of growth factors for tissue regeneration are based on the principle to release one or more than one of these factors in the site of lesion. The product that is more widely used is PRP. The characteristics of growth factors and of the platelets concentrates are widely described in the following section.

SECTION 2

The role of platelets concentrates in tendons' and ligaments' healing process

Chapter 1

Platelets

Besides the well known hemostatic role, platelets are also able to release factors promoting tissue repair, angiogenesis, and inflammation: in a site of lesion platelets release a wide range of inflammatory and mitogenic molecules involved in all of the phases of the healing process of wounds (Rozman and Bolta, 2007).

FORMATION, COMPOSITION, PHYSIOLOGY AND BIOLOGICAL ROLE OF PLATELETS

Platelets are pieces of cells without nucleus and with secretory activity. They derive from megakaryocytes of the bone marrow through a controlled process of cellular fragmentation (Rozman and Bolta, 2007).

Megakaryocytes develop from multipotent progenitors CD 34+, myeloid cells belonging to the hemopoietic tissue and blood. Megakaryocytes represent approximately 0.1-.05% of nucleated bone marrow cells and are sited under the capillary twits of bone marrow. Megakaryocytes produce cytoplasmic

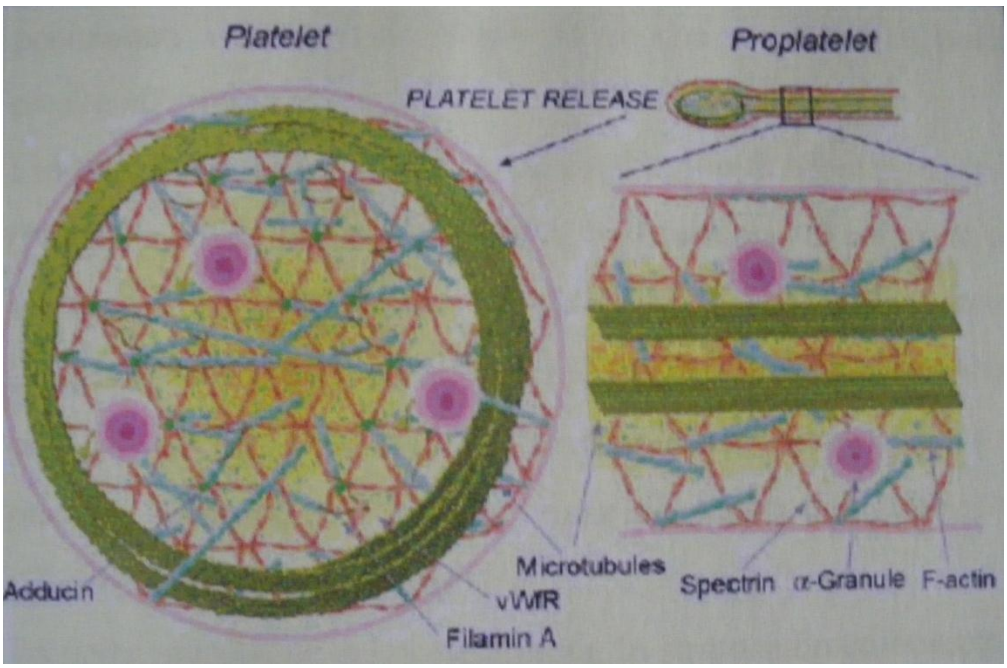
projections which represent the pro-platelets and that are in contact with blood. These projections are afterwards cut off to release platelets in the blood stream (Hartwing and Italiano, 2003).

The proliferation of megakaryocytes is mandatory to have platelets genesis. Thrombopoiesis is regulated by a serum factor, thrombopoietin, responsible for increasing not only the production of platelets but also of megakaryocytes. Also some interleukins (IL-3, IL-6, IL-11) have thrombopoietic activity (Hartwing and Italiano, 2003).

Mature megakaryocytes continuously produce cytoplasmic expansions that are filled with cytoskeleton proteins (α- and β-tubulin, actin, myosin, actin-binding proteins – ABP – gelosin, profillin, taline, vinculine, and tropomyosin) with specific granules and membrane systems. Despite the knowledge that platelets are the derivatives of megakaryocytes cytoplasmic projections, the biological process leading to the their production is nowadays not completely clear (Hartwing and Italiano, 2003).

Human platelets have discoid shape and a dimension of 1-4 μm of diameter per 1μm of thickness. In a healthy adult human their number ranges between 150,000 and 400,000/mm^3. Equine platelets are also discoid fragments 5-7μm long and 1-3μm large, even though in blood they can be as large as 20μm.

When firstly released in the blood flow platelets are larger and with a greater hemostatic power than the circulating mature particles (Hartwig and Italiano, 2003). The average life of a platelet is 7-9 days, which is the time lapse a platelets spends circulating in blood in an inactive form. Afterwards it is caught by hemocateretic organs and phagocytized by the system of mononuclear phagocytes (Hartwig and Italiano, 2003).



Platelet structure (Hartwig and Italiano, 2003)

Platelets' membrane. The membrane of platelets is composed of three layers: the glycocalice, the phospholipid layer and the sub-membranous layer (Tablin, 2000). The glycocalice is the outer layer and is compounded by polysaccharides, lipoproteins and glycoproteins; it has glycoproteic receptors involved in the adhesion and the activation of the protein. Glycoproteins constitute platelets' membranous antigens and are divided in three classes: integrin, leucine rich proteins and selectin. The main glycoproteins present on the outer surface are the collagen receptor, the fibronectine receptor, the vitronectine receptor, the fibrinogen receptor, the laminin receptor and the von Willebrand factor receptor (Tablin, 2000).

The central layer of this structure is made of a double layer of phospholipids with anticoagulant properties, identical to that of other cells, with trans-membranous proteins and peripheral proteins acting like membrane receptors.

Phospholipids are arranged in the double layer with the polar parts toward the outside.

Negative polar lipids, amino-phospholipids (phosphatidilserine-PS) and phosphatidil hetanolamine (PH) constitute the inner part of the phospholipid membrane. Neuter phospholipids (phosphatidilcoline – PC) and sphingosine (SP) are present in the outer side of the phospholipid membrane. Other lipids are present inside the membrane: phosphatidil inositol and lisolectine (Gentry and Nyarko, 2000).

The inner layer of the membrane, the sub-membranous layer, is actually a part of the cytoskeleton binding some proteins of the outer layer. The membrane folds out forming a series of cliffs which constitutes the open canalicolar system (OCS). This system determines an increase of the surface available for the exchanges with the surrounding environment thus a quick way of secretion of the granules once the platelet is activated (Tablin, 2000).

Integrins. Integrins are proteins involved in a large number of cellular interaction. Along with platelets they favor aggregation and adhesion. The basic structure of integrins is formed by two sub-units, α and β, tied by a covalent bond. Integrins are internally connected with platelets cytoplasm with a single C-terminal tail and externally with the medium by different sub-units with extracellular N-terminal domains. The inner portion of integrins is associated with signal proteins (G-proteins and tyrosine kinase) and phosphoinositides. The most important sub-units of integrins inside a platelet are β_1 and β_2. The β_1 sub-unit is associated to three different α units. The β_3 subunit is connected to the glycoprotein IIb-IIIa and to the receptor for vitronectine. The glycoprotein IIb-IIIa, also known as P-selectine and fibrinogen receptor, is on the surface of the α-granules and interacts with fibrinogen, with the von Willebrand factor, with the fibronectin and the vitronectine. The externalization of this glycoprotein is related to the activation of the platelet (Carmona, 2006).

Leucine-rich glycoproteins and other platelet receptors. Leucine-rich glycoproteins, especially the Ib-IX-V GP, contribute to the formation of the negative charge on the surface of platelets. These glycoproteins have two subunits, IbαGP and IbβGP. The outer domains have receptors for thrombine and for the von Willebrand factor. The inner cytoplasmic tail is associated to actin through its binding protein (ABP). When the von Willebrand factor binds to these glycoproteins the cytoplasmic concentration of calcium increases and the phosphoatidil-inositol-3-kinase is activated. These phenomena produce the activation of the A2 phospholipases and the synthesis of arachidonic acid and A2-tromboxane. Other membrane receptors of platelets include receptors for the thrombin, for immunoglobulins and for thrombospondin (Tablin, 2000).

Platelets' cytoplasm. Platelets' cytoplasm is compounded of the same cytoplasmic proteins of megakaryocytes. In platelets there are two types of actin building up the cytoplasm net: globular and linear actin. The strands of actin act as a structural support for the different platelet granules and for mitochondria. The platelet response is mediated by a contractile activity thanks to the polymerization of actin-myosin.

Other elements of the cytoskeleton are represented by microtubules and intermediate filaments. Microtubules are arranged in a twisted fashion to maintain the discoid shape of inactive platelets. After platelets' activation microtubules reorganize and arrange linearly. The adequate assembly of the cytoskeleton is fundamental to the centralization of the granules in the platelets, the secretion and the retraction of the coagulum (Carmona, 2006).

Platelets' granules. The platelets of mammals contain three types of granules: lysosomal granules, dense granules (also called δ-granules), and α-granules (Hartwig and Italiano, 2003).

Lysosomal granules contain acid hydrolases, guanine, phospholipases and kinases working as protolithic and hydrolytic enzymes. They can degrade intracellular material or they can merge into the OCS's membrane to release their content in the extracellular space (Tablin, 2000).

Dense granules represent the storing site for adenylyl nucleotides, as they store ADP and ATP. They also contain calcium, phosphorus, and serotonin. The ADP induces platelets' migration and, along with serotonin, provokes the contraction of damaged arteries. The ATP thwarts ADP's action. Dense granules are less numerous than α-granules (Tablin, 2000). Alpha-granules contain adhesion proteins (fibronectin, vitronectin, thrombospondin), coagulation factors (fibrinogen, factor-V, factor-IV), fibrinolysis factors, mitogen growth factors, cytokines, chemokines, angiogenesis regulation factors, anti-proteases, bactericide proteins, and glycoproteins of the membrane (Tablin, 2000).

Alpha granules contain numerous growth factors, seven of which are greatly involved in wounds' healing process. These factors are the platelet derived growth factor (PDGF), the transforming growth factor (TGF-β1, TGF-β2), the epidermal growth factor (EGF), the vascular endothelial growth factor (VEGF), the insulin-like growth factor (IGF-I) and the hepatocyte growth factor (HGF) (Anitua *et al.*, 2004).

In the cytoplasm of platelets there are also free-moving glycogen granules. Glycogen is the principal source of energy for the metabolism of inactivated platelets (Tablin, 2000).

There are many adhesive proteins: fibrinogen (FG), fibronectin (FN), vitronectin (VN) and thrombospondin-1 (TSP-1). During the hemostatic process part of these factors attaches to platelets receptors and participates directly to the

creation of the thrombus. Fibrinogen potentiate the effect of interleukin-3 on the hemopoietic progenitors in humans, while fibronectin and vitronectin take part to the wound healing process. Among fibrinolytic proteins the PAI-1 is the natural inhibitor of the activator of tissue plasminogen, main inducer of vascular fibrinolysis. The PAI regulates fibrinolysis and can bind vitronectin to improve the interaction between matrix and cells.

Osteonectin is another protein derived by platelets and is also produced by osteoblasts. It is capable to create a complex with plasminogen and tie it to the collagen.

Platelets' activation and adhesion. Platelets can be activated by various mechanisms: by direct contact and adhesion to the sub-endothelial layer for an endothelial lesion, by the action of physiologic agonists (thrombin, ADP, collagen, A2 thromboxane, epinephrine, platelets activation factors) interacting with specific receptors of the membrane associated to the G-protein, and by other mechanisms that produce intracellular signals (Rozman and Bolta, 2007).

Platelets adhere to the sub-endothelium thanks to the integrins of the membrane and to the help of the adhesion proteins of the endothelium (Carinci, 2002). In these conditions the coagulation cascade follows and thrombin is formed. Thrombin contributes to stimulate platelets' activation with a positive feedback response (Rozman and Bolta, 2007). After platelets' activation has occurred a series of events follow: platelets change in shape, secrete the content of granules (including ADP, fibrinogen and serotonin), aggregate, and then the coagulum is retracted (Rozman and Bolta, 2007).

Platelets' shape modification. Platelets quickly pass from their discoid shape of the inactive phase to an irregular spherical figure, with pseudopods which are

firstly very short and then become thinner and longer. This new shape allows the contact between platelets. This mechanism takes place thanks to the great majority of the molecules of the cytoskeleton: microtubules rearrange and the actin strands associated to the membrane contract. The shape rearrangement is strictly dependent on ATP. If the stimulus activating the platelet is either week or too short, the platelet quickly regains its initial status becoming completely morphologically identical to a platelet which has never been stimulated. Platelets regaining the inactive status may not respond, in a refractory phase, to a second stimulus, even when this second one is stronger and longer than the previous. This probably is a mechanism of control tending to self-limit the platelet activation process. When the stimulus is effective instead, a series of other actions follow to the shape modification, such as the reaction of granules' content release and aggregation. From the functional point of view the shape changing has consequences of great importance as it makes the platelet factor 3 (PF3) available on the membrane of the platelet.

The platelet factor 3 is a phospholipid (phosphatidilserin) mainly sited on the inner side of plasmatic membrane in an inactive platelet being thus unapproachable: the change of shape reorganizes the membrane which exposes on the outer side the PF3. The PF3 is involved in some reactions of coagulation (Gentry, 2000).

Secretion. The secretion process of platelets is an active ATP-dependent procedure following adhesion. The secretion of the content of platelets' granules occurs through a mechanism of exocytosis and is related to the increase of calcium concentration in the platelet (Rozman and Bolta, 2007).

While the shape changing takes place, granules are carried close to the OCS (centralization of granules) and their membrane merges with the canalicolar membrane allowing secretion (Tablin, 2000).

Aggregation. Platelets aggregation is mediated by fibrinogen molecules or the vWF, which connect the platelets binding the glycoproteic IIb/IIIa complexes to adjacent platelets creating a platelet aggregate. Each platelet has 50 000-80 000 IIb/IIIa glycoproteins on its own surface. To be able to bind the fibrinogen glycoproteins must be first converted from a status of low affinity to the one of high affinity. This happens through a process called inside-out signaling that starts right after the activation process (Rozman and Bolta, 2007). In inactive platelets the glycoproteic complex IIb/IIa is presented in its inactive form as the two glycoproteins (IIb and IIIa) are separated. The heterodimer GPIIb/IIIa, which is the active form of the complex, is formed only after the stimulation of the platelet and in the presence of calcium ions. Afterward platelets expose this active form of the complex which is able to bind fibrinogen, in turn binding to the glycoproteic receptors of surrounding platelets (Gentry, 2000).

Inside the granules there is a high concentration of molecules capable of maintaining and amplify the aggregation, which is limited to few platelets at first. ADP, Ca^{++}, serotonin, fibrinogen, thrombospondin, and the thrombin generated by the concurrent activation of coagulation are all powerful agonists of platelets' aggregation. Platelets' surface is in fact provided of receptors for all of these molecules which induce a strong biochemical response. Other agonists are the molecules newly synthetized by activated platelets (PAF – platelet activating factor – and thromboxane A2). All of these act together to speed up the formation of an insoluble platelet aggregate: they promote activation and cohesion of platelets recruited from the blood stream to tie to those already attached (Gentry, 2000).

Thrombospondin increases the dimension of platelets' aggregates; it has been proposed that it would work by producing a cross-linking to stabilize the aggregates of platelets and fibrinogen, or that it would co-work with fibrinogen

in positioning itself as a bridge among the glycoproteic complexes GPIIb-IIIa on the surface of platelets (Gentry, 2000).

GROWTH FACTORS

Growth factors are small polypeptides synthetized by various types of cells belonging the immunitary and muscular-skeletal systems. Growth factors bind to specific cell receptors to start cycles of intracellular transduction of the signal and stimulate the production of proteins involved in wound healing. Every single growth factor has a different effect on different cells in the lesion environment. Some factors regulate the expression and the concentration of other factors through feedback responses both autocrine and paracrine. These modulations mainly happen thanks to the power to modulate the expression of cell receptors and their affinity (Woo *et al.*, 2000).

Extracellular signals deriving by growth factors and other active molecules activate the membrane. Afterwards the signal is transduced by the tyrosine kinase receptor. The cells of tissue, stimulated by various PDGFs activate enzymatic chains of signal transformation, trespassing the eventual lack of amplification cascade in the cases of old age and pathological processes in progress. This mechanism gives a possible explanation to the massive effects of the application of platelet derivatives on soft tissues' lesions (Borzini and Mazzucco, 2005).

The contribution of growth factors is complied with the regulation of cellular metabolism, the division, the migration and differentiation of cells, the expression of proteins and the production of enzymes. Potentially they have the capability to make wounds heal, as they stimulate angiogenesis and cell proliferation, influence the production and degradation of the extracellular matrix, and they also have chemotactic action towards inflammatory cells and fibroblasts (Komarcevic, 2000).

Growth factors are nearly innumerable. The factors that have been studied more are:

- Epidermal growth factor (EGF)
- Transforming growth factor BETA (TGFβ)
- Insulin-like growth factor (IGF)
- Platelet-derived growth factor (PDFG)
- Basic fibroblast growth factor (bFGF)
- Vascular endothelial growth factor (VEGF)
- Platelet factor 4 (PF-4)
- Brain derived neurotropic factor (BDNF)
- Interleukins (ILs)
- Colony-stimulating factor (CSF).

The concentration of growth factors in the site of lesion varies in time; this gives a sign on how complex is the process to "arrange" a wound healing (Pietramaggiori *et al.*, 2006).

Acute lesions contain numerous growth factors that have a crucial role in the initial phases of wound healing. A well balanced environment is established to achieve scarring quickly and easily. With the chronicization process this balance is lost. Many studies have evaluated the iatrogenic application of growth factors on cutaneous chronic lesion. Knighton demonstrated that the topic application of various growth factors (PDGF, TGF-β, PDAF, PF4, PDEGF) could improve the quality and speed up the wound healing in an uncontrolled way (Komarcevic, 2000). Similarly growth factors intervene in the healing of tendons and ligaments promoting cells proliferation and differentiation, increasing the synthesis of extracellular matrix and stimulating angiogenesis.

The growth factors that have been more thoroughly studied for their involvement in the healing process of tendons and ligaments are the PDGF, the IGF-1, the TGF-β, the VEGF and the bFGF (Virchenko *et al.*, 2006).

These factors can be potentially produced both by intrinsic cells (i.e. epitendon) and by extrinsic cells (i.e. macrophages); they often have dose-dependent effects, require specific receptors to work, and usually act in synergy with other signal-molecules (Molloy *et al.*, 2003). In vivo researches have shown how the genic expression for IGF-1 and TGF-β1 is decreased in the initial phases of tendon healing. This suggests the possible therapeutic action of an exogenous implementation of them (Haupt *et al.*, 2006).

IGF-1. The molecule is made of a single polipeptidic chain homologue in structure to proinsuline. It is involved in the physiologic body growth and in healing processes. It can bind to two types of receptors: mannose-6-phosphate type 1 and 2. Specific binding proteins tie IGF-1 keeping it in ad inactive form and protecting it from degradation. Probably the reserve of IGF is kept in extracellular environment up to the income of a tissue damage, when the release of enzymes frees the factor activating it.

IGF-1 is an important mediator in every phase of the healing process of a wound, especially during the inflammatory and proliferative phases. It favors fibroblasts migration and proliferation to the site of lesion, and later on it increases the production of collagen. Its action of promoting cell proliferation is amplified by the presence of other growth factors, as PDGF-BB. In vitro studies have shown how the mitosis, thus the cell division, of fibroblasts and superficial cells of tendons are strongly increased when both the growth factors are applied, compared to their application alone.

In vivo studies on rats have shown the capability of IGF-1 to reduce inflammation and the normal functional deficit that follows a tendon lesion. Even if the exact mechanism through which IGF-1 modulates inflammation is unknown, the hypothesis is that a feed-back response is built up where high

concentrations of this factor would inhibit the cellular genes responsible for the inflammatory cascade in the site of lesion (Molloy *et al.*, 2003).

Among growth factors only IGF-1 has been studied for clinical use in equine medicine. It has been demonstrated that a series of intra-tendinous injections of IGF-1 can improve the healing of a collagenase-induced tendonitis. There aren't any collateral effects following the injection of IGF-1, which seems to also have a powerful anti-inflammatory effect just like an anabolic one. The only inconvenient of such therapeutic option is due to limited access to IGF-1 itself thus it is still used for research purposes. IGF-1 is available as a lyophilized powder to be reconstituted with sterile saline solution and stored at -80°C. The treatment consists in a series of 3 or 4 injections every 3 days (Dahlgren, 2009).

TGF-β. There are three homodimeric isoforms (β1, β2, β3) each one of which has its own proper phenotype. They are produced by many cells involved in the healing process and they bind to three different classes of membrane receptors (RI, RII, RIII). The bond with the receptor allows the factor to carry out its own job. The expression of TGFβ-1 mRNA quickly increases after a tendon lesion occurs and it is believed to play a fundamental role in the initial inflammatory response of the damaged tissue. Lactate is one of the mediators more precociously involved in wound healing: some studies show how the cellular production of TGFβ-1 is directly stimulated by it (Molloy *et al.*, 2003).

Once activated, TGF-β influences the various aspects of tissue repair: it promotes cellular differentiation and proliferation (Carter *et al.*, 2002). Klein demonstrated that all three isoforms of TGF-β produced some effects on the proliferation of collagen and cells (Klein, 2002).

The group of TGFs is considered the first responsible for the development of cutaneous exuberant scars, especially the isoforms β-1 and β-2. TGF β-3 has been more lately described and could have an inhibitory function on the

formation of the scar, being a natural antagonist of TGF β-1 and -2 (Komarcevic, 2000).

TGF-β works in synergy with other growth factors. In vitro studies on fibroblasts derived from dog cranial cruciate ligaments have demonstrated that low doses of TGFβ-1 facilitate the isomer PDFG-AB in stimulating the proliferation of fibroblasts, while high concentrations have the opposite effect. In vivo studies have furthermore shown that high levels of TGF β-1 increase the development on adhesions in the tendon (Molloy *et al.*, 2003).

VEGF. There are various isoforms all with the same biological activity. The receptors they bind to are three and present great affinity with tyrosine-kinase (VEGFR-1, -2, -3). The main functions of VEGF are guaranteed by the bond with VEGFR-2.

Even if VEGF has some kind of role in the initial migration and proliferation of cells, it reaches the highest concentrations only after the inflammatory phase when it manifests its great angio-genetic power. The gene expression of VEGF increases in response to biological and biomechanical stimuli like hypoxia, other growth factors, interleukins and bone distraction (Molloy *et al.*, 2003).

PDGF. It is a group of dimeric polypeptides (PDGF-AA, PDGF-AB, PDGF-BB) formed by three types of subunits similar in their structure. Their activity is mediated by the interaction with two receptors close to the tyrosine-kinase, one of which binds all three kinds of PDGF chains while the other one can bind only one.

PDGF is produced right after the tendon is damaged and at the same time it induces the production of other growth factors like IGF-1. It also has a role in remodeling the tissue (Molloy *et al.*, 2003).

In vivo studies on the application of PDGF on ligament lesions have ascertained an increase in the strength and the stiffness of the ligament itself, while the application of PDGF+IGF-1 or PDGF+bFGF has not brought any improvement compared to those seen in controls (Molloy *et al.*, 2003).

Chapter 2

Platelets' Concentrates

Platelet rich plasma (PRP) is an autologous platelet concentrate obtained by processing whole blood and containing red and white blood cells, plasma and platelets (Dahlgren, 2009). Because of the presence of so many platelets it is considered a vehicle and source of growth factors (Van Den Dolder *et al.*, 2006).

In the last decades a significant attention has been put on platelets concentrates and great expectations have risen about these products as platelets' α-granules contain a huge number of growth factors many of which have quite positive effects in the healing process of tissues. PRP has been studied for its potential benefits on all sorts of tissues, including tendons and ligaments more recently.

Platelets have been studied and used either plainly as platelet concentrate, like PRP, or as a jelly product (platelet gel, PG) or as platelet lysate; these last two as a derivation of PRP. The term gel has been used to describe a jelly-like product which results from the addiction of thrombin and calcium to the PRP to provoke the break of fibrinogen. Fibrin is formed and its polymerization produces a gel slightly sticky and pasty. Platelets are trapped in the gel and activate releasing their bioactive molecules. These factors are temporarily held in the gel but afterwards they slowly diffuse in the surrounding area.

The term lysate is used to describe a liquid product obtained both by platelet activation through thrombin addiction and by platelet lyses after a freezing-thawing process. The term lysate, though, is preferably used to refer to the product obtained by freezing and thawing of platelets, while the thrombin addiction produces what should be called relysate (Borzini and Mazzucco, 2005). Platelets lyses, thus the release of their factors, is achieved when they are stored at -80°C and then thawed at 37°C. The lysate is also used as a scaffold mean to apply stem cells. This combination of products has shown brilliant results in vitro (Del Bue *et al.*, 2007). Clinical studies have demonstrated the positive effect of the application of platelet concentrates on the healing process of hard and soft tissue.

Platelet concentrates are used in odontoiatry, in maxilla-facial surgery, and recently also in diabetic patients with serious skin ulcers and in plastic, reconstructive, ophthalmologic and orthopedic surgery (Anitua *et al.*, 2004; Virchenko *et al.*, 2006). The use of platelet concentrates was demonstrated to reduce the need for drainages and elastic or compressive bandages, along with reducing the incidence of complications, infections and hospitalization time. PRP has anti-inflammatory, bactericide and hemostatic properties. It also is used in many surgical procedures, as the lumbar arthrodesis, the reduction of complicated bone fractures, the repositioning of joints after luxation, arthroscopic treatment of traumatic osteo-chondral defects, treatment of the Achilles tendon's lesions, intrarticular treatment of osteoarthritis. PRP is used also to repair the retina of patients affected by idiopathic macular holes (Anitua *et al.*, 2004).

Use in odontoiatry and maxilla-facial surgery. In 1997 Whitman and colleagues first reported the effects of platelet gel to speed up healing after oral surgery. One year later Marx *et al.* used the PRP in combination with autologous bone implants to rebuild mandibles affected by loss-of-substance. Their study demonstrated that this therapeutic option could achieve a faster maturation of bone and a higher density of the matrix compared to the bone implant alone (Marx *et al.*, 1998). Since then the effects of PRP on bone regeneration have been widely studied (Whitman *et al.*, 1997; Kassolis *et al*, 2000; Camargo *et al.*, 2002; Lekovic *et al.*, 2003; Nikolidakis *et al.*, 2006) and PRP has been used for many clinical procedures in odontoiaatry (Plachokova *et al.*, 2008).

In 1999 Anitua performed a study on 20 odontoiatric patients. Some of them had vertical fractures and dental extraction had been indicated as the only possible option to solve the problem; other patients had severe periodontal pathologies to be solved by positioning an implant. Ten of these patients were treated with topic treatment with GFRP (Growth Factors Rich Plasma) while other ten were used as controls. The results obtained in the GFPR patients were of an excellent rehepitelization, an almost complete regeneration of the treated area, the formation of trabecular bone organized and mature. The control group showed a good reparation of the damaged area but the loss of substance was filled in with connective tissue, and only a few cases had mature bone (Anitua, 1999).

Use in plastic surgery. The use of autologous platelets in reconstructive and facial plastic surgery has ported many advantages: there is a general decrease of operative time, need for drainages, compressive bandages and incidence of complications. Valbonesi performed a study on 14 patients affected by either traumatic or pathologically chronic loss of soft tissues and skin and reported a reduction of post-operative infections and time of hospitalization.

The anti-inflammatory properties of platelets concentrates were also demonstrated in a study on 8 women undergoing facial lifting, and that showed a lower rate of edema and ecchymosis (Anitua *et al.*, 2004).

Use for treatment of ulcers. In 2002 Tarroni and colleagues successfully used a platelet gel obtained by the activation of two traditional hemo-derivatives: a platelet concentrate and the cryo-precipitated activated with an enzyme with coagulant action, batroxobine, with the addiction of chloride calcium. The product was applied on a chronic ulcer on a foot of a 62 years old man. The ulcers recovered after 8 applications and the foot had not to be amputated any more.

The application of a platelet lysate as a source of growth factors was of great success also in a patient affected by β-thalaxemia with an ulcer older than 4

years on his ankle. Complete recovery was achieved in one month (Anitua *et al.*, 2004).

Use in orthopedic surgery. There is a number of studies in this field (), the most interesting of which regard the spine. Autologous concentrates of growth factors have been used in patients supposed to undergo lumbar arthrodesis with encouraging outcomes. Lowery and colleagues performed a study on 39 patients, 19 of which were followed up for 6 months. Among these 5 showed a steady fusion of the bones (Anitua *et al.*, 2004).

Treatment of tendons and ligaments. In recent studies Anitua demonstrated that the injection of autologous PRP and calcium facilitated the regeneration of the anterior cruciate ligament and the reattachment of the articular cartilage of the knee in humans. The fundamental role of endogenous growth factors (like the IGF-1, TGF-β, VEGF, PDGF, and bFGF) in the healing of tendons and ligaments has been documented: these factors in fact take part to the inflammatory, proliferative and remodeling phases of the tissue. Furthermore Anitua experimented the use of autologous platelet concentrates activated with calcium for the reparation of muscle ruptures and of the Achilles' tendon with satisfactory results (Anitua *et al.*, 2004).

Use in ophthalmic surgery. Another surgical field where the positive effects of platelets concentrates have been reported is ophthalmology. In 1999 Gehring and colleagues applied an hyper concentrated platelet product to treat macular retinoic holes achieving a quicker closure. Hartwig and colleagues more recently (2004) have applied PRP on corneal epithelium defects (Borzini and Mazzucco, 2005).

In a study, 110 French patients have been subjected to surgical treatment to reduce idiopathic macular holes and half of them have been injected with autologous platelet concentrate during the treatment. One month after surgery

the anatomic success of the closure of retinoic holes was significantly higher in the PRP treated patients (Anitua *et al.*, 2004).

Potential risks of the use of PRP in human medicine. Platelets release low molecular weight compounds that can diffuse. They also release a great number of micro-particles that carry pro-thrombotic proteins (like TF and Il-1β). The application of platelet concentrates nearby blood vessels must be very careful, especially in patients at risk of thrombosis. The contemporary use of anti-aggregation drugs may theoretically reduce the risk of development of thrombosis, but further studies are still needed on this topic (Anitua *et al.*, 2004).

The preparation of platelet concentrates must be performed in proper transfusion centers to respect asepsis and security standards to avoid the risk of infection. Anyways no cases of infected wounds after platelet gel application have been so far reported.

The risk of an immunitary reaction or transmission of infectious diseases (i.e. hepatitis and HIV) must be excluded because the product is autologous.

Even if growth factors have mitogen properties there is no evidence they may promote development of tumors or have cancerogenous power. Scott and Pawson have also demonstrated that growth factors act on cell membranes and not on the nuclei; furthermore the gene expression induced by growth factors doesn't present any anomaly (Everts *et al.*, 2006).

Studies on animal models. The studies about PRP application on animal models confirm the results obtained in humans (Anitua *et al.*, 2004).

Anitua and Ortiz performed a study on goats using titanium implants covered by PRP and obtained improvements both in the quality and the quantity of regenerated bone tissue around the implant (Anitua *et al.*, 2004).

Histologic studies on bone defects around titanium implants in dogs have proven that PRP increases the local production of bone (Kim *et al.*, 2002). With human

PRP the distance between the pores of hydroxyapatite could be reduced (Siebrecht *et al.*, 2002).

The application of PRP in ligaments was proposed for dogs with cranial cruciate ligament rupture and in rats with patellar ligament lesion (Dahlgren, 2009).

A study on muscular tissue reported consistent results of muscle regeneration in rabbits treated with PRP (Jodezyk *et al.*, 1986). In another study lesions of the femur trochlear cartilage were induced in rabbits and the intrarticular injection of PRP favored the formation of a tissue maintaining the viscoelastic features proper of the original cartilage tissue (Serra *et al.*, 2006).

Cullinane and colleagues demonstrated that treatments with platelet concentrates improved the cell proliferation in the healing of retinoic lesions in rabbits (Anitua *et al.*, 2004).

APPLICATION OF PRP IN HORSES

Use in orthopedic surgery. In a preliminary study platelet gel was applied in two horses with a vertical fracture of the navicular bone. The PG was applied on the line of fracture and after 4 months radiographic controls showed the complete recovery. In a filly with an olecranon fracture and subjected to PG application two weeks after the osteo-synthesis surgery the healing of the bone was complete and uneventful (Spadari *et al.*, 2006).

Treatment of skin wounds. In the treatment of skin lesions the application of PRP resulted in an increase of formation of granulation tissue, fibrous tissue and epithelium. Carter and colleagues have compared the time and quality of the healing of skin wounds treated with local application of jelly PRP and non-treated wounds. The analysis of bioptic samples has demonstrated how the PRP induced a faster differentiation of the epithelium and a greater production of collagen with braided strands and better organization. Seven days after lesions the treated tissues were more epithelized than the controls and after 79 days their collagen had more parallel fasces, while in the control they were fewer and randomly oriented (Carter *et al.*, 2003).

Monteiro and colleagues proposed again the use of PRP on surgically created skin wounds in the distal portion of the limbs of 6 horses. PRP induced the development of an excessive quantity of granulation tissue and the healing was significantly slowed 1, 2, and three weeks after surgery. Monteiro deduced that the local application of PRP did not accelerate or improve the healing of small wounds of limbs in horses but he sustained its application for lesion with massive loss of substance or chronic wounds (Monteiro *et al.*, 2009).

Prp in equine teno-desmic lesions. The available data about the use of PRP in tendon and ligament lesions in horses are still too few; thus nowadays it is only possible to suppose the intra-lesional injection of such product can supply the damaged tissue with a large amount of growth factors and support the healing. To recommend the use of platelet concentrates to treat equine tendinopathies a lot of issues must be taken into consideration: the choice of the method of preparation, the eventual need to activate platelets at the time of injection, the quantity of growth factors to make it effective in promoting the regeneration of the tissue (Dahlgren, 2009).

Many in vitro studies have been performed. Tablin and colleagues studied the effect of platelet growth factors (obtained from supernatant frozen with dry ice and then rehydrated) and applied on fibroblast cell cultures. The obtained results were an improvement of the proliferation and migration of fibroblasts and an increase in the production of collagen (Tablin *et al.*, 2008).

PRP improved the gene expression of type I and type III collagen and of COMP when used in vitro on SDFT explants (Dahlgren, 2009).

Other studies on cultured fibroblasts deriving from SLs have shown the PRP ability to increase the synthesis of COMP and other proteins (Schnabel *et al.*, 2008).

Haupt and colleagues instead applied a human growth factor (PDGF-BB) on equine tendon explants. They observed an increase in type I collagen and a temporary increase of the expression of PDGF-BB itself. The study lasted 6 days and the registered effects were very limited compared to the total content of collagen of SDFTs (Haupt *et al.*, 2006).

The good perspectives designed by in vitro studies have led to in vivo trials. Waselau and colleagues treated with intra-lesional injection of PRP 9 standardbred horses affected by SL desmitis. After treatment a fitting rehabilitation protocol was set up and the horses got back to racing. Their start-

ups, the total wins and the wins per start-up were compared to those obtained by an equal pool of horses not treated with PRP. The results obtained were quite in favor for the first group, especially in the second year of racing but the study was unable to discriminate whether if the positive outcome had to be attributed to the PRP treatment, to the rehabilitation protocol, or to a combination of the two things (Waselau *et al*, 2008).

Arguelles and colleagues used PRP in 2 horses affected by acute SDFT tendonitis and 3 with a chronic proximal SL desmitis. An ultrasound-guided intra-lesional injection was performed 3 times every two weeks. Horses were clinically controlled after 2, 12, and 20 months after injection. Clinical signs and ultrasound appearance were taken into consideration for a complete evaluation. The ultrasound appearance improvement regarded mainly the horses with SDFT lesions; in any case all horses improved clinically and got back to racing in a short time (2 to 6 months after treatment) without relapsing within the 20 months of the study (Arguelles *et al.*, 2008).

QUANTIFICATION OF GROWTH FACTORS IN PRP

The knowledge of the quantity of growth factors available in PRP is highly predictive to outline the prognosis for the subject to be treated, thus of great importance for a proper clinical use. Unfortunately there are no available procedures at this time to have an esteem on the amount of growth factors present and biologically active in a PRP sample. Neither the whole blood nor the platelet count can help in understanding the GF concentration in the platelets of an individual. The GF present in platelets of mammals are basically of the same kind among species, but there is a difference in their relative concentration. Furthermore, each subject has a different total amount of GFs, even if they are in proportion according to the specie.

In a study on human blood samples, after proper centrifugation and automatic separation of the fractions, the quantification of some GFs (PDGF, TGF-β1 and 2, IGF-1) was obtained using specific ELISA kits (Quantikine Elisa Kit, R&D Systems). The analysis of variance for the PDGF-AB, PDGF-BB, TGF-β1 and TGF-β2 showed that there is no correlation at all with sex or age and the quantity of growth factors. Only the levels of IGF-1 seemed to be slightly lower in older patients. A high variability among subjects has thus been recognized, and the relation for the quantity of GFs and other individual biological factors yet to be discovered has been suspected (Weibrich *et al.*, 2002).

Weibrich and colleagues sustain that the platelet concentration in human PRP is related to the platelet count of the whole blood (Van Den Dolder *et al.*, 2006).

In a comparative study, PRP from humans, rats, and goats were compared. The result was that human PRP contains higher concentrations of GFs than the other two species. Furthermore, goat's PRP has more GFs than the rat's, exception

made for the PDGF-BB which is more concentrated in rat's PRP. These result may then somehow justify the difficulty obtained in achieving clearly positive results of PRP application in animal models (Van Den Dolder *et al.*, 2006).

Platelet count. The normal number of platelets in human blood varies between 150,000 and 350,000/mm^3. A concentration of 1,000,000/mm^3 in 5 mL of plasma could be considered the ideal concentration of platelets for an effective human PRP: lower concentrations haven't brought any improvement to wound healing, while higher concentrations do not achieve proportionally higher results (Van Den Dolder *et al.*, 2006; Everts *et al.*, 2006).

The reported concentrations of platelets in equine PRP vary a lot among studies. Carter and colleagues successfully used a PRP produced by apheresis with a platelet concentration of 490,000/mm^3 platelets (Carter *et al.*, 2003). Sutter obtained a 855,000/ mm^3 platelet concentrate with the apheresis method and a 1,472,000/ mm^3 platelet concentrate using the buffy coat system.

ACTIVATION OF PRP

PRP itself is an inactive product because the growth factors are stored inside the α-granules of the cytoplasm. Activation of PRP can be achieved with the aid of many different bioactive molecules; among these, thrombin is the most powerful one. Thrombin is a protease of the serum involved in the coagulation process; although, it has many other biological properties similar to those of growth factors: it has chemotactic action toward neutrophils, monocytes and macrophages, it stimulates fibroblasts' proliferation, accelerates wound healing. In vitro studies suggest that thrombin's role in the healing process is separated by its role in coagulation. Thrombin is responsible for the conversion of fibrinogen into fibrin. Its action is allowed thanks to the bond with specific superficial cell receptors that can activate proteases while the non-protolithic portions of the molecule can activate other receptors (Virchenko *et al.*, 2006).

Bovine thrombin is the most commonly used in practice but its application in humans has been correlated to the development of antibodies against some coagulation factors (V, XI) eventually resulting in a coagulopathy (Landesberg *et al.*, 2000). To overcome to this problem a synthetic product, batroxobine, has been afterwards used.

Batroxobine is a thrombin-like enzyme that induces jellification in a different way than thrombin does. While thrombin activates platelets, thus induces a quick release of growth factors and accelerates the retraction of the coagulum, batroxobine traps the platelets in a net of fibrin without activating them. For this reason the release of growth factors and the retraction of the coagulum happen slowly, in a gradual process. Batroxobine, differently from thrombin, doesn't stimulate the immunitary system and is not inhibited by heparin. It has also been demonstrated that the survival and the availability of cytokines released by platelets change depending on the type of polymerization of fibrin. The use of

batroxobine other than thrombin is especially preferred in those cases where growth factors are strongly preferred to stay in the site of lesion for a long time (Mazzucco *et al.*, 2008).

In disguise of thrombin calcium chloride can be preferred: it is an inert substance thus it cannot induce any immunitary reaction (Arguelles *et al.*, 2006). The use of thrombin and calcium together induces the production of a jelly substance called platelet gel (PG) (Zimmermann *et al.*, 2008).

The levels of GFs are influenced by the degree of platelets' activation, thus they may change depending on the substance used to activate them (Zimmermann *et al.*, 2003; Tablin *et al.*, 2008).

Another way to induce platelet activation is freezing (-80°C or -196°C). This irreversible activation is known as "cold lesion" and can be avoided with a process of biochemical stabilization using amiloride, adenosine and sodium nitropruxiate (Pietramaggiori *et al.*, 2006). The method consists in freezing the platelet concentrate and then thawing it at 37°C. This physical treatment induces cell membranes to break and release the chemotactic and growth factors without needing further activation (Del Bue *et al.*, 2007).

The release of GFs from the granules is never complete, neither after the formation of the coagulum nor after freezing-induces lisys. This might be due to the presence of platelets' micro particles (PMPs), which are small fragments of the platelet membrane, and of WBCs deriving micro particles. Micro particles seem to contain growth factors that cannot be detected with immunologic techniques (Zimmermann *et al.*, 2003).

Another way to jellify PRP is the use of ITA jelling agent (Natrex Technologies Inc., Greenville, NC). This method requires simpler material and can be used also in ambulatory environment (Landsberg *et al.*, 2000).

For human medicine ready-to-use kits have been produced: they supply the activators for the platelet concentrate and often also the instruments to aseptically inject the product (Smart PreP2 by Harvest, Plateltex).

METHODS FOR THE PREPARATION OF AUTOLOGOUS PRP IN EQUINE MEDICINE

The traditional methods to prepare platelet concentrates are: a) the buffy coat method; b) the platelet rich plasma method; c) the apheresis. Nowadays there are many systems available to prepare PRP that cannot be classified in any of these categories. The simplest possible classification is to them in automatic and manual methods. Manual systems (buffy coat and apheresis) are those where the operator can see the interface plasma-red blood cells having the possibility to modify the procedure based on the single case (Sutter *et al.*, 2004).

1) **Buffy coat method**. Fifty milliliters of blood are collected from the jugular vein with a 12G needle into a citrated blood-tube with valves for the separation of blood fractions. The tube is centrifuged at 2100g per 9 minutes in a special centrifuge with a support for the tube. The fraction with the red blood cells is afterwards extracted from the bottom through a blood port, leaving exactly 3 cm of RBC and platelets packed in the tube. The tube is then centrifuged again at 2100g per 3 minutes. The plasma is afterwards discharged through the plasma port which is set to leave in situ 5 milliliters of product (10% of the initial volume) that contain the final product (Sutter *et al.*, 2004).
2) **Apheresis method**. An apheresis system is used to obtain the platelet concentrate with a process of discontinuous centrifugation. A bowl set with a basic volume of 125 milliliters is installed into the apheresis unit and connected to blood separation bag with a scaled tube. In the separation line there are three bags: one for the plasma, one for the buffy coat, and one for the air. The bowl set is connected to the blood bag of the donor. The apheresis unit is initially set at 5650 rpm, with a pumping rate of 60 ml/min. The donor bag is rocked in continuous to prevent

sedimentation of RBC. The bowl is filled in until the RBC reach a preset level; at this point the platelet poor plasma (PPP) enters the plasma bag and the pump stops. The centrifugation speed decreases to 2400rpm and the tap of platelet concentrate and the plasma bag are opened. At this point the pumping unit is reactivated and 3mls are pumped in the bag of the buffy coat. The pump is turned off for 10 sec before it is turned on again to pump 3 more milliliters of platelet concentrate in the collection bag. This process of platelet extraction continues until 45 ml of concentrate have been obtained (Sutter *et al.*, 2004).

3) **Method of filtration after apheresis**. The concentrate obtained with apheresis is concentrated again with a 30 KD pores filter that filtrates water and low molecular weight molecules. The 45 ml of concentrate gained with the apheresis are pumped through the filter in both directions to reduce the volume to the final 15 ml (Sutter *et al.*, 2004).

Comparison between results of methods 1, 2, and 3 and whole blood. The time and the costs of apheresis method are higher than the buffy coat one, but the possibility of bacterial contamination is lower. The apheresis technique followed by filtration registered the higher degree of platelet concentration in horses: it reaches a 13 times fold, against the 8.9 of the buffy coat and 5.2 of the apheresis compared to the platelet count of whole blood. The buffy coat system though starts with a small quantity of blood (50 ml) thus the obtained PRP is little compared to the other methods.

The levels of TGF-β1 of the buffy coat PRP aren't very different from those obtained with apheresis, whilst apheresis concentrates more the TGF-β2. The concentrations of IGF-1 are not increased compared to the whole blood, and they are way lower in the buffy coat PRP than in the apheresis PRP or in whole blood.

With apheresis there are many more less RBCs thus a lower hematocrit. The effects of RBCs in the platelet concentrate are yet to be investigated but they

would certainly be a problem having to use the product in a different subject other than the donor (Sutter *et al.*, 2004).

4) **Method of the single centrifugation (tube method).** Forty milliliters of whole blood are harvested by jugular venipuncture with a 23G butterfly needle in sodium citrate. The 40 ml of citrate blood are collected in 8 tubes with 5 ml each; they are then centrifuged at 120g per 5 min. Two fractions of platelet concentrate are obtained: PC-A and PC-B. PC-A is arbitrarily considered the 50% of the supernatant just above the buffy coat, and the PC-B the 50% over the PC-A. Five ml of each PC are separately put in 10 ml polypropylene tubes.

   In preliminary studies to set up this protocol Anitua's centrifugation schedule was followed, which is at 460g per 8 min. Afterwards it demonstrated inapplicable in equine medicine as the effect on sedimentation of equine platelets is different from that of human platelets (Arguelles *et al.*, 2006).
5) **Method of the double centrifugation (tube method)**. Eighty milliliters of whole blood are harvested by jugular venipuncture with a 23G butterfly needle in sodium citrate. The 80 ml of citrate blood are collected in 16 tubes with 5 ml each; they are then centrifuged at 120g per 5 min. The PC-A fraction (averagely 20 ml) is collected from each tube and put in two 10 ml tubes. This fraction is centrifuged at 240g per 5 min. The centrifuged PC-A is then arbitrarily divided in two fractions: PC-C and PC-D. The PC-C is the lowest 25% of the centrifuged PC-A and the PC-D the remaining 75% (Arguelles *et al.*, 2006).

Comparison of the methods 4, 5 and whole blood. In equine blood after a single centrifugation (120g per 5 min) platelets and white blood cells are distributed almost uniformly in PC-A and PC-B. The results obtained by Anitua on human blood instead presented a higher concentration of platelets in the 75%

of the plasma thus Anitua called it growth factors rich plasma and the remaining 25% growth factors poor plasma. This is why Anitua's protocol to obtain a good platelet concentrate from equine blood, while the buffy coat and the apheresis methods can be as well used for horses without modifications (Arguelles *et al.*, 2006).

The levels of TGF-β1 calculated in the PCs result higher compared to the starting whole blood. In PC-B and PC-C the levels are similar and higher than PC-A and PC-D. The levels of TGF-β1 are not related to the platelet count or the number of white blood cells.

The levels of MPV (Medium Platelet Volume), MPC (Medium Platelet Concentration) and MPCDW (Medium Platelet Concentration Distribution Weight) calculated with a hematologic flow cytometer in the PCs of both methods result to be very different: much higher levels are in the PCs after single centrifugation, while after double centrifugation they are low and similar to the starting whole blood.

PCs obtained with one centrifugation have significantly higher level of platelet activation compared to double centrifugation PCs and whole blood. It can then be stated that the PC-B and the PC-C are to be preferred for clinical use in equine patients with muscular-skeletal pathologies (Arguelles *et al.*, 2006).

Comparison of methods 1, 2, 3, 4, 5, and whole blood. The number of concentrated platelets is higher with the apheresis with filtration, followed by buffy coat, then by the apheresis and finally by PC-D, PC-C, PC-B, PC-A and whole blood.

In the tube method leukocytes are less concentrated compared to the methods of the buffy coat and the apheresis.

The concentration of TGF-β1 varies depending on the method used: it is higher in the apheresis with filtration to decrease progressively with apheresis, buffy

coat, in the PC-C and PC-B, PC-D and PC-A, with the lowest levels in whole blood (Arguelles *et al.*, 2006).

Different methods of centrifugation to obtain PRP. Many variations on the method of the double centrifugation are described in literature. The time and the speed of centrifugation proposed by authors are all different.

Virchenko and colleagues obtained a platelet concentrate from rats: the blood with the anticoagulant was first centrifuged at 220g for 20 min. The supernatant was collected and centrifuged at 480g per 20 min (Virchenko *et al.*, 2006).

Landesberg and colleagues tried to obtain a higher platelet concentration from human blood. They evaluated the number of platelets in relation to the time and the speed of centrifugation and the best results were obtained with 200 g per 10 min for both centrifugations (Landesberg *et al.*, 2000).

ROLE OF WHITE BLOOD CELLS IN THE PLATELET CONCENTRATE

The gradient of density of some categories of leukocytes is very close to the platelets'. This is why some leukocytes, especially lymphocytes and monocytes, stratify along with platelets when PRP or PG are prepared, while granulocytes stratify with red blood cells. Thus PRP and PG contain a certain amount of WBCs (Zimmermann *et al.*, 2008). Everts and colleagues point out to the wrong terminology used in the papers so far published about PRP and other platelet concentrates, where the product that is used is actually a platelet-leukocyte rich plasma (Everts *et al.*, 2007). The contamination with WBCs incises on the total amount of growth factors but very little is yet known about the in vivo effects of such contamination. For sure the quote of growth factors in a platelet concentrate produced with common methods are not to be attributed only to platelets, as WBCs play a role in the release of GFs. Only applying platelet concentrates obtained by platelet-pheresis the effects of platelets alone can be distinguished by the effects of platelets and WBCs together (Zimmermann *et al.*, 2008).

Another role often imputed to leukocytes in PRP is the antimicrobial activity. Neutrophils and monocytes are rich in granules containing myeloperoxidase, an enzyme catalyzing the oxidation of chlorite to produce hypo-chloric acid and other reactive derivatives of oxygen acting as toxic oxidant agents, thus being disruptive toward bacteria and funguses (Everts *et al.*, 2007).

POTENTIAL RISKS OF THE USE OF PRP IN HORSES

The risk of thrombosis, already reported for humans, doesn't represent a problem for horses treated with PRP, as thrombotic pathologies are neither likely nor frequent in this specie. Also for horses, as for humans, it is quite important to respect aseptic conditions in all of the phases of preparation and injection of the product. There are in any case no reports of post-operative complications due to contamination.

SECTION 3

Use of autologous platelet rich plasma for tendons' and ligaments' injuries in horses: a clinical trial

DESIGNATION OF A PROTOCOL FOR THE PREPARATION OF AUTOLOGOUS PLATELET RICH PLASMA IN HORSES

As previously described in literature there are at least three standardized methods for the preparation of PRP: buffy coat method (semi-automatic), apheresis system (automatic), and of the single or double centrifugation (manual) (Monteiro *et al.*, 2009) each of which has been in time modified by different authors to the purpose.

The system proposed in this study was designed adapting to horse blood the protocol used for human blood in the Laboratory of Hematology of the Rizzoli Hospital of Bologna, as suggested by Dr. Dallari (pers. comm., 2005).

The protocol designed is the result of a series of attempts to refine a procedure that could be easy to apply, moderate in costs, repeatable, fast to perform and that could guarantee the wanted characteristics of the final product: high concentration of platelets, no contamination, low WBCs and RBCs counts.

Thus the choice was to use a double transfusion bag with collection needle already inserted and membranes to separate the bags. This is a closed, sterile system that allows to easily process and handle the product without any possible contamination or contact with outer environment through the whole process.

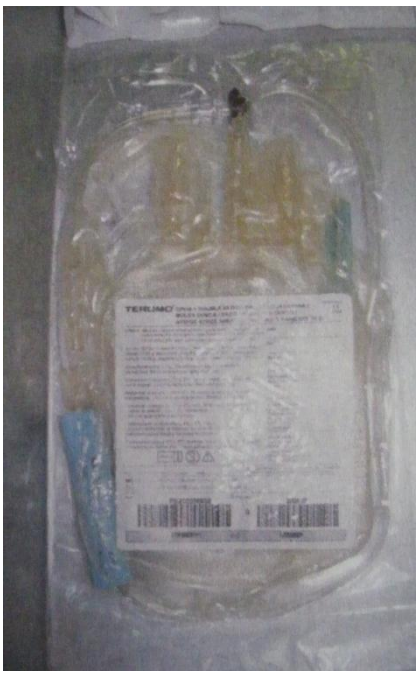
Preparation procedure

A small squared area of the upper-mid jugular region was clipped and cleaned with Betadine. A subcutaneous injection of 0.5ml of 2% Lidocaine was

performed to reduce the itching of the collection needle. The skin was afterwards aseptically prepared with serial passages of a betadine solution (10%) and clorexidine. Skin was finally washed and dried with sterile water and gauzes.

Three-hundred-fifty milliliters of whole blood were collected by jugular venipuncture in a double blood bad pre-filled with citrate to prevent coagulation. The first bag (collection bag) is connected to the needle through which the harvesting was performed. In this first phase the second bag remains closed. The collection happens per gravity, and throughout the bag is continuously rocked to prevent the formation of micro-aggregates. After the harvesting, the collection bag was hermetically closed with plastic pins, and the skin of the horse treated with antimicrobial paste.

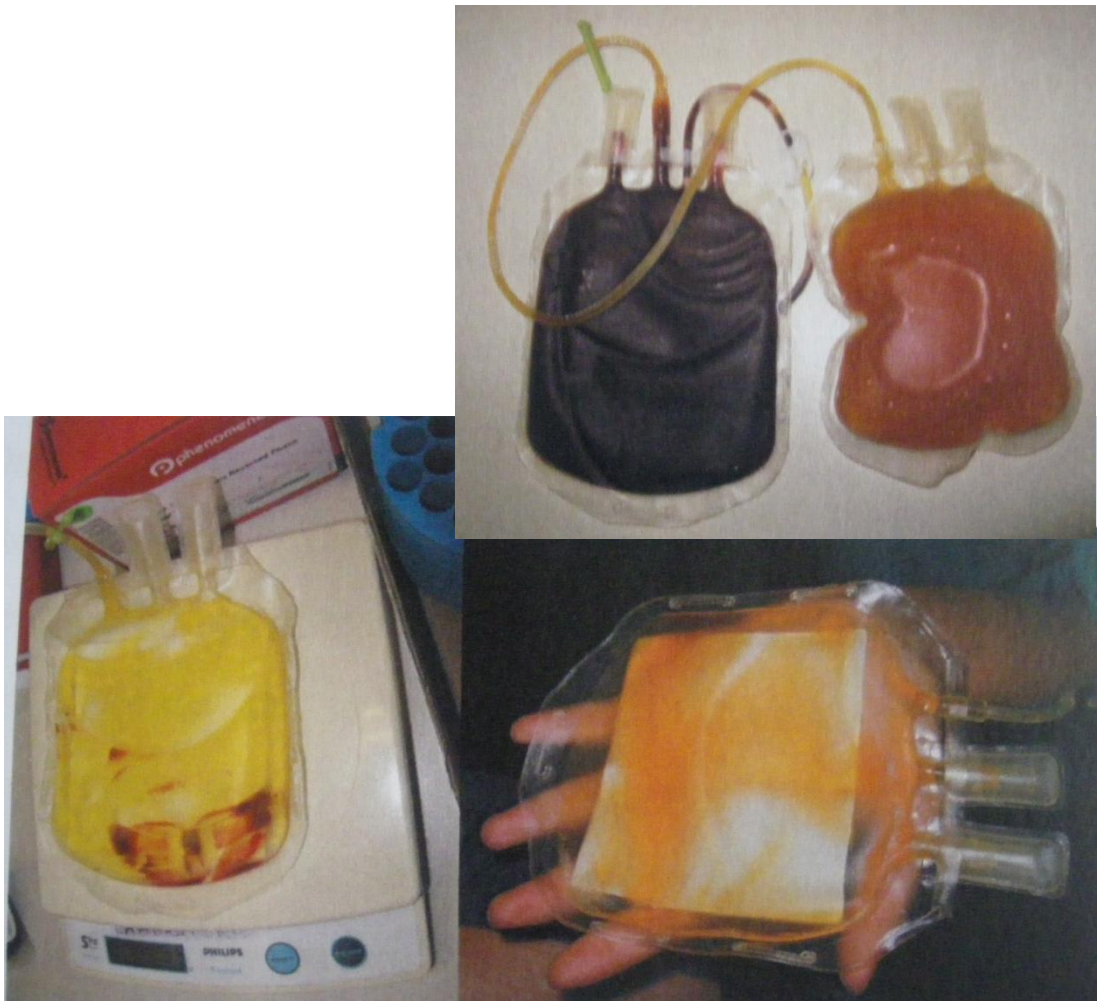
The centrifuge used in the study (GPK Centrifuge, Beckam Instruments Inc.) is calibrated in RPM other than in g, thus a conversion was needed following the conversion formula g=11.18r (M rpm/1000)2, where g is the relative centrifuge force, r is the revolution ray (the distance between the particle and the rotation axis), N rpm is the number of revolutions per minute. The maximum ray of the used centrifuge was 0.19 mt.



After centrifugation the various hemocomponents (RBCs, plasma, platelets, and WBCs) stratify on the basis of their weight: RBCs at the bottom, the buffy coat (mainly WBCs and some platelets), platelets and then supernatant plasma.

The bag system is the centrifuged a first time at 1000 RPM per 20 minutes. It has been sometimes necessary to prolong this time up to the 100% to allow a better separation of the fractions. The second bag is afterwards opened and the supernatant is pushed to flow into this second bag. The amount of buffy coat let into the second bag was different depending on the type of product wanted.

After closing the communication between the two bags with metal clamps the second centrifugation is performed at 3000 RPM for 10 min. Afterwards the metal clamps are removed and the greatest amount of the content of the second bag is manually pushed back into the first one. The quantity that must remain in the second bag is the 10% of the initial volume of blood and represents the PRP.



The obtained PRP was finally collected in syringes and transferred in 2 ml sterile cuvettes (in a sterile set-up) to divide it into aliquots. A small quantity of it (2 ml) was used and analyzed to evaluate the quantity of platelets and WBCs obtained. The count was performed with an automatic analyzer -- LASER Cell-Dyn 3500 (Abbott Diagnostics).

Injection

The injection procedure requires the clipping and aseptic preparation of the area where the lesion has been ultrasonographically detected. The horse is sedated with acetilpromazine (0.02 mg/kg), detomidine (0.02 mg/kg) and butorphanol (0.01 mg/kg).

A 7.5 MHz linear ultrasound probe is covered with a sterile glove to visualize the lesion and perform the ultrasound-guided intra-lesional injection. A 21G needle is inserted into the lesion and then connected to the syringe filled with PRP. The insertion of the needle must be carefully supervised ultrasonographically to direct the point of the needle in the wished direction. When the procedure is carried out correctly the incidental injection of PRP in the in sub-cutis, in other tendons or other surrounding structure is avoided.

The injection itself must be performed with a smooth constant pushing of the bucket of the syringe until the resistance is too high. Small movements of the needle are allowed within the lesion to fill it in properly. In case of multiple injections instead a new needle is needed at each time, and the insertion must be each time ultrasonographically checked. The injected quantity varies between 2 and 15 ml depending on the size of the lesion and how disruptive it has been.

After the treatment a simple soft bandage with elastic bands is put and left on for a couple of days.

Follow-up

After the injection the horse was hospitalized for two days to control the clinical condition and exclude the possible occurrence of infections.

After dismissal a rehabilitation protocol was suggested to be completed at the stable. Often though the referring veterinarian was assigned to schedule the rehabilitation protocol for the horse, limiting the possibilities to have uniform treatment in the rehabilitation phase.

The standard rehabilitation protocol suggested in the study was the following:

1st week: 5 min walk once a day
2nd week: 5 min walk twice a day
3rd week: 10 min walk twice a day
4th week: 15 min walk twice a day
5th week: 20 min walk twice a day
6th – 8th week: 15 min walk and 5 min slow trot twice a day.

This protocol ensures that at least for the first two months the horse is not pushed too hard through training.

Each owner was requested to come back to perform a clinical and ultrasonographic control at the end of the two months (and also later on) but only a few horses were submitted to the clinic again for a complete follow-up.

CLINICAL CASES

Twenty-one horses referred to the Service of Acceptance and Hospitalization for Large Animals of the Veterinary Clinical Department of the University of Bologna were included in the study.

Each horse was referred by a practitioner veterinarian with suspect of distal limb soft tissue injury. At arrival the anamnesis was collected, including data as the time of insurgence, the clinical course, the clinical signs at the first visit and the eventual changes up to the time of referral. The anamnesis was unfortunately not always thorough and complete, as especially trot and race horses are managed by numerous people and sometimes it isn't possible to gather the information each one could give.

In any case the horses were afterwards submitted to clinical investigation and ultrasonography to diagnose the lesion and, if the characteristics of the lesion would fit with a PRP treatment, they would be included in the study.

The cases here reported are of horses affected by lesions of SDFT, DDFT, ICL, and SL both in fore and hind limbs. Lesions higher than the carpus/tarsus or lower than the fetlock were excluded because, as previously described, the characteristics of the tendons (and ligament) are too different from those of the metacarpal/tarsal region.

Case #1: Horse 020002928

Breed: Standardbred

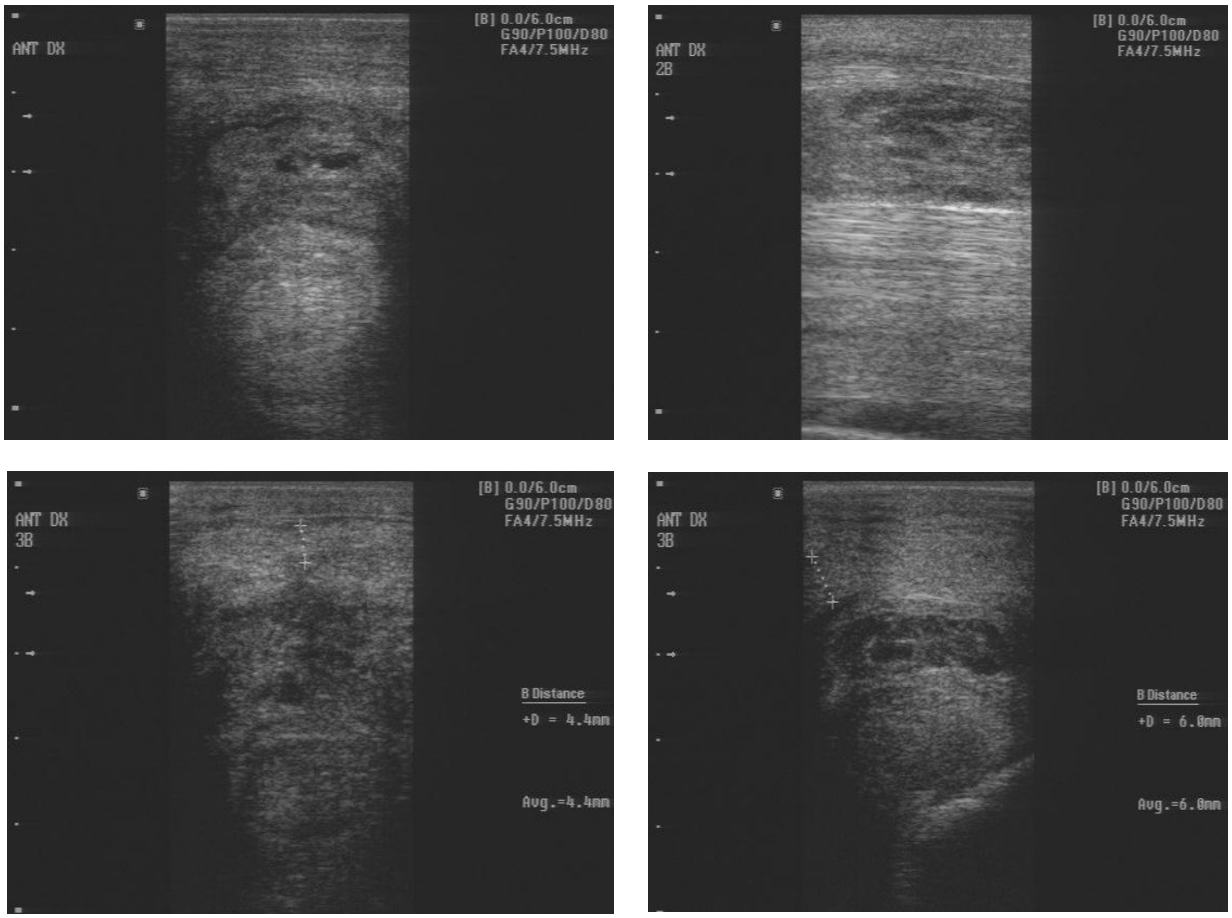
Sex: Gelding

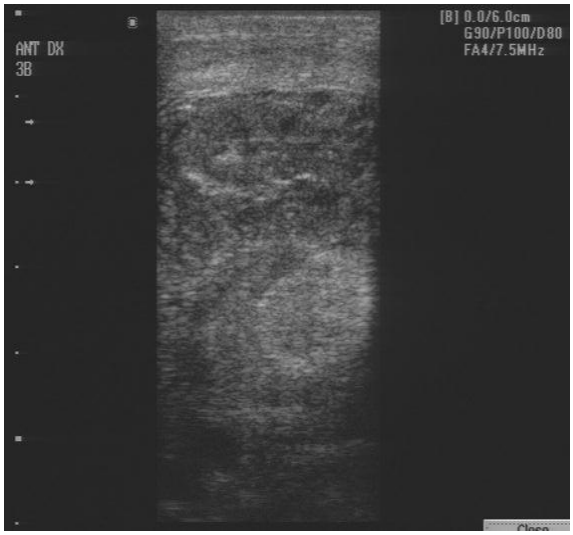
Age: 8 y. o.

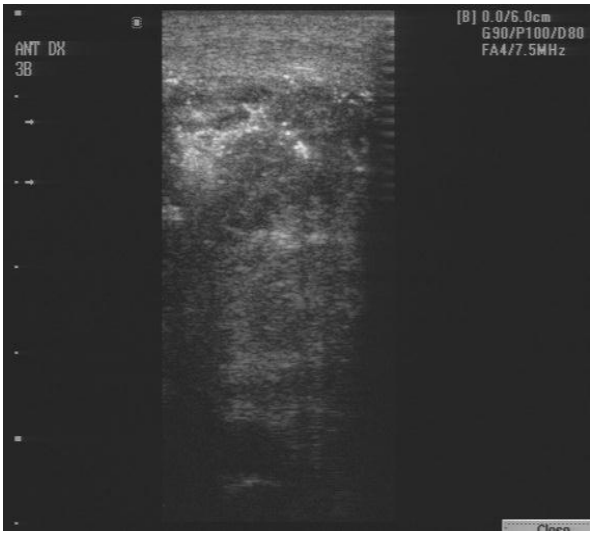
Activity: trot races

Anamnesis: From two weeks the horse is presenting a painful, hot swelling of the palmar aspect of the right forelimb. Third degree lameness. The horse has been suffering of tendonitis in the same limb.

Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree). The area is still swollen, warm and slightly painful.
The SDFT is generally hypo-echoic with altered echo-structure in the zones 2A and 3B. The longitudinal scan highlighted the presence of a large anechoic area with total absence of fibers in the zone 2B. The whole length of the tendon presented a serious misalignment of fibers.







Diagnosis: acute tendonitis (relapse) of the right forelimb SDFT.

Therapy: ultrasound guided intralesional injection of PRP.

PRP features:

Platelet count: 481000/ µl

WBCs count: 15600/ µl

Whole blood counts: PLT 147000/ µl and WBCs 8610/ µl

Platelets had a three times fold concentration and WBCs a two times fold.

Clinical course: the horse was submitted to a rehabilitation protocol for 4 months. Got back to race and after 3 start ups the pathology relapsed with the same characteristics.

Case #2: Horse 020002952

Breed: Standardbred

Sex: Male

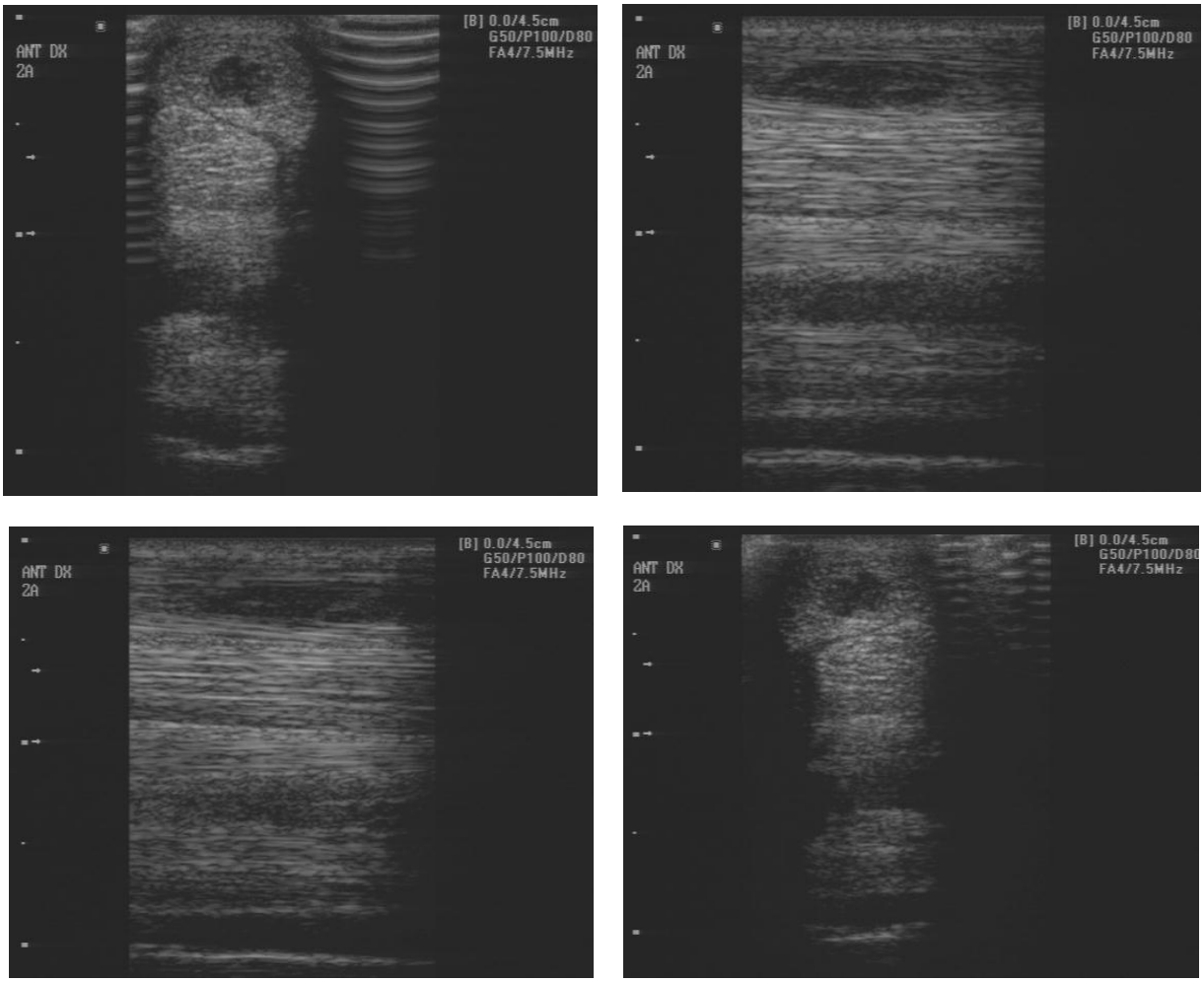
Age: 6 y. o.

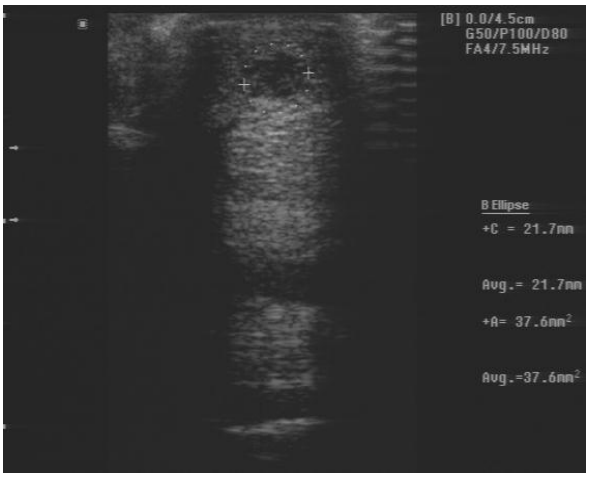
Activity: trot races

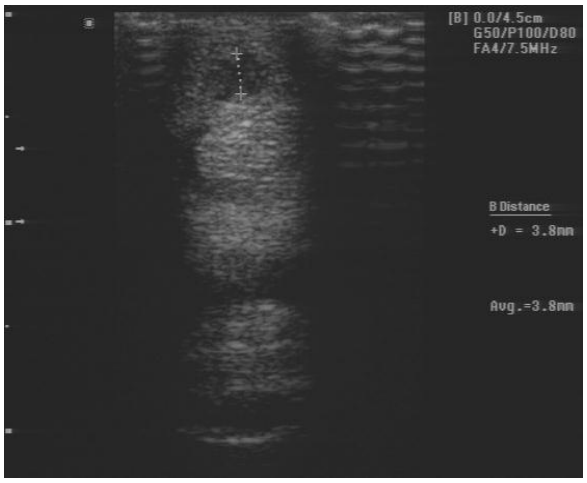
Anamnesis: From some days the horse is presenting a moderate swelling of the palmar aspect of the right front metacarpus. Slight lameness.

Examination and ultrasound: the lameness is present at the time of clinical examination (1/5 degree). The area is still slightly swollen.

The SDFT is presents a core lesion in the zone 2A. In longitudinal scan the lesion has a CSA of 37.6mm^2 (75% of the whole tendon; score 2/3), while it is 2.3cm long in longitudinal scan. The tendon has a slightly hypo-echoic aspect in the area surrounding the lesion.







Diagnosis: acute tendonitis of the right forelimb SDFT.

Therapy: ultrasound guided intralesional injection of PRP.

PRP features:

Platelet count: 706000/µl

WBCs count: 18700/µl

Whole blood counts: PLT 70700/ µl and WBCs 4810/ µl

Platelets had almost a ten times fold concentration and WBCs almost four times fold.

Clinical course: the horse was submitted to a rehabilitation protocol for almost 6 months. Got back to competition at the same previous level. After some months it was sold and the final outcome is unknown.

Case #3: Horse 020002969

Breed: Holstein

Sex: Gelding

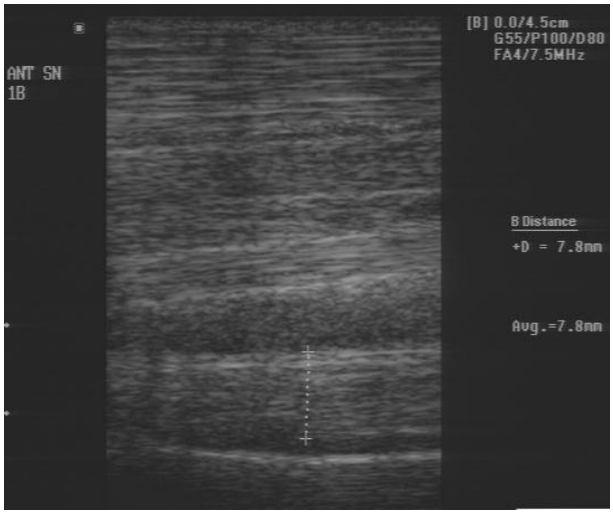
Age: 5 y. o.

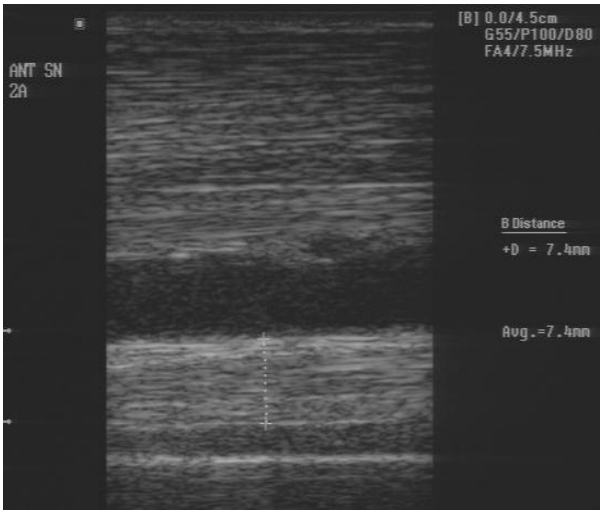
Activity: jumping

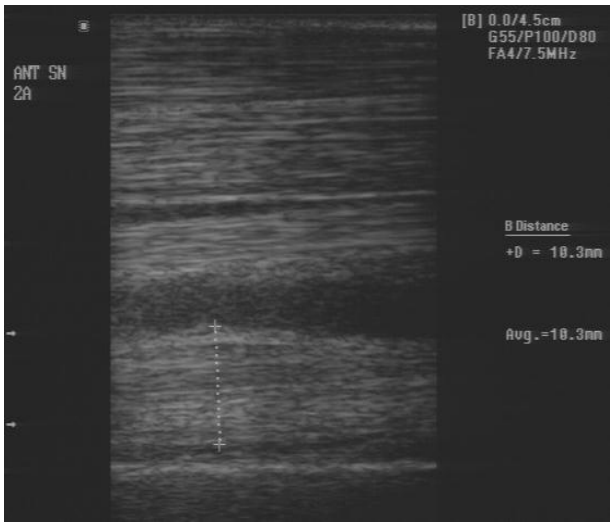
Anamnesis: the horse is referred for chronic lameness of the left forelimb, intermittent for gravity. The suspect is of a chronic left forelimb SL desmitis.

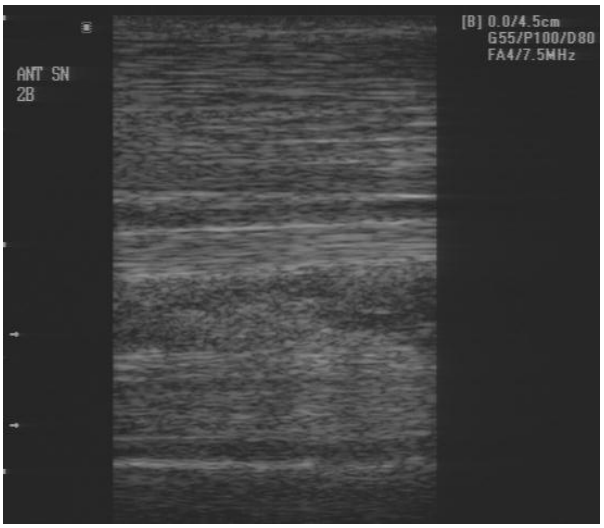
Examination and ultrasound: the lameness is present at the time of clinical examination (1/5 degree).

In longitudinal scanning the SL presents dishomogenous echogenicity and increased thickness, starting from zone 1B.









Diagnosis: chronic desmitis of the left forelimb SL.

Therapy: ultrasound guided intra-lesional injection of fresh PRP.

PRP features:

Platelet count: 735000/µl

WBCs count: 4570/µl

Whole blood counts: PLT 109000/µl and WBCs 7400/µl

Platelets had almost a seven times fold concentration and WBCs almost halved.

Clinical course: the horse was kept in stable for one week and started a 4 months rehabilitation protocol. Got back to competition at the same previous level. After two months the horse injured seriously during a competition but the lesion did not involve at all the previous pathology.

Case #4: Horse 0200003066

Breed: Standardbred

Sex: Male

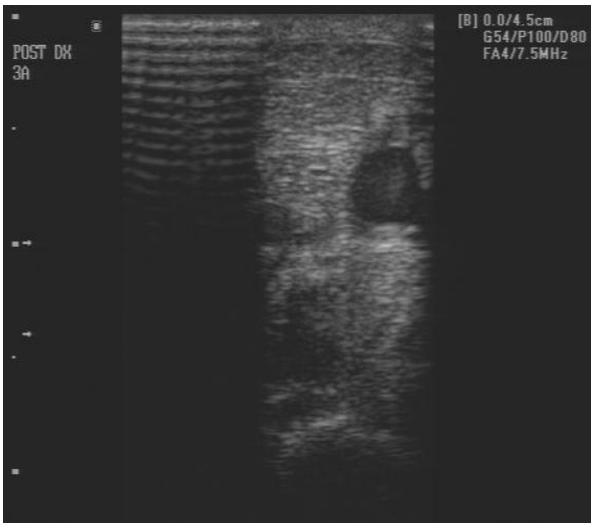
Age: 3 y. o.

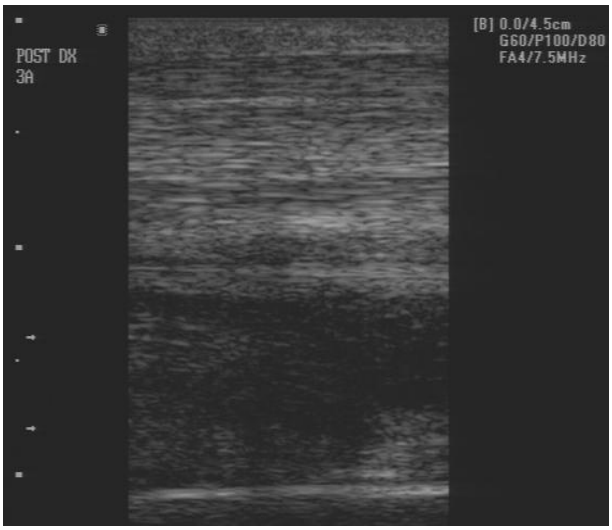
Activity: trot races

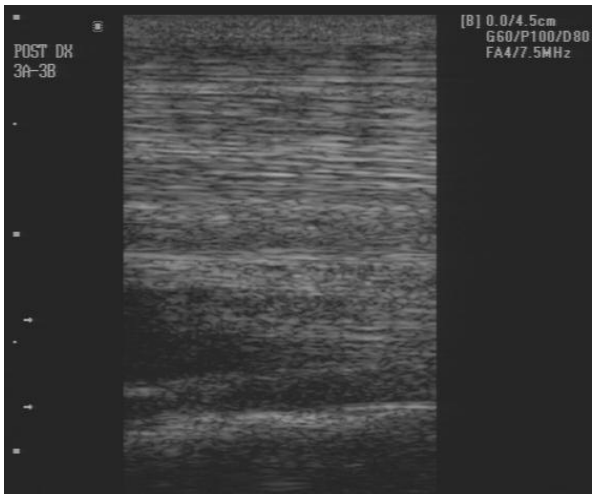
Anamnesis: the horse is referred for lameness of the right hind limb from a couple of weeks. The suspect is of acute right hind limb SL desmitis.

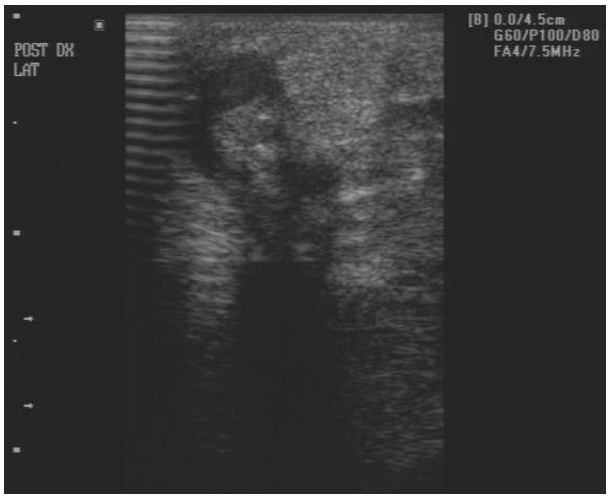
Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

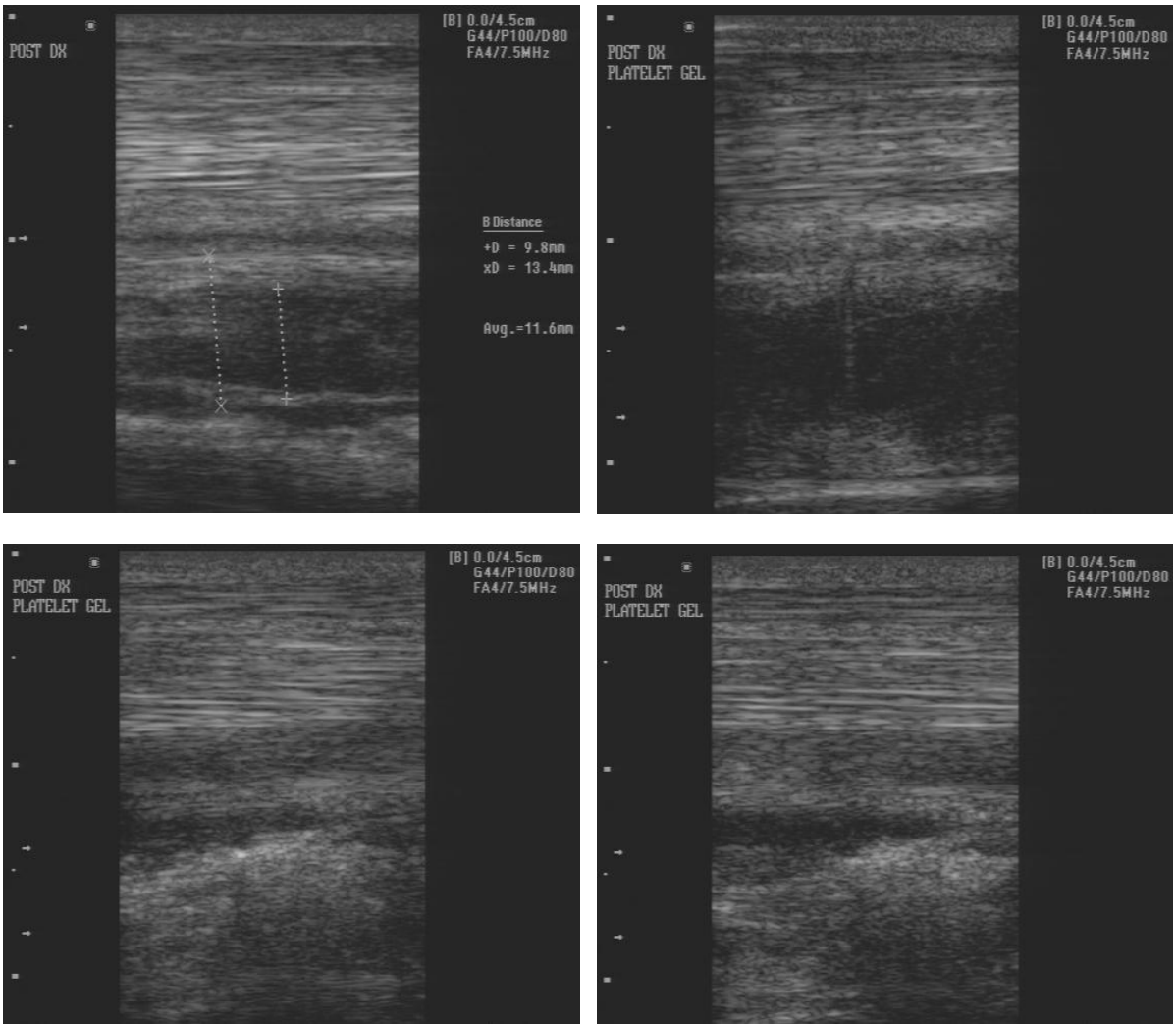
In longitudinal scanning the SL presents a hypo-anechoic area starting from zone 2B until zone 3B. In transverse scanning the lesion occupies the 60% of the whole thickness of the ligament.











Diagnosis: severe desmitis of the right hind limb SL and lateral branch.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 1471000/µl

WBCs count: 25800/µl

Whole blood counts: PLT 147000/µl and WBCs 8300/µl

Platelets had ten times fold concentration and WBCs concentrated of 3 times.

Clinical course: the horse was kept in stable for two weeks and then started a rehabilitation protocol. After 4 months the pathology relapsed in the same area.

Case #5: Horse 0100034644

Breed: Standardbred

Sex: male

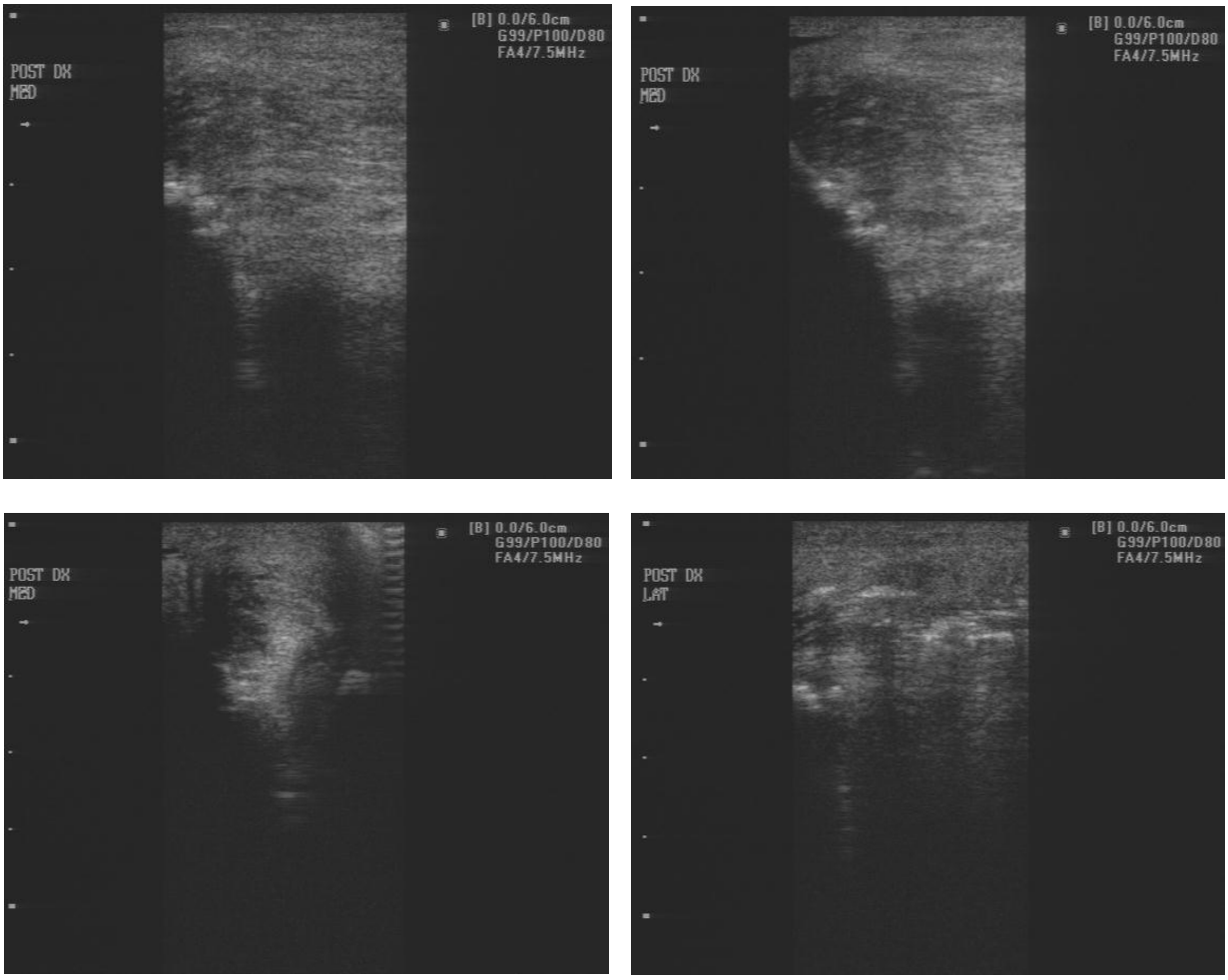
Age: 10 y. o.

Activity: trot races

Anamnesis: the horse is referred for a three weeks old lameness of the right hind limb. A hot swelling is appreciated at palpation at the palmar aspect of the metatarsus.

Examination and ultrasound: the lameness and the swelling are present at the time of clinical examination (lameness 2/5 degree).

A hypo-echoic area is visible at the insertion of the medial branch of the right hind limb SL. In transverse section the score is 2/3. Misalignment of fibers is evaluated in longitudinal scanning and scored 2/3.



Diagnosis: right hind limb SL medial branch desmitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 1105000/µl

WBCs count: 18500/µl

Whole blood counts: PLT 154000/µl and WBCs 8730/µl

Platelets had a seven times fold concentration and WBCs were little more than doubled.

Clinical course: the horse followed a rehabilitation protocol for 2 months. He competed for almost 6 months and was then retired due to the lack of results and a relapse of the pathology. Never got back to competition again.

Case #6: Horse 0100034657

Breed: Standardbred

Sex: male

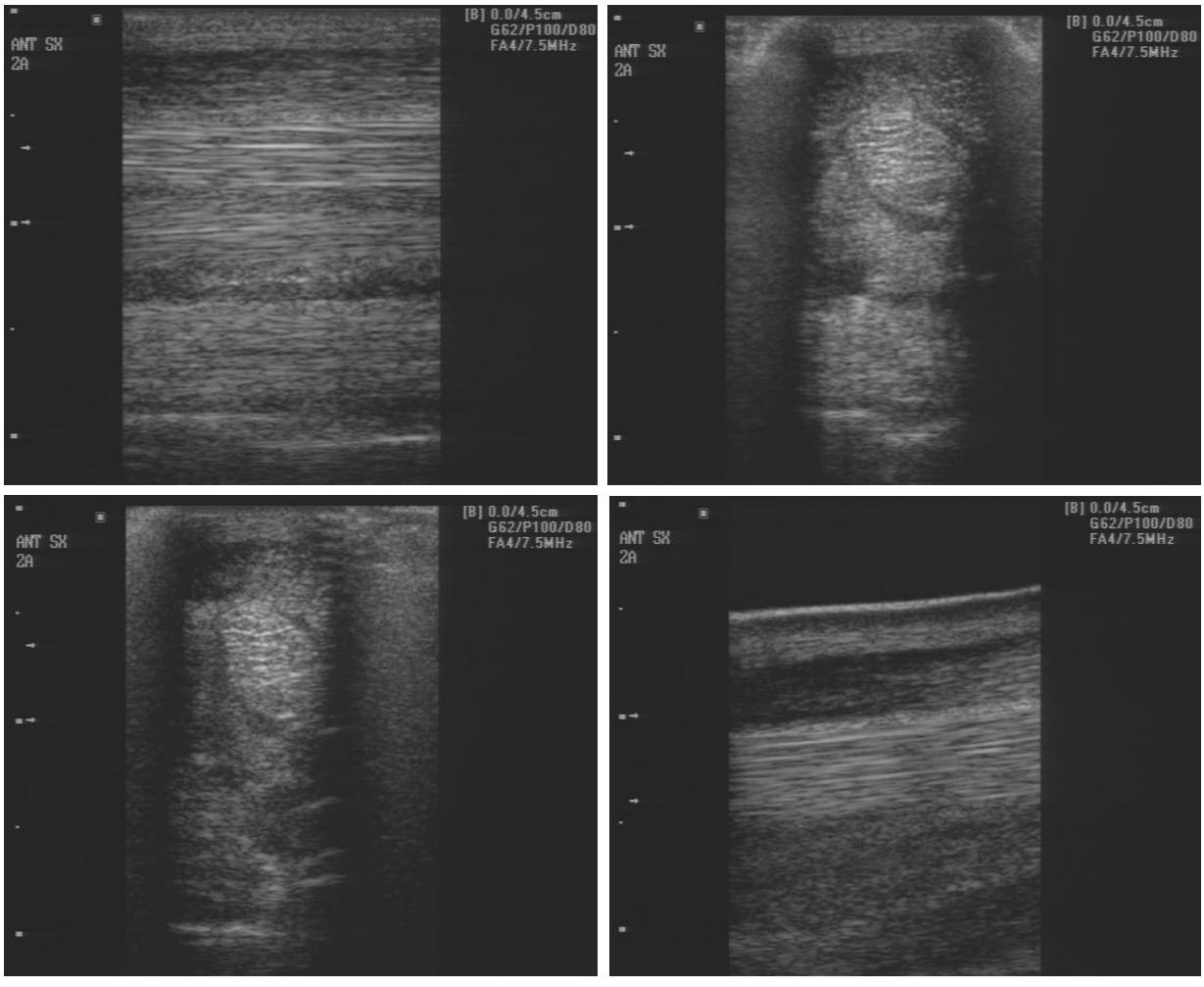
Age: 2 y. o.

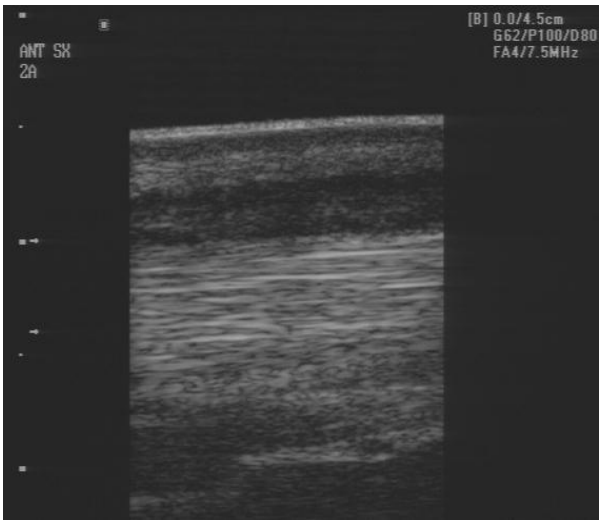
Activity: trot races

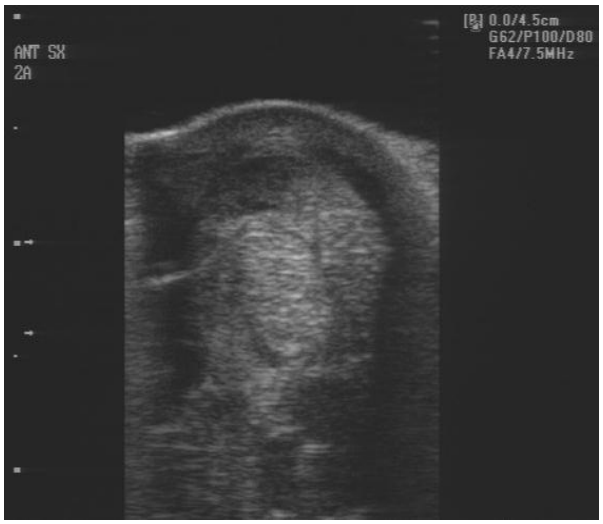
Anamnesis: the horse is referred for acute lameness of the left forelimb. A hot, painful swelling is visible on the palmar aspect of the correspondent metacarpus.

Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

An anechoic area of the SDFT is visible in zone 2A. The transverse section shows it occupies almost the 50% of the total thickness of the tendon. In longitudinal scanning a lack or severe misalignment of fibers (2/3) is visible in the same area with swelling of the surrounding tissues.







Diagnosis: left forelimb acute SDFT tendonitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

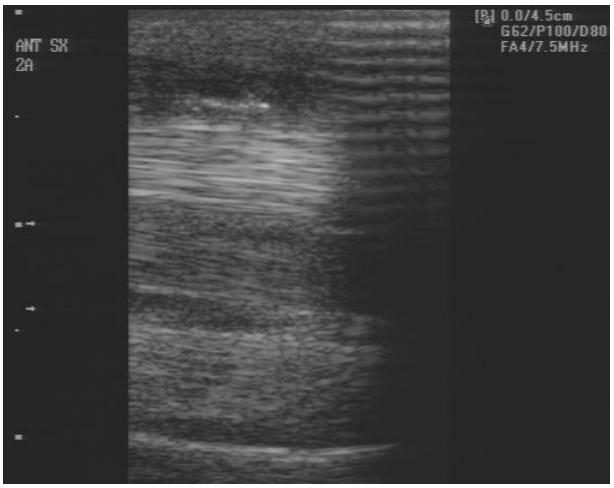
Platelet count: 1597000/µl

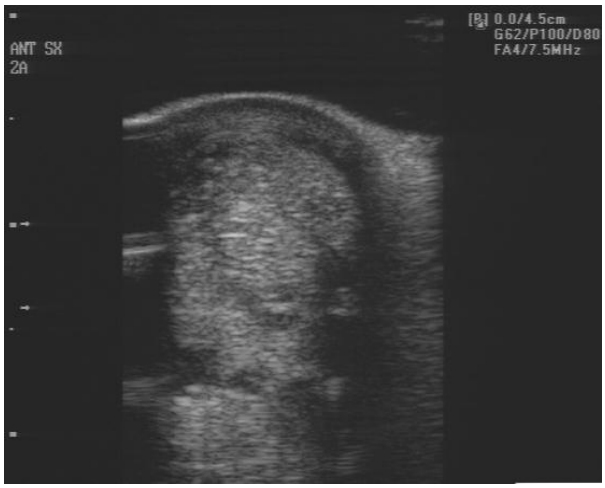
WBCs count: 25700/µl

Whole blood counts: PLT 154000/µl and WBCs 8600/µl

Platelets had a ten times fold concentration and WBCs increased of three times.

Clinical course: the horse was subjected to a personalized rehabilitation protocol: 2 months of stall rest and 2 months of training with increasing intensity. He was submitted to ultrasound controls that showed no alteration of the area. The horse got back to competition at a higher level than before without needing any other pharmacological treatment.





Case #7: Horse 0100034670

Breed: Standardbred

Sex: male

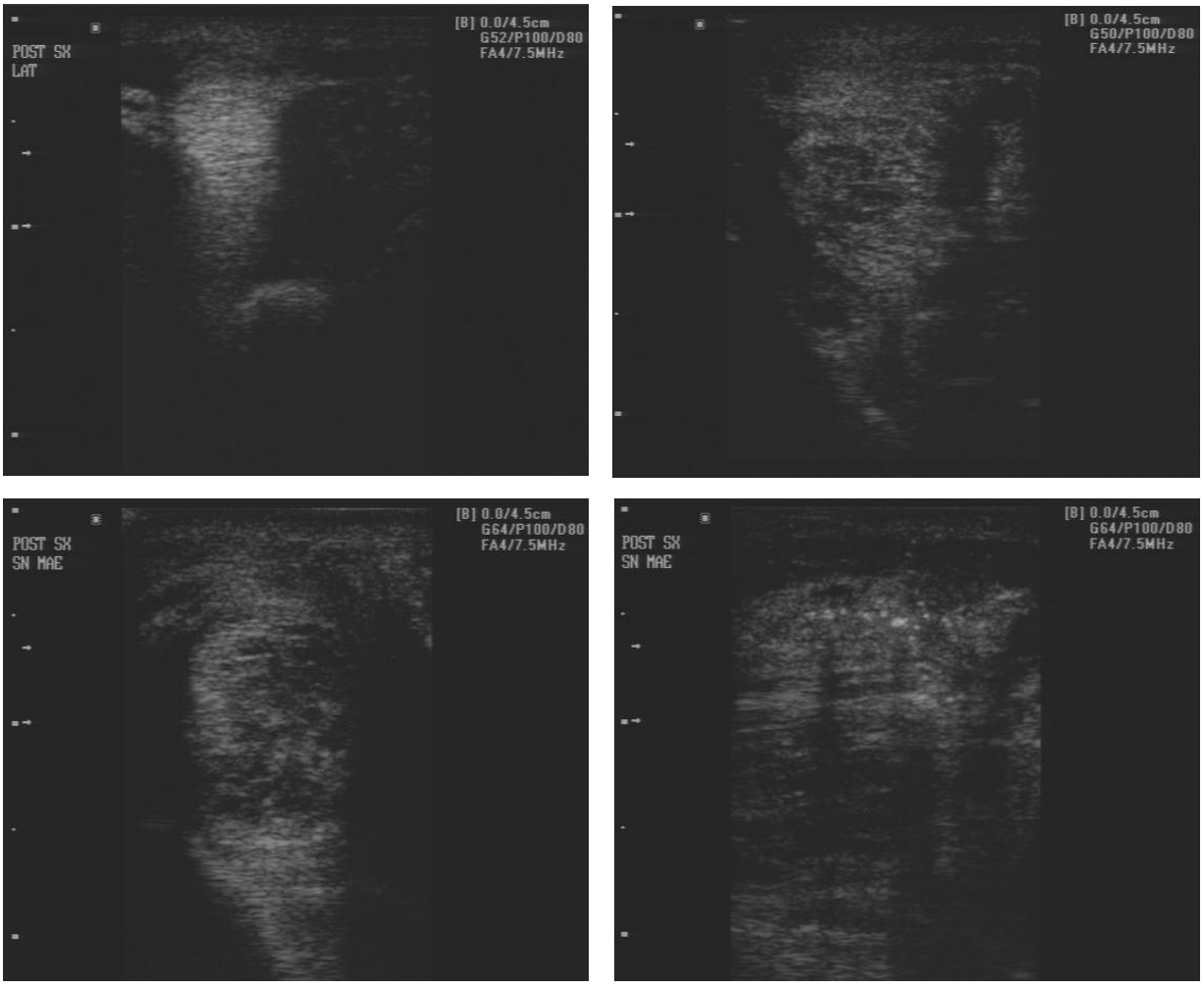
Age: 7 y. o.

Activity: trot races

Anamnesis: the horse is referred for a two weeks old lameness of the left hind limb.

Examination and ultrasound: the lameness is present at the time of clinical examination (lameness 2/5 degree).

An anechoic area (almost 50% of the thickness of the ligament in transverse section) is visible at the insertion of the medial branch of the left hind limb SL. The whole branch is swollen and edematous.



Diagnosis: left hind limb SL medial branch desmitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 970000/µl

WBCs count: 20800/µl

Whole blood counts: PLT 125000/µl and WBCs 6500/µl

Platelets had an eight times fold concentration and WBCs were thrice as many.

Clinical course: the horse followed a rehabilitation protocol with one month of stall rest and then let to paddock, with 15 days of simple walks and then reintroduction to increasing training. The returned to his previous level of training in 4 months. He competed for some months and was then retired due to the lack of results and a relapse of the pathology. Never got back to competition again.

Case #8: Horse 0100034904

Breed: Italian saddle

Sex: gelding

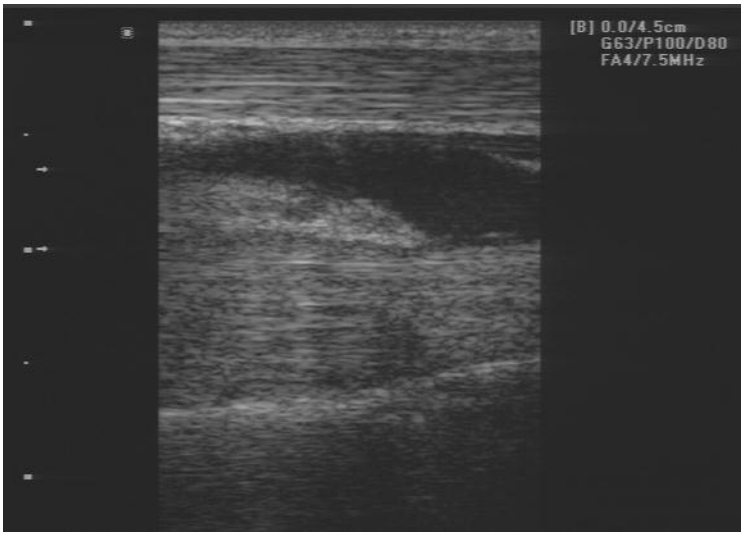
Age: 5 y. o.

Activity: jumping

Anamnesis: the horse is referred for right hind limb lameness from a couple of weeks. A SL desmitis was suspected.

Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

The SL of the right hind limb has uneven echostructure with misalignment scored 2/3 in zones 2A-3B.



Diagnosis: right hind limb SL desmitis.

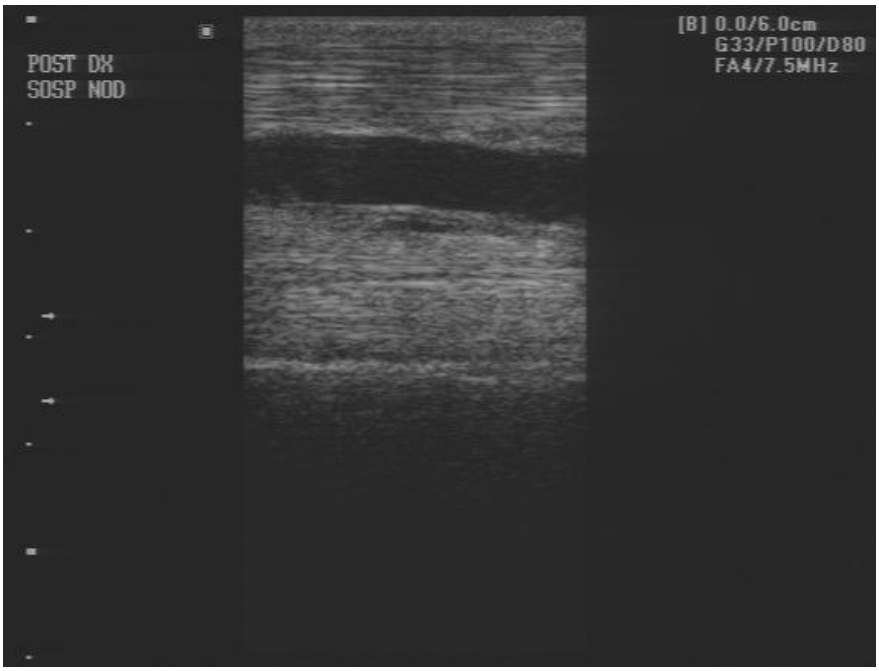
Therapy: ultrasound guided intralesional injection of PRP.

PRP features:

The counts were unavailable for a failure of the blood counter.

Clinical course: the horse followed a personalized rehabilitation schedule. Two months after the injection the ultrasound control showed an improvement of the

echographic appearance of the ligament. The following control (4 months after treatment) showed a SL almost normal. After one year the horse presented a lameness in the same hind limb and was submitted to fasciotomy. The ultrasound controls after that relieved a complete restore of the tissue despite the horse has now a vice of dragging the tip at gait.



One year post-op control.

Case #9: Horse 0100034919

Breed: English Warmblood

Sex: gelding

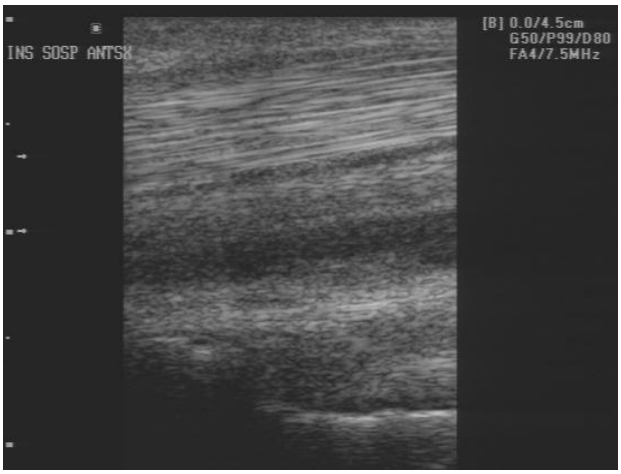
Age: 11 y. o.

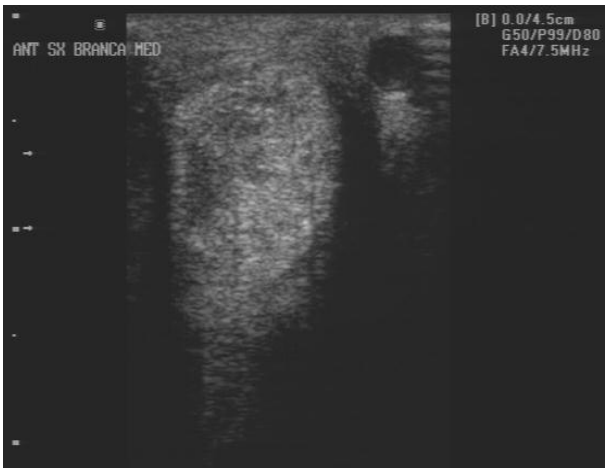
Activity: jumping

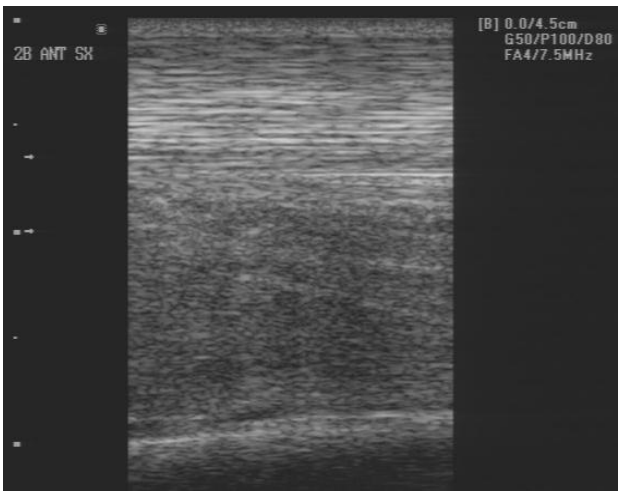
Anamnesis: the horse is referred for a left forelimb hot, painful swelling in the proximal palmar aspect of the metacarpus from a couple of weeks. Lameness is present and scored 3/5.

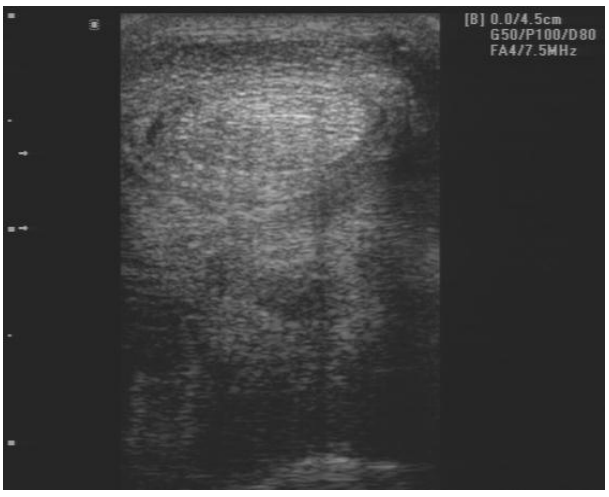
Examination and ultrasound: the lameness is present at the time of clinical examination (3/5 degree).

At ultrasound examination the SL is increased in thickness starting from the zone 2B. The medial branch is quite swollen too. Both structures show a severe architectural inhomogeneity.









Diagnosis: left forelimb SL and medial branch desmitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 248000/µl

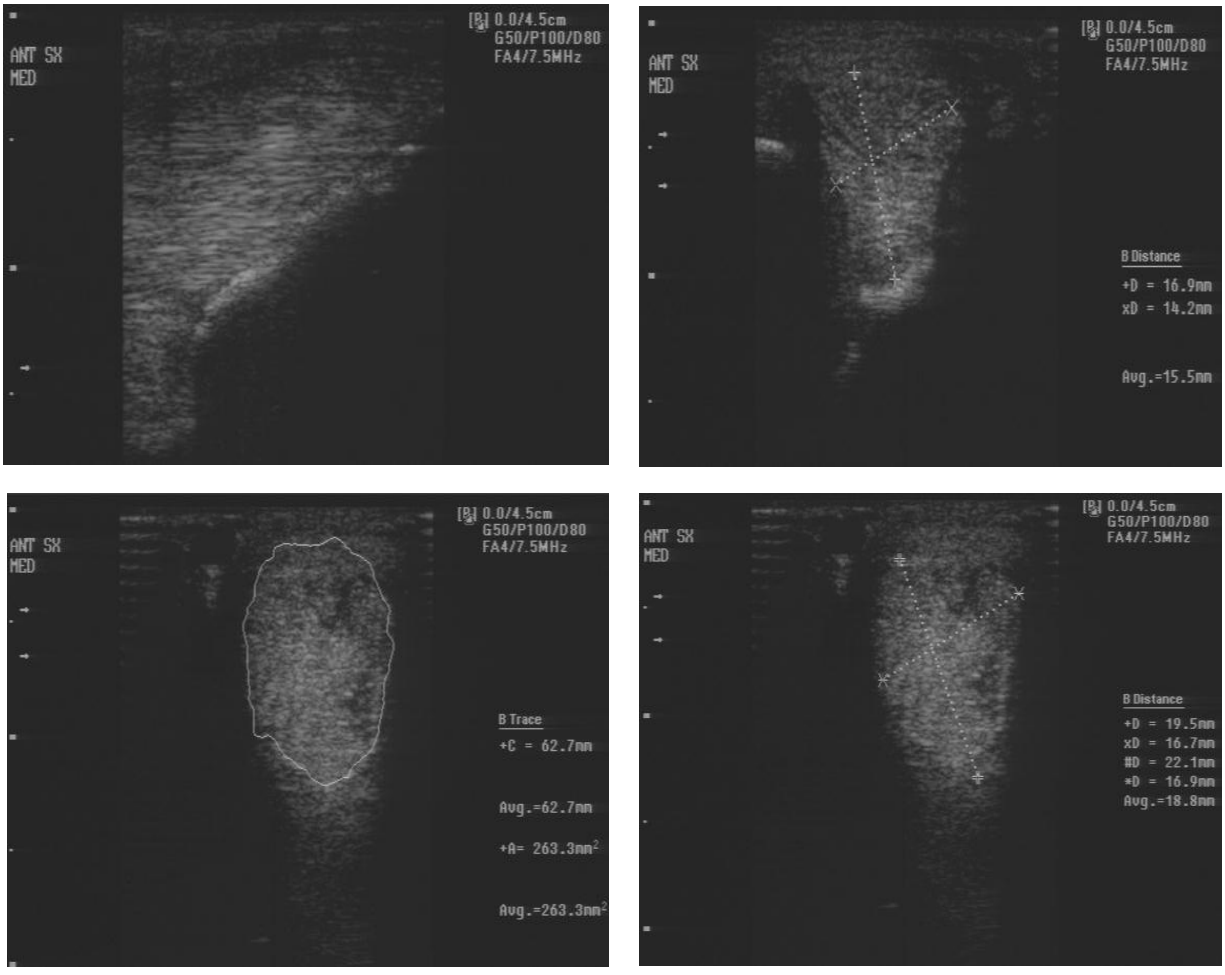
WBCs count: 2080/µl

Whole blood counts: PLT 122000/µl and WBCs 6340/µl

Platelets were only doubled and WBCs halved.

Clinical course: the horse was allowed two weeks of stable rest and then started the rehabilitation protocol. Two months after the injection the body of the SL didn't show any alteration. The medial branch was still increased in volume and presenting some hypo-anechoic areas in transvers scanning.

A second injection of PRP in the medial branch was performed. PRP was remade to obtain a better product than the first one.



PRP features:

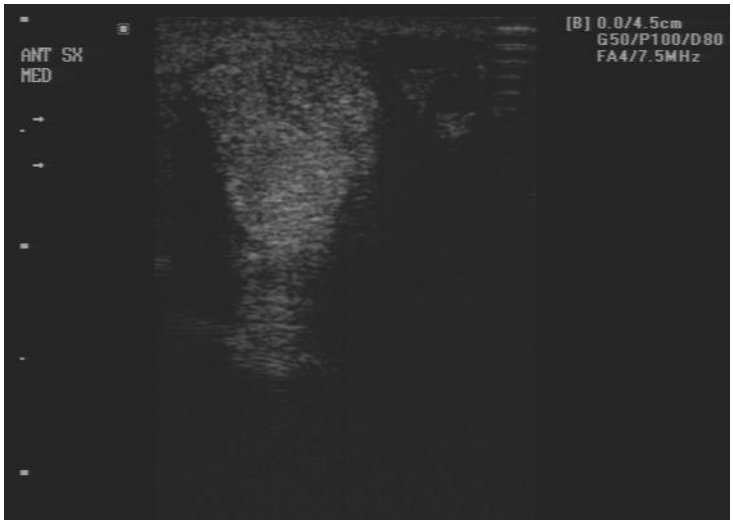
Platelet count: 1354000/µl

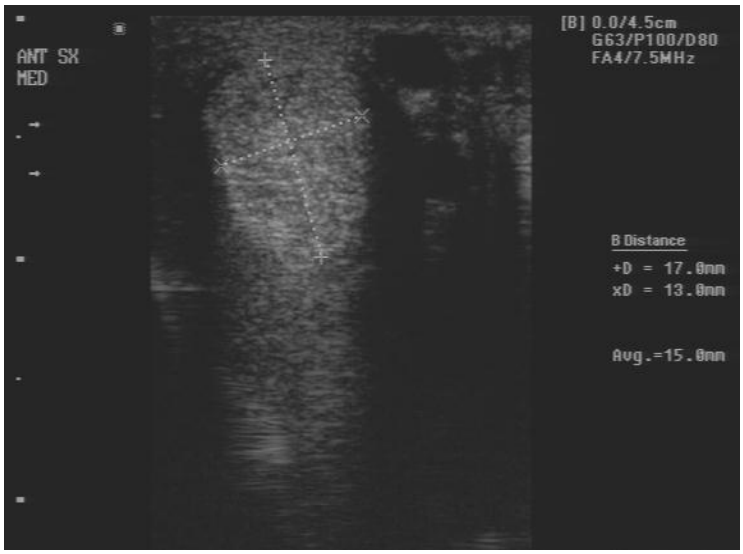
WBCs count: 14200/µl

Whole blood counts: PLT 122000/µl and WBCs 9400/µl

Platelets were concentrated 11 times and WBCs didn't even double.

Clinical course: five months after the second injection the horse was examined and the synovial pouches felt uniformly increased in volume at palpation. The swelling was not either hot nor painful. The ultrasound examination showed a good alignment of the fibers of the SL. The medial branch appeared slightly increased in volume with a mild alteration of the echo-structure in transverse section (score 1/3) while the alignment in longitudinal scan is normal.





Case #10: Horse 0200003582

Breed: English Warmblood

Sex: gelding

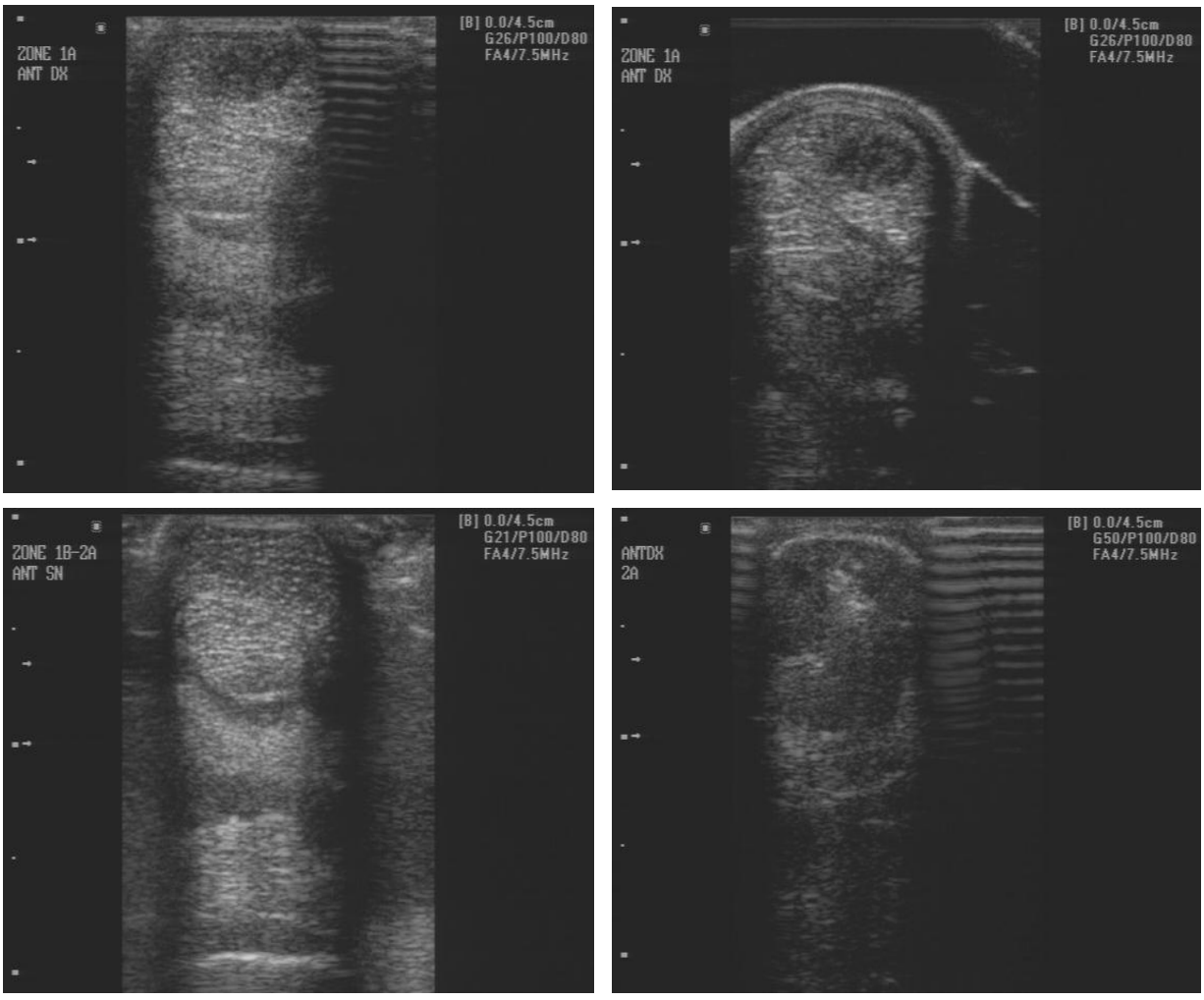
Age: 5 y. o.

Activity: gallop races

Anamnesis: the horse is referred for a right forelimb lameness from a few weeks. A hot, painful swelling is visible on the palmar aspect of the correspondent metacarpus.

Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

The ultrasound examination showed both in longitudinal and in transverse section the presence of various hypo-anechoic areas extending from 1A to 2B and occupying from the 30% to the 60% of the thickness of the SDFT.



Diagnosis: right forelimb severe SDFT tendonitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 1043000/µl

WBCs count: 34000/µl

Whole blood counts: PLT 55700/µl and WBCs 3200/µl

Platelets had almost a twenty times fold concentration and WBCs increased of ten times.

Clinical course: the horse was subjected to a personalized rehabilitation protocol. He got back to racing after about six months and was then retired for recurrent lameness. The lesions he reported may be considered in the pool of pathologies that actually are relapses of the original one.

Case #11: Horse 0100035160

Breed: Oldenburg

Sex: female

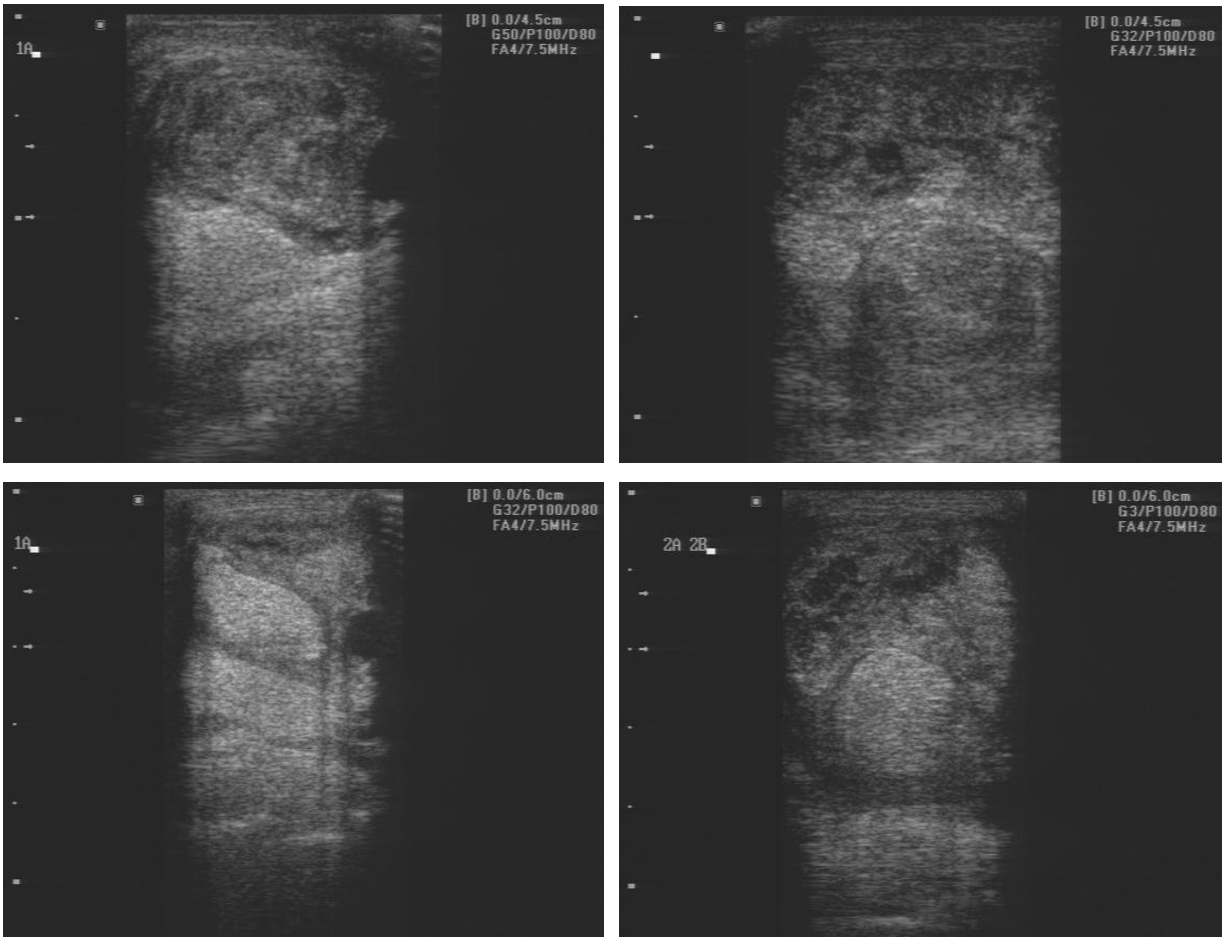
Age: 23 y. o.

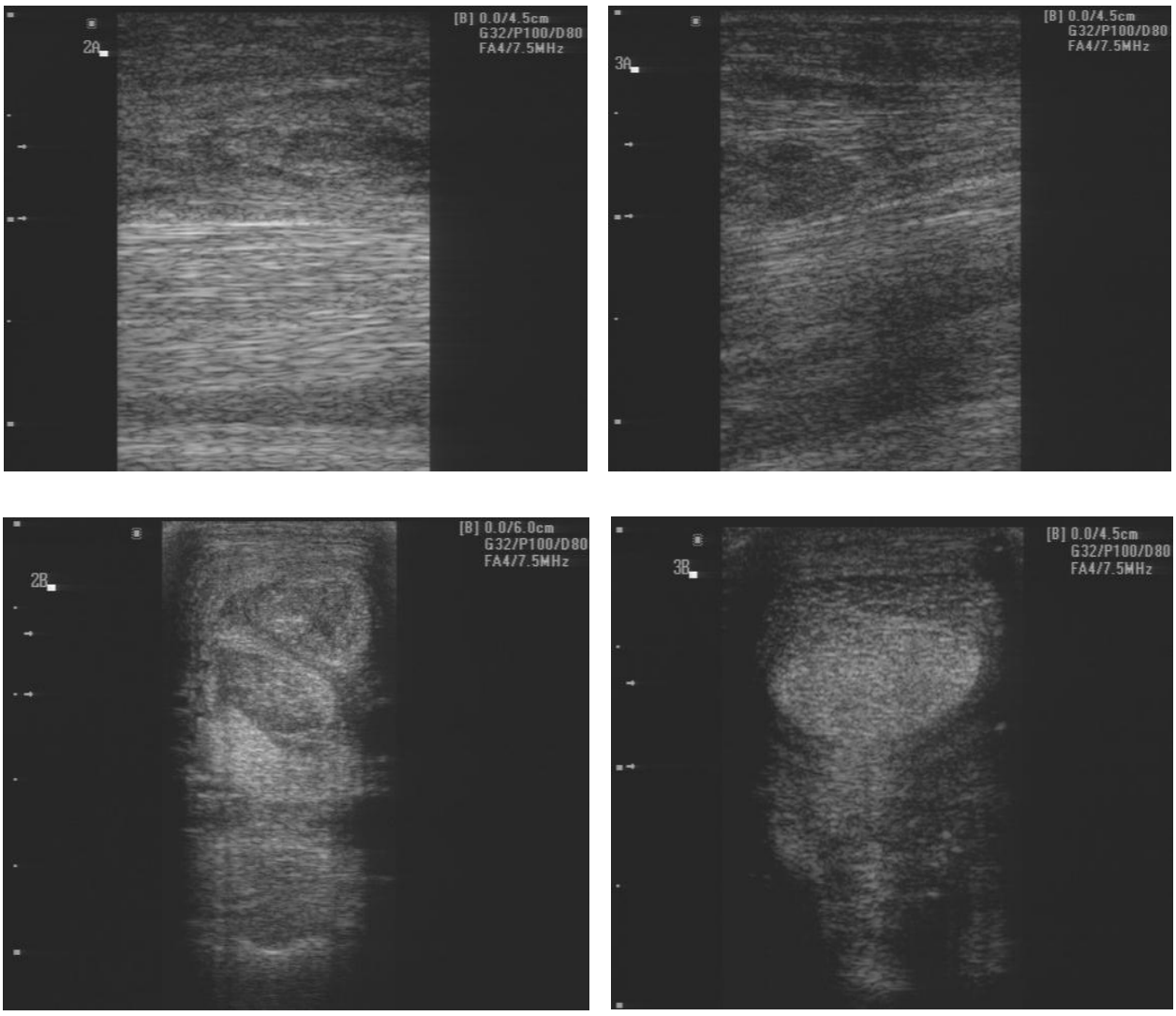
Activity: country riding

Anamnesis: the horse is referred for recurrent lameness of the left forelimb. A hot, consistent, slightly painful swelling is visible on the palmar aspect of the correspondent metacarpus.

Examination and ultrasound: the lameness is present at the time of clinical examination (4/5 degree).

Multiple, severe lesions of the SDFT are visible from the zone 1Bthrough zone 3A. The series of large anechoic area are visible both in transverse and longitudinal scanning. A severe misalignment of fibers and completely uneven pattern is presented through the whole length of the lesion.





Diagnosis: left forelimb acute SDFT tendonitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 312000/µl

WBCs count: 1240/µl

Whole blood counts: PLTs 68900/µl and WBCs 7200/µl

Platelets had almost a five times increase while WBCs increased were six times less.

Clinical course: the horse was subjected to a rehabilitation protocol after a period of stable rest. After six months the horse was able to get back to mild activity but was never reused as before also due to her age.

Case #12: Horse 0200003343

Breed: Standardbred

Sex: female

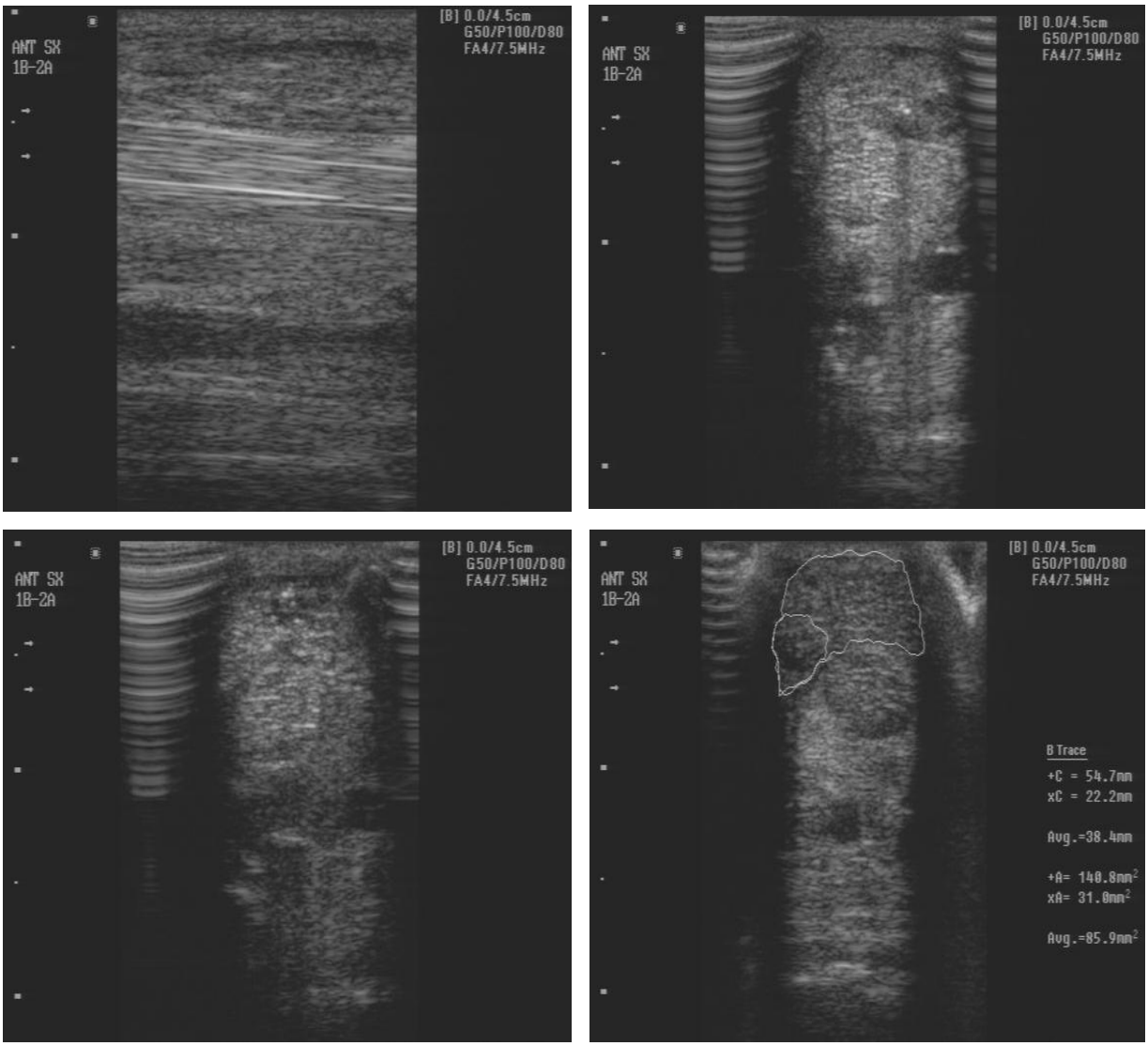
Age: 3 y. o.

Activity: trot races

Anamnesis: the horse is referred for acute lameness of the left forelimb. A mild warm swelling is visible on the palmar aspect of the correspondent metacarpus.

Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

In longitudinal scanning the portion 1B-2A of the SDFT is presenting a hypo-echoic area about 32mm^2 wide. Alignment of fibers is scored 2/3.



Diagnosis: left forelimb acute SDFT tendonitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Whole blood counts: PLTs 119000/µl and WBCs 4700/µl

Due to a tilt of the blood counter the PRP count could not be performed.

Clinical course: the horse was subjected to rehabilitation protocol for almost six months with a workload of increasing intensity. The protocol was quite close to the one suggested. The horse got back to competition at the same level than before without needing any other pharmacological treatment.

Case #13: Horse 0100035131

Breed: Dutch Saddle

Sex: gelding

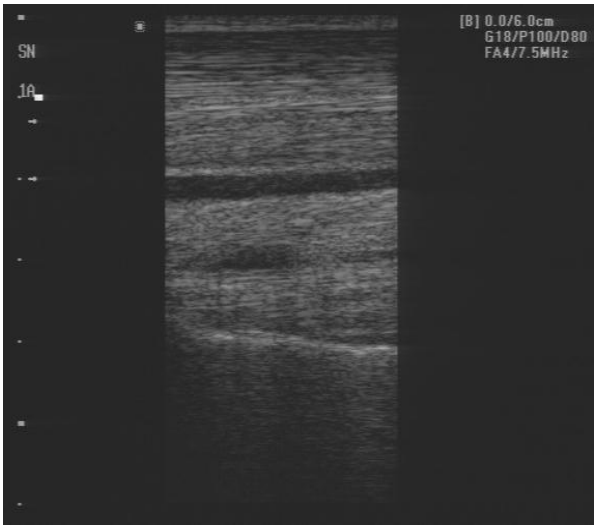
Age: 10 y. o.

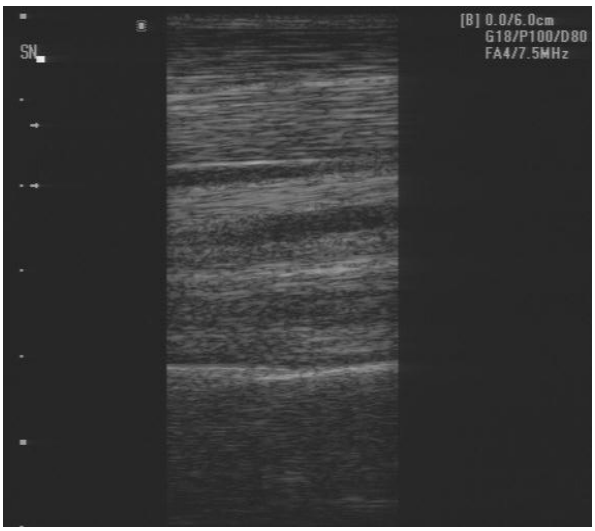
Activity: jumper

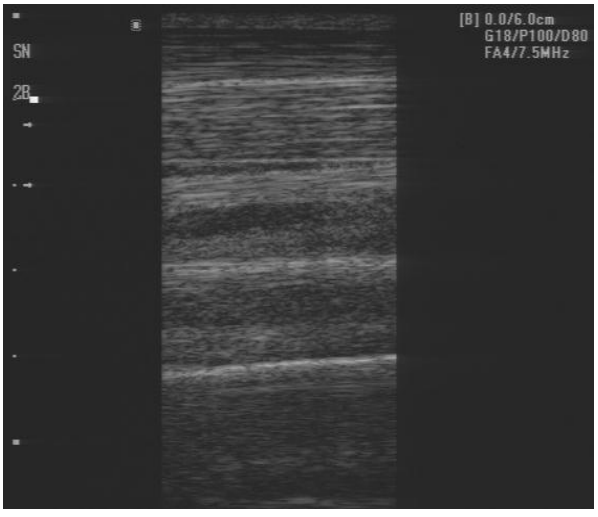
Anamnesis: the horse is referred for chronic lameness of the left forelimb. From one year the horse is suffering of SL recurrent desmitis.

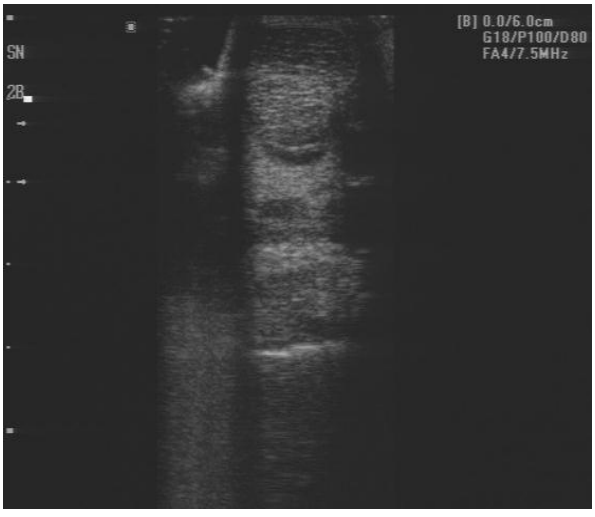
Examination and ultrasound: the lameness is present at the time of clinical examination (1/5 degree).

A hypo-echoic area of the SL is visible at its proximal insertion and in zone 2B. The transverse and longitudinal sections show a consistent edema and misalignment of fibers (1/3) is visible in the same and the surrounding areas.









Diagnosis: left forelimb recurrent SL desmitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

Fasciotomy and neurectomy of the deep branch of the left ulnar nerve.

PRP features:

Platelet count: 735000/µl

WBCs count: 4570/µl

Whole blood counts: PLTs 129000/µl and WBCs 7400/µl

Platelets had almost a six times fold and WBCs almost halved.

Clinical course: the horse was subjected to a personalized rehabilitation protocol: 1 months of stall rest and a 5 minutes walk per day. He then followed a regular training with increasing intensity. He was submitted to ultrasound controls that showed improvement of the appearance of the area. The horse got back to competition at a higher level than before with sporadic need for rest from training.

Case #14: Horse 0200003378

Breed: Italian Saddle

Sex: gelding

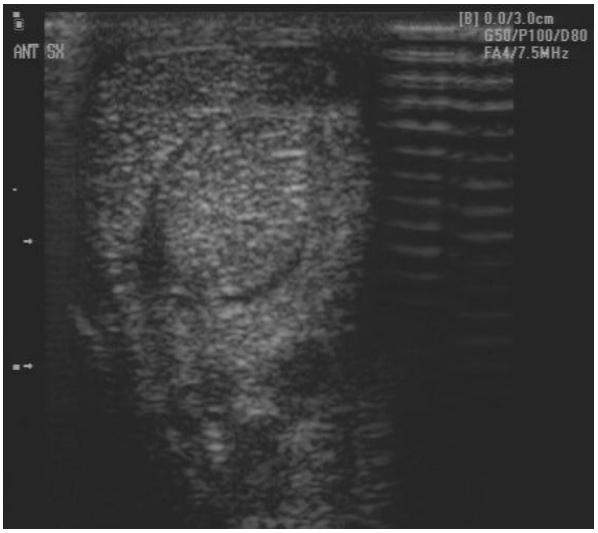
Age: 10 y. o.

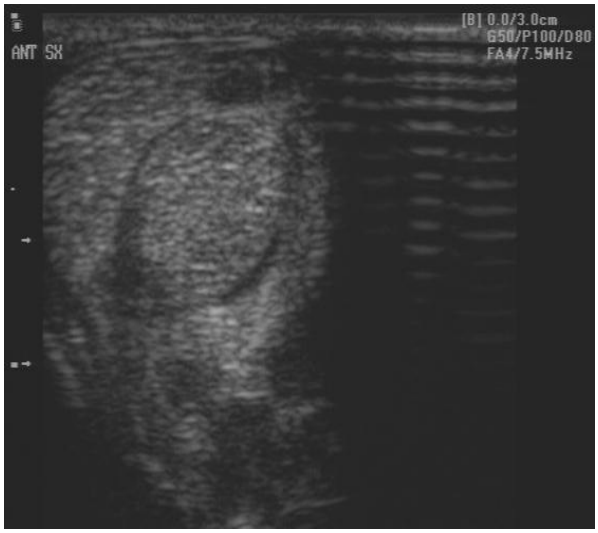
Activity: jumper

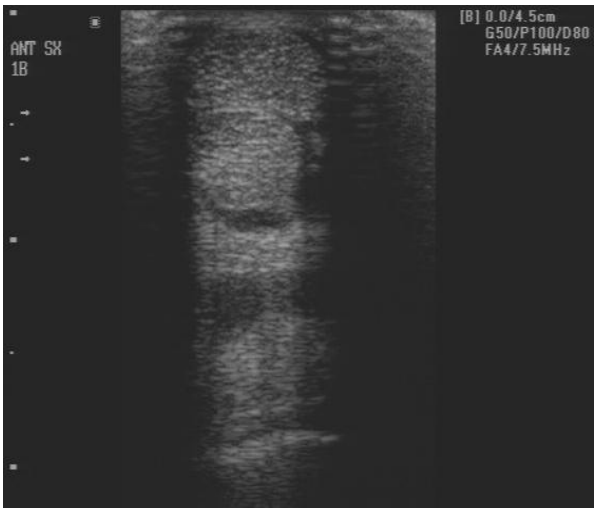
Anamnesis: the horse is referred for acute lameness (1/5) of the left forelimb.

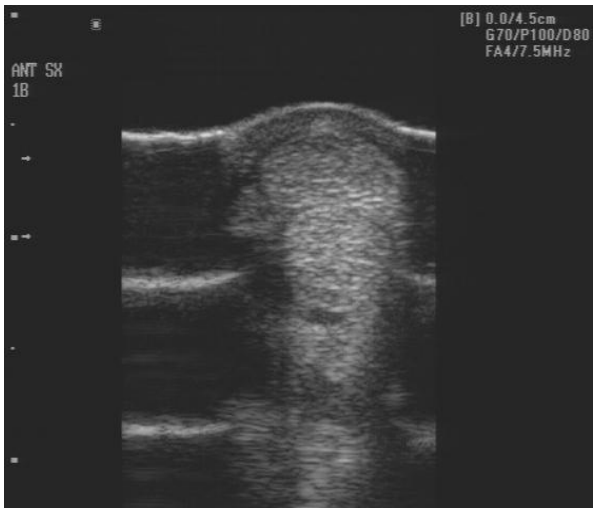
Examination and ultrasound: the lameness is present at the time of clinical examination (1/5 degree).

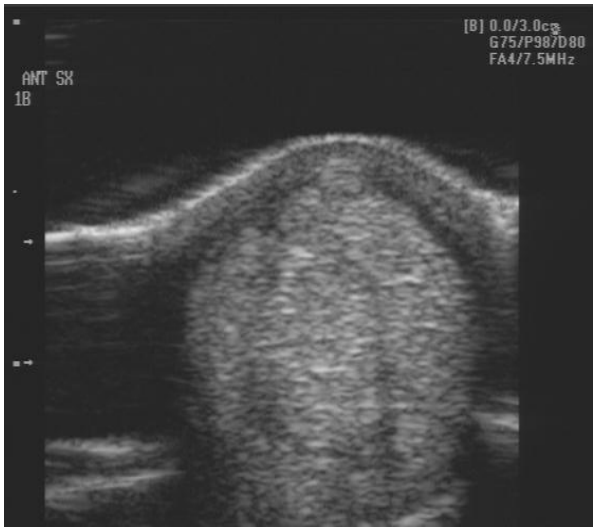
The transverse scan of the SDFT shows a hypo-echoic area at the lateral edge of the tendon 3mm thick, in the region 1B-2A. The alignment of fibers is scored 1/3.

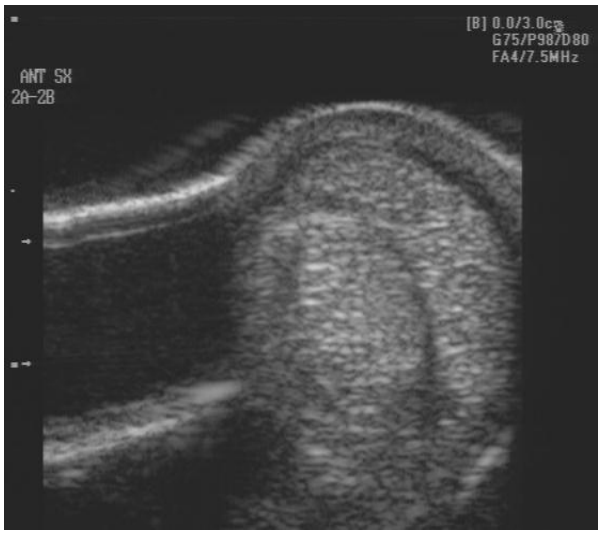












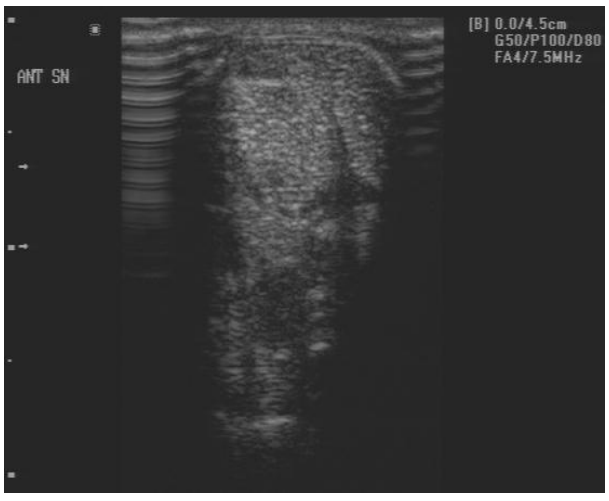
Diagnosis: left forelimb acute SDFT tendonitis.

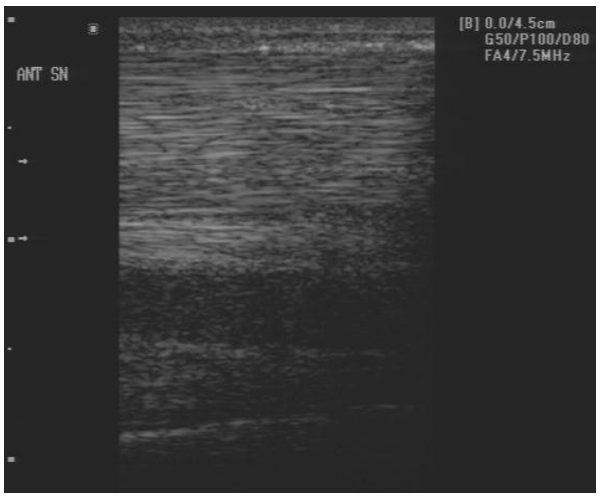
Therapy: the horse was brought back for stable rest. Less than three weeks later he was submitted again to ultrasound control and the lesion had become 7 mm wide and the alignment scored 2/3. An ultrasound guided intra-lesional injection of PRP was then decided and the pathology recorded as chronic SDFT tendonitis.

PRP features:

No platelet count was available due to a break of the blood counter.

Clinical course: the horse was subjected to a rehabilitation protocol and followed by the referring veterinarian. After about eight months he got back to competition at a lower level.





Case #15: Horse 0100034835

Breed: Italian Saddle

Sex: gelding

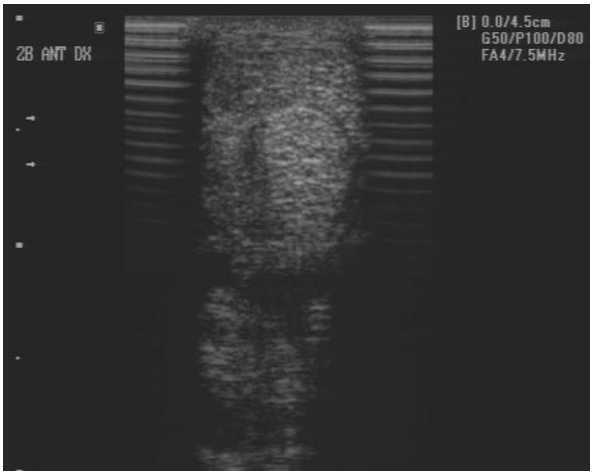
Age: 4 y. o.

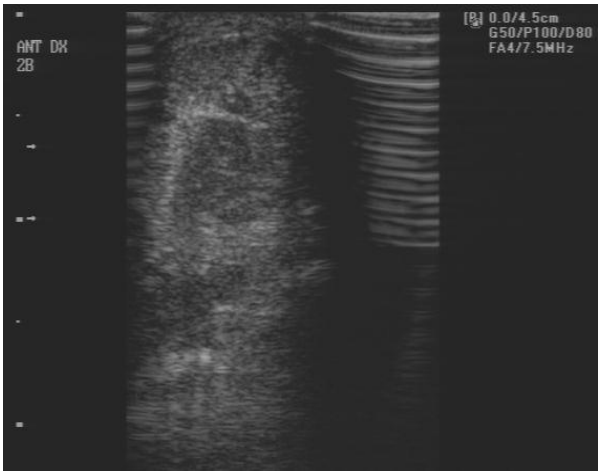
Activity: jumper

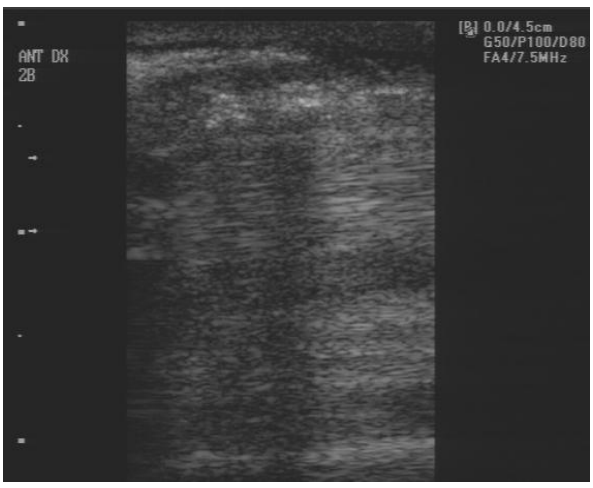
Anamnesis: the horse is referred for a suspect of right forelimb SDFT tendonitis.

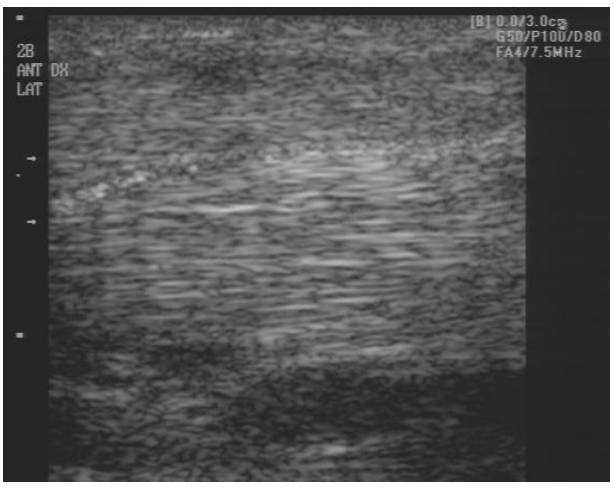
Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

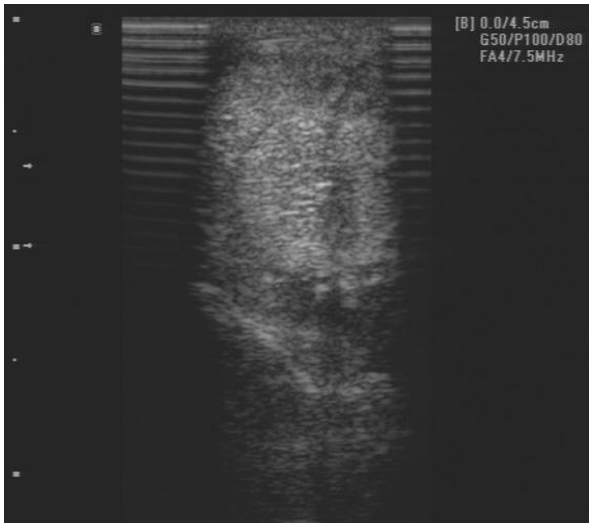
An anechoic area of the SDFT is visible in zone 2A. The transverse section shows it occupies almost the 50% of the total thickness of the tendon. In longitudinal scanning a sever misalignment of fibers (2/3) is visible in the same and the surrounding areas.

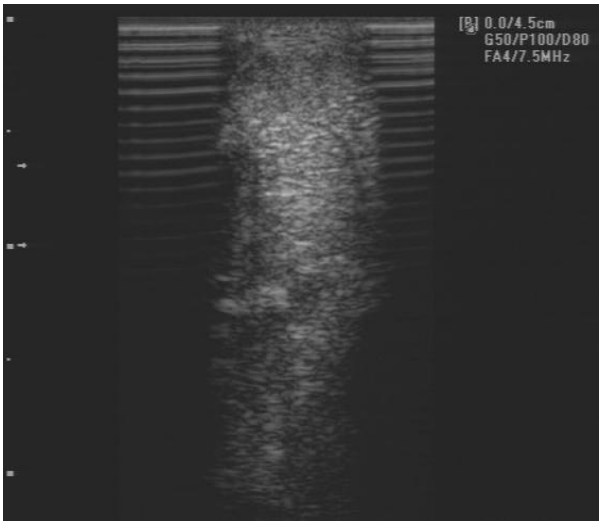












Diagnosis: left forelimb acute SDFT tendonitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 1597000/µl

WBCs count: 25700/µl

Whole blood counts: PLT 154000/µl and WBCs 8600/µl

Platelets had a ten times fold concentration and WBCs increased of three times.

Clinical course: the horse was subjected to a personalized rehabilitation protocol: 2 months of stall rest and 4 months of training with increasing intensity. The referring veterinarian refers a period of almost three weeks of severe, acute inflammation of the area after injection requiring further pharmacological treatments. The horse recovered but competed at lower level than before. Afterwards (more than 12 months) he was stopped for orthopedic problems of unknown nature.

Case #16: Horse 0200003069

Breed: Quarter Horse

Sex: gelding

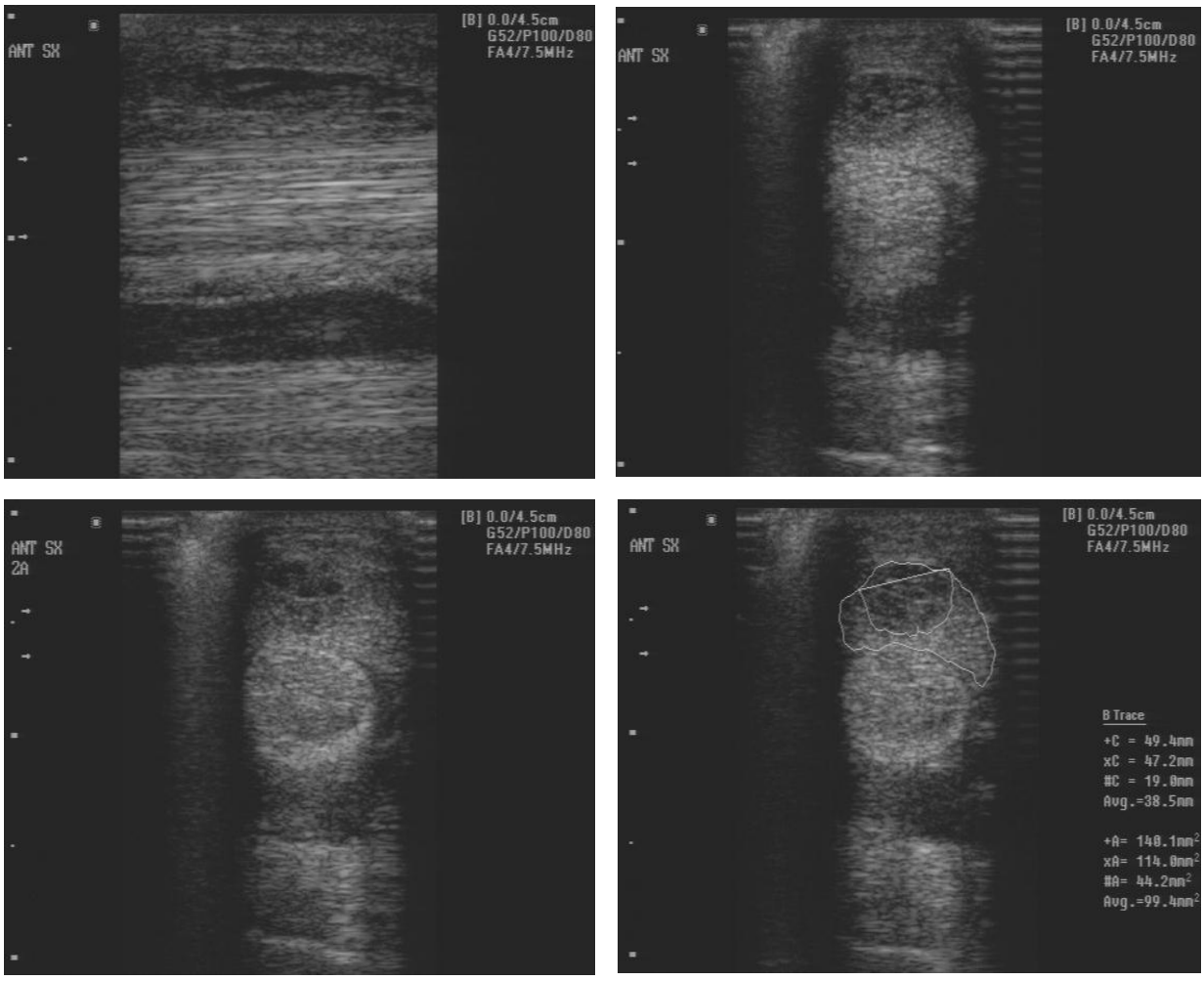
Age: 5 y. o.

Activity: saddle/jumping

Anamnesis: the horse is referred for the acute comparison of a hot swelling on the palmar aspect of the left forelimb associated to lameness (3/5) from one week.

Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

The transverse scan of the SDFT shows a hypo-echoic area (scored 2/3) in the zones from 1B to 2B, with the worst aspect in 2A. In this zone the CSA of the lesion is about 50 mm^2 wide over the 120mm^2 of the whole tendon thickness.



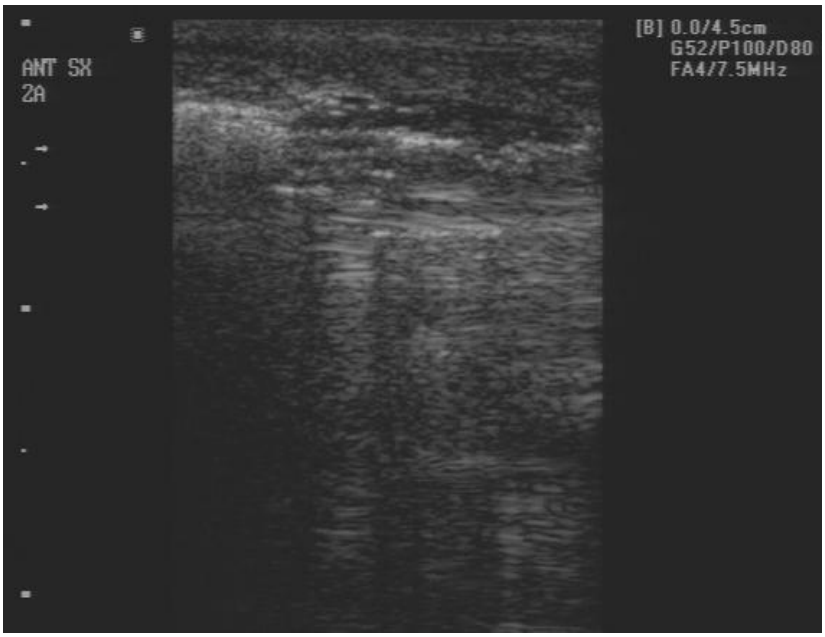
Diagnosis: left forelimb acute SDFT tendonitis.

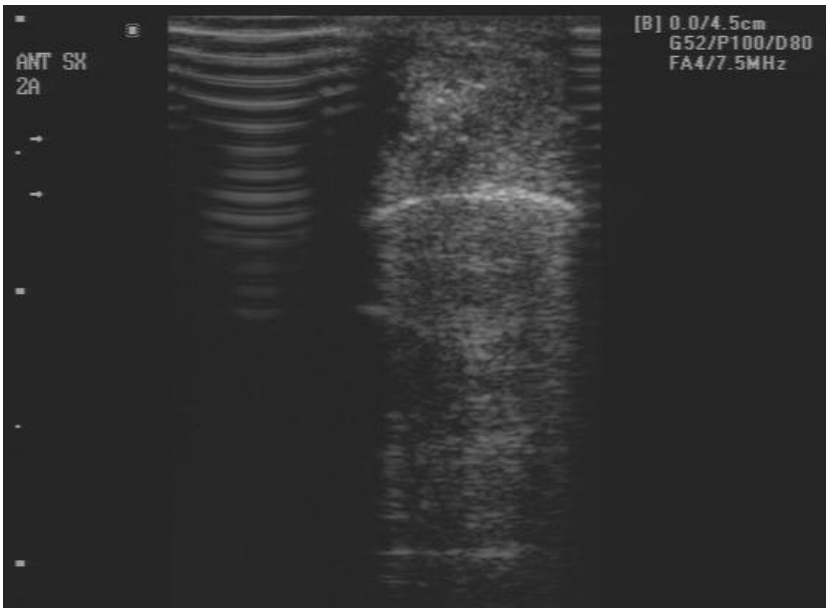
Therapy: ultrasound guided intralesional injection of PRP

PRP features:

No platelet count was available due to a break of the blood counter.

Clinical course: the horse was subjected to rehabilitation protocol and followed by the referring veterinarian. After about six months he got back to competition at a lower level.





Case #17: Horse 0200003067

Breed: Camargue

Sex: female

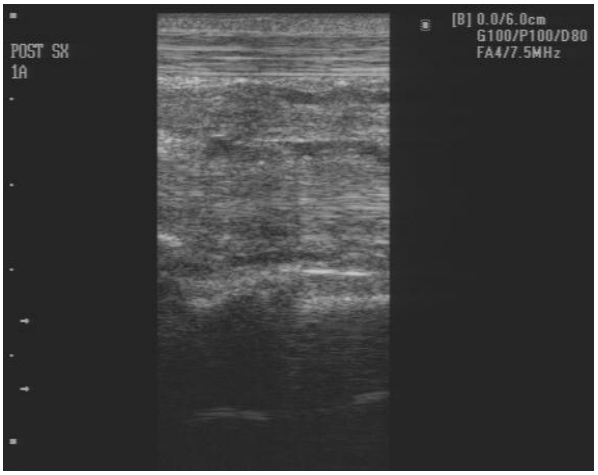
Age: 14 y. o.

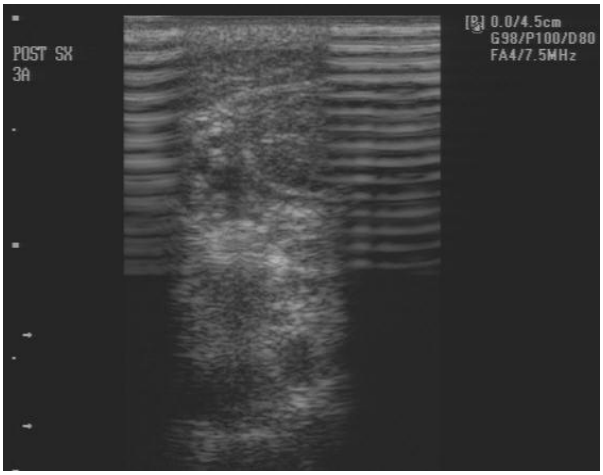
Activity: saddle/jumping

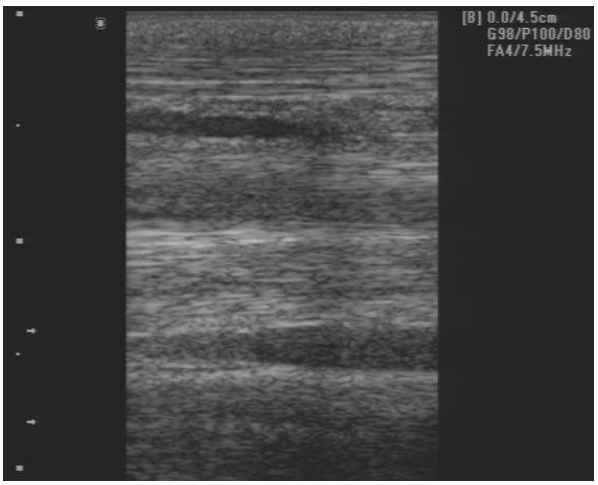
Anamnesis: the horse is referred for chronic lameness (1/5) of the left hind limb. The suspect is a SL lesion.

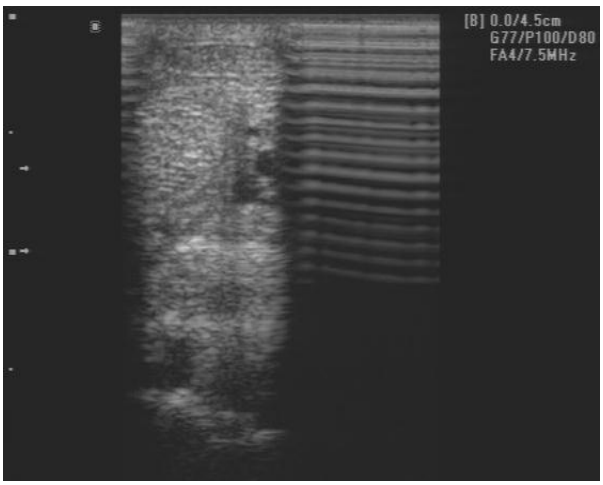
Examination and ultrasound: the lameness is present at the time of clinical examination (3/5 degree).

The transverse scan of the SL shows a small round anechoic area within the thickness of the ligament in zone 3B. In longitudinal scanning the lesion appears fusiform and extended through the whole zone. Misalignment of fibers and general edema is visible in zone 1A too.









Diagnosis: left hind limb chronic SL desmitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 579000/µl

WBCs count: 4230/µl

Whole blood counts: PLTs 128000/µl and WBCs 5890/µl

Platelets had almost a four times fold and WBCs decreased of 2/5.

Clinical course: the horse was subjected to 1 months of stall rest and then let in a paddock with 5 minutes walk per day. After two months the horse was still lame and came back for ultrasound control. At the time an ossification of the SL was detected in correspondence of the distal extremity of the IV metatarsal bone, previously fractured and in way of bony rearrangement. The owner was recommended for one month of stable rest with light walks starting two weeks later to prevent adhesions and reintroduction to work with a very light schedule. The horse recovered completely in 9 months.

Case #18: Horse 0200003333

Breed: Italian Saddle

Sex: female

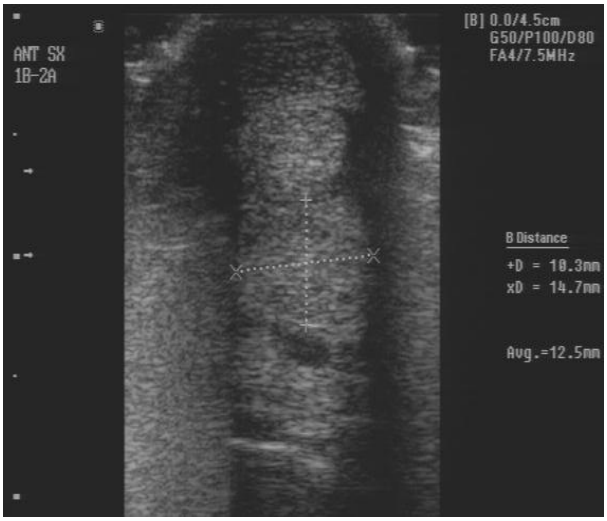
Age: 11 y. o.

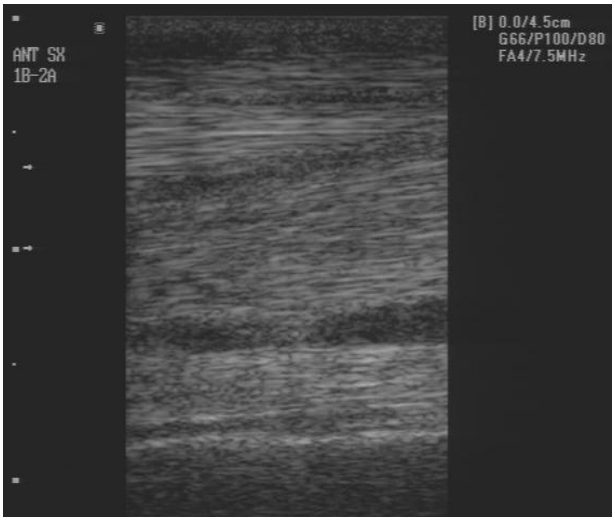
Activity: jumping

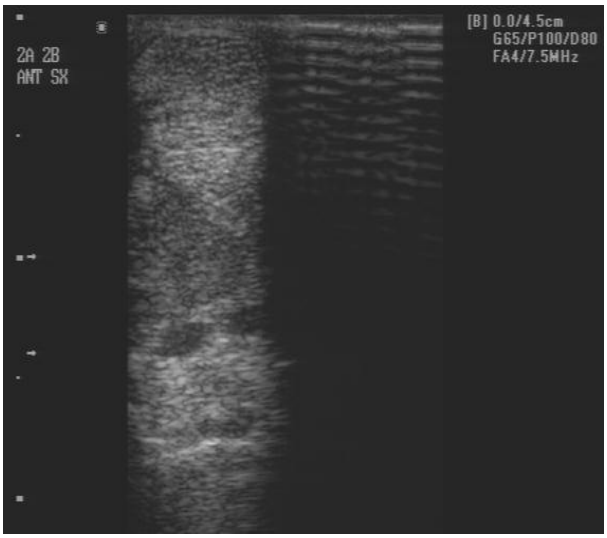
Anamnesis: the horse is referred for acute lameness (3/5) of the left forelimb. The horse had trained for a jumping competition few days before.

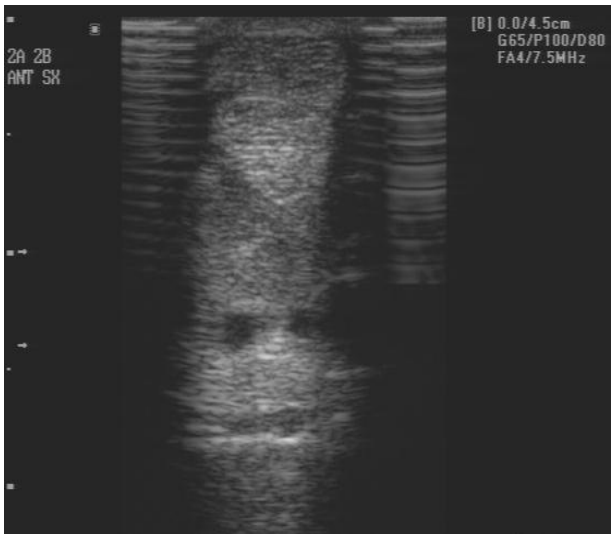
Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

The ultrasound examination showed an ICL increased in volume in zones 1B-2A, with inhomogenous pattern and a central hypo-echoic area.









Diagnosis: left forelimb acute ICL desmitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 951000/µl

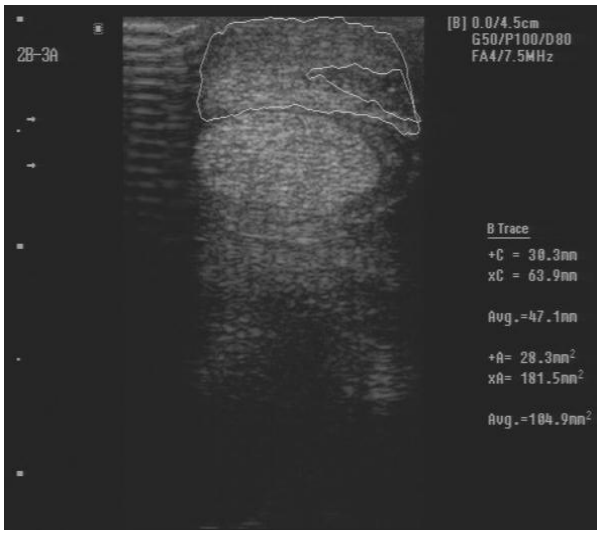
WBCs count: 11000/µl

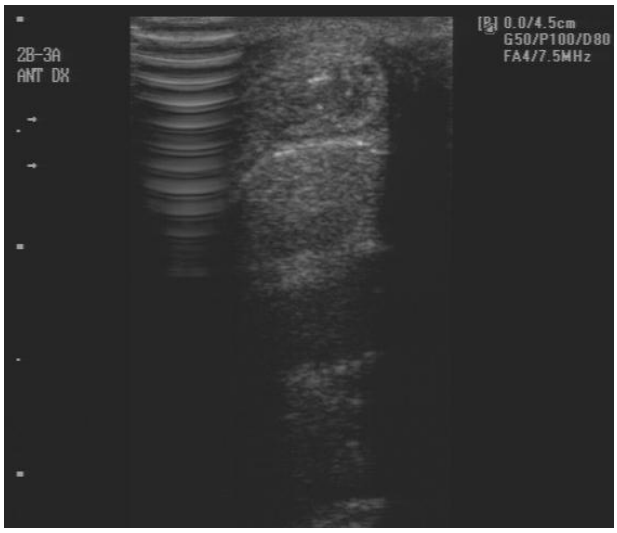
Whole blood counts: PLTs 107000/µl and WBCs 6100/µl

Platelets had about a nine times fold and WBCs doubled.

Clinical course: the horse was subjected to the standard rehabilitation protocol and got back to competition at a lower level after four months. She competed well for a couple of months and was brought back to clinic for lameness of the right forelimb seven months after the first injury.

She was submitted to lameness investigation (lameness score 2/5) and ultrasound examination. The SDFT at this time presented an elliptic hypo-echoic area in the zone 2B-3A, scored 2/3, covering an area of 28mm^2 in transverse scanning. In longitudinal scanning the lesion was 40mm long and scored 2/3. The diagnosis was of a sub-acute lesion of the right forelimb SDFT. The horse was treated with frozen PRP injection and submitted to rehabilitation protocol again. She got back to competition at the same level as before.





Case #19: Horse 0200003517

Breed: Italian Saddle

Sex: female

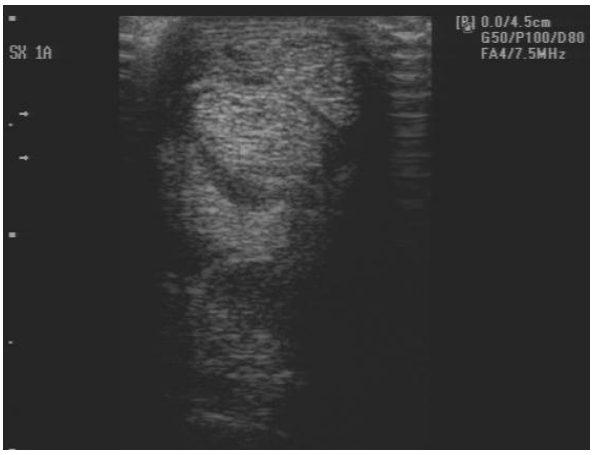
Age: 9 y. o.

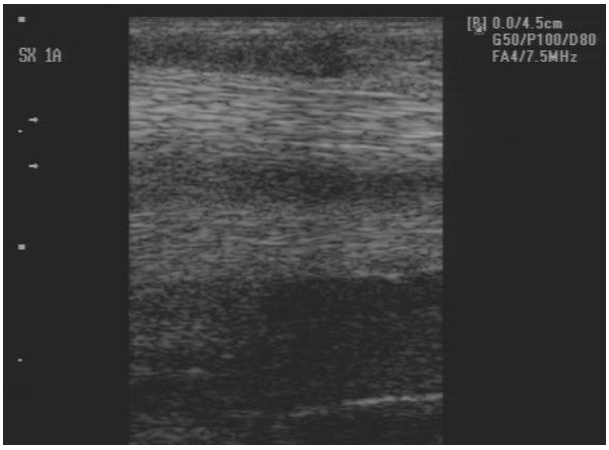
Activity: saddle/jumping

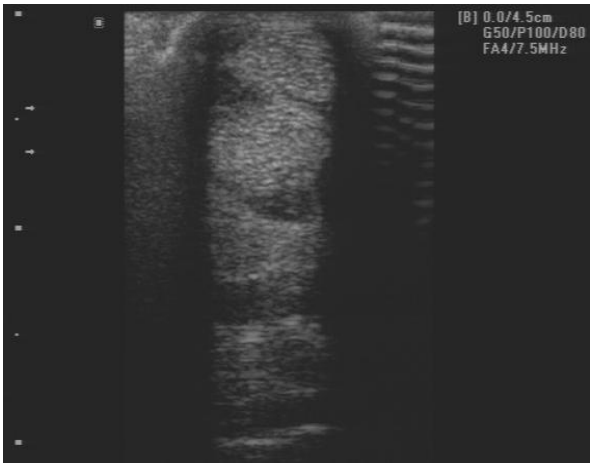
Anamnesis: the horse is referred for gait impairment of both forelimbs.

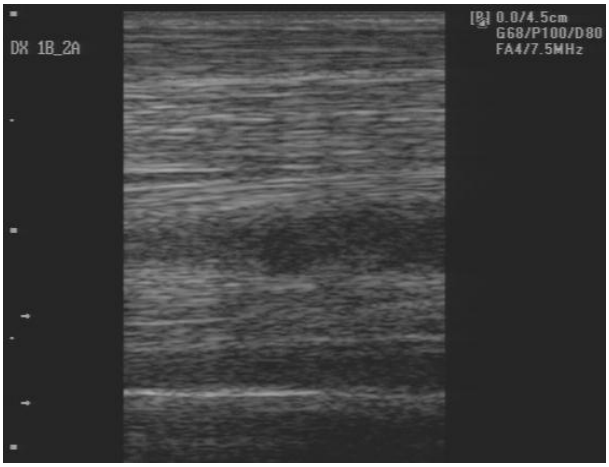
Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

The ultrasound examination of the left forelimb revealed in transverse scan the presence of an anechoic area in the medial portion of the SDFT in zone 1A (score 2/3); in longitudinal scanning the alignment of fibers was scored 2/3. In the right forelimb both in longitudinal and transversal scanning a hypo-echoic area was evident in the mid-body of the SL, having CSA of 30% of the whole thickness of the ligament.









Diagnosis: left forelimb SDFT tendonitis and right forelimb SL desmitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

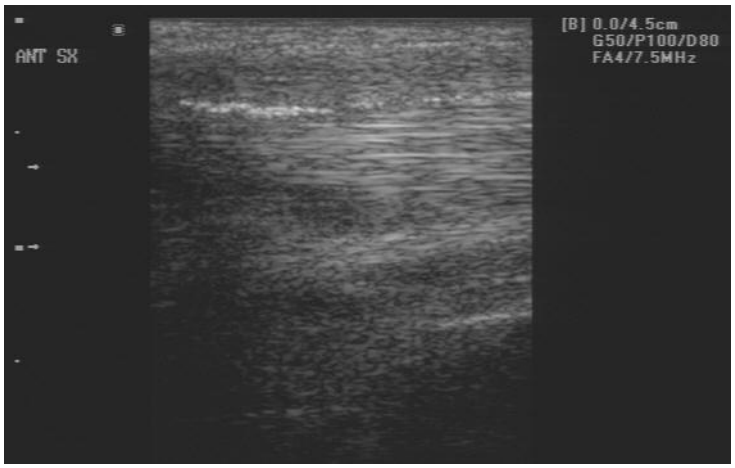
Platelet count: 842000/µl

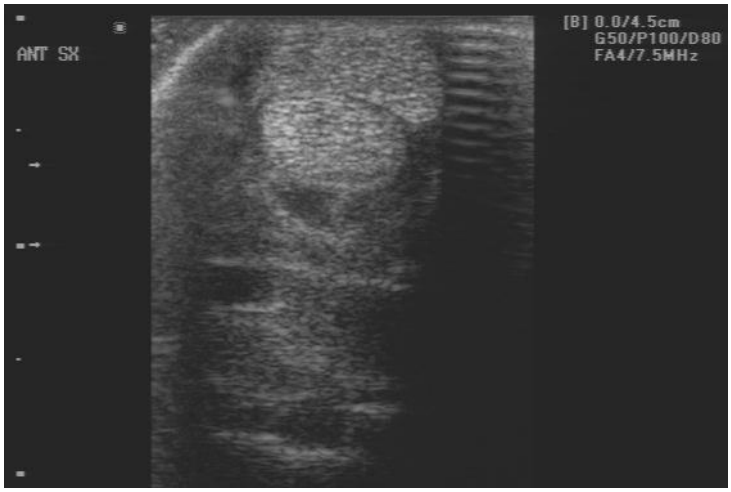
WBCs count: 17100/µl

Whole blood counts: PLTs 115000/µl and WBCs 7800/µl

Platelets had a seven times fold and WBCs increased 2.5 times.

Clinical course: the horse was subjected to 1 months of stall rest and then let in a paddock with 5 minutes walk per day. After two months the horse back for ultrasound control and a second injection of PRP was performed in the right forelimb SL. The appearance of the left SDFT was in way of recovery with a better alignment of fibers. The horse was subjected to standardized rehabilitation protocol and got back to activity at a lower level in six months.





Case #20: Horse 0200003498

Breed: English Warmblood

Sex: male

Age: 9 y. o.

Activity: gallop races

Anamnesis: the horse is referred for acute lameness of the left forelimb.

Examination and ultrasound: the lameness is present at the time of clinical examination (2/5 degree).

The ultrasound examination of the left forelimb revealed in transverse scan the presence of an anechoic area in the mid-body of the SL (score 2/3); in longitudinal scanning the alignment of fibers was scored 2/3.

Diagnosis: left forelimb SL acute desmitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 1362000/µl

WBCs count: 24200/µl

Whole blood counts: PLTs 130000/µl and WBCs 8100/µl

Platelets had a ten times fold and WBCs increased 3 times.

Clinical course: the horse was subjected to a rehabilitation protocol quite similar to the one proposed. After two months the horse had an ultrasound control and the veterinarian was cautiously optimist because of the appearance of the lesion. The horse got back to activity after six more months (8 months after treatment) at the same level of competition as before. No relapse was reported within the 12 months follow-up.

Case #21: Horse 0200003429

Breed: Arabian

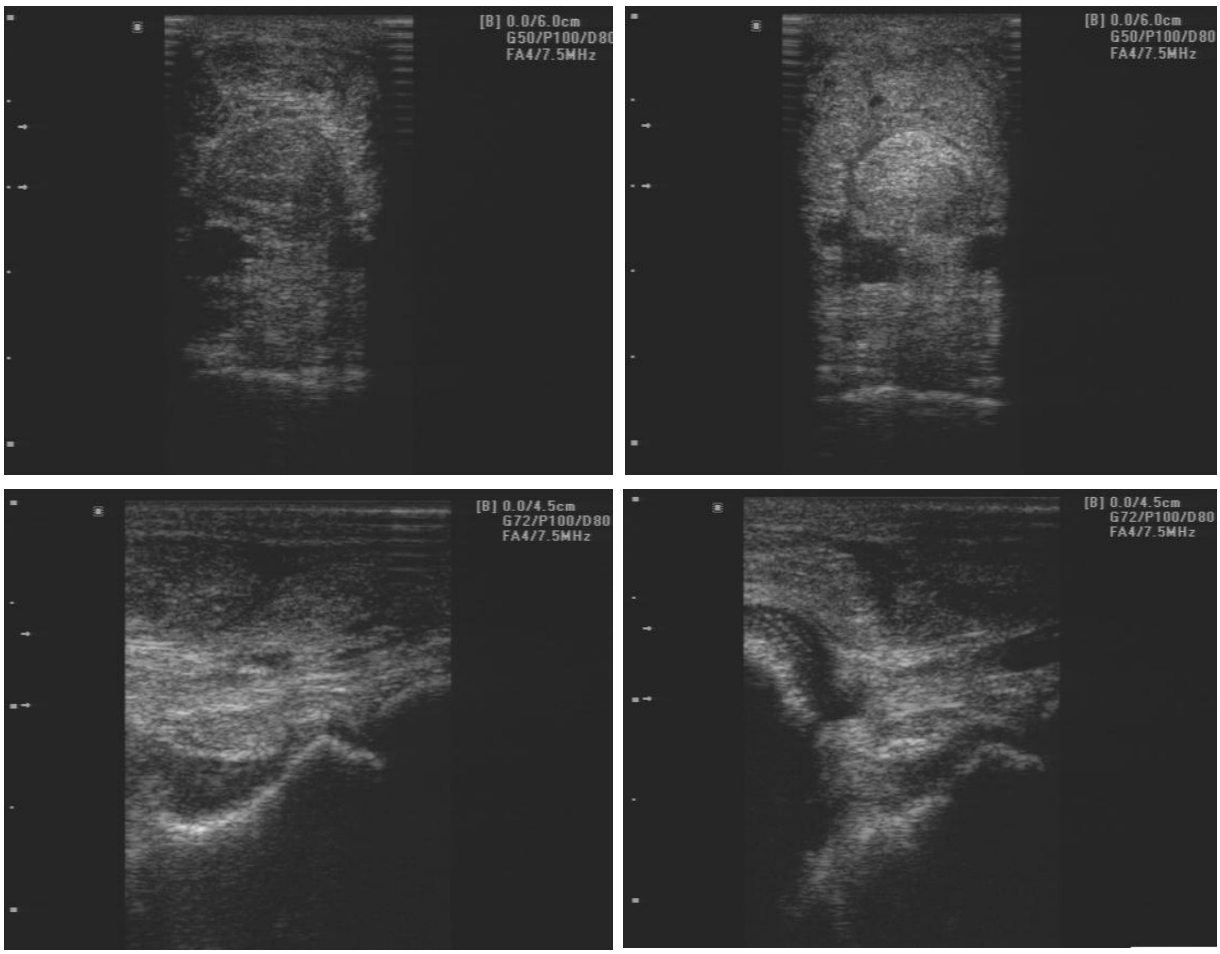
Sex: male

Age: 3 y. o.

Activity: trekking/cross country

Anamnesis: the horse is referred for a traumatic almost complete (90% of thickness) severing of the right forelimb SDFT.

Examination and ultrasound: the horse was obviously not subjected to lameness investigation. A tenorraphy was performed and the limb was casted in flexed position for one month. Afterwards the cast was removed, and the ultrasound and PRP were performed. The examination revealed both in transverse and longitudinal scan the formation of some tissue alternated to large anechoic areas, in sort of a beehive fashion in some points, from zone 2A through 3B.



Diagnosis: right forelimb chronic SDFT tendonitis.

Therapy: ultrasound guided intra-lesional injection of PRP.

PRP features:

Platelet count: 1506000/µl

WBCs count: 27200/µl

Whole blood counts: PLTs 166000/µl and WBCs 10200/µl

Platelets had a nine times fold and WBCs increased almost three times.

Clinical course: after the first month with the cast the horse was applied a corrective shoeing with high heels and a soft simple bandage to protect the digit. The horse started the suggested rehabilitation protocol with the exception that the trotting was not introduced for four months. The horse was allowed to a really small paddock though, four months after injury.

Seven months after injury the horse was able to trot regularly and one year after injury he was ridden again. He showed some gait impairment but the gross appearance of the limb was quite normal. The ultrasound controls showed a surprising normality both of fiber echogenicity and alignment.

DISCUSSION

Results for this study will only be reported as descriptive considerations throughout the discussion. The choice is due to the impossibility to perform a significant statistic analysis for the issues already discussed and later on taken again into consideration. This can be considered a limit to the research but is the most correct way to treat the reported data: producing numbers out of non-standardized information is considered both scientifically and mathematically incorrect.

Lesions of tendons and ligaments in horses are a consistent problem both for the frequency and for the difficulty to achieve complete healing and functional recovery. Therapeutic treatments so far proposed haven't yet brought to completely satisfactory outcomes. Traditional treatments lead to the formation of a scar which is stronger than the original tissue and quite less elastic. This is the reason why often lesions relapse in the areas surrounding the scar. Relapses are still nowadays a majorly occurring problem.

The use of PRP for the treatment of tendons and ligaments lesions in horses has been proposed on the spite of the enthusiasm of its use in human medicine (Anitua *et al.*, 2003; Marx *et al.*, 2004; Everts *et al.*, 2006), with the aim of obtaining tissue regeneration and not only simple reparation of tendons and ligaments.

Aim of this study was to try to evaluate clinically the efficacy of the intra-lesional injection of PRP for tendons and ligaments lesions in the limb of horses, after having the demonstration of its effectiveness on the healing of other tissues (Carter *et al.*, 2003; Spadari *et al.*, 2006).

In this study another issue was the designing of a protocol for the preparation of the PRP that would be effective, quick, easy, repeatable and cheap. Meanwhile, limit since the start of the project, it was known that the greatest limit would be the impossibility to quantify the growth factors; the evaluation of its quality could only be made on the platelet count which is not significant itself, even though in human medicine it has been shown that it can be considered appropriate for concentrations of 1.000.000 platelets/mm^3 (Van Den Dolder *et al.*, 2006).

The method for the preparation of the PRP that was finally set up is a manual method based on the double centrifugation system. Compared to other methods (buffy coat, apheresis, filtration after apheresis) or to the use of commercial kits this was the cheapest, the easiest, and the faster to be used in the reality of our clinic.

The biggest issue though to design a research project on this subject was the impossibility to perform a programmed study in regards of the number of cases, the asset of interventions, the control of the follow up and outcome. The clinical trial isn’t for sure the best method to perform research as the subjects, spontaneously afferent clinical cases, present a huge variability of specimen. The lesions differed for site, time of occurrence, gravity and extensions; the

rehabilitation protocols were quite uncontrollable, the availability of owners to cooperate in the post-op hard to quest, the assessment of ultrasound controls difficult to standardize. Thus the efficacy of the treatment couldn't be straightly related to any standard variable, such as the method of preparation or the type of lesion, but only on the outcome of the treatment itself.

Furthermore the clinical trial lacks of experimental studies on standardized models to compare the effectiveness of the treatment to other therapeutic options.

The setting up of the method passed through some trials which would not avoid, in any case, the need for an adjustment of the time of centrifugation (first centrifugation) to obtain a good separation of the hematic fractions. The evaluation was based on the observation of the relative proportion of the fractions compared to that of the blood count. A difficulty of separation of the fractions is usually to be imputed to a higher viscosity of the blood (thus to its rheological characteristics) or the impairment between the quantity of anticoagulant and coagulation properties of the blood.

The method itself usually produced a concentration of the platelets of 5-10 times fold, while WBC averagely increased 2-3 times. It thus turned out to be quite effective as the concentration of platelets was averagely above the seven times fold.

In this study the PRP was injected without activation. Many studies have been performed using PRP activated with calcium, thrombin, or through freezing (Anitua *et al.*, 2004; Borzini and Mazzucco, 2005; Everts *et al.*, 2006; Del Bue *et al.*, 2007; Zimmermann *et al.*, 2008). The general lack of literature about its use in tendons and ligaments in horses though led to the choice to use it as it is, given the fact of its instability. The activation through freezing would have been the other option of choice but it doesn't allow the treatment to be performed right after the diagnosis.

The management of the horse in the post-operative period and the rehabilitation protocol have a crucial role in the final outcome. It was decided and followed either by the owner or the referring veterinarian with impossibility to modification on our behalf. Similarly, the impossibility to have periodical standardized ultrasound controls has limited the ability to adjust the schedule properly. To optimize the rehabilitation period ultrasound controls should be ideally performed every 2-3 months at the most (Smith, 2008).

Besides the clinical and ultrasonographic evaluation, the soundness of a sport horse is mainly based on the level of competition, the number of start-ups and the money gained in competition. Thus in this study the outcome was evaluated on the basis of the return to activity, the level of competition and the time of rehabilitation. The presence of clinical symptoms and the need for other treatment were investigated and reported when the owner would let the information. The difficulties in obtaining more certain data about the events that follow the intervention are not only to be imputed to unavailability or bad will of owners. Transportation and clinical handling in general is both an expense and a stress for the horse, sometimes requiring sedation for the containment; furthermore the practitioners having the horses in cure must be trusted as competent colleagues. The possibility to have a mobile unit to follow the horses would help in the future to have more thorough and consistent follow-ups.

Given the difficulties above reported but considering the outcomes, the treatment should be considered quite effective: 5/22 treatments (one horse treated for two completely different lesions) had a straightforward negative outcome, with 1/22 treatment with unknown exitus. This number is above any other reported in literature, but many considerations will follow on its trustfulness.

In this study the follow up is usually up to twelve months after injury. This choice was made to be able to consider equally the majority of the cases, otherwise the horse treated in the last year couldn’t be inserted in the study.

Some horses have relapsed before the first year had passed and were considered as clearly negative outcomes. They can be ascribed either to a bad or too short rehabilitation protocol or to the ineffectiveness of the treatment itself. It is to be considered though that the majority of relapses occurred was in those horses (mainly trotter horses) that are erroneously pushed to get back to activity too soon. There is the general belief that the so called "regenerative treatments" would speed up the healing and a gradual reintroduction to training is superfluous. It is instead true that healing can be speed up and improved in the quality of the tissue produced but the proper reintroduction of a horse to sport activity is still considered to be happening in a lapse of time between 9 and 18 months, depending on the severity of the lesion. Even the smallest lesions (only echographically evident) should be let at least six months to heal (Smith, 2008): these conditions are incompatible with the reality of the horse racing world. Relapses we know about also occurred in the first year after treatment, yet we know that in 5/21 more cases the sport activity has been afterwards performed at a lower level. The sum of the ten cases with the two with uncertain outcome (12/22) pretty much resembles the results presented by other studies.

The platelet count of the PRPs applied in the relapsing cases was quite consistent, and this would support the thesis that the correlation between platelet count and outcome is not straight forward. The platelet concentrate that was actually used is a platelet-leucocyte rich plasma and the clinical effects depend on the combination of both PLTs and WBCs about which too little is still known (Everts *et al.*, 2007).

One issue the debate is still opened on is on the number of applications of PRP to be performed. Only in one case it was decided to perform a double injection, while all other horses have been treated once, or in two different sites. The consideration made was that pulling exogenous material into an healing tissue may alter the arrangement of the fibrils in shaping and potentially jeopardize the regular processes of healing that may be ongoing.

Some cases have been considered with positive outcome even if the horse had to interrupt the sport activity before the one year had passed because of other causes. The horses that got back to activity with good result within one year after treatment have had quite different rehabilitation periods and protocols but differ widely for platelet count. The good result can also be attributed to the young age of subjects (case 2, having also a relatively low platelet count), which certainly helps the regeneration of the tissue, whereas older horses may have good results because their activity is much less pushed (cases 11, 17).

For the horses whose long term destination is unknown the outcome was considered either positive or negative up to the time it is known. This choice was made on the basis that the cases in which the ultrasound control where performed at two and four months after treatment already showed an almost total normality in their appearance where a scar was not even visible. The tendon or the ligament thus seem to reacquire their original aspect.

The positive follow up after one year doesn't mean a relapse may occur later on (see case 7). Thus the positive outcomes so far investigated may turn out in negatives on a longer term, as it is shown in a study similar to this. In that case, the results reported for the first two years after treatment were excellent but after three years the results were worse than after 24 months (Waselau *et al.*, 2008). Arguelles and colleagues have had positive results with the application of PRP in tenodesmic lesions but the cases have been likewise followed for only one year after treatment (Arguelles *et al.*, 2008).

Similarly the application of mesenchymal stem cells, having had positive results on the regeneration of tendons and ligaments (Crovace *et al.*, 2007; Fortier and Smith, 2008) still doesn't guarantee long term results. Having similar results between PRP and MSCs the advantages of PRP must be considered as it is less invasive, cheapest, quickest and much easier to be applied (Fortier *et al.*, 2008).

CONCLUSIONS

The results here reported do not allow to assess that the intralesional application of PRP is an effective therapy in course of tendons' and ligaments' lesions in sport horses.

The variables interfering in the study were definitely too many to give certain results, and the small number of horses, even with some data about, only allow to produce some hypotheses. The ultrasonographic improvements of the tissues and to possibility for at least 14 horses out of 21 to return to activity can only make us suppose the treatment for effective for the purpose. It must be remembered though that later than 12 months relapses stay unknown.

PRP must be recognized as an excellent therapeutic option among the many, as it is quick, cheap, repeatable, and with no complications so far reported.

In the future effort should be put in the directions to improve the separation method to have only platelets in the product and eventually have them purified, so that they could be used on a horse different from the donor. The other aim is the quantification of GFs and how effective they can be in relation to the time of usage. Obviously the timing of multiple injections is to be addressed too.

The periodic ultrasonographic evaluation is a good mean of investigation and may demonstrate the improvements in the speed and the quality of the healing. This results seem to be quite far to be standardized though until research studies on live horses, other than clinical trials, will be allowed on larger numbers.

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