Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Dissertação

Intralesional treatment with mesenchymal stem cells in horses with suspensory ligament desmitis and superficial digital flexor tendonitis: a retrospective study

Rui Miguel Van Raay Ferraz de Menezes

Orientador(es) | Susana Monteiro

Marco de Bruijn

Évora 2021

Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Dissertação

Intralesional treatment with mesenchymal stem cells in horses with suspensory ligament desmitis and superficial digital flexor tendonitis: a retrospective study

Rui Miguel Van Raay Ferraz de Menezes

Orientador(es) | Susana Monteiro

Marco de Bruijn

Évora 2021

A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

Presidente	|	Rita Payan-Carreira (Universidade de Évora)
Vogais	|	Pedro Miguel Pires de Carvalho (Escola Universitária Vasco da Gama) (Arguente) Susana Monteiro (Universidade de Évora) (Orientador)

Évora 2021

Acknowledgements

I would like to express my gratitude to my supervisor Marco de Bruin, for the opportunity to do my externship at Dierenkliniek Wolvega, for the opportunity to make this study, for all the help and patience both during my externship and the writing of this dissertation. And to Don van de Winkel, and Berit Boshuizen, who were also fundamental during my externship at Dierenkliniek Wolvega, as well as during the writing of this dissertation.

I would like to thank my supervisor Susana Monteiro, for all her help and advice throughout the the final stages of my academic journey.

I would also like to thank Catherine Delesalle, for all her assistance and guidance through the elaboration of this thesis.

I have to extend my gratitude to Aart Schutrups, Waling Haytema, and everyone at Dierenkliniek Wolvega, for everything they have taught me, but especially for all the kindness and hospitality with which they have received me. In particular to the interns at DKW, Floor, Evelyn, Elizabeth, and Bram, who had the patience to endure me for 4 months, almost every day, around the clock.

My thank you to the team at the *Serviço de Urgências e Cirurgia de Equinos* of the *Faculdade de Medicina Veterinária* in Lisbon, who have played a great role in my education and shaping as a future veterinarian.

I also have to thank Carlos Santana for the opportunities given in the last years to gain experience in other fields of veterinary work.

This dissertation is the culmination of many years of work. Years that have been shaped by many people, to whom I have to give a special thank you:

My parents and Laura, who always believed in me, supported me at all moments, and made everything possible.

My friends, who made these years one of the best experiences in my life. Without you none of this would be the same!

Susana Burnay, who has been present since the beginning and has taught me almost everything I know about horses.

And finally, I would like to thank one of the most important persons that this journey has brought to me, Joana. Thank you, for all the love, support, and infinite friendship.

Abstract

Mesenchymal stem cells (MSCs) promote the regeneration of scarless tendon tissue after injury. Various sources have been described and used to obtain MSCs, adipose tissue and bone-marrow are one of the most used.

A population of 49 horses, that suffered superficial digital flexor tendonitis or suspensory ligament desmitis and were treated either with the combination of stromal vascular fraction and platelet rich plasma, or with allogenic bone marrow derived MSCs, was analyzed retrospectively. The influence of different factors (age, breed, discipline, injury chronicity, treated structure and type of MSCs used) on the outcome of the therapy was evaluated. The re-injury rate observed on the total population was 4% six months after MSC therapy, and 17,8% after 12 months.

The results obtained are similar to results obtained in other studies that evaluated the therapeutic effect of MSCs in horses, encouraging the use of these cells in the treatment of tendinoligamentous disorders.

Keywords: Mesenchymal stem cell; equine; suspensory ligament; superficial digital flexor tendon; regenerative medicine.

Resumo

Tratamento intralesional com células estaminais mesenquimatosas em cavalos com desmite do ligamento suspensor e tendinite do tendão flexor superficial digital: um estudo retrospetivo.

As células estaminais mesenquimatosas (MSCs) promovem regeneração de tecido tendinoso saudável após a lesão. Vários tecidos têm sido usados para obter MSCs, mas o tecido adiposo e a medula óssea têm sido os mais populares.

Este estudo analisou retrospetivamente uma população de 49 cavalos, com tendinite do tendão flexor digital superficial ou desmite do ligamento suspensor, que foram tratados com uma combinação de fração estromal vascular com plasma rico em plaquetas, ou com células estaminais mesenquimatosas alogénicas derivadas de medula óssea, avaliando a influência de diversos fatores (idade, disciplina, raça, cronicidade da lesão, estrutura tratada e tipo de células estaminais usadas) sobre o resultado da terapia. A taxa de lesões recidivas na população em estudo era de 4% 6 meses depois do tratamento com células estaminais, e 17,8% após 12 meses.

Os resultados obtidos neste estudo são semelhantes a outros estudos que avaliaram o mesmo tipo de terapias.

Palavras-chave: Células estaminais mesenquimatosas; equino; ligamento suspensor; tendão flexor digital superficial; medicina regenerativa.

Table of contents

List of abbreviations

AD	Adipose
ALDDFT	Accessory Ligament of the Deep Digital Flexor Tendon
ALSDFT	Accessory Ligament of the Superficial Digital Flexor Tendon
BM	Bone Marrow
CD	Cluster of Differentiation
CDET	Common Digital Extensor Tendon
COMP	Cartilage Oligomeric Matrix Protein
COX	Cyclooxygenase
CSA	Cross Sectional Area
CSL	Collateral Sesamoidean Ligament
DC	Dentritic Cell
DDFT	Deep Digital Flexor Tendon
DKW	Dierenkliniek Wolvega
DNA	Desoxirribonucleic Acid
G	Gauge
GAG	Glycosaminoglycan
HGF	Hepatocyte Growth Factor
IGF	Insuline-Like Growth Factor
ISCT	International Society for Cellular Therapy
MC	Metacarpus
MHC	Major Histocompatibility Complex
miRNA	micro Ribonucleic Acid
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
MT	Metatarsus
NSAID	Non-Steroidal Anti-Inflammatory Drug
OSL	Oblique Sesamoidean Ligament

PDGF	Platelet Derived Growth factor
PGE	Prostaglandin E
PRP	Platelet Rich Plasma
PSB	Proximal Sesamoid Bone
PSGAG	Polysulfated Glycosaminoglycan
SDFT	Superficial Digital Flexor Tendon
SL	Suspensory Ligament
SsL	Short Sesamoidean Ligament
SSL	Straight Sesamoidean Ligament
SVF	Stromal Vascular Fraction
TGF-beta	Transforming Growth Factor Beta
TH	T Helper Cell
VEGF	Vascular Endothelial Growth Factor

List of figures

List of tables

List of annexes

Preface

For a long time, tendon and ligament injuries have been one of the most devastating injuries for any athlete, the equine athlete being no exception. The intrinsic characteristics of tendon and ligament tissues do not allow for a complete regeneration of injured tissue, what means that once an injury has occurred the probability that the same injury will return after apparent healing is high. Since the beginning of this century investigators have been busy in trying to obtain a therapy that would allow for a better regeneration of tendon tissue. One of the therapies that has been successful in return equine athletes to their performance levels, after injury, is mesenchymal stem cell therapy. The following literature review attempts to gather and deliver to the reader a comprehensive vision about MSC therapy and its impact on equine veterinary medicine. To add information to further advances in MSC therapy, the literature review is followed by a retrospective study that aims to observe the effects that two different MSC sources have on superficial digital flexor tendonitis and suspensory ligament desmitis. The influence of discipline, breed, age, and the interval of time between injury and MSC administration is also tested.

1 Tendon/Ligament anatomy & physiology

1.1 Clinical anatomy

1.1.1 Suspensory ligament

The suspensory apparatus has three major components: the suspensory ligament (SL), the proximal sesamoidean bones (PSB) and the four distal sesamoidean ligaments. The main component of this apparatus is the SL, a strong tendinous band that originates from the palmar surface of the third metacarpal bone (McIII), with some fibres arising from the axial aspect of the fourth metacarpal bone (McIV) (Nagy *et al*., 2012), and a minority of these fibres coming from the deep carpal ligament (Budras *et al.*, 2011). The proximal and mid-body regions of the SL run between the axial aspects of McII and McIV, and palmar to McIII (Denoix, 1994). The SL bifurcates into two branches in the mid-metacarpal region, the lateral and the medial suspensory ligament branches. These branches insert in the abaxial surface of the apex of the PSBs, making a wide attachment along this surface and joining the origin of the ipsilateral collateral sesamoidean ligament (Fails, 2020). From the distal surface of the PSB a thin extensor branch leaves, running forward in an oblique direction across the proximal phalanx, merging with the common digital extensor on its dorsoproximal surface. The suspensory apparatus (Figure 1 and 2) continues in a distal direction through the distal sesamoidean ligaments: the oblique sesamoidean ligament (OSL), the short sesamoidean ligament (SsL), the straight sesamoidean ligament (SSL) and the cruciate sesamoidean ligament (CSL) (S. J. Dyson *et al*., 1995). All these ligaments originate from the base of the PSBs and the proximal scutum, as well as the palmar ligament (Denoix, 1994; Budras *et al*., 2011). The SSL inserts on to the palmar proximal aspect of the middle phalanx via the scutum medium. The medial and lateral OSL converge to insert on the proximal surface of the middle phalanx. The CSL constitutes the palmar border of the distal palmar synovial recess of the metacarpophalangeal joint. Finally, the SsLs have a diminutive body and insert on the palmar proximal surface of the proximal phalanx, because of their size and location they are difficult to distinguish from the deep portion of the OSL (Denoix, 1994; Sue J. Dyson *et al*., 2010).

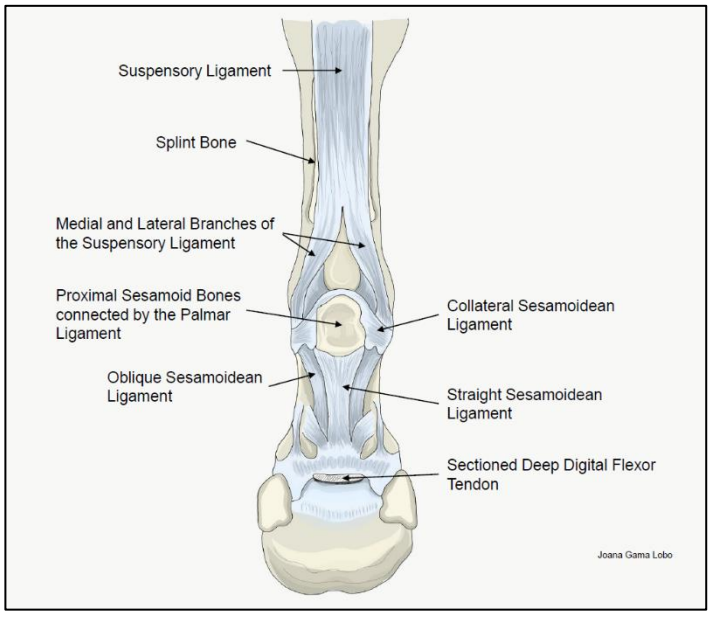


Figure 1 - Caudal view of the distal limb soft tissues (adapted from Fails, 2020)

When comparing the front limb and hind limb SL, important differences can be identified, for example the front limb SL has a rectangular and strong structure, that measures between 20 and 25 centimetres, while the hind limb SL is a thinner and rounder ligament, that measures 25 to 30 centimetres (Denoix, 1994). In the front limb the SL can be observed as a bilobed structure, right from the level of the origin. Usually the medial lobe is thinner and wider than the lateral lobe. As the ligament goes distally the lobes join to form an oval shaped structure, this occurs normally five to six centimetres distal to the carpometacarpal joint. Until the bifurcation this oval shape is maintained (Werpy *et al*., 2012).
In the hindlimb, the attachment to the plantar tubercle of the first, third and fourth tarsal bones is less prominent than in the front limb (Denoix, 1994). At the level of the proximal McIII, the SL starts off with a triangular shape. This triangle is thinner medially than it is laterally. Along the metacarpal groove the SL becomes gradually heart shaped, then rounded and finally oval, until the bifurcation of the SL branches. The apex of the triangular shape, as well as the heart shape, is directed in the plantar lateral direction (Werpy *et al*., 2012).

There are two main arteries that supply the front limb SL with blood, these are the lateral and medial metacarpal arteries, that descend at the palmar aspect of McIII, and originate in the deep palmar arch, which can be found close to the carpometacarpal joint. This arch is the result of the junction of the radial artery (medial) and the ulnar collateral artery (lateral) (Denoix, 1994; Budras *et al*., 2011; Werpy *et al*., 2012). The venous drainage of the SL is obtained through a satellite venous deep arch, between the medial and lateral metacarpal veins, that is both on the palmar and the dorsal aspects of the ligament. This arch is in turn drained by the ulnar vein (lateral) and the cephalic vein (medial) (Werpy *et al*., 2012).
In the front limb the SL receives innervation from a deep branch of the plantar branch of the ulnar nerve. This deep branch originates in the region of the distal carpus. These branches receive nerve fibres from the median nerve (Muylle *et al*., 1998).
In the hindlimb the medial and lateral plantar arteries are responsible for the blood supply. These arteries derive from the tarsal artery that perforates the tarsal canal after it branches of the dorsal pedal artery in the region of the dorsolateral hock. The venous return is obtained by the second plantar metatarsal vein, that joins the cranial tibial vein after it perforates the tarsal canal (Denoix, 1994; Budras *et al*., 2011; Werpy *et al*., 2012)

1.1.2 Superficial digital flexor tendon

The superficial digital flexor tendon (SDFT), together with its accessory ligament (ALSDFT), is one of the most important anatomo-functional components of the equine limb, along with the deep digital flexor tendon (DDFT) and its accessory ligament (ALDDFT), and the previously described suspensory apparatus (Denoix, 1994). The SDFT arises from the superficial digital flexor muscle, through a musculotendinous junction that is located between the distal antebrachium and the

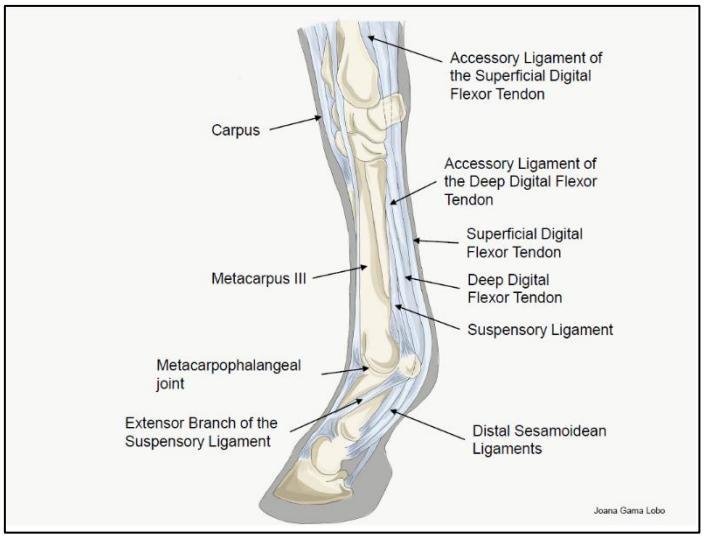


Figure 2 - Lateral view of the distal limb soft tissues (adapted from Fails, 2020)

proximal portion of the accessory carpal bone. In the carpal region, proximal to the antebrachiocarpal joint, the ALSDFT, also called the superior check-ligament, joins the SDFT from a dorsomedial and oblique direction, originating from the dorsomedial aspect of the radius. As it runs distally, the SDFT passes through the carpal canal as a round shaped tendon, that in the proximal metacarpus flattens and becomes half-moon shaped, with a sharp lateral margin and a rounded medial border. It maintains its shape along the metacarpus until the distal metacarpal region is reached, where it turns into a symmetrical and wide tendon, becoming thinner in a dorsopalmar direction and extending into a fibrous ring, called the *manica flexoria,* that encircles the DDFT (Roger K W Smith, 2007). Distal to the PSBs, the SDFT continues as a thin and wide structure, until it branches at the level of the mid proximal phalanx. These branches thicken distally and end between the palmar ligaments of the proximal interphalangeal joint, on the proximopalmar aspect of the middle phalanx, through the middle scutum (Denoix, 1994; Roger K W Smith, 2007; Budras *et al*., 2011).

The SDFT is supplied with blood by various arteries. One of these is a “nutrient artery”, that originates from the median artery, along the ALSDFT and enters the tendon in the musculotendinous junction. The proper digital artery also provides blood to the SDFT through an arterial branch that branches off near the proximal margin of the palmar annular ligament. Together these arteries supply the intratendinous arterial network and two arteries that run on each side of the SDFT (Denoix, 1994).

1.2 Structure and composition of tendon and ligament

Tendon is an elaborate structure that is the product of a complex organization between tendon cells (tenocytes), proteins of the extracellular matrix, blood vessels, lymphatic vessels and nerves (Birch *et al*., 2013). This complex organization, together with a specialized molecular composition, results in a high strength structure, capable of resisting profound uni-directional forces (Chavaunne Thandiwe Thorpe, 2010). The extracellular matrix is composed of collagen (mainly type I), non-collagenous proteins, and a high water content (65% (Kümmerle *et al*., 2019)), which is important to ensure the elastic properties of the tendon tissue (Birch *et al*., 2013).

Non collagenous proteins are mainly proteoglycans and glycoproteins. Proteoglycans can be subdivided in large proteoglycans and small leucine rich proteoglycans. An important glycoprotein is the cartilage oligomeric matrix protein (COMP), a protein that has a structural role in tendon development, as it has been shown *in vitro* that COMP can accelerate collagen fibrillogenesis. It accumulates during growth, reaching its highest concentration at maturity. As the animal ages COMP concentration decreases, explaining partially the reduced healing ability of older tendons (Halász *et al*., 2007; Birch *et al*., 2013).

Proper maintainance of the extracellular matrix is realized by a small population of fibroblast like cells, better known as tenocytes (Kümmerle *et al*., 2019). They produce tropocollagen, that self assembles into fibrils, in a parallel regular organization, along the tensional axis of the tendon. These fibrils are the start of the basic structure of the tendon, and they organize into collagen fibres, that are surrounded by endotendon to form fibre bundles called fascicles. The whole tendon is surrounded by a fine layer of connective tissue, called the epitendon (Birch *et al*., 2013). This hierarchical structure can be observed in Figure three, which illustrates all the main components of collagen organization within the tendon. A feature, specific for tendons is seen at the microscopic level, where collagen fibrils adopt a straight organized pattern, oriented along the direction of the main stress load of that tendon. This is in contrast to the right-handed helical path followed by collagen fibrils in tissues like the skin (Birch *et al*., 2013; Franchi *et al*., 2010). However, collagen fibrils in some regions of the tendon show fibrillar crimps, that are formed as the fibrils first twist leftwards, changing their plane of running, and then bend sharply also changing the direction of coursing. This functions as a biological hinge, that opens when tensional load is applied on the tendon and recoiling when the load is removed (Franchi *et al*., 2010; Birch *et al*., 2013).

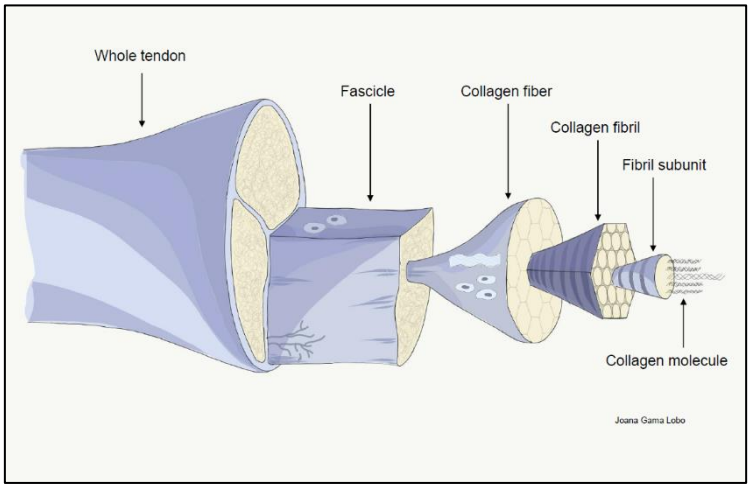


Figure 3 - Hierarchical structure of collagen in the tendon (adapted from Birch, 2013)

Looking at the tenocytes we can distinguish three types, according to nuclear morphology, localization, and presence in either juvenile or adult tissues (Banes *et al*., 1988; Roger K.W. Smith *et al*., 2008). Type I tenocytes have flattened, cigar shaped nuclei. They are the “satellite” tenocytes that lie between the collagen fibres. Type II tenocytes are the active type. They have a

rounded nucleus and can be found in linear groupings between collagen fibres. Type III collagen fibres are chondrocyte like and are found in regions of the tendon that are submitted to the biggest compressive forces (Goodship *et al*., 1994; Stanley *et al*., 2008). The true extension of the synthetic activity of tenocytes is unknown, but it is known that the turnover of non-collagenous components is higher than the collagenous components, suggesting that the plumper cells, present in the endotendon, are more active than the cells present between the collagen fibers, with elongated nucleus (Chavaunne T. Thorpe *et al*., 2010; Birch *et al*., 2013).

The SL is a modified muscle with tendinous characteristics. The main difference when compared with the SDFT and other tendons is the percentage of present muscle fibers (Pimenta, 2018) which can reach percentages between 2% and 11%, explaining its anatomical identification as "the interosseus muscle" (Denoix, 1994). The main muscle fibres that compose the ligament are type I fibres, which are slow twitch fibres. The muscle portion of the SL likely contributes to forelimb stability and elastic energy storage during locomotion (Soffler *et al*., 2006).

1.3 Functional role of tendons and ligaments

Tendons and ligaments have a primary role of connecting muscle to bone and bone to bone, respectively. But the SDFT and the SL are specialized structures, with specialized roles. They are the product of an evolutionary adaptation that allowed the horse to become one of the fastest runners on the planet. This anatomical adaptation to reach faster speeds is expressed by a simplified distal extremity structure, with reduction of the muscle mass in the distal limb, development of accessory ligaments to strengthen the function of the limbs, both in the active and passive phase of locomotion, and development of a specialized tendon and ligament apparatus that can withstand the forces being exerted on the distal limb. The function of the digital flexor tendons and the SL has phylogenetically evolved into a biological spring, which "winds up" as it bears the weight of the fully supported limb, preventing the hyperextension of the metacarpophalangeal joint (Denoix, 1994), store the energy that is generated, and release the energy once the limb is protracted (Wilson *et al*., 2001) (Figure

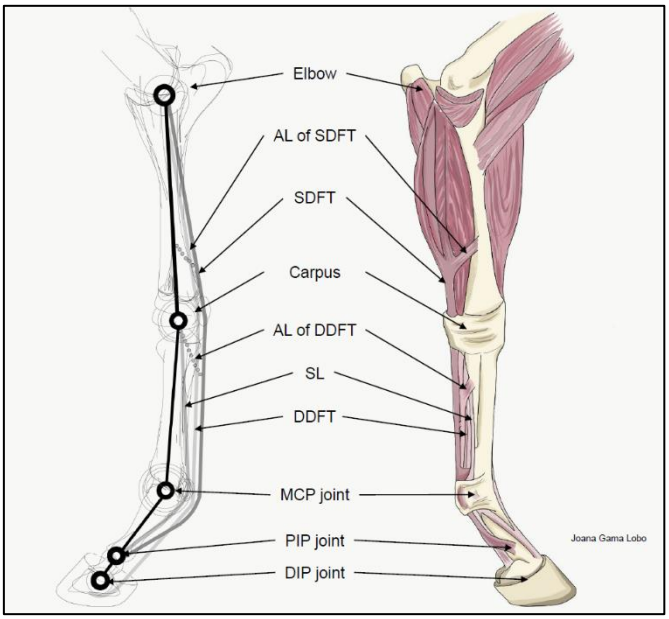


Figure 4 - Anatomic structure vs bio-mechanic structure of the limb (adapted from Wilson et al*., 2011)*

4). It has been shown that this mechanism buffers up to 36% of the mechanical work during gallop, and 40% during the transition from walk to trot (Biewener, 1998). The accessory ligaments further reduce the energy spent by muscle work by providing a direct bone-to-bone tendinous connection when the limb is bearing the full weight, as they attach the tendon directly to the bone (Birch *et al*., 2013). The SDFT has an added function as it flexes the digit during the swing phase of locomotion (Batson *et al*., 2003).

1.4 Tendon biomechanics

During locomotion, the flexor tendons are subjected to tension during the weight bearing phase, stretching and stressing the tendon tissue. It allows the flexor tendons to show their main physical properties: high tensile strength, flexibility, and elasticity (Goodship *et al*., 1994).

A diagram that illustrates the relationship between the force applied per unit of area (stress) and percentage of elongation (strain), shows how the tendon behaves according to the tension applied on it. There are four distinct regions in the diagram: 1) the start episode, called “the toe phase”, is characterized by a non-linear stretch of the tendon, that does not comply with Hooke’s law, a common feature in collagenous structures (Goodship *et al*., 1994). This means that although a relatively small load is applied, the tendon tissue shows great extension. The extension occurs due to sliding of the fascicles. The toe phase is associated with the elimination of the crimp pattern in the fascicles because of this sliding mechanism (Chavaunne *et al*., 2012). The crimp length and angle is still recoverable in the toe phase, if the load is removed, meaning that the tendon is still elastically recoverable (Goodship *et al*., 1994); 2) When the load applied to the tendon further increases, the resistance to further elongation of the tissue also increases (i.e. increase in stiffness). Here we see a linear relationship between stress and strain. It is in this phase that the structural property of stiffness is determined (load divided by deformation) (Kümmerle *et al*., 2019). The elongation mechanism occurs by elongation of the collagen fibrils, a step further from the elongation of the fascicle fibre elongation in the toe region. The fibrillar crimp is straightened out and there is sliding of fibrils and fibers relatively to one another; 3) A continued increase in strain applied to the tendon will lead to irreversible lengthening of the tendon, the consequence of slippage of collagen fibrils, fibers or fascicles (Birch *et al*., 2013), and covalent crosslink rupture (Kümmerle *et al*., 2019). This region of the curve is called the yield point and is characterized by a reduction in stiffness of the tendon. Strains of 12% to 20% have shown to be responsible for these effects on the tendon (Stephens *et al*., 1989); 4) The final region of the curve represents rupture of the tendon, when the stress-strain curve fails (Birch *et al*., 2013) the collagen cross-links or fibrils rupture (Roger K.W. Smith, 2010). As determined *in vitro,* these events occur at strains around 25% (Birch, 2007).

Studies have shown that the strain applied to the equine SDFT during gallop can reach values as high as 16% (Stephens *et al*., 1989), which shows how close physiological and failure strains approach each other during maximal exercise, only allowing for a narrow biomechanical safety window (Riemersma, 1989; Stephens *et al*., 1989; Goodship *et al*., 1994). This can be a possible

explanation for the high incidence of tendon injury seen in the horse, and especially the athletic horse.

Equine tendons are highly effective spring mechanisms, with low energy dissipation, absorbing up to 93% of the muscular work performed in the limb movement through an elastic recoil. The remainder of the energy is dissipated as heat (Alexander, 2002). This energy dissipation can be classified as hysteresis, the result of the difference between the stress/strain ratio of the loaded tendon compared with the unloaded tendon (Roger K.W. Smith *et al*., 2008) (Figure 5). The heat produced by energy dissipation rises the temperature within the tendon core when the tendon is repeatedly put under tension, as in the exercising horse (Kümmerle *et al*., 2019). It is hypothesised that this is one of the main causes of tendon injury in the athletic horse (Wilson 1994).

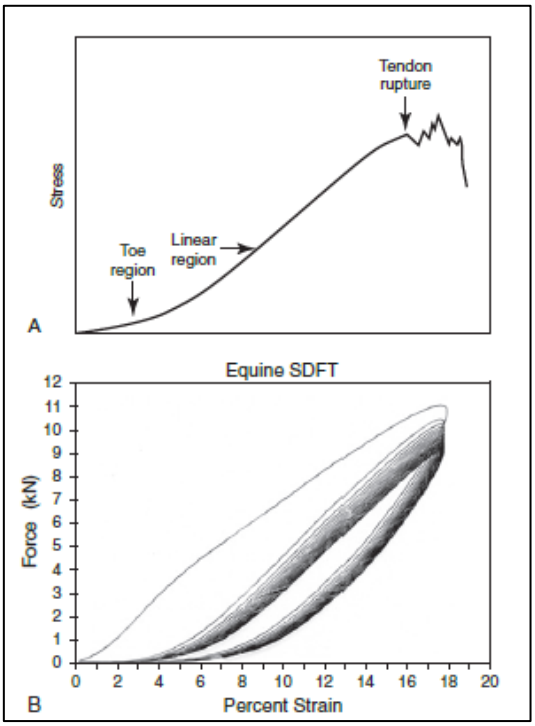


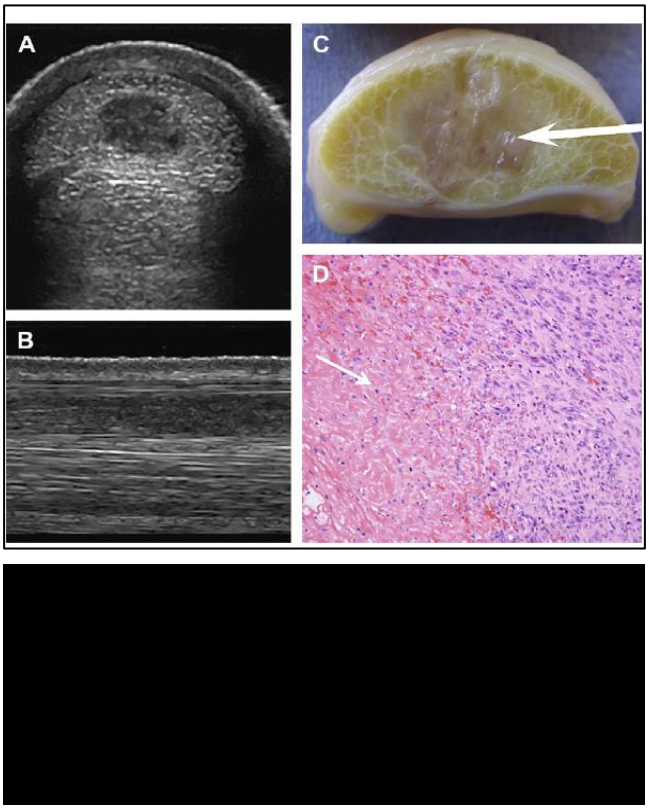
Figure 5 - A: stress-strain curve; B: hysteresis and conditioning. Hysteresis is shown as the difference between the loading and unloading curves. Conditioning is the result of the viscoelastic properties of the tendon. As the tendon is repeatedly loaded and unloaded it becomes more elastic, shifting the stress-strain curve to the right (Birch, 2013; Kümmerle, 2019)

When comparing the SDFT to a tendon with less weight supporting tasks, like the CDET, a positional tendon (Kümmerle *et al*., 2019), the SDFT has a higher CSA, and has a higher structural stiffness, which means that it has a higher failure load, as well as a lower mean elastic modulus (Roger K.W. Smith, 2010). These properties allow the SDFT to store a greater amount of elastic energy in comparison to the CDET. Studies have shown that the SL has comparable characteristics to the SDFT and shares a role in storing elastic energy during locomotion. The SDFT and the SL have a lower elastic modulus than the CDET, which means that they are less rigid structures, returning more elastic energy during locomotion (Birch, 2007; Alexander, 2002). The SDFT and the SL have a very similar extracellular matrix composition and configuration: both contain high COMP levels and a combination of small and large collagen fibrils. In contrast, the CDET and the DDFT, tendons with different roles in the limb, have lower levels of COMP and mostly large collagen fibrils (Kümmerle *et al*., 2019).

1.5 Tendon injury and repair

The most common orthopaedic injury in equine athletes is strain induced tendon and ligament injury (Birch *et al*., 2013). The type of injury can be related with certain disciplines, for example

show-jumping horses have a greater prevalence of SDFT and DDFT injuries, while dressage horses have more hindlimb SL injuries (Murray *et al*., 2006). The prevalence of flexor tendon and SL injuries on UK racetracks (flat racing and National Hunt racing), in 1997, 1998 and 1999, represented 46% of all reported limb injuries, and subsequent clinical evaluation revealed that these injuries where more common among older horses (Williams *et al*., 2001). Another study gathered information of 1223 horses, that spent 9466 months at risk of injury, and revealed that the SDFT and SL injury rate was accountable for almost two injuries per 100 months of training. Approximately 89% were SDFT injuries, and the remaining injuries were SL injuries (Ely *et al*., 2009).



Tendinopathies occur due to extrinsic or intrinsic factors that negatively affect the tendon structure and tissue. Extrinsic factors include percutaneous trauma, while intrinsic factors are mainly triggered by the strain applied to the tendon (Kümmerle *et al*., 2019). Injuries with an intrinsic origin can occur in two ways, either through a sudden overload of the tendon, or through the progressive degeneration of the tendon tissue (Birch *et al*., 2013). In most cases tendon degeneration precedes any clinical injury, progressively weakening the structure, eventually leading to clinical lesions (R. K. Smith *et al.*, 2002; R. K. Smith *et al*., 1999). Several studies have established that degeneration effectively precedes most clinical injuries (R. K. Smith *et al.*, 2002; R. K. Smith *et al*., 1999).

The degeneration of tendon tissue is usually attributed to cyclical over loading, which leads to cumulative fatigue and microdamage (Kümmerle *et al*., 2019). It is known that tendon degeneration is characterized macroscopically by a change in coloration of the tendon core, along with changes in the matrix itself, such as elevated levels of GAGs, collagen type III fibres and also an increase in cellularity, as well as decreased collagen linked fluorescence, a marker of matrix age (Birch *et al*., 1998) (Figure 6). These changes that come along with tendon degeneration indicate an elevated matrix turnover speed, that can be the result of a failure to establish a good healing response or an inability of cellular response (Thorpe *et al*., 2010) by the resident cell population (Kümmerle *et al*., 2019). High speed locomotion, such as canter and gallop, leads to

high frequency and powerful repetitive strain power imposed on the soft tissues of the palmar surface of the distal limb, mainly the SDFT and the SL (Kümmerle *et al*., 2019; Thorpe *et al*., 2010). These important strain forces can result in localised physical damage to the tendon matrix, rupturing of fibres and crosslinks, affecting matrix proteins, and as a consequence, induce production of proteolytic enzymes, such as MMP-1 (Arnoczky *et al*., 2007; Lavagnino *et al.*, 2006), which on their turn induce secondary damage. The older the animal is, the more pronounced the effects of this damage are (Dudhia *et al*., 2007). Although this damage is localised, it is likely to alter cell matrix interactions (Arnoczky *et al*., 2008) and accumulate more damage, as the tenocytes are unable to achieve a complete repair of the microdamage (C. T. Thorpe *et al*., 2010). The primary effects of physical damage and the secondary damage by proteolytic enzymes can generate cleaved matrix proteins, that can also induce further damage to the matrix. This chain of events can result in a vicious circle of matrix degeneration, induced by exercise (Dakin *et al*., 2014; Johnson *et al*., 2004). Another mechanism that can result from primary physically induced microdamage is the "reduction of loading tenocytes" (Kümmerle *et al*., 2019). When there is microdamage to the tendon tissue the damaged fibrils are unloaded (Lavagnino *et al*., 2006) and as a consequence are shielded from stress. It has been shown *in vitro* that stress deprivation results in elevation of proteolytic enzymes, such as collagenase MMP-13, as well as changes in cell morphology, pericellular environment (Arnoczky *et al*., 2008) and upregulation of protein synthesis in tendon cells (Egerbacher *et al*., 2006). The under stimulated tenocytes react differently to mechanical stimulus, being unable to respond normally to microdamage (Thorpe *et al*., 2010).

Temperature changes in the tendon can also have a negative effect on the tendon matrix. As referred previously, tendons store energy during locomotion, returning most of it as elastic energy to the movement of the limb, but about 7% of this energy is released as heat, elevating temperatures of the tendon core up to 45 degrees Celsius (Goodship *et al*., 1994). Although it has been shown *in vitro* that tenocytes of the SDFT can resist temperatures of as much as 48 degrees Celsius (Birch *et al*., 1997), it may be possible that, *in situ*, the ability of the cells to communicate via their gap junctions renders the tendon cells more susceptible to heat (Burrows *et al*., 2003). Other studies have shown an increase in pro-inflammatory cytokines when equine tenocytes where subjected to temperatures above 45 degrees Celsius (Hosaka *et al*., 2006).

SDFT injury may also be linked with fatigue of the deep digital flexor muscle. This muscle is rich in fast twitch muscle fibres, making it more susceptible to fatigue (Takahashi *et al*., 2014; Valberg, 2008). The DDFT acts to stabilize the metacarpophalangeal joint during locomotion, impeding it from hyperextending, but as the muscle fatigues this stabilization decreases, allowing for an increase in extension of the metacarpophalangeal joint, and as a consequence the associated palmar structures (SDFT and SL) suffer more strain (Butcher *et al*., 2009).

As previously mentioned, weight bearing tendons operate at their functional limits during high speed exercise (Kümmerle *et al*., 2019). Because of this, even small changes in the structural integrity of the tendon tissue, can result in irreversible damage, through physical disruption of the tendon matrix (Roger K.W. Smith, 2010). This disruption can vary from fibrillar slippage to complete separation of tendon tissues, which on its turn is expressed by different degrees of clinical injury. When this occurs, the organism tries to repair the damage in three phases:

1) immediately after the disruption of the tendon matrix an acute inflammatory phase starts off, which usually lasts for a few days. The ultimate onset is an intra-tendinous haemorrhage, followed by infiltration of the injury site by neutrophils, macrophages and monocytes, and subsequent release of proteolytic enzymes. Also blood platelets infiltrate the injury site and release growth factors and endothelial chemo-attractants (Thomopoulos *et al*., 2015; Voleti *et al*., 2012). Macrophages remove cellular debris, while growth factors recruit tenocytes to the wounded area and stimulate them to proliferate (Voleti *et al*., 2012).The inflammatory reaction is designed to remove damaged tendon cells, but it can exceed its effects, further damaging the tendon (Smith, 2010). It has been shown that the modulation of inflammation, through macrophage depletion, in the early stages of inflammation after tendon reconstruction resulted in improved healing (Hays *et al*., 2008). Macrophages have a dual role, as they induce and resolve inflammation, but also facilitate and moderate tendon healing (Thomopoulos *et al*., 2015). It is known that macrophages can behave abnormally, inducing fibrosis, and diminishing tendon structure quality (Nichols *et al*., 2019). Keeping in mind reported results of studies looking into the role of inflammation and immune response in tendon healing and fibrosis, it is important to develop therapies that will moderate this first inflammatory phase and immune reaction, rather than invest in therapies that completely block the inflammatory chain (Nichols *et al*., 2019).

2) The second phase is the fibroblastic phase, also called the subacute phase, and is characterized by strong synthetic activity orchestrated by macrophages and tenocytes (Thomopoulos *et al*., 2015). They induce a profuse angiogenic response, along with fibroblast accumulation (Birch *et al*., 2013). These fibroblasts come from extrinsic and intrinsic sources (Juneja *et al*., 2013) and are different from normal tenocytes, being responsible for the formation of scar tissue, which is also different from normal tendon tissue. It has a higher collagen type III content, is more hydrated, and has more GAGs (Nichols *et al*., 2019; Smith *et al*., 2013). The temporary matrix of collagen type III provides a scaffold for the migration of subsequently recruited cells into the wounded area (Nichols *et al*., 2019).

3) The reparative phase begins one to two months after the onset of the injury and merges with the remodelling phase (Thomopoulos *et al*., 2015). During this phase there is a gradual transformation of collagen type III into collagen type I, as granulation tissue matures into scar tissue (Nichols *et al*., 2019; Williams *et al*., 1980). This transformation is incomplete, because tenocytes in the adult tendon are not able to effectively remodel collagen type III matrix into

collagen type I matrix (Nichols *et al*., 2019). The presence of extrinsic cells is also believed to be a cause for incomplete transformation. Myofibroblastic cells have been detected in scar tissue of naturally occurring superficial digital flexor tendinopathy (Williams *et al*., 1980), and a prolonged activity of this type of cells is linked with fibrosis in organs like kidneys and lungs (Meran *et al*., 2011; Phan, 2002). The new collagen I fibres that are indeed produced, become thicker, the crosslinks that bind them increase in number, and fibre alignment improves. Within and around the tendon there are deposited large amounts of fibrous tissue, enlarging the whole tendon, and making it a stiffer structure (Kümmerle *et al*., 2019). The increase in structural stiffness and persistently deficient structural organization and composition of the matrix has a negative impact on the biomechanics of the tendon. It reduces the efficiency of the spring mechanism, compromising the performance of the horse and adding the risk of re-injury (Crevier-Denoix *et al*., 2010).

The cells that are recruited for repair of damaged tendon come from extrinsic cell populations, originating in the peripheral circulation, as well as neighbouring tissues like the paratendon and tendon sheath. Intrinsic cell populations also contribute to the cellular infiltrate of the lesion. They are derived from tendon parenchyma, epitendon and endotendon (Nichols *et al*., 2019). It is important to know the way these two cell sources impact tendon healing. Extrinsic cells promote the formation of scar tissue and adhesions (Nichols *et al*., 2019), while a cell population dominated by intrinsic cells results in a scarless regenerated tissue (Beredjiklian *et al*., 2003), as shown in foetal tendon healing, where intrinsic healing dominates over extrinsic healing.

The various factors that influence tendon healing have to be well known in order to obtain a therapy for tendon injury that fully regenerates original tendon tissue, restoring its normal biomechanical and functional properties.

2 Current orthopaedic therapies

A brief description of the current therapies that can be applied to SDFT tendonitis and SL desmitis will be provided in this section. Only medical treatment and rehabilitation methods will be described as surgical therapies are beyond the scope of this thesis.

Medical treatment of tendon and ligament injury should start as soon as possible after the lesion occurs in order to prevent overzealous expression of acute swelling and inflammation (Eggleston *et al*., 2020).

This especially applies for suspensory ligament desmitis. The body of this ligament is confined in a narrow canal between McII, McIII and McIV. The rapid resolution of swelling is crucial to prevent further ligament damage and to prevent compression neuropathy of the lateral plantar nerve (C. Gillis, 2011).

2.1 Medical management

Non-steroidal anti-inflammatory drugs are recommended for the treatment of acute stage injury. NSAIDs work through the inhibition of cyclooxygenase (COX) enzymes, promoting analgesic and anti-inflammatory effects (Bentz, 2015). COX enzymes are involved in the conversion of arachidonic acid into important elements of inflammation, prostaglandins and thromboxanes (Baxter, 2011), so their inhibition will prevent the synthesis of these inflammatory factors.

Different NSAIDs seem to behave differently depending on the injured system. While flunixin meglumine is the strongest NSAID to alleviate visceral pain in the horse with colic, phenylbutazone is considered the best NSAID specifically for musculoskeletal lesions (Kirker-Head *et al*., 2013).

Beside systemic administration of NSAIDs, also topical application of NSAIDs can be considered, such as diclofenac liposomal cream. When applied, this drug penetrates the skin and has direct local anti-inflammatory effects through COX enzyme inhibition and analgesic effects through its capacity to block sodium channels of noci-receptive afferent fibres (Nair *et al*., 2010).

Corticosteroids are an option during the first 24 to 48h after lesion induction, however beyond this time window their use is not recommended as they can inhibit fibroplasia and impede the normal healing of the tendon as a consequence (Kümmerle *et al*., 2019). There are scientific sources that advise against the topical use of perilesional dexamethasone during the acute phase, as it may delay collagen formation. Other sources indicate that the histological appearance of equine tendon injected with corticosteroids resembles the histological image of tendinopathy (McIlwraith, 2010). Depot corticosteroids like methylprednisolone should not be administered directly in the tendon or ligament, because of the risk of dystrophic mineralization and tissue necrosis, caused by the carrier of the drug (Smith, 2007).

Polysulfated glycosaminoglycan (PSGAG) can be considered as a soft-tissue anti-inflammatory agent (Kümmerle *et al*., 2019) because of its inhibitory effect on enzymes such as collagenases and metalloproteinases, as well as inhibiting macrophage activation and promoting a suppression of inflammation (Moraes *et al*., 2009). These properties make it an adequate product to be used in the acute stage of injury (Dowling *et al*., 2000). Although it has no effect on the synthesis of fibroblasts (Kümmerle *et al*., 2019) PSGAG has an effect on the metabolism of tenocytes and fibroblasts, which consequently influences the production of collagen and non-collagen proteins (Moraes *et al*., 2009). Despite these known characteristics of PSGAGs, a long-term clinical study has found no difference in the recurrent injury rate of horses whose tendons were treated with systemic or intralesional PSGAGs, compared to horses that were treated with controlled exercise alone (Dyson, 2010; Dyson, 1997).

A recommended treatment plan with PSGAGs consists of the intramuscular administration of 500mg of this drug, every four days for a total of seven treatments (Gillis, 2011).

Hyaluronan is a natural component of tendon matrix. It has a direct influence on the formation of collagen fibrils and their aggregation, as it stimulates synthesis of collagen type I (Ross *et al*., 2011). When administered intrathecally hyaluronan may diminish the formation of adhesions during tendon repair, along with reducing the inflammatory cell infiltrate as well as the intra-tendinous haemorrhage (Kümmerle *et al*., 2019).

2.2 Physical Therapies

Before medical treatment is begun there are basic procedures that can diminish the progress of inflammation and further damage to the tendon. Cold therapy can be installed in the acute phase of the inflammation, as it has an immediate anti-inflammatory and analgesic effect. Cold temperatures promote vasoconstriction, reduce both enzymatic activity and synthesis of inflammatory mediators, and diminishes nerve conduction (Petrov, Hoogmoed, 2003).

Bandaging is useful to reduce the oedema that forms in the surrounding tissue following tendon injury (C. Gillis, 2011). It may also assist healing as stimulation of mechanoreceptors occurs (Baxter, 2011).

Foot conformation and shoeing can help diminish the strains applied to the affected tendon. To reduce strain on the SL and SDFT it is important to have a straight pastern/hoof axis, putting the metacarpophalangeal joint in a normal position. Elevation of the heel is contraindicated as it increases strains on the SL. It is best to shorten the toe and increase the hoof angle (Dyson *et al*., 2010). If the horse doesn't have enough support from his foot shape, an egg bar shoe can be used (Gillis, 2011). It can also be helpful to raise the toe or apply a shoe with a wider width in the toe region, impeding the toe from sinking too much in soft terrain, this way transferring the load from the SL and SDFT to the DDFT (Kümmerle *et al*., 2019).

Several different physical techniques for the treatment of tendon disorders have been developed. Modalities such as extracorporeal shockwave therapy and laser therapy claim to stimulate the regeneration process, through reduction of inflammation and swelling, increase in blood circulation and alleviation of pain (Bergh, 2011).

Extracorporeal shockwave therapy works through the transmission of shockwaves into the tendon tissue. The exact effect that these waves produce is unclear, but it is most likely that they induce analgesia and decreasing nerve conduction properties in sensory nerves (Bolt *et al*., 2004). It has been noticed that in normal tissue the shockwaves can be deleterious, but in diseased tendon the induction of tendon matrix disorganization can be a stimulus for repair in chronic injuries (Bosch *et al*., 2007). A study investigating the use of extracorporeal shockwave as a therapy for chronic SL desmopathy concluded that 41% of the treated patients resumed full work six months after the lesion was diagnosed (Crowe *et al*., 2004). This is a positive result when compared to studies that

showed a 13% return to full work in the same time frame when the patients were treated conservatively (Dyson, 1994).

Low-power laser therapy has been shown to promote cellular metabolism, besides enhancing fibroblast proliferation and collagen synthesis *in vitro* (Henninger, 1994).

The physiological effects of laser therapy include anti-inflammatory effects, more organized fibroblasts, increased cellularity, increased collagen formation, vasodilation, increased concentration of capillaries, DNA synthesis, RNA production and pain reduction through the influence of the pain gate and nerve velocity (Bergh, 2011).

The variety in treatment protocols of laser therapy makes it difficult to show consensual results between different studies (Schindl *et al*., 2000). There are studies that report positive results when infrared laser was applied in tendonitis cases. While other investigations suggest negative results regarding tendon healing in horses (Bergh, 2011). The ambiguous results that are associated with low-power laser may be related to the insufficient depth of penetration (Ryan *et al*., 2007). In recent years veterinary medicine has been adopting high-power laser therapy from human medicine, hoping for more consistent results in the treatment of soft-tissue orthopaedic injuries. A recent study with 150 horses showed that this therapy was both safe and efficacious, with significant improvement in lameness scores and low re-injury rates (Pluim *et al*., 2018).

Stall confinement is mandatory during the rehabilitation phase. It is important to control the exercise the horses undergo, as excessive exercise can further damage the tendon and impede its normal healing. Box rest combined with controlled walking exercise for two months is normally recommended. Exercise helps resolve residual inflammation, maintain gliding function, and promote optimal collagen remodelling (Gillis, 1997; Smith, 2007). During this exercise program it is important to make serial ultrasonographic evaluations to control the tendon healing and eventually detect any setbacks (Eggleston, 2020) .

3 Regenerative medicine

None of the accepted treatment methods has been consistently better than any other for the long-term return to athletic activity without re-injury. After tendon injury, extensive scar tissue replaces the original tendon tissue, which means that the new tendon structure is architecturally and functionally inferior to the original structure. Regenerative medicine aims to overcome this obstacle in returning full normal function to the tendon, while also diminishing the risk of re-injury. The ideal regenerative therapy “avoids the formation of excessive fibrous tissue and is able to regenerate normal tendon matrix”. It tries to replicate the events that occur during tissue development, allowing spatial and temporal interaction between three main factors: 1) scaffold 2) growth factors 3) cells (Smith, 2008; Smith, 2020). These three factors can be applied alone but ideally in combination with each other, along with mechanical stimulation, that is delivered through rehabilitation programs.

3.1 PRP

Platelet rich plasma has been emerging in recent years as a promising treatment for tendon and ligament injuries. It is an autologous whole blood product, that contains two to four times the concentration of platelets that are present in normal venous blood (Fortier, 2010; Smith, 2007). Platelets are one of the main sources of growth factors and bioactive proteins such as cytokines and chemokines. When delivered at the injury site, platelets contact the exposed basement membrane, which stimulates the platelets to aggregate and degranulate, releasing the anabolic bioactive substances, that promote tissue repair, regulate inflammation, and stimulate recruitment of stem cells (Fortier, 2010). The growth factors released by platelets, after the degranulation of alpha-granules, include platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-beta), vascular endothelial growth factor (VEGF), and insulin like growth factor one (IGF 1) (Koch *et al*., 2019). All these growth factors are protein signalling molecules, that regulate cellular metabolism, stimulating cell proliferation, increasing cellular matrix synthesis, promoting vascular in-growth, while also downregulating catabolic matrix degrading cytokines (interleukin and matrix metalloproteinases for example). The aggregation of platelets results in the formation of a platelet clot, a fibrin scaffold that allows for cellular migration into the injury and retains the growth factors at the injury site. (Foster *et al*., 2009).

PRP can be prepared either by centrifugation or filtration by gravity of the autologous blood (platelets are smaller and less dense than erythrocytes and leukocytes), but it is highly variable between individuals and preparatory techniques. There are several commercial systems, so there will be important variations between the different preparations, whether in platelet and leukocyte concentration, whether in fibrin architecture. Some authors believe that leukocyte poor PRP is preferential, as white blood cells may induce further inflammation through the release of inflammatory cytokines (Fortier, 2010).

PRP has advantages in its ease of use, the fact that it administrates autologous peptides (diminishing the risk for immune reactions), along with a combination of growth factors that help the natural healing process, and it has a low cost when compared to stem cells (Ortved, 2018).

The disadvantages of PRP lye with the lack of stem cells within the preparations, and the variability between the various products that are available, both in platelet concentration and residual leukocyte content (Fortier, 2010; Middleton *et al*., 2012).

Platelet concentrations are positively correlated with growth factor concentrations, as well as with tendon and ligament matrix gene expression. More platelets, more growth factors, more extracellular matrix expression, more collagen type one cartilageoligomeric matrixprotein.

More white blood cells mean more collagen type three, associated with scar tissue (Ortved, 2018).

The optimal time for PRP treatment after lesion development has yet to be determined, but it has been accepted that the acute phase of inflammation is an adequate timing for the injection of

PRP, due to the anti-inflammatory factors present in the solution (Ortved, 2018). It has been shown that tendons treated with PRP seven days after SDFT lesion onset, had earlier improvement of ultrasonographic parameters, than tendons treated 14 days after injury (Fonseca *et al*., 2014).

3.2 Mesenchymal stem cells

3.2.1 Historical perspective

In the 1890's scientists proposed the existence of a cell which originated in the bone marrow and migrated along the blood stream, to injury sites across the organism, in order to participate in the tissue regeneration (Bianco, 2009). This was the first time a concept of mesenchymal stem cells was introduced, but only in the 1970s the first breakthrough was made in this field, as Friedenstein cultured bone forming cells from guinea-pigs (Friedenstein *et al*., 1970), characterising them subsequently as a minor subpopulation of marrow derived plastic-adherent cells, with osteogenic, chondrogenic and hematopoietic supportive potential (Friedenstein *et al*., 1974), challenging the previous theories that assumed a hematopoietic origin of these cells. Subsequent studies established that these cells could be isolated by plastic adherence, and that they could form osteoblasts, chondrocytes, adipocytes and myoblasts (Friedenstein *et al*., 1987; Owen *et al*., 1988; Piersma *et al*., 1985). In 1992 the first isolation and culture of human MSCs was reported by Caplan and Haynesworth (Haynesworth *et al*., 1992), who eventually went on to implant these cells in humans in 1993 (Lazarus *et al*., 1995). Arnold Caplan was also the one who introduced the term "mesenchymal stem cells", comparing these cells with the stem cells who originate from the mesodermal tissues of the embryo (Caplan, 1991). Later in the 90's the ligands of the SH-2 and SH-3 antibodies were described as CD105 and CD73 respectively (Barry *et al*., 2001; Barry *et al*., 1999).

From here on it became possible to characterize MSCs according to their ability to adhere to plastic, the expression of cell surface markers, and their capacity to give rise to mesodermal cell lineages in vitro.

These were the pioneers of the field of MSCs, soon to be followed by equally important pioneers of equine MSCs. Fortier and her team were able to isolate and cultivate for the first time equine bone stem cells from bone marrow aspirate, in 1998 (Fortier *et al*., 1998). And in 2003 Smith was able to implant autologous, in vitro expanded, bone marrow MSCs, into a damaged SDFT (Smith *et al*., 2003), laying the foundation for the future of this regenerative therapy in equine medicine.

3.2.2 Definition

Stem cells are commonly defined as being undifferentiated cells that are able to self-renew and differentiate into different cell types (Ortved, 2018), as illustrated by the mesogenic process. Mesenchymal stem cells (MSCs) specifically are multipotent cells that originate in the mesoderm (Ortved, 2018). In order to have an uniform concept of mesenchymal stem cells, the International Society for Cellular Therapy (ISCT) has established three criteria by which MSCs are

recognisable: 1) adherence to plastic 2) specific surface antigen expression 3) multipotent differentiation potential (Dominici *et al*., 2006).

These definitions imply that the cells that are used in regenerative medicine are able to differentiate into the predominant cell type of the injured tissue (Caplan, 2017), but this is not the true action of the infused cells in the lesions. Instead they act as carriers of paracrine factors that have an immunomodulatory effect (Bruno *et al*., 2015). As such, there have been advocates to change the name of Mesenchymal Stem Cells, into Medical Signalling Cells, or Mesenchymal Stromal Cells (Horwitz *et al*., 2005; Caplan, 2017), maintaining the MSC acronym, but eliminating the term “stem”, that induced an erroneous thought about the properties of these cells.

3.2.3 Mechanisms of action

The original though behind the therapeutic effect of MSCs was that these cells would directly replace the damaged tissues by differentiating into the predominant cell type at the injury site and multiplying subsequently. It is now known that this is not the main therapeutic effect of MSCs (Caplan, 2017; Ortved, 2018). The infused cells exert an immunomodulatory effect at the injury site, through paracrine action (Ortved, 2018). This paracrine action is carried out by a group of molecules and factors that MSCs secrete, called “the secretome” (Al Naem *et al*., 2019). The secretome is composed of growth factors, cytokines, soluble proteins, free nucleic acid, lipids, and extracellular vesicles (Al Naem *et al*., 2019). The growth factors include transforming growth factor beta (TGF-beta), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), interleukin 10, interleukin one receptor antagonist, interleukin six, human leukocyte antigen G, leukocyte inhibitory factor, indoleamine two, three-dioxygenase, nitric oxide, galectines one and nine, tumour necrotic factor alpha stimulated gene six (Pittenger *et al*., 2019) (Figure 7). The extracellular vesicles are made up of a lipidic bilayer, that encloses various cytoplasmic components, like regulatory miRNA, DNA, structural and functional proteins, cytokines, growth factors and signalling lipids (Bruno *et al*., 2015; Al Naem *et al*., 2019). All these secreted factors have individual effects on the immune answer of the host, but the synergistic action makes the immunomodulation completer and more effective (Ortved, 2018).

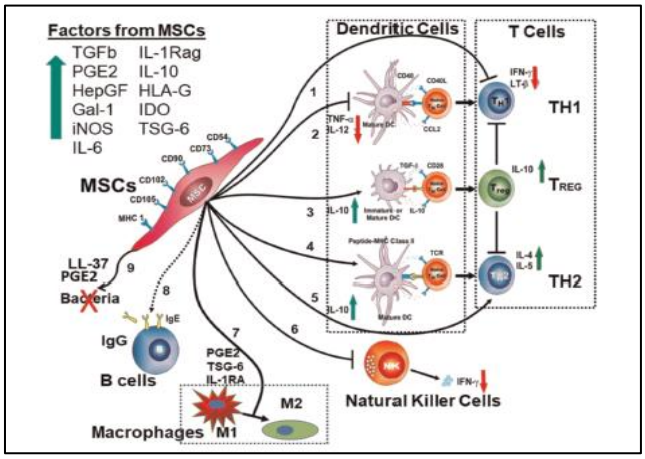


Figure 7 - MSC-immune cell interactions (Pittenger, 2019)

There is a differential effect on the maturing immune cell populations that are present at the injury site. The natural killer cells, the dentritic DC1 cells and the proinflammatory TH1 cells are inhibited, while the dentritic DC2 cells, the anti-inflammatory TH2 cells and the regulatory T cells are upregulated (Bruno *et al*., 2015; Pittenger *et al*., 2019).

The anti-proliferative action of MSCs is dependent on cell-to-cell contact, secreted factors and the factors that are carried by the extracellular vesicles, as this is the way MSCs communicate with neighboring cells (Bruno *et al*., 2015; Al Naem *et al*., 2019).

Whatever the origin of the stem cells is, they always have an important role in the maintenance of cellular homeostasis, as well as in tissue regeneration. This is the basis of the therapeutic effect of MSCs (Al Naem *et al*., 2019). MSCs promote normal healing instead of scarring (Carrade *et al*., 2012).

It has been suggested that MSCs may differentiate into two different types, according to the environment they are exposed to. Toll-like receptor three (TLR3) priming would induce the differentiation into an anti-inflammatory phenotype (MSC type two), while TLR4 priming conditions would shift MSCs into a pro-inflammatory phenotype (MSC type1). MSC type one would then activate the innate and adaptive immune system elements (eg. M1 macrophages and T lymphocytes), and type two MSCs would promote immunomodulatory and trophic activities after the activation of M2 macrophages and secretion of specific mediators. This means that type one MSCs may contribute to the first reparative action against tissue injury, and type two MSCs contribute in a later phase through a regenerative response (Somoza *et al*., 2015) (Figure 8).

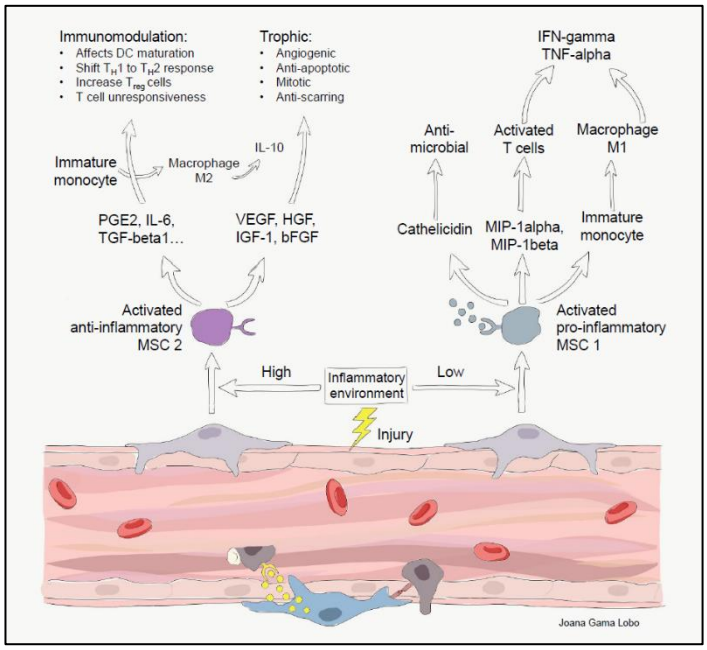


Figure 8 - MSC secreted factors and mechanism of action in vivo (adapted from Somoza et al*., 2015)*

Another interesting in vitro finding showed that inflammation causes downregulation of stem cell migration related genes and increases gene expression of cellular adhesion. This may explain the propensity of MSCs to localise to sites of inflammation (Barrachina *et al*., 2016).

3.2.4 Sources

MSCs can be isolated from virtually every vascularized tissue, due to their close relationship to pericytes (Esteves *et al*., 2017; Pittenger *et al*., 2019; Gomez-Salazar *et al*., 2020). In fact, there are studies that have showed a correlation between stem cell yields and vascular density in adipose tissue (Da Silva Meirelles *et al*., 2009). At the moment, various tissues from the equine

organism have been used to isolate MSCs: bone marrow, adipose tissue (Romero *et al*., 2017; Vidal *et al*., 2012), embryonic tissue (Guest *et al*., 2010), synovial fluid and membrane, umbilical cord (Carrade *et al*., 2011), peripheral blood (Carvalho *et al*., 2013), periosteum, muscle (Radtke *et al*., 2013), dental pulp, periodontal ligament (Mensing *et al*., 2011), endometrium (Rink *et al*., 2017), and even hair follicles (Michler *et al*., 2017) (Figure 9).

The main sources that have been clinically used to harvest MSCs have been bone marrow and adipose tissue. These options will be explored in detail in the following paragraphs.

3.2.4.1 Bone marrow derived MSCs

The cancellous portions of bones contain bone marrow, a semi-solid tissue that gives origin to a population of stromal, fibroblast like, cells. A subpopulation of these cells, the MSCs, can be found on the endosteal surface of the marrow space (Alves *et al*., 2011; Stewart *et al*.., 2011). The MSCs found within bone marrow can be harvested through aspiration from the tuber coxae or the sternum (Arnhold *et al*., 2007; Goodrich *et al*., 2008; Kasashima *et al*., 2011). Usually the first 5ml of the aspirate contain the most MSCs, but in practice 10 to 20ml are collected (Kasashima *et al*., 2011; Stewart *et al*., 2011).

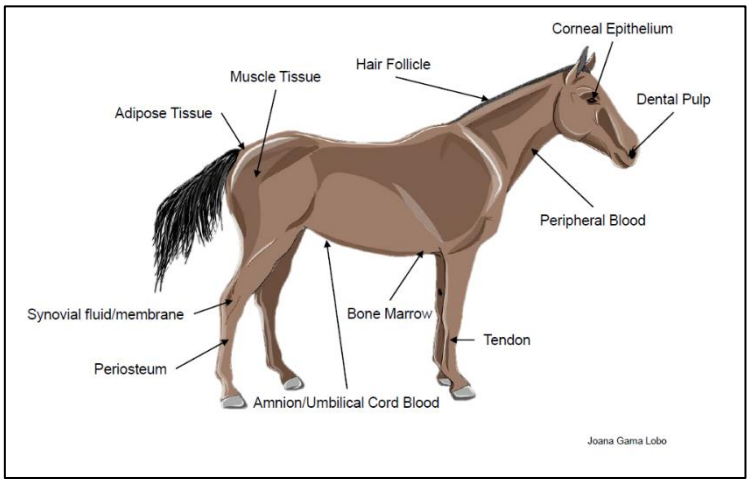


Figure 9 - Undifferentiated MSC sources in the horse (adapted from Gugjoo et al*., 2019)*

The sample is collected into a heparinized container (Alves *et al*., 2011), and isolated in the laboratory. There are various isolation procedures for BM-MSCs, but the most convenient are the ones that are based on the plastic adherence characteristics of MSCs. With this protocol, the fraction of the BM aspirate, rich in mononuclear cells, is placed on plastic culture dishes and left to adhere for five days. When the five days are over, the cells that did not adhere, mostly hematopoietic cells, are discarded. The adherent cells that remain are further cultured for 14 days, after which they are detached through trypsinization (Al Naem *et al*., 2019; Stewart *et al*., 2011). Another method of isolation of BM-MSCs uses density-gradient centrifugation to separate initially the mononuclear cells from the hematopoietic cells. This is achieved using a Ficoll-gradient emulsification of the bone marrow sample. The mononuclear cell fraction is cultured afterwards at low densities, allowing the plastic adherent cells to form colonies, after two to three weeks, that derive from MSCs in a ratio of 1:1 (Pacini *et al*., 2007; Schnabel *et al*., 2014). Another density-gradient centrifugation method uses a Percoll colloidal solution that consists of silica particles (Bourzac *et al*., 2010). The Ficoll method has a significant downside however, as it depletes highly

regenerative cells and impairs cell function, as it decreases the expression of chemokine receptors (Pösel *et al*., 2012). Beside this downside, density gradient centrifugation is also a lengthy procedure, that only allows for the recovery of 15 to 30% of the initial stem cell population (Al Naem *et al*., 2019).

If the cultured cells are for autologous use, they are shipped back to the veterinarian after isolation (3 weeks), usually at a concentration of about 10 x 10^6 cells, or 50 x 10^6 when the defect is very large. The cells may be suspended in citrated bone marrow supernatant in order to supplement the MSCs with the growth factors present in the supernatant (Alves *et al*., 2011).

3.2.4.2 Adipose tissue derived MSCs

Adipose tissue originates in the mesoderm, contains adipocytes and a fraction of stromal cells that include vascular smooth muscle cells, endothelial cells, fibroblasts, monocytes, macrophages, pre-adipocyte lymphocyte and AD-MSCs (Miana *et al*., 2018). These last cells, the AD-MSCs appear to be almost indistinguishable from BM-MSCs, are easier to collect, and have high initial cell yields, making them a good alternative for BM-MSCs, although some studies have shown that they do not differentiate so well into specific cell lineages (Vidal *et al*., 2007; Toupadakis *et al*., 2010; Alves *et al*., 2011). The most important argument to support this, is the fact that they show an inferior capacity to generate osseous or cartilaginous tissues when compared to BM-MSCs or other MSCs (Stewart *et al*., 2011). This lack of differentiation capacity may be compensated by the potent immunomodulatory effects that these cells appear to have, as demonstrated in studies with immune mediated arthritis (Gonzalez-Rey *et al*., 2010) and in vitro lymphocyte activation assays (Bochev *et al*., 2008). Samples may be collected from various sites, (Alves *et al*., 2011), but the most used areas are the head of the tail and supragluteal region (Al Naem *et al*., 2019). Usually five to 10ml of adipose tissue is collected (Alves *et al*., 2011), this sample is then digested using a enzymatic collagenase solution, over three to 18h, depending on the needs (Al Naem *et al*., 2019). After this, the mononuclear cell fraction is separated and concentrated, being available for direct administration or to be further

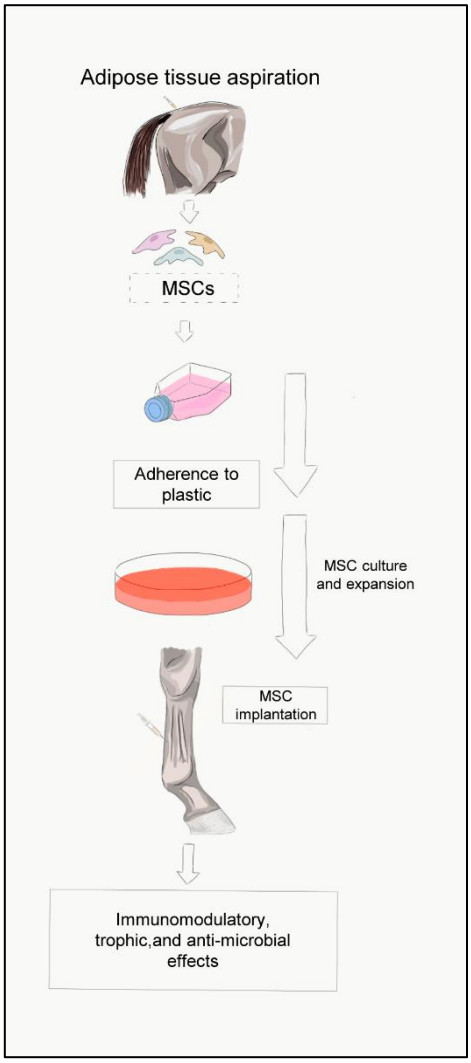


Figure 10 - The process of MSC therapy (adapted from Somoza et al*., 2015)*

cultured. If they are further cultured and expanded a similar process to the one used with BM-MSCs is applied (Alves *et al*., 2011) (Figure 10). If the cells are directly administered to the patient without further culture they are referred to as the stromal vascular fraction (SVF). They are not pure MSCs, instead it is a mixed population composed of endothelial cells, preadipocytes and MSCs (Vidal *et al*., 2007).

3.2.5 Autologous vs Allogenic

Initially MSC therapies used mainly autologous cells. However, this approach entailed several disadvantages such as the time frame between the diagnosis of the lesion, harvesting the cells and finally administrating the MSCs. It normally takes up to three weeks to culture and expand the cells in the laboratory, which is not ideal in a great deal of cases. Besides this big disadvantage, it has been shown that age and health of the donor horse influences the beneficial properties of the MSCs. So, an allogenic donor might provide better quality MSCs when compared to the autologous MSCs. Donors of younger ages appear to have cells with the best healing properties like an increased proliferative activity, a better differentiation ability and a different gene expression when compared to donors of an older age (Colbath *et al*., 2020; Khong *et al*., 2019; Myneni *et al*., 2019). Keeping in mind all the aforementioned, there has been an increasing interest towards allogenic MSCs, an “off the shelf” product, that can be administered right at the time of the diagnosis of the lesion. The MSCs with an allogenic donor also have the advantage to be further cultured and expanded beyond the three week time frame, making it possible to obtain well characterised cell lines, which allows for a more predictive outcome, as the administered cells are from a more uniform population (Colbath *et al*., 2020).

The main concern with respect to allogenic MSCs is the safety of their repetitive administration. Introducing biological material derived from a different individual always poses a risk for immunological reactions to foreign MHC antigens (Colbath *et al*., 2020). To address this issue several studies have been performed. In vitro studies, using mixed leucocyte reactions, showed a significant decrease in lymphocyte proliferation (Carrade *et al*., 2012; Colbath *et al*., 2017; Holt *et al*., 2014; Paterson *et al*., 2014), no difference in immune suppressive ability between allogenic and autologous MSCs (Colbath *et al*., 2017), and BM-MSCs of a mismatched haplotype were able to significantly reduce lymphocyte proliferation (Ranera *et al*., 2016). In vivo studies are less abundant but showed positive results. When administrated intravenously and subcutaneously there was little evidence for adverse reactions (Kol *et al*., 2015;Williams *et al*., 2016), and the same occurred when allogenic MSCs where administrated intratendinously in healthy tendons (Guest *et al*., 2008; Souza *et al*., 2018). (Guest *et al*., 2008; Souza *et al*., 2018).

The reduced evidence for immune responses to allogenic MSCs may be related to the inherent immune suppressive characteristics and low level of MHC expression by MSCs (Barberini *et al*., 2014; Holt *et al*., 2014; Tessier *et al*., 2015).

3.2.6 Administration

MSCs for treatment of tendinoligamentous lesions are ideally administerd intratendinously, within the lesion itself if it is a contained lesion. Frequently the injection is done blindly, but the best result is obtained when ultrasonographic guidance is used. The horse should be sedated, and local analgesia applied, the area should be aseptically prepared as well (Kümmerle *et al*., 2019). It is important to use a large gauge needle to minimize damage to the cells that are administrated, preferably with a needle gauge above 20G (Garvican *et al*., 2014; Lang *et al*., 2017)**.** Intralesional therapy should not be started before three days after lesion onset, because of risk for increasing the haemorrhage. Large volumes should be avoided as they can be damaging to the healing of the tendon (van den Belt *et al*., 1993). The most common concentration for administration is 10 x 10^6 cells, although this isn't a consensual dosing (Ortved, 2018), in fact, there is yet to be determined a consensual dosing of MSCs in the treatment of tendinoligamentous lesions. An optimal timing for MSCs therapy is also yet to be determined. Some authors have the opinion that the administration of MSCs could be done right at the inflammatory stage, as the MSCs have immunomodulatory effects, reducing inflammation and stimulating local stem cells and tenoblasts (Docheva *et al*., 2015).

4 Objective of the study

An increasing clinical interest is developing around regenerative therapies in equine practice, especially around mesenchymal stem cell therapies. The most common use of MSCs in equine practice is the treatment of tendon injuries (Koch *et al*., 2019). Studies regarding the use of MSCs in equine tendinopathy and desmitis indicate that this regenerative therapy has a positive effect on this type of injuries. A study made in 2008 noted histologic improvement of tendon structure and cellular composition after the administration of AD-MSCs on collagenase induced tendonitis (Nixon *et al*., 2008). Studies that used bone-marrow derived mesenchymal stem cells also had good results. In a small study of 11 horses with naturally occurring SDF tendinopathy, 9 recovered completely and returned to competition, through the treatment with BM-MSCs (Pacini *et al*., 2007). While another study regarding similar lesions, that were treated with BM-MSCs as well, demonstrated a re-injury rate of 27,4% (Godwin *et al*., 2012). A larger study, that encompassed various tendon and ligament disorders, including SDF tendinopathy and SL desmitis, and used allogenic equine mesenchymal stem cells derived from umbilical cord blood, observed that 77% of the treated horses returned to work (Van Loon *et al*., 2014). And another study with allogenic AD-MSCs in combination with PRP, used to treat 19 horses affected by SDF tendinopathy, observed that 24 months post-treatment 89,5% of the horses returned to their previous level of competition, and 10,5% had re-injury (Ricco *et al*., 2013).

This study tries to show the efficiency of MSCs for this type of injuries, comparing two different cell sources. It also tries to show in which conditions stem cells are best used, relative to structure, limb, chronicity and discipline or breed. The ultimate objective is to add information to the previous

studies that were done regarding this subject, hopefully contributing to a better understanding of this novel treatment.

5 Material and methods

5.1 Study population

The study population was obtained through the analysis and retrieval of cases treated with mesenchymal stem cells at “Dierenkliniek Wolvega” (DKW). These clinical cases were obtained through the veterinary management software (Animana®). From all 209 cases collected the ones chosen for the present study complied to the following criteria: SDFT or SL lesions, with no other lesions present at the moment of treatment; lesions treated solely with SVF+PRP or BM-MSCs; sufficient information available on Animana®.

The information recorded encompassed: gender, age, discipline, affected structure, limb, lesion, chronicity of the lesion, other treatments, type of MSCs used, lameness at zero, six and 12 months, ultrasonographic examination at zero, six and 12 months, if there was re-injury at six or 12 months, and work level after 12 months. The information that was not possible to be retrieved through clinical information that was available was collected through telephonic enquiry and enquiry of the treating veterinarians.

Lameness

Lameness was evaluated according to the AAEP lameness scale (0-5): grade 0 – sound horse with no lameness perceptible; grade 1 – the horse shows an inconsistent and difficult to observe lameness, independent of the circumstances; grade 2 – lameness continues to be difficult to detect at walk and trot in a straight line, but becomes more apparent under certain circumstances, like trotting in a circle, inclines, etc; grade 3 – the horse shows lameness consistently at a trot, in all circumstances; grade 4 – the horse is visibly lame at a walk; grade 5 – the horse has minimal weight bearing on the affected limb (Baxter *et al*., 2020). The evaluation of the lameness was made by the treating veterinarians at the moment of diagnosis (zero months), six months after treatment and 12 months after treatment.

Lesion category

Ultrasonographic evaluations from each horse were retrospectively obtained from the records of each veterinarian. These observations were categorized in a scaled based on the ultrasonographic score described by Rantanen (Rantanen *et al*., 2010). This score was modified, and it was determined that scores from one to three were clinically irrelevant, while scores from four to six were clinically relevant. Reducing the categories from six to two was useful to simplify the statistical analysis and making an easier appreciation of the clinical evolution of the patients.

Follow-up

Follow up information was obtained from the records of the treating veterinarians in Animana®, together with information obtained directly from the veterinarians, and telephonic enquiries made with the owners.

Information gathered included re-injury (yes or no), work level (lower, same, or higher), reasons for eventual lower work level (original lesion, another orthopaedic problem, or other issues) and lameness (AAEP scale for veterinarians, and observable lameness or not, for owners.

Re-injuries included lesions on the same limb and lesions on the contralateral limb, as strain injuries have a bilateral component.

5.2 Ultrasonographic evaluation

Ultrasonographic examinations where evaluated and categorized according to the classification proposed by Rantanen (Rantanen *et al*., 2010). This classification takes in account quantitative measurements (size, shape, echogenicity, fiber pattern and surrounding inflammatory reaction), along with clinical presentation of each individual. The lesion classification by Rantanen is made up of six different categories:

> *Category I* – Category I implies that no quantitative or qualitative abnormalities are detected on ultrasound examination;
>
> *Category II* – No hypoechoic or anechoic lesions are detected, but the tendon cross sectional area is slightly enlarged, due to unusual development, a new low-grade tendonitis/desmitis, or an old tendon/ligament injury that healed and is now stable;
>
> *Category III* – Small hypoechoic lesions are present, and the structure's cross-sectional area may be enlarged, or not. Category III lesions may be an incidental finding, a minimal tendon/ligament injury, or a healed and clinically stable tendon/ligament injury. To determine their clinical relevance these findings have to be evaluated along with clinical history, physical examination and serial monitoring;
>
> *Category IV* – Characterized by focal hypoechoic or anechoic lesions, and the tendon/ligament may, or may not, be substantially enlarged in its cross-sectional area. Associated with slight injury or an important compromise of tendon/ligament fiber bundles;
>
> *Category V* – Injuries included in this category are characterized by a substantial enlargement of the cross-sectional area of the tendon/ligament, accompanied by focal hypoechoic or anechoic lesions. Normally associated with moderate tendon/ligament lesions, horses may exhibit lameness from this category forward;

Category VI – This is the most severe lesion category. Ultrasonographic examinations may show extensive hypoechoic or anechoic lesions, along with a substantially enlarged cross-sectional area (Rantanen *et al*., 2010).

In order to simplify the statistical analysis of the ultrasonographic scores attributed to each lesion these six categories were gathered into two different classes of lesions:

Class 1 – includes category I, II and III, making up all the lesions that had no clinical significance at the time of examination;

Class 2 – includes category IV. V and VI, gathering all the lesions that had clinical relevance at the time of examination.

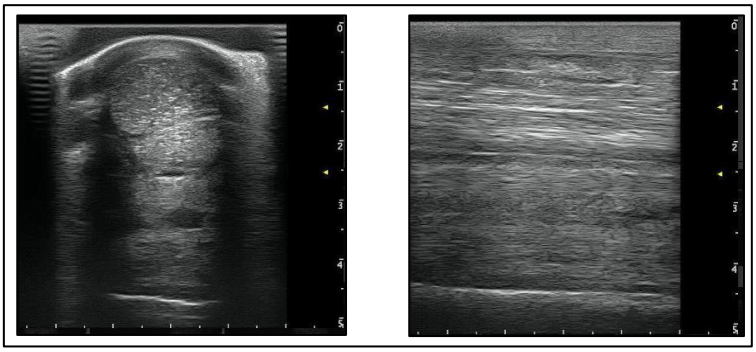


Figure 11 - Class 1 lesion. The CSA is close to normal and the fibers have a good alignment.

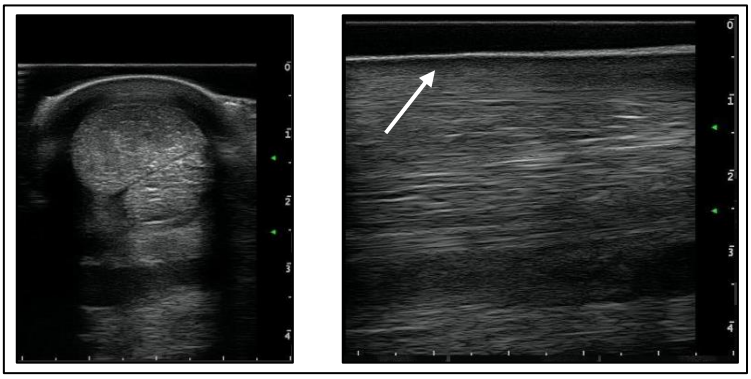


Figure 12 - Class 2 lesion. SDFT lesion with enlarged CSA and fiber alignment disruption (arrow).

5.3 MSC treatment protocol

Two different MSC protocols were used in the time this retrospective study covers. Between 2013 and 2017 the clinic used Stromal Vascular Fraction in combination with Platelet Rich Plasma (Medivet®), and between 2017 and 2019 Bone-Marrow derived Mesenchymal Stem Cells were used. The first product was an autologous preparation, obtained through liposuction, from the tail base, of the patient itself. Around 20 to 40 grams of fat were collected, which would be prepared through enzymatic neutralization. This process separated the mature adipocytes from the cellular fraction. The cellular fraction is a primary, heterogenous, mesenchymal cell suspension (Brown *et al*., 2019). At the same time PRP would be prepared from a blood sample collected from the patient as well. The PRP was produced using the commercial system made available by Medivet®.

The cells used between 2017 and 2019 were obtained from the bone marrow of donor horses at the National Research Institute of Animal Production, in Balice, Poland. These allogenic MSCs were isolated from bone marrow aspirates from the iliac crests of the donor horses. The isolation was done through the Ficoll-Paque centrifugation protocol, and the resulting cells were cultured

on Dulbecco's Modified Eagle's Medium, after which they were digested into single cell suspension using a tripsin solution, and frozen (Opiela *et al*., 2013).

5.4 Administration

Before administration of the MSCs the injection site was clipped and prepared with iodopovidone and alcohol. With ultrasound guidance the MSCs were injected intra-tendinously. Core lesions were treated with a single intralesional injection, while diffuse lesions were treated with multiple injections in the tendon. Around four mL of MSC suspension was administered, which was aspirated using a 19G needle, and injected with a two and a half cm 20G hypodermic needle. No standard exercise program was applied, as these programs were adapted to the horse and the nature of its lesion.

5.5 Statistical analysis

Pearson's Chi-square test was used to assess the effect of age, breed, discipline, lesion chronicity, structure, and type of MSCs used on the outcome. P values <0.05 were taken to be significant.

6 Results

Population characteristics

The studied population (*n*=49) had a median age of 9,47 years at the time of the treatment, and was mainly made up of warmblood horses (*n*=17; 34,6%), but also Friesian horses (*n*=12; 24,4%) and Standardbred horses (*n*=14; 28,5%). Other breeds included Haflingers, Quarter-Horses, Pura Raza Española, and Icelandic (*n*=7; 14,3%). The horses were particularly active in dressage (*n*=18; 36,7%), and harness racing (*n*=14; 28,5%), along with jumping (*n*=7; 14,3%), and leisure activities (*n*=11; 22,4%). The population had an almost even distribution of lesions with SDFT lesions representing 48,9% (*n*=24) and SL lesions 53% (*n*=26). Most of these lesions were between two and four weeks old (*n*=23; 46,9%), but there were also injuries with less than two weeks (*n*=13; 26,5%) and more than four weeks (*n*=14; 28,5%). Front limbs were the most affected limbs (*n*=35; 71,4%), while hindlimbs had a smaller representation (*n*=15; 30%). The MSCs used had an almost even distribution, with SVF+PRP treated horses representing 53% (*n*=26) of the population, and BM-MSC treated horses representing 48,9% (n=24).

6.1 Overall population

Lameness

Six months after treatment 85,7% (*n*=42) of the horses did not show any signs of lameness, what meant an improvement relative to the 51% (*n*=25) of horses that did not show signs of lameness at the moment of treatment. But after 12 months the percentage of horses that had no signs of lameness decreased to 84,4% (*n*=38) (Figure 13).

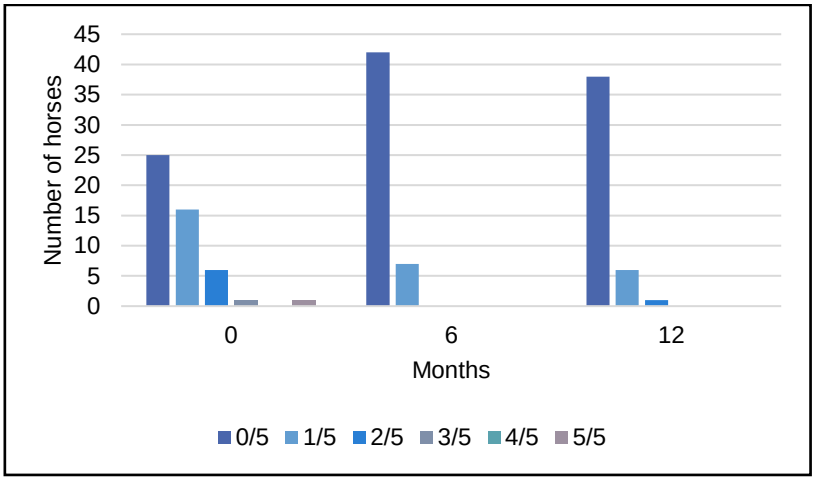


Figure 13 - Lameness scores for the total study population at the moment of treatment (0 months), 6 months after treatment and 12 months after treatment.

Ultrasonographic lesion classification

At six months 69,4% (*n*=34) of the horses had a clinically irrelevant US image (ultrasonographic class 1), and at 12 months this percentage was 84,4% (*n*=38). Re-injury rate was 4,1% (*n*=2) six months after the treatment, and 17,8% (*n*=8) 12 months after treatment (Figure 14). Only two horses suffered re-injury on the contralateral limb (4,1%), both after 12 months.

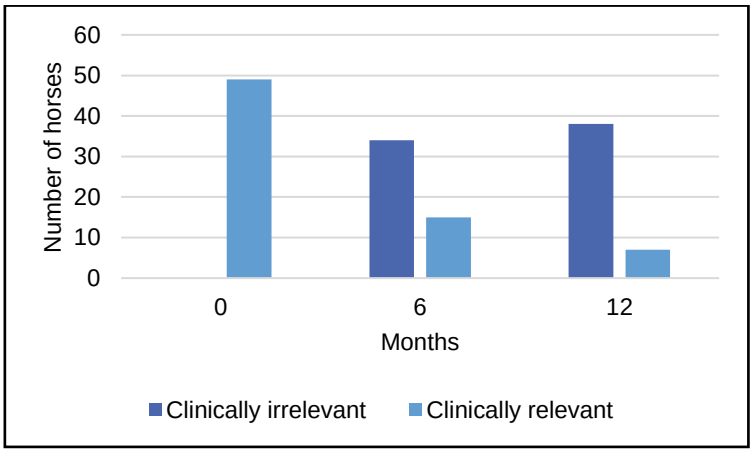


Figure 14 - Ultrasonographic scores for the total study population at the moment of treatment (0 months), 6 months after treatment and 12 months after treatment.

Work level

One year after the treatment 44,9% (*n*=22) of the horses had not returned to the work level they had before the injury. It has to be noted that 27,3% (*n*=6) of these horses had a lower work level due to reasons unrelated to the original injury.

Re-injury

Re-injury rate was 4,1% (*n*=2) 6 months after the treatment, and 17,8% (*n*=8) 12 months after treatment. Only two horses suffered re-injury on the contralateral limb (4,1%), both after 12 months.

The results obtained for the overall population are summarized on Annex 1.

6.2 Breed

Lameness

Friesian horses had the best improvement of lameness score 6 months after treatment, together with the group that included various breeds. Only 33,3% (*n*=4) of Friesian horses had no lameness (0/5 lameness score) at the moment of treatment, and six months after treatment 91,1% (*n*=11) of these horses did not show lameness, an improvement of almost 60%. The group that included various breeds went from 42,9% (*n*=3) of the horses with no lameness at treatment, to 100% (*n*=7) of the horses with no lameness at six months and 12 months after treatment. The breed that had the worse improvement of lameness was the Standardbred. At treatment 78,6% (*n*=11) of the horses did not show signs of lameness, but six months after treatment only 71,4% (*n*=10) had no signs of lameness, and 12 months after treatment this percentage was 69,2% (*n*=9).

Ultrasonographic lesion classification

Warmblood horses and Friesian horses improved 75% (*n*=12 Warmblood, *n*=9 Friesian) in terms of ultrasonographic scores, six months after treatment. From the group that included various breeds 85% (*n*=6) of the horses improved their ultrasonographic scores. The breed with the worse ultrasonographic scores after six months was the Standardbred, as 50% (*n*=7) of the horses still had a clinically relevant image at US.

Standardbred horses remained the group with the highest rate of clinically relevant images at US 12 months after treatment, as only 61,5% (*n*=8) of the horses had a good ultrasonographic score. At 12 months Friesian horses and the heterogeneous group had 100% (*n*=11 Friesian, *n*=6 other) of the horses with clinically irrelevant US images. While 85,7% (*n*=13) of warmblood horses had a clinically irrelevant US image.

After statistical analysis it was revealed that the breed of the horses had a significant influence on the ultrasonographic lesion classification after 12 months (*p*=0,039).

Re-injury

Six months after treatment the re-injury rate was extremely low, across all breeds. Only warmblood and standardbred horses registered a re-injury (6,3%; *n*=1 and 7,1%; *n*=1 respectively).

Standardbred horses also had the most re-injuries 12 months after treatment, while the rest of the breeds had similar re-injury rates, between 9% and 16%.

Work level

All the breed groups had a distribution of around 50% in terms of return to previous work level. From the 16 warmblood horses, nine (56,3%) returned to the same work level or had a higher work level than before the injury. The standardbred horses had a 50% (*n*=7) return to the same, or higher, work level. Within the heterogeneous group three horses (42,9%), from a total of seven, returned to the previous work level, or had a higher work level. The Friesian horses stood out as 66,7% (*n*=8) of the horses had a similar or better work level than before the injury.

Most of the horses had a lower work level due to the treated injury. The only exception was in the warmblood group, were 42,9% (*n*=3) of the horses that had a lower work level, had so due to another reason.

The results obtained for the breed groups are summarized on Annex 2.

6.3 Age

Lameness

After six months the age groups of horses between six and 10 years, 11 and 15 years, and above 20 years showed improvement in lameness scores. The group with horses between 11 and 15 years old had the biggest improvement, rising from 66,7% (*n*=2) of the horses in the group with no lameness at zero months, to 92,3% (*n*=12) of horses in the group with no lameness. The groups of horses with ages between 16 and 20 years, and less than five years, maintained their percentage of horses with no signs of lameness, showing 77,8% (*n*=7) and 66,7% (*n*=2) respectively.

At 12 months two groups had an increase in their lameness scores, what means their percentage of horses with no signs of lameness decreased. The group of horses aged less than five years had 66,7% (*n*=6) and the group of horses between 11 and 15 years old had 83,3% (*n*=10). The other groups increased their percentages of horses with no lameness. The group with horses between six and 10 years old had 90% (*n*=18), the group of horses aged between 16 and 20 had 100% (*n*=3), and the group of horses older than 20 years also had 100% (*n*=1).

Ultrasonographic lesion classification

In terms of ultrasonographic scores the age group that had the least improvement after six months was the one with horses aged between 11 and 15 years (61,5%; *n*=8). The horses older than 16 years (group 16-20 and group 20<) all had clinically irrelevant US scores.

After 12 months the group with horses aged between six and 10 years had less horses with clinically irrelevant US scores (85%; *n*=17) when compared with the six-month evaluation (86,4%;

n=19). But maintained the same number of horses with clinically relevant US scores (*n*=3). From the group of horses with ages under five years 88,9% (*n*=8) of the horses had good US scores, and in the group of horses aged between 11 and 15 years 75% (*n*=9) of them also had good US scores. The horses aged above 16 maintained all their good US scores.

Re-injury

Only the groups of horses with ages between six and 10 years and 11 and 15 years had re-injuries after six months. Both these groups had one horse re-injured (4,5% and 7,7%, respectively). After 12 months these two groups had re-injuries as well, along with the group of horses under five years. The group of horses younger than five years had a re-injury rate of 22,2% (*n*=2), the group with horses between six and 10 years old had a re-injury rate of 15% (*n*=3), and the group of horses aged between 11 and 15 years had a re-injury rate of 25% (*n*=3).

Work level

The group of horses with ages between 11 and 15 years had the biggest rate of horses that did not return to their previous work level (69,2%; *n*=9).

The results obtained for the age groups are summarized on Annex 3.

6.4 Chronicity

Lameness

Sub-acute injuries (2-4 weeks old) recovered better in terms of lameness, both six months after treatment as 12 months after treatment. At six months 95,5% (*n*=21) of horses with these injuries had no signs of lameness, an improvement of 54,6% compared to the moment of treatment (0 months). And 12 months after treatment 95% (*n*=19) of the horses with subacute injuries had not signs of lameness.

Acute injuries (less than two weeks old) did better after six months and chronic injuries (more than four weeks old) did better after 12 months. There was an improvement in lameness score in the group of horses with acute injuries at six months, as it went from 69,2% (*n*=9) of the horses with no signs of lameness, to 84,6% (*n*=11) of the horses with no signs of lameness, six months after the treatment. But 12 months after the treatment only 61,5% (*n*=8) of the animals with acute injuries had no signs of lameness. Horses with chronic injuries had a steady improvement over time, going from 50% (*n*=7) of the horses with no lameness, at the moment of treatment, to 71,4% (*n*=10) of the horses with no lameness, six months after treatment. And finally, 12 months after treatment, 91,7% (*n*=11) of the horses did not exhibit signs of lameness.

Ultrasonographic lesion classification

Looking at ultrasonographic scores six months after treatment we see that horses with sub-acute injuries had the best improvements, as 81,8% (*n*=18) of the horses showed clinically irrelevant

US images. Horses with acute and chronic injuries had similar improvements, as 61,5% (*n*=8) of horses with acute injuries, and 57,1% (*n*=8) of horses with chronic injuries, had good US images.

Twelve months after treatment horses with sub-acute and chronic injuries had similar results in terms of US scores, as 90% (*n*=18) of the horses with sub-acute injuries and 91,7% (*n*=11) of the horses with chronic injuries, had clinically irrelevant US images. While only 69,2% (*n*=9) of horses with acute injuries had sound US images.

Re-injury

Only horses with chronic injuries had re-injuries 6 months after treatment (14,3%; *n*=2).

The re-injury rates 12 months after treatment were 38,5% (*n*=5) for horses with acute injuries, 10% (*n*=2) for horses with sub-acute injuries, and 8,3% (*n*=1) for horses with chronic injuries.

Work level

Work level was similar across all groups. It was showed that 46,2% (*n*=6) of the horses with acute injuries, 45,5% (*n*=10) of horses with sub-acute injuries, and 42,9% (*n*=6) of horses with chronic injuries, did not return to their previous level of work.

But if we look at the reasons behind the lower work level, we see that 40% (*n*=4) of the horses with sub-acute injuries had a lower work level due to reasons unrelated to the injury they were treated on. A higher percentage compared to the acute and chronic injury groups, where only 16,7% (*n*=1) of the horses had another reason for their lower work level.

The results obtained for the chronicity groups are summarized on Annex 3.

6.5 Discipline

Lameness

All disciplines, except harness racing horses, improved their lameness score from treatment to six months after. Show-jumping horses went from 71,4% (*n*=5) of the horses showing no lameness, at treatment, to 85,7% (*n*=6) at six months after treatment. Dressage horses had the biggest improvement, going from 41,2% (*n*=7) of the horses showing no lameness, to 94,1% (*n*=16), in six months. The category that included various other disciplines also had a big improvement, 18,2% (*n*=2) to 90,9% (*n*=10), after six months. Harness racing was the exception, as 78,6% (*n*=11) of the horses showed no signs of lameness at the moment of treatment, and six months after treatment this percentage was 71,4% (*n*=10). This tendency continued 12 months after the treatment, when only 69,2% (*n*=9) of the harness racing horses had no sign of lameness. At the same moment 85,7% (*n*=6) of show-jumping horses, 87,5% (*n*=14) of dressage horses, and 100% (*n*=9) of the undifferentiated horses showed no signs of lameness.

Ultrasonographic lesion classification

After six months all the discipline groups had improvements in their US scores. All the show-jumping horses (100%; *n*=7) had clinically irrelevant US images. From the dressage group, 76,5% (*n*=13) of the horses had good US images, and the group with various disciplines had 85,7% (*n*=7) of the horses with good US scores. The discipline group with less improvement was the harness racing group, where only 50% (*n*=7) of the horses improved their US score.

Show-jumping horses did not improve their US images after 12 months, in fact the percentage of horses with good US scores decreased from 100% at six months to 85,7% (*n*=6) at 12 months. This was the only group were a percentual decrease was observed in relation to clinically irrelevant images. In the dressage group 100% (*n*=16) of the horses had clinically irrelevant US images after 12 months, and 88,9% (*n*=8) of the horses from the undifferentiated group had good US scores. Harness racing horses remained the group with the worse US images, as 38,5% (*n*=5) of these horses still had clinically relevant US images.

Similar to the breed category, statistical analysis showed a significant influence of discipline on the outcome of ultrasonographic lesion classification 12 months after treatment (*p*=0,04).

Re-injury

In terms of re-injury there were only two re-injuries observed after six months, one in the harness racing group (7,1%) and one in the undifferentiated group (9,1%). After 12 months all the groups had re-injuries. The biggest re-injury rate was observed in the harness racing group (30,8%; *n*=4). Show-jumping horses had a re-injury rate of 14,3% (*n*=1), dressage horses 12,5% (*n*=2), and the undifferentiated group 11,1% (*n*=1).

Work level

The horses in the undifferentiated group had the biggest rate of horses that did not return to their previous level of work (63,6%; *n*=7). While 50% (*n*=7) of harness racing horses, 35,3% (*n*=6) of dressage horses, and 28,6% (*n*=2) of show-jumping horses had a lower work level than before the injury. Most of the horses that had a lower work level had so due to the injury they were treated for. Only the undifferentiated group had a higher percentage of horses (42,9%; *n*=3) that had a lower work level due to a reason unrelated to the injury.

The results obtained for the discipline groups are summarized on Annex 5.

6.6 Type of MSCs

Lameness

No significant differences were noted in terms of lameness scores between the two types of MSCs, at six and at 12 months after treatment. After six months 88% (*n*=22) of SVF+PRP treated horses, and 83,3% (*n*=20) of BM-MSC treated horses had no sign of lameness. At 12 months

these rates decreased to 87,5% (***n***=21) in the SVF+PRP group, and 81% (*n*=17) in the BM-MSC group.

Ultrasonographic lesion classification

After six months 41,7% (*n*=10) of BM-MSC treated horses still had clinically relevant US images, while only 20% (*n*=5) of SVF+PRP treated horses had the same negative scores. Twelve months after treatment 87,5% (*n*=21) of SVF+PRP treated horses, and 81% (*n*=17) of BM-MSC treated horses had positive US scores, meaning that their US images had no clinical relevance.

Re-injury

Six months after treatment both groups had one re-injury, meaning that the re-injury rate for the SVF+PRP group was 4% and for the BM-MSC group was 4%. Twelve months after treatment the re-injury rate was higher in the BM-MSC group, 23,8% (*n*=5) compared to 12,5% (*n*=3) from the SVF+PRP group.

Work level

Work level after 12 months was better in horses treated with SVF+PRP, as only 36% (*n*=9) of the horses had a lower work level, and 54,2% (*n*=13) of the BM-MSC had a lower work level. But most of the horses that had a lower work level and were treated with SVF+PRP had a lower work level due to the lesion itself (88,9%; *n*=8), while a great part of the BM-MSC treated horses had a lower work level due to other reasons (38,5%; *n*=5).

The results obtained for the type of MSCs used are summarized on Annex 6.

6.7 Structure

Lameness

Horse with SDFT lesions had a better improvement of lameness after six months, as 91,7% (*n*=22) of these horses had no signs of lameness. While 80% (*n*=20) of the horses with SL injury had no detectable lameness. The SDFT group had an increase in lameness rate after 12 months, only 81,8% (*n*=18) of the horses had no lameness. The SL group maintained the number of horses that had no lameness (*n*=20), but increased the percentage of horses with no lameness (87%) as two horses from this group were eliminated due to euthanasia or re-injury at six months.

Ultrasonographic lesion classification

No significant difference was observed between the SDFT and the SL group six months after treatment, as both groups had 17 horses with good US scores. This meant that 68% of the SL horses and 70,8% of the SDFT horses had improved their US images in six months. After 12 months the SL group had the most horses with good US scores (91,3%; *n*=21). The SDFT group had 17 horses with good US scores (77,3%).

Re-injury

Low percentage of re-injury at six months, overall, with no significant difference between the two groups. Both groups had 1 horse with re-injury in the six-month period, meaning that the SL group had a 4% re-injury rate and the SDFT group had a 4,2% re-injury rate.

At 12 months the SL group showed less re-injuries, as only 12,5% (*n*=3) of the horses re-injured, and 23,8% (*n*=5) of the horses from the SDFT group re-injured.

Work level

SL lesions had a better work level after 12 months, as only 36% (*n*=9) of the horses had a lower work level, compared to 54,2% (*n*=13) of the SDFT group. But the 9 horses that had a lower work level (100%) did so due to their lesion, while 46,2% (*n*=6) of the SDFT horses had a lower work level due to a problem unrelated with the original lesion.

The only variables that appeared to have influence on any outcome were breed and discipline, that both showed to have a statistically significant influence on lesion score at 12 months (breed *p*=0.039 and discipline *p*=0.04). Standardbred horses and horses that competed in Harness racing were the ones who had the most significant difference, compared to the other groups. No other variable showed any statistically significant influence on the outcome.

The results obtained for the different structures treated are summarized on Annex 7.

Table 1 - Re-injury rates at 6 and 12 months, for SVF+PRP and BM-MSC treated horses, and horses with SDFT and SL lesions.

RE-INJURY								
MONTH	**Type of MSC**	**NO**	**YES**	**Total**	**Structure**	**NO**	**YES**	**Total**
6	**SVF+PRP**	96% (*n*=24)	4% (*n*=1)	*n*=25	**SL**	96% (*n*=24)	4% (*n*=1)	*n*=25
	BM-MSC	95,8% (*n*=23)	4,2% (*n*=1)	*n*=24	**SDFT**	95,8% (*n*=23)	4,2% (*n*=1)	*n*=24
12	**SVF+PRP**	87,5% (*n*=21)	12,5% (*n*=3)	*n*=24	**SL**	87,5% (*n*=21)	12,5% (*n*=3)	*n*=24
	BM-MSC	76,2% (*n*=16)	23,8% (*n*=5)	*n*=21	**SDFT**	76,2% (*n*=16)	23,8% (*n*=5)	*n*=21

In the previous table it is possible to see a similarity in numbers on both groups (type of MSCs and structures). This is a coincidental incidence, as both groups were equally distributed after case selection.

Table 2 - Re-injury rates of structure coupled with type of MSCs used.

		RE-INJURY		
MONTH	**Type of MSC**	**NO**	**YES**	**Total**
6	**SL and SVF+PRP**	100% (*n*=16)	0	*n*=16
	SL and BM-MSC	88,89% (*n*=8)	11,11% (*n*=1)	*n*=9
	SDFT and SVF+PRP	88,89% (*n*=8)	11,11% (*n*=1)	*n*=9
	SDFT and BM-MSC	100% (*n*=15)	0	*n*=15
12	**SL and SVF+PRP**	93,75% (*n*=15)	6,25% (*n*=1)	*n*=16
	SL and BM-MSC	77,78% (*n*=7)	22,22% (*n*=2)	*n*=9
	SDFT and SVF+PRP	77,78% (*n*=7)	22,22% (*n*=2)	*n*=9
	SDFT and BM-MSC	80% (*n*=12)	20% (*n*=3)	*n*=15

When looking at the difference between treatments, within each structure, no significant findings were noted. The re-injury rates varied between 0 and 11,11% six months after treatment, and 6,25% and 22,22% after 12 months (Table 2).

6.8 Statistical analysis

Beside the significant influence of breed and discipline on the ultrasonographic lesion classification at 12 months, no other variable showed any statistically significant influence on any of the outcomes.

7 Discussion

The results of MSC treatment, whether with autologous SVF whether with allogenic BM-MSCs, were positive, with a re-injury rate of 4,1%, six months after treatment, and 17,8%, 12 months after treatment. Comparing the values obtained in the present study with other similar studies may not have the ideal statistical and clinical value, but similarities in criteria, variables and outcomes between this study and other published works may highlight the effects of MSCs in the treatment of SDF tendonitis and SL desmitis.

When analysing the effect of the different variables (age, breed, discipline, lesion chronicity, structure and type of MSC used) it was noted that Standardbred horses and horses that competed in harness racing, both revealed influence on the outcome of ultrasonographic scores after 12 months (breed p=0.039 and discipline p=0.04). The fact that these two variables had similar p values, and consequently similar effect on outcome, may be explained by the fact that they are two very similar populations, most likely composed by the same individuals. All the other variables did not have any significant influence on the outcome. Relative to the type of MSCs used there was no significant difference between the outcome of horses treated with autologous SVF+PRP and horses treated with allogenic BM-MSCs, as re-injury at six months (p=0,976) and re-injury at

12 months (p=0,322) did not reveal any statistically significant differences between treatments. This was not expected as SVF preparations have a heterogeneous composition in terms of cells, and less MSCs, when compared to BM-MSCs, that are cultured and expanded in a laboratory setting (Metcalf *et al*., 2016; Opiela *et al*., 2013).

The effect of the MSC therapy, whether with SVF+PRP or with BM-MSCs, appeared to be similar to other studies conducted with other regenerative therapies, such as autologous BM-MSCs (Godwin *et al*., 2012) and high powered laser (Pluim *et al*., 2018). Re-injury rate of horses treated with autologous SVF+PRP was 16% (n=4) and with allogenic BM-MSCs was 25% (n=6), a similar result to horses treated with autologous BM-MSC, that had a re-injury rate of 27,4% (Godwin *et al*., 2012). Horses with tendinopathy and desmopathy treated with high-powered laser, had a re-injury rate of 16,8%, while the present study obtained a re-injury rate of 4% (n=1) in the same time period. At 12 months 21% of the high-powered laser treated horses re-injured while 23,8% of the horses treated with MSCs re-injured (Pluim *et al*., 2018).

Work level of 40 horses (77%), from a population of 52, was the same or higher after treatment with allogenic umbilical cord blood-MSCs (Van Loon *et al*., 2014). In the present study the percentage of horses that returned to their previous work level or went on to a higher work level was lower than the aforementioned study (55,1%; n=27), but six of the horses that had a lower work level (27,3%) had so due to a reason unrelated to the injury they were treated for. This has may have had a negative influence on the final work level rates of the study.

Comparing with horses that were treated conservatively, with hyaluronan, and PSGAGs, the horses treated with MSCs had half of the re-injuries, as the horses studied by Dyson had a re-injury rate of 42,9% (Dyson, 2004), and the horses from the present study had a 20,4% re-injury rate. The same study made by Dyson divided horses in disciplines, when looking at the re-injury

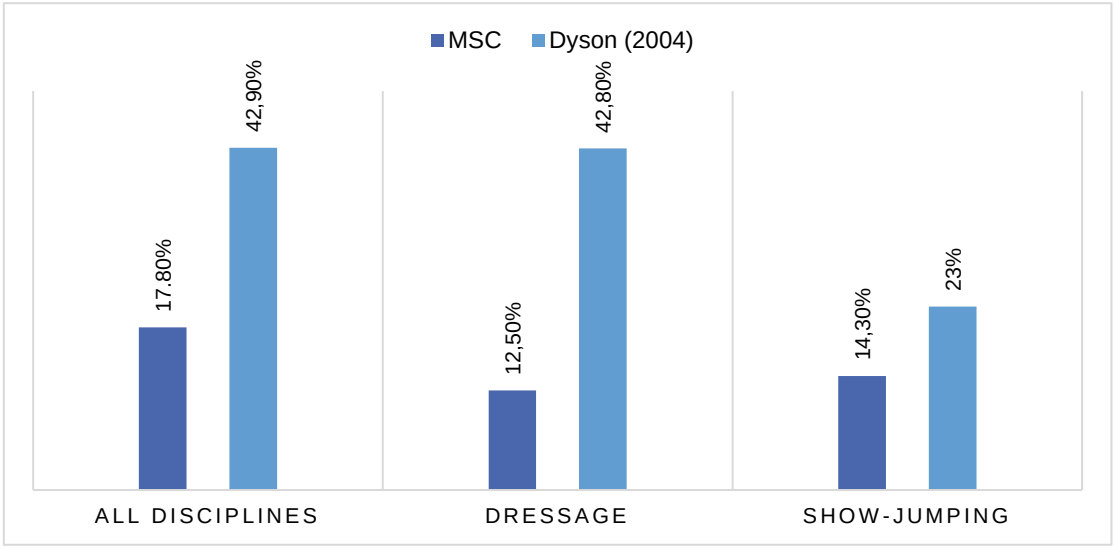


Figure 15 - Re-injury rates from the present study and from a study performed by Dyson and colleagues between 1992 and 2002 (Dyson, 2004). Horses in this last study were treated conservatively, with hyaluronan, and PSGAGs.

rates of the same discipline groups in this study it is visible that they are lower than the ones observed by Dyson. Dressage horses had a re-injury rate of 42,8% in the study made in 2004, and in this study this rate was 12,5%. And show-jumping horses had a 23% re-injury rate in 2004, while 14,3% of show-jumping horses in this study re-injured.

The interval of time between injury and implantation of MSCs (chronicity) did not appear to influence the outcome of the therapy, as chronicity of the injury did not have a statistical significant effect on re-injury at six months (p=0.074) and at 12 months (p=0.068). This was also observed by a larger study with BM-MSC therapy, were it was only noted that the average time between injury and implantation was longer in the horses that re-injured (Godwin *et al*., 2012). In the present study the group of horses with the longest time between injury and implantation (more than four weeks) had the only registered re-injuries after six months (14,3%; n=2) but the least re-injury cases (8,3%; n=1) after 12 months. As these findings fail to show any statistical significance it is not possible to attribute a cause to these numbers.

Adverse effects were noted only a few days after SVF implantation, as the horses became sore on the site of administration and showed lameness. These signs disappeared after two to three days. This may be due to the heterogeneous cell population of the administered suspension, composed of perivascular cells, leukocytes, and endothelial cells (Brown *et al*., 2019). Allogenic BM-MSCs did not show any adverse effects. This was expected, as more clinical trials showed no adverse reactions after administration of allogenic MSCs, from various sources (umbilical cord blood, amnion, bone marrow, adipose tissue and blood) (Lange-Consiglio *et al*., 2013; Ricco *et al*., 2013; Van Loon *et al*., 2014; Beerts *et al*., 2017).

As the population for this study was obtained through a retrospective analysis of records from the clinic it was not possible to build a valid control group, the main reason being a lack of patients with the same injuries and only treated in a conservative manner. Because of this limitation it was chosen to compare the present study to other published works. This method is however a statistically weak exercise, due to the differences in population characteristics, inclusion criteria, study variables and study outcome.

8 Conclusion

Mesenchymal stem cells, in the form of autologous stromal vascular fraction combined with platelet rich plasma, and allogenic bone marrow derived MSCs, are a safe and efficacious therapy for the treatment of superficial digital flexor tendonitis and suspensory ligament desmitis. The re-injury rates accompanied the tendency set by previously published works regarding MSC therapy, a trend that consistently remains below the numbers shown by conventional treatments. There was no conclusion to whether one of the therapies had an increased success in contrast to the other one. Also, it was unclear if the treated structure (SDFT or SL) influenced the success of the treatment. The same applied to the timing of the treatment. Breed and discipline affected the

outcome of the therapy, as Standardbred and Harness racing horses had a negative outcome in terms of ultrasonographic score, in contrast to the other groups. The fact that this is a retrospective study carries a great deal of limitations to the value of the conclusions drawn by this study. With that in mind it can be said that the results shown may bring a positive value to further work that will be done in the field of equine regenerative medicine.

9 Bibliography

Al Naem, M., Bourebaba, L., Kucharczyk, K., Röcken, M., & Marycz, K. (2019). Therapeutic mesenchymal stromal stem cells: Isolation, characterization and role in equine regenerative medicine and metabolic disorders. *Stem Cell Reviews and Reports*. https://doi.org/10.1007/s12015-019-09932-0

Alexander, R. M. N. (2002). Tendon elasticity and muscle function. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *133*(4), 1001–1011. https://doi.org/10.1016/S1095-6433(02)00143-5

Alves, A. G. L., Stewart, A. A., Dudhia, J., Kasashima, Y., Goodship, A. E., & Smith, R. K. W. (2011). Cell-based Therapies for Tendon and Ligament Injuries. *Veterinary Clinics of North America - Equine Practice*, *27*(2), 315–333. https://doi.org/10.1016/j.cveq.2011.06.001

Arnhold, S. J., Goletz, I., Klein, H., Stumpf, G., Beluche, L. A., Rohde, C., Addicks, K., & Litzke, L. F. (2007). Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. *American Journal of Veterinary Research*, *68*(10), 1095–1105. https://doi.org/10.2460/ajvr.68.10.1095

Arnoczky, S. P., Lavagnino, M., & Egerbacher, M. (2007). The mechanobiological aetiopathogenesis of tendinopathy: Is it the over-stimulation or the under-stimulation of tendon cells? *International Journal of Experimental Pathology*, *88*(4), 217–226. https://doi.org/10.1111/j.1365-2613.2007.00548.x

Arnoczky, S. P., Lavagnino, M., & Egerbacher, M. (2008). The Response of Tendon Cells to Changing Loads: Implications in the Etiopathogenesis of Tendinopathy. *Tendinopathy in Athletes*, *12*, 46–59. https://doi.org/10.1002/9780470757987.ch4

Arnoczky, S. P., Lavagnino, M., Egerbacher, M., Caballero, O., Gardner, K., & Shender, M. A. (2008). Loss of homeostatic strain alters mechanostat “set point” of tendon cells in vitro. *Clinical Orthopaedics and Related Research*, *466*(7), 1583–1591. https://doi.org/10.1007/s11999-008-0264-x

Banes, A. J., Donlon, K., Link, G. W., Gillespie, Y., Bevin, A. G., Peterson, H. D., Bynum, D., Watts, S., & Dahners, L. (1988). Cell populations of tendon: A simplified method for isolation of synovial cells and internal fibroblasts: Confirmation of origin and biologic properties. *Journal of Orthopaedic Research*, *6*(1), 83–94. https://doi.org/10.1002/jor.1100060111

Barberini, D. J., Freitas, N. P. P., Magnoni, M. S., Maia, L., Listoni, A. J., Heckler, M. C., Sudano, M. J., Golim, M. A., Da Cruz Landim-Alvarenga, F., & Amorim, R. M. (2014). Equine mesenchymal stem cells from bone marrow, adipose tissue and umbilical cord: Immunophenotypic characterization and differentiation potential. *Stem Cell Research and*

Therapy, *5*(1). https://doi.org/10.1186/scrt414

Barrachina, L., Remacha, A. R., Romero, A., Vázquez, F. J., Albareda, J., Prades, M., Ranera, B., Zaragoza, P., Martín-Burriel, I., & Rodellar, C. (2016). Effect of inflammatory environment on equine bone marrow derived mesenchymal stem cells immunogenicity and immunomodulatory properties. *Veterinary Immunology and Immunopathology*, *171*, 57–65. https://doi.org/10.1016/j.vetimm.2016.02.007

Barry, F., Boynton, R., Murphy, M., & Zaia, J. (2001). The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. *Biochemical and Biophysical Research Communications*, *289*(2), 519–524. https://doi.org/10.1006/bbrc.2001.6013

Barry, F. P., Boynton, R. E., Haynesworth, S., Murphy, J. M., & Zaia, J. (1999). The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). *Biochemical and Biophysical Research Communications*, *265*(1), 134–139. https://doi.org/10.1006/bbrc.1999.1620

Batson, E. L., Paramour, R. J., Smith, T. J., Birch, H. L., Patterson-Kane, J. C., & Goodship, A. E. (2003). Are the material properties and matrix composition of equine flexor and extensor tendons determined by their functions? *Equine Veterinary Journal*, *35*(3), 314–318. https://doi.org/10.2746/042516403776148327

Baxter, G. M. (2011). Therapeutic options. In *Manual of Equine Lameness* (pp. 405–427).

Baxter, G. M., Stashak, T. S., & Keegan, K. G. (2020). Examination for Lameness. In *Adams and Stashak's Lameness in Horses* (pp. 67–188). Wiley. https://doi.org/10.1002/9781119276715.ch2

Beerts, C., Suls, M., Broeckx, S. Y., Seys, B., Vandenberghe, A., Declercq, J., Duchateau, L., Vidal, M. A., & Spaas, J. H. (2017). Tenogenically induced allogeneic peripheral blood mesenchymal stem cells in allogeneic platelet-rich plasma: 2-year follow-up after tendon or ligament treatment in horses. *Frontiers in Veterinary Science*, *4*(SEP). https://doi.org/10.3389/fvets.2017.00158

Bentz, B. (2015). Clinical pharmacology of the equine musculoskeletal system. In *Equine Pharmacology* (1st ed., pp. 218–253). John Wiley & Sons, Ltd.

Beredjiklian, P. K., Favata, M., Cartmell, J. S., Flanagan, C. L., Crombleholme, T. M., & Soslowsky, L. J. (2003). Regenerative versus reparative healing in tendon: A study of biomechanical and histological properties in fetal sheep. *Annals of Biomedical Engineering*, *31*(10), 1143–1152. https://doi.org/10.1114/1.1616931

Bergh, A. (2011). Physical treatment of the equine athlete. In *Equine Sports Medicine and*

Surgery: Second Edition (pp. 1230–1241).

Bianco, P. (2009). The great MSC History review. *Cell*, *2*(4), 313–319. https://doi.org/10.1016/j.stem.2008.03.002.Mesenchymal

Biewener, A. A. (1998). Muscle-tendon stresses and elastic energy storage during locomotion in the horse. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, *120*(1), 73–87. https://doi.org/10.1016/S0305-0491(98)00024-8

Birch, H. L. (2007). Tendon matrix composition and turnover in relation to functional requirements. *International Journal of Experimental Pathology*, *88*(4), 241–248. https://doi.org/10.1111/j.1365-2613.2007.00552.x

Birch, H. L., Bailey, A. J., & Goodship, A. E. (1998). Macroscopic “degeneration” of equine superficial digital flexor tendon is accompanied by a change in extracellular matrix composition. *Equine Veterinary Journal*, *30*(6), 534–539. https://doi.org/10.1111/j.2042-3306.1998.tb04530.x

Birch, H. L., Sinclair, C., Goodship, A. E., & Smith, R. K. W. (2013). Tendon and ligament physiology. In *Equine Sports Medicine and Surgery: Second Edition* (Second Edi). Elsevier Ltd. https://doi.org/10.1016/B978-0-7020-4771-8.00009-0

Birch, H. L., Wilson, A. M., & Goodship, A. E. (1997). The effect of exercise-induced localised hyperthermia on tendon cell survival. *Journal of Experimental Biology*, *200*(11), 1703–1708.

Bochev, I., Elmadjian, G., Kyurkchiev, D., Tzvetanov, L., Altankova, I., Tivchev, P., & Kyurkchiev, S. (2008). Mesenchymal stem cells from human bone marrow or adipose tissue differently modulate mitogen-stimulated B-cell immunoglobulin production in vitro. *Cell Biology International*, *32*(4), 384–393. https://doi.org/10.1016/j.cellbi.2007.12.007

Bolt, D. M., Burba, D. J., Hubert, J. D., Pettifer, G. R., & Hosgood, G. L. (2004). Evaluation of cutaneous analgesia after non-focused extracorporeal shock wave application over the 3rd metacarpal bone in horses. *Canadian Journal of Veterinary Research*, *68*(4), 288–292.

Bosch, G., Lin, Y. L., Van Schie, H. T. M., Van De Lest, C. H. A., Barneveld, A., & Van Weeren, P. R. (2007). Effect of extracorporeal shock wave therapy on the biochemical composition and metabolic activity of tenocytes in normal tendinous structures in ponies. *Equine Veterinary Journal*, *39*(3), 226–231. https://doi.org/10.2746/042516407X180408

Bourzac, C., Smith, L. C., Vincent, P., Beauchamp, G., Lavoie, J. P., & Laverty, S. (2010). Isolation of equine bone marrow-derived mesenchymal stem cells: a comparison between three protocols. *Equine Veterinary Journal*, *42*(6), 519–527. https://doi.org/10.1111/j.2042-3306.2010.00098.x

Brown, J. C., & Katz, A. J. (2019). Stem Cells Derived From Fat. In *Principles of Regenerative Medicine* (pp. 295–305). Elsevier. https://doi.org/10.1016/b978-0-12-809880-6.00019-9

Bruno, S., Deregibus, M. C., & Camussi, G. (2015). The secretome of mesenchymal stromal cells: Role of extracellular vesicles in immunomodulation. *Immunology Letters*, *168*(2), 154–158. https://doi.org/10.1016/j.imlet.2015.06.007

Budras, K.-D., Sack, W. O., Röck, S., Horowitz, A., & Berg, R. (2011). *Anatomy of the Horse* (6th ed.). Schlüterschee.

Burrows, S., Patterson-kane, J. C., Fleck, R. A., & Becker, D. L. (2003). *Alterations in gap junction communication in tenocyte monolayers following an episode of hyperthermia School of Veterinary Science , The University of Queensland , St Lucia , QLD , Australia ; 2 The Royal Veterinary College , Hatfield , United Kingdom ; 3*. *1*(323), 1998.

Butcher, M. T., Hermanson, J. W., Ducharme, N. G., Mitchell, L. M., Soderholm, L. V., & Bertram, J. E. A. (2009). Contractile behavior of the forelimb digital flexors during steady-state locomotion in horses (Equus caballus): An initial test of muscle architectural hypotheses about in vivo function. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *152*(1), 100–114. https://doi.org/10.1016/j.cbpa.2008.09.007

Caplan, A. I. (1991). Mesenchymal Stem Cells. *Journal of Orthopaedic Research : Official Publication of the Orthopaedic Research Society*, *9*(5), 641–650. https://doi.org/10.1016/0009-2614(70)87009-9

Caplan, A. I. (2017). Mesenchymal stem cells: Time to change the name! *Stem Cells Translational Medicine*, *6*(6), 1445–1451. https://doi.org/10.1002/sctm.17-0051

Carrade, D. D., Affolter, V. K., Outerbridge, C. A., Watson, J. L., Galuppo, L. D., Buerchler, S., Kumar, V., Walker, N. J., & Borjesson, D. L. (2011). Intradermal injections of equine allogeneic umbilical cord-derived mesenchymal stem cells are well tolerated and do not elicit immediate or delayed hypersensitivity reactions. *Cytotherapy*, *13*(10), 1180–1192. https://doi.org/10.3109/14653249.2011.602338

Carrade, D. D., Lame, M. W., Kent, M. S., Clark, K. C., Walker, N. J., & Borjesson, D. L. (2012). Comparative Analysis of the Immunomodulatory Properties of Equine Adult-derived Mesenchymal Stem Cells. *Cell Medicine*, *4*(37), 1–11.

Carvalho, A. D. M., Yamada, A. L. M., Martins, J. R. B., Maia, L., Golim, M. A., Deffune, E., Hussni, C. A., Alves, A. L. G., M, A. C. A., Yamada, A. L. M., Martins, J. R. B., Maia, L., Golim, M. A., & Deffune, E. (2013). *Isolation and characterization of equine peripheral blood-derived multipotent mesenchymal stromal cells 1*. *33*(9), 1151–1154.

Colbath, A. C., Dow, S. W., McIlwraith, C. W., & Goodrich, L. R. (2020). Mesenchymal stem cells for treatment of musculoskeletal disease in horses: Relative merits of allogeneic versus autologous stem cells. *Equine Veterinary Journal*, 0–3. https://doi.org/10.1111/evj.13233

Colbath, A. C., Dow, S. W., Phillips, J. N., McIlwraith, C. W., & Goodrich, L. R. (2017). Autologous and Allogeneic Equine Mesenchymal Stem Cells Exhibit Equivalent Immunomodulatory Properties In Vitro. *Stem Cells and Development*, *26*(7), 503–511. https://doi.org/10.1089/scd.2016.0266

Crevier-Denoix, N., Collobert, C., Pourcelot, P., Denoix, J. M., Sanaa, M., Geiger, D., Bernard, N., Ribot, X., Bortolussi, C., & Bousseau, B. (2010). Mechanical properties of pathological equine superficial digital flexor tendons. *Equine Veterinary Journal*, *29*(S23), 23–26. https://doi.org/10.1111/j.2042-3306.1997.tb05046.x

Crowe, O. M., Dyson, S. J., Wright, I. M., Schramme, M. C., & Smith, R. K. W. (2004). Treatment of chronic or recurrent proximal suspensory desmitis using radial pressure wave therapy in the horse. *Equine Veterinary Journal*, *36*(4), 313–316. https://doi.org/10.2746/0425164044890562

Da Silva Meirelles, L., Sand, T. T., Harman, R. J., Lennon, D. P., & Caplan, A. I. (2009). MSC frequency correlates with blood vessel density in equine adipose tissue. *Tissue Engineering - Part A*, *15*(2), 221–229. https://doi.org/10.1089/ten.tea.2008.0103

Dakin, S. G., Smith, R. K. W., HeinegArd, D., Önnerfjord, P., Khabut, A., & Dudhia, J. (2014). Proteomic analysis of tendon extracellular matrix reveals disease stage-specific fragmentation and differential cleavage of COMP (cartilage oligomeric matrix protein). *Journal of Biological Chemistry*, *289*(8), 4919–4927. https://doi.org/10.1074/jbc.M113.511972

Denoix, J. M. (1994). Functional anatomy of tendons and ligaments in the distal limbs (manus and pes). *The Veterinary Clinics of North America. Equine Practice*, *10*(2), 273–322. https://doi.org/10.1016/S0749-0739(17)30358-9

Docheva, D., Müller, S. A., Majewski, M., & Evans, C. H. (2015). Biologics for tendon repair. *Advanced Drug Delivery Reviews*, *84*, 222–239. https://doi.org/10.1016/j.addr.2014.11.015

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., Deans, R. J., Keating, A., Prockop, D. J., & Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, *8*(4), 315–317. https://doi.org/10.1080/14653240600855905

Dowling, B. A., Dart, A. J., Hodgson, D. R., & Smith, R. K. W. (2000). Superficial digital flexor

tendonitis in the horse. *Equine Veterinary Journal*, *32*(5), 369–378. https://doi.org/10.2746/042516400777591138

Dudhia, J., Scott, C. M., Draper, E. R. C., Heinegård, D., Pitsillides, A. A., & Smith, R. K. (2007). Aging enhances a mechanically-induced reduction in tendon strength by an active process involving matrix metalloproteinase activity. *Aging Cell*, *6*(4), 547–556. https://doi.org/10.1111/j.1474-9726.2007.00307.x

Dyson, S. J. (1994). Proximal suspensory desmitis in the hindlimb: 42 cases. *British Veterinary Journal*, *150*(3), 279–291. https://doi.org/10.1016/S0007-1935(05)80008-9

Dyson, S. J. (2004). Medical management of superficial digital flexor tendonitis: A comparative study in 219 horses (1992-2000). *Equine Veterinary Journal*, *36*(5), 415–419. https://doi.org/10.2746/0425164044868422

Dyson, S. J. (2010). Medical management of superficial digital flexor tendonitis: a comparative study in 219 horses (1992-2000). *Equine Veterinary Journal*, *36*(5), 415–419. https://doi.org/10.2746/0425164044868422

Dyson, S. J., Arthur, R. M., Palmer, S. E., & Richardson, D. (1995). Suspensory ligament desmitis. *The Veterinary Clinics of North America. Equine Practice*, *11*(2), 177–215. https://doi.org/10.1016/S0749-0739(17)30319-X

Dyson, S J. (1997). Treatment of superficial digital flexor tendinitis: A comparison of conservative management, sodium hyaluronate, and glycosaminoglycan polysulfate. *Proc Amer Assoc Eq Prac*, *43*(December), 297–300.

Dyson, Sue J., & Genovese, R. L. (2010). The Suspensory Apparatus. In *Diagnosis and Management of Lameness in the Horse: Second Edition* (Second Edi, Issue McIV). Elsevier Inc. https://doi.org/10.1016/B978-1-4160-6069-7.00072-9

Egerbacher, M., Arnoczky, S. P., Gardner, K., Caballero, O., & Gartner, J. (2006). Stress-deprivation of tendons results in alterations in the integrin profile and pericellular matrix of tendon cells. *52nd Annual Meeting of the Orthopaedic Research Society*, 2006.

Eggleston, R. B., & Baxter, G. M. (2020). Lameness of the Distal Limb. In *Adams and Stashak's Lameness in Horses* (pp. 439–596).

Ely, E. R., Avella, C. S., Price, J. S., Smith, R. K. W. W., Wood, J. L. N. N., & Verheyen, K. L. P. (2009). Descriptive epidemiology of fracture, tendon and suspensory ligament injuries in National Hunt racehorses in training. *Equine Veterinary Journal*, *41*(4), 372–378. https://doi.org/10.2746/042516409X371224

Esteves, C. L., Sheldrake, T. A., Dawson, L., Menghini, T., Rink, B. E., Amilon, K., Khan, N., Péault, B., & Donadeu, F. X. (2017). Equine Mesenchymal Stromal Cells Retain a

Pericyte-Like Phenotype. *Stem Cells and Development*, *26*(13), 964–972. https://doi.org/10.1089/scd.2017.0017

Fails, A. D. (2020). Functional Anatomy of the Equine Musculoskeletal System. In *Adams and Stashak's Lameness in Horses* (pp. 1–65). Wiley. https://doi.org/10.1002/9781119276715.ch1

Fonseca, F. A., Oliveira, F. T. A., Rajao, M. D., Dumont, C. B. S., Santos-Leonardo, A., & Lima, E. M. M. (2014). Does time matter for platelet-rich plasma treatment of equine tendinitis? *23rd Scientific Meeting of the European College of Veterinary Surgeons*.

Fortier, L. (2010). Clinical Use of Stem Cells, Marrow Components, and Other Growth Factors. In *Diagnosis and Management of Lameness in the Horse: Second Edition* (Second Edi). Elsevier Inc. https://doi.org/10.1016/B978-1-4160-6069-7.00073-0

Fortier, L. A., Nixon, A. J., Williams, J., & Cable, C. S. (1998). Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells. *American Journal of Veterinary Research*, *59*(9), 1182–1187.

Foster, T. E., Puskas, B. L., Mandelbaum, B. R., Gerhardt, M. B., & Rodeo, S. A. (2009). Platelet-rich plasma: From basic science to clinical applications. In *American Journal of Sports Medicine* (Vol. 37, Issue 11, pp. 2259–2272). Am J Sports Med. https://doi.org/10.1177/0363546509349921

Franchi, M., Ottani, V., Stagni, R., & Ruggeri, A. (2010). Tendon and ligament fibrillar crimps give rise to left-handed helices of collagen fibrils in both planar and helical crimps. *Journal of Anatomy*, *216*(3), 301–309. https://doi.org/10.1111/j.1469-7580.2009.01188.x

Friedenstein, A. J., Chailakhjan, R. K., & Lalykina, K. S. (1970). The Development of Fibroblast Colonies in Monolayer Cultures of Guinea-Pig Bone Marrow and Spleen Cells. *Cell Proliferation*, *3*(4), 393–403. https://doi.org/10.1111/j.1365-2184.1970.tb00347.x

Friedenstein, A J, Chailakhyan, R. K., & Gerasimov, U. V. (1987). *Transplantation in Diffusion Chambers*. 263–272.

Friedenstein, Alexander J., Chailakhyan, R. K., Latsinik, N. V., Panasyvk, A. F., & Keiliss-Borok, I. V. (1974). Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues: Cloning in vitro and retransplantation in vivo. *Transplantation*, *17*(4), 331–340. https://doi.org/10.1097/00007890-197404000-00001

Garvican, E. R., Cree, S., Bull, L., Smith, R. K. W., & Dudhia, J. (2014). Viability of equine mesenchymal stem cells during transport and implantation. *Stem Cell Research and Therapy*, *5*(4), 1–10. https://doi.org/10.1186/scrt483

Gillis, C. (2011). Soft tissue injuries: tendinitis and desmitis. In *Equine Sports Medicine and*

Surgery: Second Edition (pp. 399–418).

Gillis, C. L. (1997). Rehabilitation of Tendon and Ligament Injuries. *AAEP Proceedings*, *43*, 306–309.

Godwin, E. E., Young, N. J., Dudhia, J., Beamish, I. C., & Smith, R. K. W. (2012). Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon. *Equine Veterinary Journal*, *44*(1), 25–32. https://doi.org/10.1111/j.2042-3306.2011.00363.x

Gomez-Salazar, M., Gonzalez-Galofre, Z. N., Casamitjana, J., Crisan, M., James, A. W., & Péault, B. (2020). Five Decades Later, Are Mesenchymal Stem Cells Still Relevant? *Frontiers in Bioengineering and Biotechnology*, *8*(February). https://doi.org/10.3389/fbioe.2020.00148

Gonzalez-Rey, E., Gonzalez, M. A., Varela, N., O'Valle, F., Hernandez-Cortes, P., Rico, L., Büscher, D., & Delgado, M. (2010). Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *69*(1), 241–248. https://doi.org/10.1136/ard.2008.101881

Goodrich, L. R., Acvs, D., Frisbie, D. D., & Kisiday, J. D. (2008). How to Harvest Bone Marrow Derived Mesenchymal Stem Cells for Expansion and Injection. *American Association of Equine Practitioners*, *54*, 252–257.

Goodship, A. E., Birch, H. L., & Wilson, A. M. (1994). The pathobiology and repair of tendon and ligament injury. *The Veterinary Clinics of North America. Equine Practice*, *10*(2), 323–349. https://doi.org/10.1016/S0749-0739(17)30359-0

Guest, D. J., Smith, M. R. W., & Allen, W. R. (2008). Monitoring the fate of autologous and allogeneic mesenchymal progenitor cells injected into the superficial digital flexor tendon of horses: Preliminary study. *Equine Veterinary Journal*, *40*(2), 178–181. https://doi.org/10.2746/042516408X276942

Guest, D. J., Smith, M. R. W., & Allen, W. R. (2010). *Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendon*. *42*, 636–642. https://doi.org/10.1111/j.2042-3306.2010.00112.x

Halász, K., Kassner, A., Mörgelin, M., & Heinegård, D. (2007). COMP acts as a catalyst in collagen fibrillogenesis. *Journal of Biological Chemistry*, *282*(43), 31166–31173. https://doi.org/10.1074/jbc.M705735200

Haynesworth, S. E., Goshima, J., Goldberg, V. M., & Caplan, A. I. (1992). Characterization of

cells with osteogenic potential from human marrow. *Bone*, *13*(1), 81–88. https://doi.org/10.1016/8756-3282(92)90364-3

Hays, P. L., Kawamura, S., Deng, X. H., Dagher, E., Mithoefer, K., Ying, L., & Rodeo, S. A. (2008). The role of macrophages in early healing of a tendon graft in a bone tunnel. *Journal of Bone and Joint Surgery - Series A*, *90*(3), 565–579. https://doi.org/10.2106/JBJS.F.00531

Henninger, R. (1994). Treatment of superficial digital flexor tendinitis. *The Veterinary Clinics of North America. Equine Practice*, *10*(2), 409–424. https://doi.org/10.1016/S0749-0739(17)30362-0

Holt, D. D. C., Wood, J. A., Granick, J. L., Walker, N. J., Clark, K. C., & Borjesson, D. L. (2014). *Inhibit T Cell Proliferation Through Different Mechanisms Depending on Tissue Source*. *23*(11), 1258–1265. https://doi.org/10.1089/scd.2013.0537

Horwitz, E. M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Deans, R. J., Krause, D. S., & Keating, A. (2005). Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy*, *7*(5), 393–395. https://doi.org/10.1080/14653240500319234

Hosaka, Y., Ozoe, S., Kirisawa, R., Ueda, H., Takehana, K., & Yamaguchi, M. (2006). Effect of heat on synthesis of gelatinases and pro-inflammatory cytokines in equine tendinocytes. *Biomedical Research*, *27*(5), 233–241. https://doi.org/10.2220/biomedres.27.233

Johnson, A., Smith, R., Saxne, T., Hickery, M., & Heinegård, D. (2004). Fibronectin fragments cause release and degradation of collagen-binding molecules from equine explant cultures. *Osteoarthritis and Cartilage*, *12*(2), 149–159. https://doi.org/10.1016/j.joca.2003.10.008

Juneja, S. C., Schwarz, E. M., O'Keefe, R. J., & Awad, H. A. (2013). Cellular and molecular factors in flexor tendon repair and adhesions: A histological and gene expression analysis. *Connective Tissue Research*, *54*(3), 218–226. https://doi.org/10.3109/03008207.2013.787418

Kasashima, Y., Ueno, T., Tomita, A., Goodship, A. E., & Smith, R. K. W. (2011). Optimisation of bone marrow aspiration from the equine sternum for the safe recovery of mesenchymal stem cells. *Equine Veterinary Journal*, *43*(3), 288–294. https://doi.org/10.1111/j.2042-3306.2010.00215.x

Khong, S. M. L., Lee, M., Kosaric, N., Khong, D. M., Dong, Y., Hopfner, U., Aitzetmüller, M. M., Duscher, D., Schäfer, R., & Gurtner, G. C. (2019). Single-Cell Transcriptomics of Human Mesenchymal Stem Cells Reveal Age-Related Cellular Subpopulation Depletion and Impaired Regenerative Function. *Stem Cells*, *37*(2), 240–246.

https://doi.org/10.1002/stem.2934

Kirker-Head, C. A., & Feldmann, H. (2013). Pharmacotherapy of joint and tendon disease. In *Equine Sports Medicine and Surgery: Second Edition* (Second Edi). Elsevier Ltd. https://doi.org/10.1016/B978-0-7020-4771-8.00023-5

Koch, D., & Goodrich, L. R. (2019). Principles of Therapy for Lameness. In *Adams and Stashak's Lameness in Horses* (pp. 875–947).

Kol, A., Wood, J. A., Carrade Holt, D. D., Gillette, J. A., Bohannon-Worsley, L. K., Puchalski, S. M., Walker, N. J., Clark, K. C., Watson, J. L., & Borjesson, D. L. (2015). Multiple intravenous injections of allogeneic equine mesenchymal stem cells do not induce a systemic inflammatory response but do alter lymphocyte subsets in healthy horses. *Stem Cell Research and Therapy*, *6*(1). https://doi.org/10.1186/s13287-015-0050-0

Kümmerle, J. M., Theiss, F., & Smith, R. K. W. (2019). Diagnosis and Management of Tendon and Ligament Disorders. In *Equine Surgery* (pp. 1411–1445).

Lang, H. M., Schnabel, L. V., Cassano, J. M., & Fortier, L. A. (2017). Effects of Needle diameter on the viability of equine bone marrow derived mesenchymal stem cells. *Veterinary Surgery*, *46*(5), 731–737. https://doi.org/10.1111/vsu.12639.

Lange-Consiglio, A., Tassan, S., Corradetti, B., Meucci, A., Perego, R., Bizzaro, D., & Cremonesi, F. (2013). Investigating the efficacy of amnion-derived compared with bone marrow-derived mesenchymal stromal cells in equine tendon and ligament injuries. *Cytotherapy*, *15*(8), 1011–1020. https://doi.org/10.1016/j.jcyt.2013.03.002

Lavagnino, M., Arnoczky, S. P., Egerbacher, M., Gardner, K. L., & Burns, M. E. (2006). Isolated fibrillar damage in tendons stimulates local collagenase mRNA expression and protein synthesis. *Journal of Biomechanics*, *39*(13), 2355–2362. https://doi.org/10.1016/j.jbiomech.2005.08.008

Lazarus, H. M., Haynesworth, S. E., Gerson, S. L., Rosenthal, N. S., & Caplan, A. I. (1995). Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. *Undefined*.

McIlwraith, C. W. (2010). The use of intra-articular corticosteroids in the horse: What is known on a scientific basis? In *Equine Veterinary Journal* (Vol. 42, Issue 6, pp. 563–571). Equine Vet J. https://doi.org/10.1111/j.2042-3306.2010.00095.x

Mensing, N., Gasse, H., Hambruch, N., Haeger, J., Pfarrer, C., & Staszyk, C. (2011). *Isolation and characterization of multipotent mesenchymal stromal cells from the gingiva and the periodontal ligament of the horse*. 1–13. https://doi.org/10.1186/1746-6148-7-42

Meran, S., & Steadman, R. (2011). Fibroblasts and myofibroblasts in renal fibrosis. *International Journal of Experimental Pathology*, *92*(3), 158–167. https://doi.org/10.1111/j.1365-2613.2011.00764.x

Metcalf, G. L., McClure, S. R., Hostetter, J. M., Martinez, R. F., & Wang, C. (2016). Evaluation of adipose-derived stromal vascular fraction from the lateral tailhead, inguinal region, and mesentery of horses. *Canadian Journal of Veterinary Research*, *80*(4), 294–301. /pmc/articles/PMC5052881/?report=abstract

Miana, V. V., & Prieto González, E. A. (2018). Adipose tissue stem cells in regenerative medicine. *Ecancermedicalscience*, *12*. https://doi.org/10.3332/ecancer.2018.822

Michler, J. K., Hillmann, A., Savkovic, V., & Christoph, K. W. M. (2017). *Horse Hair Follicles : A Novel Dermal Stem Cell Source for Equine Regenerative Medicine*. 1–11. https://doi.org/10.1002/cyto.a.23198

Middleton, K. K., Barro, V., Muller, B., Terada, S., & Fu, F. H. (2012). Evaluation of the effects of platelet-rich plasma (PRP) therapy involved in the healing of sports-related soft tissue injuries. In *The Iowa orthopaedic journal* (Vol. 32, pp. 150–163). University of Iowa. /pmc/articles/PMC3565396/?report=abstract

Moraes, J. R. E., Facco, G. G., Moraes, F. R., Filho, J. R. E., Miyazato, L. G., & Beretta, D. C. (2009). Effects of glycosaminoglycan polysulphate on the organisation of collagen fibres in experimentally induced tendonitis in horses. *Veterinary Record*, *165*(7), 203–205. https://doi.org/10.1136/vr.165.7.203

Murray, R. C., Dyson, S. J., Tranquille, C., & Adams, V. (2006). Association of type of sport and performance level with anatomical site of orthopaedic injury diagnosis. In *Equine vet J., Suppl* (Vol. 36).

Muylle, S., Desmet, P., Simoens, P., Lauwers, H., & Vlaminck, L. (1998). Histological study of the innervation of the suspensory ligament of the forelimb of the horse. *Veterinary Record*, *142*(22), 606–610. https://doi.org/10.1136/vr.142.22.606

Myneni, V. D., Mcclain-Caldwell, I. A. N., Martin, D., Vitale-cross, L. Y. N. N., Marko, K., Firriolo, J. M., Labow, B. I., & Mezey, E. (2019). Mesenchymal stromal cells from infants with simple polydactyly modulate immune responses more efficiently than adult mesenchymal stromal cells. *Cytotherapy*, *21*(2), 148–161. https://doi.org/10.1016/j.jcyt.2018.11.008

Nagy, A., & Dyson, S. (2012). Magnetic resonance imaging and histological findings in the proximal aspect of the suspensory ligament of forelimbs in nonlame horses. *Equine Veterinary Journal*, *44*(1), 43–50. https://doi.org/10.1111/j.2042-3306.2011.00365.x

Nair, B., & Taylor-Gjevre, R. (2010). A review of topical diclofenac use in musculoskeletal

disease. In *Pharmaceuticals* (Vol. 3, Issue 6, pp. 1892–1908). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/ph3061892

Nichols, A. E. C., Best, K. T., & Loiselle, A. E. (2019). The cellular basis of fibrotic tendon healing: challenges and opportunities. *Translational Research*, *209*, 156–168. https://doi.org/10.1016/j.trsl.2019.02.002

Nixon, A. J., Dahlgren, L. A., Haupt, J. L., Yeager, A. E., & Ward, D. L. (2008). Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis. *American Journal of Veterinary Research*, *69*(7), 928–937. https://doi.org/10.2460/ajvr.69.7.928

Opiela, J., Samiec, M., Bochenek, M., Lipiński, D., Romanek, J., & Wilczek, P. (2013). DNA aneuploidy in porcine bone marrow-derived mesenchymal stem cells undergoing osteogenic and adipogenic in vitro differentiation. *Cellular Reprogramming*, *15*(5), 425–434. https://doi.org/10.1089/cell.2012.0099

Ortved, K. F. (2018). Regenerative Medicine and Rehabilitation for Tendinous and Ligamentous Injuries in Sport Horses. *Veterinary Clinics of North America - Equine Practice*, *34*(2), 359–373. https://doi.org/10.1016/j.cveq.2018.04.012

Owen, M., & Friedenstein, A. J. (1988). Stromal stem cells: marrow-derived osteogenic precursors. In *Ciba Foundation symposium* (Vol. 136, pp. 42–60).

Pacini, S., Spinabella, S., Trombi, L., Fazzi, R., Galimberti, S., Dini, F., Carlucci, F., & Petrini, M. (2007). Suspension of bone marrow-derived undifferentiated mesenchymal stromal cells for repair of superficial digital flexor tendon in race horses. *Tissue Engineering*, *13*(12), 2949–2955. https://doi.org/10.1089/ten.2007.0108

Paterson, Y. Z., Rash, N., Garvican, E. R., Paillot, R., & Guest, D. J. (2014). Equine mesenchymal stromal cells and embryo-derived stem cells are immune privileged in vitro. *Stem Cell Research and Therapy*, *5*(4). https://doi.org/10.1186/scrt479

Petrov, R., MacDonald, M. H., Tesch, A. M., & Van Hoogmoed, L. M. (2003). Influence of topically applied cold treatment on core temperature and cell viability in equine superficial digital flexor tendons. *American Journal of Veterinary Research*, *64*(7), 835–844. https://doi.org/10.2460/ajvr.2003.64.835

Phan, S. H. (2002). The Myofibroblast in Pulmonary Fibrosis. *Chest*, *122*(6), 2865–2895. https://doi.org/10.1378/chest.122.6

Piersma, A. H., Ploemacher, R. E., Brockbank, K. G. M., Nikkels, P. G. J., & Ottenheim, C. P. E. (1985). Migration of Fibroblastoid Stromal Cells In Murine Blood. *Cell Proliferation*, *18*(6), 589–595. https://doi.org/10.1111/j.1365-2184.1985.tb00702.x

Pimenta, J. (2018). *Desmite na Origem do Ligamento Suspensor do Boleto Desmite na Origem do Ligamento Suspensor do Boleto*.

Pittenger, M. F., Discher, D. E., Péault, B. M., Phinney, D. G., Hare, J. M., & Caplan, A. I. (2019). Mesenchymal stem cell perspective: cell biology to clinical progress. *Npj Regenerative Medicine*, *4*(1). https://doi.org/10.1038/s41536-019-0083-6

Pluim, M., Martens, A., Vanderperren, K., Sarrazin, S., Koene, M., Luciani, A., van Weeren, P. R., & Delesalle, C. (2018). Short- and long term follow-up of 150 sports horses diagnosed with tendinopathy or desmopathy by ultrasonographic examination and treated with high-power laser therapy. *Research in Veterinary Science*, *119*(2017), 232–238. https://doi.org/10.1016/j.rvsc.2018.06.003

Pösel, C., Möller, K., Fröhlich, W., Schulz, I., Boltze, J., & Wagner, D. C. (2012). Density Gradient Centrifugation Compromises Bone Marrow Mononuclear Cell Yield. *PLoS ONE*, *7*(12). https://doi.org/10.1371/journal.pone.0050293

Radtke, C. L., Nino-Fong, R., Esparza Gonzalez, B. P., Stryhn, H., & Mcduffee, L. A. (2013). Characterization and osteogenic potential of equine muscle tissue- and periosteal tissue-derived mesenchymal stem cells in comparison with bone marrow- and adipose tissue-derived mesenchymal stem cells. *American Journal of Veterinary Research*, *74*(5), 790–800. https://doi.org/10.2460/ajvr.74.5.790

Ranera, B., Antczak, D., Miller, D., Doroshenkova, T., Ryan, A., Mcilwraith, C. W., & Barry, F. (2016). Donor-derived equine mesenchymal stem cells suppress proliferation of mismatched lymphocytes. *Equine Veterinary Journal*, *48*(2), 253–260. https://doi.org/10.1111/evj.12414

Rantanen, N. W., Jorgensen, J. S., & Genovese, R. L. (2010). Ultrasonographic Evaluation of the Equine Limb: Technique. In *Diagnosis and Management of Lameness in the Horse: Second Edition* (Second Edi). Elsevier Inc. https://doi.org/10.1016/B978-1-4160-6069-7.00016-X

Ricco, S., Renzi, S., Del Bue, M., Conti, V., Merli, E., Ramoni, R., Lucarelli, E., Gnudi, G., Ferrari, M., & Grolli, S. (2013). Allogeneic adipose tissue-derived mesenchymal stem cells in combination with platelet rich plasma are safe and effective in the therapy of superficial digital flexor tendonitis in the horse. *International Journal of Immunopathology and Pharmacology*, *26*(1), 61–68. https://doi.org/10.1177/03946320130260S108

Riemersma, D. J. (1989). Tendon strains in horses. *American Journal of Veterinary Research*, *50*(11).

Rink, B. E., Amilon, K. R., Esteves, C. L., French, H. M., Watson, E., Aurich, C., & Donadeu, F. X. (2017). Isolation and characterization of equine endometrial mesenchymal stromal

cells. *Stem Cell Research and Therapy*, *8*(1). https://doi.org/10.1186/s13287-017-0616-0

Romero, A., Barrachina, L., Ranera, B., Remacha, A. R., Moreno, B., de Blas, I., Sanz, A., Vázquez, F. J., Vitoria, A., Junquera, C., Zaragoza, P., & Rodellar, C. (2017). Comparison of autologous bone marrow and adipose tissue derived mesenchymal stem cells, and platelet rich plasma, for treating surgically induced lesions of the equine superficial digital flexor tendon. *Veterinary Journal*, *224*, 76–84. https://doi.org/10.1016/j.tvjl.2017.04.005

Ross, M. W., Genovese, R. L., Dyson, S. J., & Jorgensen, J. S. (2011). Superficial Digital Flexor Tendonitis. In *Diagnosis and Management of Lameness in the Horse: Second Edition* (pp. 706–726).

Ryan, T., & Smith, R. K. W. (2007). An investigation into the depth of penetration of low level laser therapy through the equine tendon in vivo. *Irish Veterinary Journal*, *60*(5), 295–299. https://doi.org/10.1186/2046-0481-60-5-295

Schindl, A., Schindl, M., Pernerstorfer-Schön, H., & Schindl, H. (2000). Low-intensity laser therapy: a review. *J Investig Med*, *48*(5), 312–326.

Schnabel, L. V., Pezzanite, L. M., Antczak, D. F., Felippe, M. J. B., & Fortier, L. A. (2014). Equine bone marrow-derived mesenchymal stromal cells are heterogeneous in MHC class II expression and capable of inciting an immune response in vitro. *Stem Cell Research and Therapy*, *5*(1), 1–13. https://doi.org/10.1186/scrt402

Smith, M. M., Jacobson, E., Dart, A. J., & Little, C. B. (2013). Large Proteoglycan Metabolism in Tendinopathy. *British Journal of Sports Medicine*, *47*(9), e2.51-e2. https://doi.org/10.1136/bjsports-2013-092459.55

Smith, R. K., Birch, H. L., Goodman, S., Heinegard, D., & Goodship, A. E. (2002). The influence of ageing and exercise on tendon growth and degeneration—hypotheses for the initiation and prevention of straininduced tendinopathies. *Comparative Biochemistry and Physiology*, *133*(Part A), 1039–1050.

Smith, R. K., Birch, H. L., Patterson-Kane, J. C., Firth, E. C., Williams, L., Cherdchuthan, W., van Weeren, W. R., & Goodship, A. E. (1999). Should Equine Athletes Commence Training During Skeletal Development?: Changes in Tendon Matrix Associated with Development, Ageing, Function and Exercise. *Equine Exercise Physiology*, *30*, 201–209.

Smith, R. K. W., Korda, M., Blunn, G. W., & Goodship, A. E. (2003). Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. *Equine Veterinary Journal*, *35*(1), 99–102. https://doi.org/10.2746/042516403775467388

Smith, Roger K.W. (2008). Tendon and Ligament Injury. *AAEP Proceedings*.

Smith, Roger K.W. (2010). Pathophysiology of Tendon Injury. In *Diagnosis and Management of Lameness in the Horse: Second Edition* (Second Edi). Elsevier Inc. https://doi.org/10.1016/B978-1-4160-6069-7.00068-7

Smith, Roger K.W., & Goodship, A. E. (2008). Tendon and ligament physiology: Responses to exercise and training. In *Equine Exercise Physiology* (First Edit). Elsevier Ltd. https://doi.org/10.1016/B978-070202857-1.50007-X

Smith, Roger K W. (2007). Tendon and Ligament. *Tissue Mechanics*, *54*, 559–594. https://doi.org/10.1007/978-0-387-49985-7_16

Smith, Roger K W. (2020). Regenerative medicine in equine orthopaedics: what and when? *UK-Vet Equine*, *4*(1), 8–13. https://doi.org/10.12968/ukve.2020.4.1.8

Soffler, C., & Hermanson, J. W. (2006). Muscular Design in the Equine Interosseus Muscle. *Journal of Morphology*, *267*, 696–704.

Somoza, R. A., Correa, D., & Caplan, A. I. (2015). Roles for mesenchymal stem cells as medicinal signaling cells. *Nature Protocols*, *June*, 2015. http://www.nature.com/nprot/posters/msc/index.html

Souza, J. B. De, Alvarenga, M. L., Pfeifer, P. H., Hugo, V., Eduardo, C., Rodrigues, M., Laufer-amorim, R., Castillo, A. L., Liz, A., & Alves, G. (2018). Allogeneic mesenchymal stem cell transplantation in healthy equine superficial digital flexor tendon: A study of the local inflammatory response. *Research in Veterinary Science*, *118*, 423–430. https://doi.org/10.1016/j.rvsc.2018.03.012

Stanley, R. L., Edwards, L. J., Goodship, A. E., Firth, E. C., & Patterson-Kane, J. C. (2008). Effects of exercise on tenocyte cellularity and tenocyte nuclear morphology in immature and mature equine digital tendons. *Equine Veterinary Journal*, *40*(2), 141–146. https://doi.org/10.2746/042516408X266097

Stephens, P. R., Nunamaker, D. M., & Butterweck, D. M. (1989). Application of a Hall-effect transducer for measurement of tendon strains in horses. *American Journal of Veterinary Research*, *50*(7), 1089–1095.

Stewart, M. C., & Stewart, A. A. (2011). Mesenchymal Stem Cells: Characteristics, Sources, and Mechanisms of Action. *Veterinary Clinics of North America - Equine Practice*, *27*(2), 243–261. https://doi.org/10.1016/j.cveq.2011.06.004

Takahashi, T., Ohmura, H., Mukai, K., Matsui, A., & Aida, H. (2014). Fatigue in the Superficial and Deep Digital Flexor Muscles During Exercise in Thoroughbred Horses. *Equine Veterinary Journal*, *46*, 30–30. https://doi.org/10.1111/evj.12267_90

Tessier, L., Bienzle, D., Williams, L. B., & Koch, T. G. (2015). Phenotypic and

immunomodulatory properties of equine cord blood-derived mesenchymal stromal cells. *PLoS ONE*, *10*(4). https://doi.org/10.1371/journal.pone.0122954

Thomopoulos, S., Parks, W. C., Rifkin, D. B., & Derwin, K. A. (2015). Mechanisms of tendon injury and repair. *Journal of Orthopaedic Research*, *33*(6), 832–839. https://doi.org/10.1002/jor.22806

Thorpe, C. T., Clegg, P. D., & Birch, H. L. (2010). A review of tendon injury: Why is the equine superficial digital flexor tendon most at risk? *Equine Veterinary Journal*, *42*(2), 174–180. https://doi.org/10.2746/042516409X480395

Thorpe, Chavaunne T., Streeter, I., Pinchbeck, G. L., Goodship, A. E., Clegg, P. D., & Birch, H. L. (2010). Aspartic acid racemization and collagen degradation markers reveal an accumulation of damage in tendon collagen that is enhanced with aging. *Journal of Biological Chemistry*, *285*(21), 15674–15681. https://doi.org/10.1074/jbc.M109.077503

Thorpe, Chavaunne T., Udeze, C. P., Birch, H. L., Clegg, P. D., & Screen, H. R. C. (2012). Specialization of tendon mechanical properties results from interfascicular differences. *Journal of the Royal Society Interface*, *9*(76), 3108–3117. https://doi.org/10.1098/rsif.2012.0362

Thorpe, Chavaunne Thandiwe. (2010). *Extracellular Matrix Synthesis and Degradation in Functionally Distinct Tendons*. University College London.

Toupadakis, C. A., Wong, A., Genetos, D. C., Cheung, W. K., Borjesson, D. L., Ferraro, G. L., Galuppo, L. D., Leach, J. K., Owens, S. D., & Yellowley, C. E. (2010). Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. *American Journal of Veterinary Research*, *71*(10), 1237–1245. https://doi.org/10.2460/ajvr.71.10.1237

Valberg, S. J. (2008). Skeletal Muscle Function. *Clinical Biochemistry of Domestic Animals*, 459–484. https://doi.org/10.1016/B978-0-12-370491-7.00015-5

van den Belt, A. J. M., Dik, K. J., Keg, P. R., & Barneveld, A. (1993). The Correlation between the Dose and Distribution of Intratendinous Fluid Injections in the Flexor TendodLigaments of the Horse. *Journal of Veterinary Medicine*, *40*, 713–719.

Van Loon, V. J. F., Scheffer, C. J. W., Genn, H. J., Hoogendoorn, A. C., & Greve, J. W. (2014). Clinical follow-up of horses treated with allogeneic equine mesenchymal stem cells derived from umbilical cord blood for different tendon and ligament disorders. *Veterinary Quarterly*, *34*(2), 92–97. https://doi.org/10.1080/01652176.2014.949390

Vidal, M. A., Kilroy, G. E., Lopez, M. J., Johnson, J. R., Moore, R. M., & Gimble, J. M. (2007). Characterization of equine adipose tissue-derived stromal cells: Adipogenic and

osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. *Veterinary Surgery*, *36*(7), 613–622. https://doi.org/10.1111/j.1532-950X.2007.00313.x

Vidal, M. A., Walker, N. J., Napoli, E., & Borjesson, D. L. (2012). Evaluation of senescence in mesenchymal stem cells isolated from equine bone marrow, adipose tissue, and umbilical cord tissue. *Stem Cells and Development*, *21*(2), 273–283. https://doi.org/10.1089/scd.2010.0589

Voleti, P. B., Buckley, M. R., & Soslowsky, L. J. (2012). Tendon Healing: Repair and Regeneration. *Annual Review of Biomedical Engineering*, *14*(1), 47–71. https://doi.org/10.1146/annurev-bioeng-071811-150122

Werpy, N. M., & Denoix, J. M. (2012). Imaging of the Equine Proximal Suspensory Ligament. *Veterinary Clinics of North America - Equine Practice*, *28*(3), 507–525. https://doi.org/10.1016/j.cveq.2012.08.005

Williams, I. F., Heaton, A., & McCullagh, K. G. (1980). Cell Morphology and Collagen Types in Equine Tendon Scar. *Research in Veterinary Science*, *28*(3), 302–310.

Williams, L. B., Co, C., Koenig, J. B., Tse, C., Lindsay, E., & Koch, T. G. (2016). Response to intravenous allogeneic equine cord blood-derived mesenchymal stromal cells administered from chilled or frozen state in serum and protein-free media. *Frontiers in Veterinary Science*, *3*(JUL), 56. https://doi.org/10.3389/fvets.2016.00056

Williams, R. B., Harkins, L. S., Hammond, C. J., & Wood, J. L. N. (2001). Racehorse injuries, clinical problems and fatalities recorded on British racecourses from flat racing and National Hunt racing during 1996, 1997 and 1998. *Equine Veterinary Journal*, *33*(5), 478–486. https://doi.org/10.2746/042516401776254808

Wilson, A. M., McGuigan, M. P., Su, A., & Van den Bogert, A. J. (2001). Horses damp the spring in their step. *Nature*, *414*(6866), 895–899. https://doi.org/10.1038/414895a

10 Annexes

Annex 1 - Lameness score, lesion category, re-injury rate, work level and rate of lower work level due to lesion of the overall population.

LAMENESS SCORE							
Months	**0/5**	**1/5**	**2/5**	**3/5**	**4/5**	**5/5**	**Total**
0	51% (n=25)	32,7% (n=16)	12,2% (n=6)	2% (n=1)	0	2% (n=1)	n=49
6	85,7% (n=42)	14,3% (n=7)	0	0	0	0	n=49
12	84,4% (n=38)	13,3% (n=6)	2,2% (n=1)	0	0	0	n=45

LESION CATEGORY			
M	**Clinically irrelevant**	**Clinically relevant**	**Total**
0	0	100% (n=49)	n=49
6	69,4% (n=34)	30,6% (n=15)	n=49
12	84,4% (n=38)	15,6% (n=7)	n=45

RE-INJURY			
M	**NO**	**YES**	**Total**
6	96% (n=40)	4% (n=2)	n=42
12	82,2% (n=30)	17,8% (n=8)	n=38

WORK LEVEL			
M	**SAME/HIGHER**	**LOWER**	**Total**
12	55,1% (n=27)	44,9% (n=22)	n=49

LOWER WORK LEVEL DUE TO LESION		
YES	**NO**	**Total**
72,7% (n=16)	27,3% (n=6)	n=22

Annex 2 - Lameness score, lesion category, re-injury rate and work level of the different breed groups.

LAMENESS SCORE

MONTHS	BREED	0/5	1/5	2/5	3/5	4/5	5/5	Total
0	WB	43,8% (n=7)	31,3% (n=5)	12,5% (n=2)	6,3% (n=1)	0	6,3% (n=1)	n=16
	Fr	33,3% (n=4)	50% (n=6)	16,7% (n=2)	0	0	0	n=12
	STB	78,6% (n=11)	14,3% (n=2)	7,1% (n=1)	0	0	0	n=14
	Other	42,9% (n=3)	42,9% (n=3)	14,3% (n=1)	0	0	0	n=7
6	WB	87,5% (n=14)	12,5% (n=2)	0	0	0	0	n=16
	Fr	91,7% (n=11)	8,3% (n=1)	0	0	0	0	n=12
	STB	71,4% (n=10)	28,6% (n=4)	0	0	0	0	n=14
	Other	100% (n=7)	0	0	0	0	0	n=7
12	WB	86,7% (n=13)	6,7% (n=1)	6,7% (n=1)	0	0	0	n=15
	Fr	90,9% (n=10)	9,1% (n=1)	0	0	0	0	n=11
	STB	69,2% (n=9)	30,8% (n=4)	0	0	0	0	n=13
	Other	100% (n=6)	0	0	0	0	0	n=6

LESION CATEGORY

MONTHS	BREED	Clinically irrelevant	Clinically relevant	Total
0	WB	0	100% (n=16)	n=16
	Fr	0	100% (n=12)	n=12
	STB	0	100% (n=14)	n=14
	Other	0	100% (n=7)	n=7
6	WB	75% (n=12)	25% (n=4)	n=16
	Fr	75% (n=9)	25% (n=3)	n=12
	STB	50% (n=7)	50% (n=7)	n=14
	Other	85,7% (n=6)	14,3% (n=1)	n=7
12	WB	86,7% (n=13)	13,3% (n=2)	n=15
	Fr	100% (n=11)	0	n=11
	STB	**61,5% (n=8)***	38,5% (n=5)	n=13
	Other	100% (n=6)	0	n=6

RE-INJURY

M	BREED	NO	YES	Total
6	WB	93,8% (n=15)	6,3% (n=1)	n=16
	Fr	100% (n=12)	0	n=12
	STB	92,9% (n=13)	7,1% (n=1)	n=14
	Other	100% (n=7)	0	n=7
12	WB	86,7% (n=13)	13,3% (n=2)	n=15
	Fr	90,9% (n=10)	9,1% (n=1)	n=11
	STB	69,2% (n=9)	30,8% (n=4)	n=13
	Other	83,3% (n=5)	16,7% (n=1)	n=6

WORK LEVEL

M	BREED	SAME/ HIGHER	LOWER	Total
12	WB	56,3% (n=9)	43,8% (n=7)	n=16
	Fr	66,7% (n=8)	33,3% (n=4)	n=12
	STB	50% (n=7)	50% (n=7)	n=14
	Other	42,9% (n=3)	57,1% (n=4)	n=7

WB = Warmblood; Fr = Friesian; STB = Standardbred

***p=0.039**

Annex 3 - Lameness score, lesion category, re-injury rate and work level for the different discipline groups.

LAMENESS SCORE

MONTHS	DISCIPLINE	0/5	1/5	2/5	3/5	4/5	5/5	Total
0	SJ	71,4% (n=5)	28,6% (n=2)	0	0	0	0	n=7
	Dr	41,2% (n=7)	35,3% (n=6)	17,6% (n=3)	0	0	5,9% (n=1)	n=17
	HR	78,6% (n=11)	14,3% (n=2)	7,1% (n=1)	0	0	0	n=14
	Other	18,2% (n=2)	54,5% (n=6)	18,2% (n=2)	9,1% (n=1)	0	0	n=11
6	SJ	85,7% (n=6)	14,3% (n=1)	0	0	0	0	n=7
	Dr	94,1% (n=16)	5,9% (n=1)	0	0	0	0	n=17
	HR	71,4% (n=10)	28,6% (n=4)	0	0	0	0	n=14
	Other	90,9% (n=10)	9,1% (n=1)	0	0	0	0	n=11
12	SJ	85,7% (n=6)	14,3% (n=1)	0	0	0	0	n=7
	Dr	87,5% (n=14)	12,5% (n=2)	0	0	0	0	n=16
	HR	69,2% (n=9)	30,8% (n=4)	0	0	0	0	n=13
	Other	100% (n=9)	0	0	0	0	0	n=9

LESION CATEGORY

M	DISCIPLINE	Clinically irrelevant	Clinically relevant	Total
0	SJ	0	100% (n=7)	n=7
	Dr	0	100% (n=17)	n=17
	HR	0	100% (n=14)	n=14
	Other	0	100% (n=11)	n=11
6	SJ	100% (n=7)	0	n=7
	Dr	76,5% (n=13)	23,5% (n=4)	n=17
	HR	50% (n=7)	50% (n=7)	n=14
	Other	85,7% (n=7)	14,3% (n=4)	n=11
12	SJ	85,7% (n=6)	14,3% (n=1)	n=7
	Dr	100% (n=16)	0	n=16
	HR	**61,5% (n=8)***	38,5% (n=5)	n=13
	Other	88,9% (n=8)	11,1% (n=1)	n=9

RE-INJURY

M	D	NO	YES	Total
6	SJ	100% (n=7)	0	n=7
	Dr	100% (n=17)	0	n=17
	HR	92,9% (n=13)	7,1% (n=1)	n=14
	Other	90,9% (n=10)	9,1% (n=1)	n=11
12	SJ	85,7% (n=6)	14,3% (n=1)	n=7
	Dr	87,5% (n=14)	12,5% (n=2)	n=16
	HR	69,2% (n=9)	30,8% (n=4)	n=13
	Other	88,9% (n=8)	11,1% (n=1)	n=9

WORK LEVEL

M	D	SAME/ HIGHER	LOWER	Total
12	SJ	71,4% (n=5)	28,6% (n=2)	n=7
	Dr	64,7% (n=11)	35,3% (n=6)	n=17
	HR	50% (n=7)	50% (n=7)	n=14
	Other	36,4% (n=4)	63,6% (n=7)	n=11

SJ = Show-jumping; Dr= Dressage; HR = Harness Racing

D = Discipline; M = Month

***p=0.04**

Annex 4 - Lameness score, lesion categories, re-injury rate and work level for the different age groups.

LAMENESS SCORE

M	AGE	0/5	1/5	2/5	3/5	4/5	5/5	Total
0	0-5	77,8% (n=7)	11,1% (n=1)	11,1% (n=1)	0	0	0	n=9
	6-10	50% (n=11)	31,8% (n=7)	13,6% (n=3)	0	0	4,5% (n=1)	n=22
	11-15	38,5% (n=5)	53,8% (n=7)	0	7,7% (n=1)	0	0	n=13
	16-20	66,7% (n=2)	0	33,3% (n=1)	0	0	0	n=3
	20<	0	50% (n=1)	50% (n=1)	0	0	0	n=2
6	0-5	77,88% (n=7)	22,2% (n=2)	0	0	0	0	n=9
	6-10	86,4% (n=19)	13,6% (n=3)	0	0	0	0	n=22
	11-15	92,3% (n=12)	7,7 (n=1)	0	0	0	0	n=13
	16-20	66,7% (n=2)	33,3% (n=1)	0	0	0	0	n=3
	20<	100% (n=2)	0	0	0	0	0	n=2
12	0-5	66,7% (n=6)	33,3% (n=3)	0	0	0	0	n=9
	6-10	90% (n=18)	10% (n=2)	0	0	0	0	n=20
	11-15	83,3% (n=10)	8,3% (n=1)	8,3% (n=1)	0	0	0	n=12
	16-20	100% (n=3)	0	0	0	0	0	n=3
	20<	100% (n=1)	0	0	0	0	0	n=1

LESION CATEGORY

MO	AGE	Clinically irrelevant	Clinically relevant	Total
0	0-5	0	100% (n=9)	n=9
	6-10	0	100% (n=22)	n=22
	11-15	0	100% (n=13)	n=13
	16-20	0	100% (n=3)	n=3
	20<	0	50% (n=2)	n=2
6	0-5	77,8% (n=7)	22.2% (n=2)	n=9
	6-10	86,4% (n=19)	13,6% (n=3)	n=22
	11-15	61,5% (n=8)	38,5% (n=5)	n=13
	16-20	100% (n=3)	0	n=3
	20<	100% (n=2)	0	n=2
12	0-5	88,9% (n=8)	11,1% (n=1)	n=9
	6-10	85% (n=17)	15% (n=3)	n=20
	11-15	75% (n=9)	25% (n=3)	n=12
	16-20	100% (n=3)	0	n=3
	20<	100% (n=1)	0	n=1

RE-INJURY

M	AGE	NO	YES	Total
6	0-5	100% (n=9)	0	n=9
	6-10	95,5% (n=21)	4,5% (n=1)	n=22
	11-15	92,3% (n=12)	7,7% (n=1)	n=13
	16-20	100% (n=3)	0	n=3
	20<	100% (n=2)	0	n=2
12	0-5	77,8% (n=7)	22,2% (n=2)	n=9
	6-10	85% (n=17)	15% (n=3)	n=20
	11-15	75% (n=9)	25% (n=3)	n=12
	16-20	100% (n=3)	0	n=3
	20<	100% (n=1)	0	n=1

WORK LEVEL

M	AGE	SAME/ HIGHER	LOWER	Total
12	0-5	66,7% (n=6)	33,3% (n=3)	n=9
	6-10	63,6% (n=14)	36,4% (n=8)	n=22
	11-15	30,8% (n=4)	69,2% (n=9)	n=13
	16-20	66,7% (n=2)	33,3% (n=1)	n=3

20<	50% (n=1)	50% (n=1)	n=2

Annex 5 - Lameness score, lesion categories, re-injury rate and work level for the different lesion chronicity groups.

LAMENESS SCORE							
M	**CHRONICITY**	**0/5**	**1/5**	**2/5**	**3/5**	**4/5**	**5/5**	**Total**
	<2 weeks	69,2% (n=9)	23,1% (n=3)	7,7% (n=1)	0	0	0	n=13
0	**2-4 weeks**	40,9% (n=9)	27,3% (n=6)	22,7% (n=5)	4,5% (n=1)	0	4,5% (n=1)	n=22
	4< weeks	50% (n=7)	50% (n=7)	0	0	0	0	n=14
	<2 weeks	84,6% (n=11)	15,4% (n=2)	0	0	0	0	n=13
6	**2-4 weeks**	95,5% (n=21)	4,5% (n=1)	0	0	0	0	n=22
	4< weeks	71,4% (n=10)	28,6% (n=4)	0	0	0	0	n=14
	<2 weeks	61,5% (n=8)	30,8% (n=4)	7,7% (n=1)	0	0	0	n=13
12	**2-4 weeks**	95% (n=19)	5% (n=1)	0	0	0	0	n=20
	4< weeks	91,7% (n=11)	8,3% (n=1)	0	0	0	0	n=12

LESION CATEGORY				
M	**CHRONICITY**	**Clinically irrelevant**	**Clinically relevant**	**Total**
	<2 weeks	0	100% (n=13)	n=13
0	**2-4 weeks**	0	100% (n=22)	n=22
	4< weeks	0	100% (n=14)	n=14
	<2 weeks	61,5% (n=8)	38,5% (n=5)	n=13
6	**2-4 weeks**	81,8% (n=18)	18,2% (n=4)	n=22
	4< weeks	57,1% (n=8)	42,9% (n=6)	n=14
	<2 weeks	69,2% (n=9)	30,8% (n=4)	n=13
12	**2-4 weeks**	90% (n=18)	10% (n=2)	n=20
	4< weeks	91,7% (n=11)	8,3% (n=1)	n=12

RE-INJURY				
M	**CHRONICITY**	**NO**	**YES**	**Total**
	<2 weeks	100% (n=13)	0	n=13
6	**2-4 weeks**	100% (n=22)	0	n=22
	4< weeks	85,7% (n=12)	14,3% (n=2)	n=14
	<2 weeks	61,5% (n=8)	38,5% (n=5)	n=13
12	**2-4 weeks**	90% (n=18)	10% (n=2)	n=20
	4< weeks	91,7% (n=11)	8,3% (n=1)	n=12

WORK LEVEL				
M	**CHRONICITY**	**SAME/ HIGHER**	**LOWER**	**Total**
	<2 weeks	53,8% (n=7)	46,2% (n=6)	n=13
12	**2-4 weeks**	54,5% (n=12)	45,5% (n=10)	n=22
	4< weeks	57,1% (n=8)	42,9% (n=6)	n=14

Annex 6 - Lameness score, lesion categories, re-injury rate and work level for the different types of MSCs used.

LAMENESS SCORE

M	MSCs	0/5	1/5	2/5	3/5	4/5	5/5	Total
0	SVF+PRP	56% (n=14)	40% (n=10)	4% (n=1)	0	0	0	n=25
	BM-MSCs	45,8% (n=11)	25% (n=6)	20,8% (n=5)	4,2% (n=1)	0	4,2% (n=1)	n=24
6	SVF+PRP	88% (n=22)	12% (n=3)	0	0	0	0	n=25
	BM-MSCs	83,3% (n=20)	16,7% (n=4)	0	0	0	0	n=22
12	SVF+PRP	87,5% (n=21)	12,5% (n=3)	0	0	0	0	n=24
	BM-MSCs	81% (n=17)	14,3% (n=3)	4,8% (n=1)	0	0	0	n=21

LESION CATEGORY

M	MSCs	Clinically irrelevant	Clinically relevant	Total
0	SVF+PRP	0	100% (n=25)	n=25
	BM-MSCs	0	100% (n=24)	n=24
6	SVF+PRP	80% (n=20)	20% (n=5)	n=25
	BM-MSCs	58,3% (n=14)	41,7% (n=10)	n=24
12	SVF+PRP	87,5% (n=21)	12,5% (n=3)	n=24
	BM-MSCs	81% (n=17)	19% (n=4)	n=21

RE-INJURY

M	MSCs	NO	YES	Total
6	SVF+PRP	96% (n=24)	4% (n=1)	n=25
	BM-MSC	95,8% (n=23)	4,2% (n=1)	n=24
12	SVF+PRP	87,5% (n=21)	12,5% (n=3)	n=24
	BM-MSC	76,2% (n=16)	23,8% (n=5)	n=21

WORK LEVEL

M	MSCs	SAME/HIGHER	LOWER	Total
12	SVF+PRP	64% (n=16)	36% (n=9)	n=25
	BM-MSC	45,8% (n=11)	54,2% (n=13)	n=24

Annex 7 - Lameness score, lesion categories, re-injury rate and work level for the different treated structures.

LAMENESS SCORE

M	ST	0/5	1/5	2/5	3/5	4/5	5/5	Total
0	SL	52% (n=13)	32% (n=8)	16% (n=4)	0	0	0	n=25
	SDFT	50% (n=12)	33,3% (n=8)	8,3% (n=2)	4,2% (n=1)	0	4,2% (n=1)	n=24
6	SL	80% (n=20)	20% (n=5)	0	0	0	0	n=25
	SDFT	91,7% (n=22)	8,3% (n=2)	0	0	0	0	n=24
12	SL	87% (n=20)	13% (n=3)	0	0	0	0	n=23
	SDFT	81,8% (n=18)	13,6% (n=3)	4,2% (n=1)	0	0	0	n=22

LESION CATEGORY

M	ST	Clinically irrelevant	Clinically relevant	Total
0	SL	0	100% (n=25)	n=25
	SDFT	0	100% (n=24)	n=24
6	SL	68% (n=17)	32% (n=8)	n=25
	SDFT	70,8% (n=17)	29,2% (n=7)	n=24
12	SL	91,3% (n=21)	8,7% (n=2)	n=23
	SDFT	77,3% (n=17)	22,7% (n=5)	n=22

RE-INJURY

M	ST	NO	YES	Total
6	SL	96% (n=24)	4% (n=1)	n=25
	SDFT	95,8% (n=23)	4,2% (n=1)	n=24
12	SL	87,5% (n=21)	12,5% (n=3)	n=24
	SDFT	76,2% (n=16)	23,8% (n=5)	n=21

WORK LEVEL

M	ST	SAME/HIGHER	LOWER	Total
12	SL	64% (n=16)	36% (n=9)	n=25
	SDFT	45,8% (n=11)	54,2% (n=13)	n=24