UNIVERSITÀ DEGLI STUDI DI CAMERINO

School of Advanced Studies

DOCTORAL COURSE IN LIFE AND HEALTH SCIENCES
XXXIII° cycle

TREATMENT OF SPONTANEOUS OSTEOARTHRITIS WITH MICRO-FRAGMENTED ADIPOSE TISSUE GRAFT IN DOG

PhD Student
Botto Riccardo

Supervisors
Prof.ssa Palumbo Piccionello Angela

INTRODUCTION

Osteoarthritis (syn: OA, degenerative joint disease, osteoarthritis) can be defined as a disease of the movable joints [1] characterized by chronic deterioration of the articular cartilage with altered subchondral bone metabolism, the formation of periarticular osteophytes, a degree variable synovial inflammation leading to progressive joint stiffness and pain [2]. It is the most common form of arthritis in dogs and cats and also reported in human species as common as well [3]. In 2005, 26.9 million adults in the USA were estimated to have OA [4], up from 21 million in 1990. As reported from the Osteoarthritis Research Society International (OARSI) the osteoarthritis accounts for 2.4% of all years lived with disabilities (YLD) and has been ranked as the 10th leading contributor to global YLDs [5].

In Veterinary Medicine, although robust epidemiological data are lacking, it has been estimated that approximately 20% of adult dogs are affected by OA [6] and also reported as well in more than 60% of adult cats which showed radiographic evidence of this condition in the appendicular skeleton [7]. Pet dogs share both an environment and lifestyle with their owners, and a growing public awareness is developing and also among researchers of the One Health Medicine. Dog's OA is generally considered to bear a close resemblance to human OA, which regards anatomic similarity, disease heterogeneity and progression [8]. For this reason, several human researchers used dogs with spontaneous OA and ranks this species as the best animal model adopted for research [9]. Furthermore, mutual co-studies on osteoarthritis in veterinary and human medicine could be beneficial in both humans and dogs itself. One Health Medicine is emerging as a holistic paradigm wherein veterinary and human medical researchers and clinical practitioners collaborate to increase their understanding of shared diseases and to develop new therapies [10].

OA is not a single disease, and is often misperceived as a disease of only cartilage, but it is a complex degenerative joint disease which have to be controlled with a multimodal therapies [11]. Multimodal management of canine osteoarthritis is based on several therapeutic approach including non-steroidal anti-inflammatory medications

(NSAIDs), joint supplements, weight management, and rehabilitation therapy [6;12]. When common conservative therapies fail to provide a long-term response, and a surgical management is not the proper decision to make, regenerative medicine is another option in the multimodal approach to manage OA [13]. Several publication reports the use of different interarticular regenerative medications in dogs affected by OA, such as hyaluronic acid, non transfusional hemocomponent (PRP, PRF, IRAP) mesenchiamal stem cells, and stromall vascular fraction. [13-15]

In the last few years, medical researchers on the Regenerative field focus on the therapeutic properties of the mesenchymal stem cells (MSCs) to know the feasibility and the efficacy of their use [16-17].

Although mesenchymal stem cells were originally isolated from bone marrow [18-19], similar populations have been reported in other tissues. Human MSC have been isolated from adipose tissue [20], umbilical cord blood [21–24], peripheral blood [25-26], connective tissues of the dermis, and skeletal muscle [27].

Due to the characteristic high concentration of stem cells, the simple isolation, and the plastic properties, the scientific community shows a particular interest in MSCs derived from adipose tissue, which is called Adipose-Derived Stem Cells [28-31]. The adipose tissue once processed, allow to extract the aqueous fraction consisting of ASCs and the stromal vascular fraction (SVF). SVF contains precursors of endothelial cells (EPCs), macrophages, smooth muscle cells, lymphocytes, pericytes, pre-adipocytes [32]. The clinical efficacy in the treatment of OA through SVF infiltration is linked to its anti-inflammatory and immunoregulatory effects, alongside the regenerative capacity of ASCs [28]. Mesenchymal stem cells (MSCs) are considered to be a promising candidate for cartilage regeneration, due to their ability to differentiate towards cartilage and bone cells and secrete trophic factors with regenerative functions [33]. The paracrine effect and anti-apoptotic, anti-inflammatory and anti-aging functions of these stem cells, is fundamental for the regeneration process. ASCs are a positive alternative treatment for OA, as in vitro studies have proven they contain CD73, CD90, CD105, and CD106 markers, which are necessary for cell differentia-

tion into cartilage, moreover, in vivo studies have also reported good results, despite with some limits yet [34-35].

Over the past decade, research has brought new insights into the effects of ASCs, and more new mechanical disintegration technologies promise to achieve rapid, ready-to-use stem cell collection.[36-38].

The following research aims to validate a novel system for the disintegration of adipose tissue in dogs and evaluate the effectiveness of intra-articular injection of micro-fragmented adipose tissue (MFAT) for the treatment of spontaneous osteoarthritis in dogs by comparing the use of hyaluronic acid, currently considered standard of care for the treatment of OA.

1 OSTEOARTHRITIS

Osteoarthritis (OA) may be defined, in pathologic terms, as the aberrant repair and eventual degradation of articular cartilage in association with alterations in subchondral bone metabolism, periarticular osteophytosis, and a variable degree of synovial inflammation [39]. OA should be thought of as a disease process rather than a disease entity because it appears to be a common final pathway for the failing joint. It is a pathological condition of the entire diarthrodial joint, including articular cartilage (hyaline), synovial membrane, synovial fluid, subchondral bone, and surrounding supporting structures (muscles and ligaments). The joint can be considered an "organ" in which all components of it are affected by the pathological process. Historically recognized as "types of non-inflammatory arthritis" *(Fig.1.1)*, due to evidence of the lack of active inflammation on synovial cytology, OA is now considered an inflammatory clinical condition, without an increase in white blood cells in the fluid synovial as in other types of arthritis [40]. In pathologic terms, OA has been defined as "an inherently noninflammatory disorder of movable (synovial) joints characterized by deterioration of articular cartilage and by the formation of new bone at the joint surfaces and margins.

OA is associated with a progressive destruction and loss of cartilage, remodeling of bone, and intermittent inflammation. Changes in subchondral bone, synovium, and ligaments are detectable at an early stage, and initially an increase in cartilage matrix synthesis occurs concurrently with increased degradation [5;39]. Synovial and cartilage-derived proteases are major players in cartilage matrix degradation, with matrix metalloproteinases (MMPs) and aggrecanases seemingly key catabolic agents. Articular cartilage is considered the key tissue in osteoarthritis, but it must be remembered that the synovial joint as an organ, has cross-talk between various tissues (cartilage, synovium, bone, ligament, synovial fluid, fat). The relative importance of this cross-talk is still non completely know [40]. In recent decades, research studies on OA have focused on the characterization of molecular biomarkers in order to highlight the osteoarthritis process in the initial stages and be able to prevent the trigger of cartilage deterioration [41].
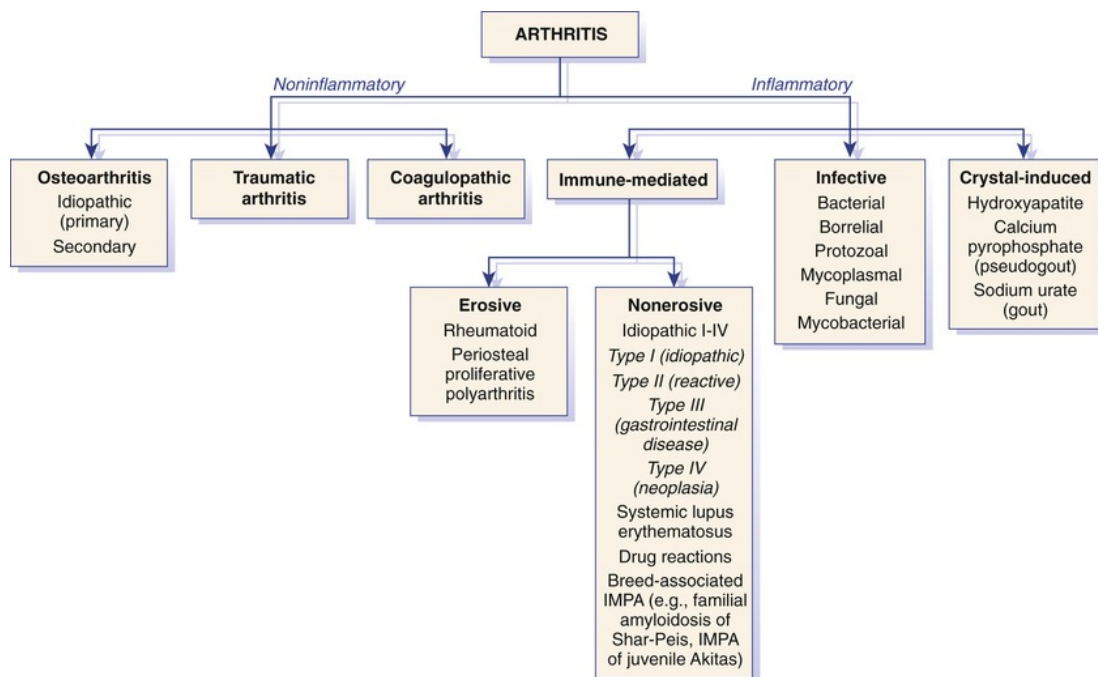
fig.1.1 Classification of canine and feline arthritis [41]

1.1 PREVALENCE IN DOG

Osteoarthritis affects more than 80% of Americans over age 55 [42] years and approximately 1 in 5 adult dogs in the USA [43]. It is the number one cause of chronic pain in dogs, and approximately 10–12 million dogs in the USA show signs of OA. The 'average' veterinary practice sees approximately 45 arthritic dogs per month, 21% of which are considered 'severe', 38% are considered 'moderate', and 41% are considered 'mild' as assessed by their clinical presentation [44]. The demographics of dogs with OA are broad-reaching. Although the condition tends to be over-represented in older, heavy dogs, it can be a clinical problem in any dog. The 'poster child' for OA in dogs is the middle-aged to older (>4 years), large breed (>50 lb [>22.5 kg]) dog that is overweight to obese. In the case of obesity, which is often seen in older dogs, abnormal stress on the joints is accentuated.

1.2 CLASSIFICATION OF OSTEOARTHRITIS

The OA onset and development in dogs is a complex mechanism, and both the causative causes of the disease and the factors that support its progression are not yet fully understood. Despite the challenge of the full understand of disease, osteoarthritis can be classified as *idiopathic* (syn: *primary*) or *secondary* [45].

Primary OA is associated to joint aging, whose causes of cartilage degeneration are not yet understood at all; it is described as a joint disease caused by wear of the synovial components due to prolonged use and to predisposing OA factors. *Secondary OA* onset itself as a response to anomalies or trauma that cause joint instability and can be associated with genetic factors predisposing to the development of pathologies [45].

The initiation of OA is not well understood, but several theories have been extended to explain how different stimuli may give rise to a progressive, degenerative disorder. These hypotheses can mainly be divided into those that propose abnormal stresses

acting on normal cartilage (e.g. Trauma) and those that propose a consequence of normal stresses acting on abnormal cartilage (e.g Dysplasia).

In dogs, the disorder most frequently occurs secondary to an identifiable abnormality of the joint such as a developmental disorder, joint instability, or trauma (e.g., osteo-chondritis dissecans, hip dysplasia, cruciate ligament rupture).

Once established, both primary and secondary OA progress along a "final common pathway" of anatomic and biochemical changes in the joint.

1.3 ETIOLOGY

The etiology of osteoarthritis might be not clear, at least in the case of idiopathic osteoarthritis, or obviously, in the case of secondary osteoarthritis, the complexity of the condition extends well beyond the identification of its cause. The inevitable progression of OA can largely be attributed to enzymatic degradation of the articular cartilage. Current models of arthritic etiology indicate that each individual has an intrinsic susceptibility to the development of the disease, associated with an overlap of local factors at the joint level, as reported in Figure 1.2 [46].
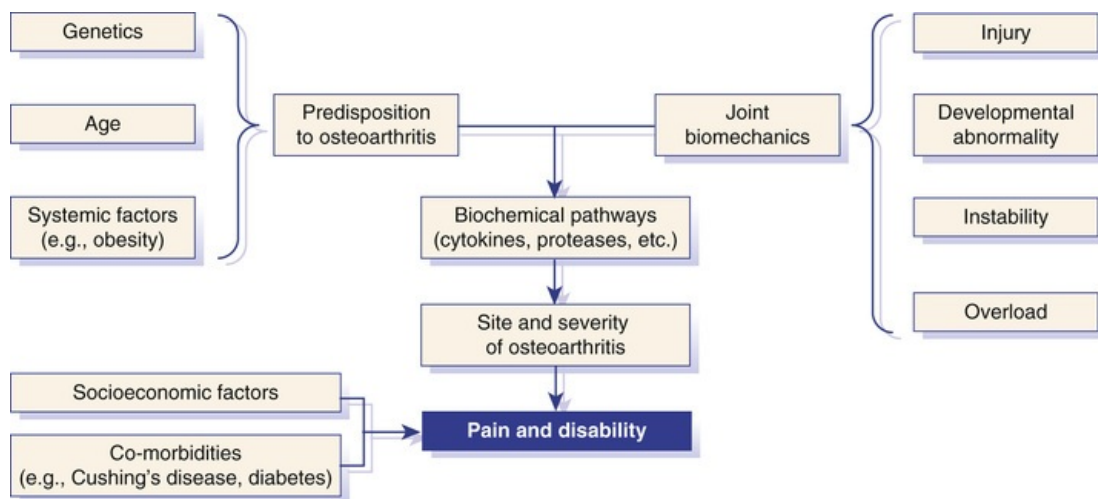

fig.1.2 Model of canine and feline osteoarthritis [46].

Therefore the individual's susceptibility is summed up by genetic, age, and systemic factors, such as obesity. The genes that control this susceptibility have not yet been well identified in dogs, although progress has been made in improving our comprehension of the genetic basis of the diseases that cause secondary osteoarthritis [47-50].

PREDISPOSITION FACTOR

There is a significant variation in the severity and rate of progression of the OA between individual animals compared with the same initialing injuries. This may be influenced by the environment (e.g., amount of exercise, bodyweight), genetic, and comorbidities factors.

Genetics.

In humans, idiopathic or *primary* osteoarthritis is very common, and a genetic predisposition has been clearly highlighted, already reported in the early 1960s [51]. However, due to the prevalence of osteoarthritis in the human population, and the marked heterogeneity in the clinical onset, it has been hard to correlate the precise genetic contribution to the etiology of osteoarthritis. Furthermore, it is clear that polygenic factors can contribute to the incidence and severity of osteoarthritis, which these can differ depending on the specific joint, gender, and race [52].Several candidate genes encoding proteins of the extracellular matrix of the articular cartilage have been associated with early onset osteoarthritis [53]. In addition to point mutations in type II collagen, inherited forms of human osteoarthritis can be caused by mutations in other genes expressed in cartilage, such as the type IV, V, and VI collagen gene, as well as the oligomeric matrix protein of cartilage (COMP).

In addition to structural proteins, genes have been identified that encode non-structural proteins associated with the risk of osteoarthritis, for example, growth differentiation factor (GDF) -5 [54-56]. In the canine species, breed predisposition to the development of OA has been reported, due to hereditary genetics defects related to the development of predisposing diseases (e.g. CHD) [47,58,59].

Age.

Age also appears to influence the susceptibility of osteoarthritis, due to the structure of joint tissues, including joint cartilage. As chondrocytes age, they synthesize smaller, less uniform aggregate molecules and less functional link proteins, their mitotic and synthetic activities decline, and anabolic stimuli and growth factor decrease [60-62]. Accumulation of advanced glycation end-products within the type II collagen network is also another aspect of aging. [63-64]. These age-related cross-links of collagen appear to reduce turnover of the collagen network. Such shorter aggregate molecules contain fewer chondroitin sulfate side chains but greater amounts of keratan sulfate and therefore have less ability to absorb water into the tissue, which alters the biomechanical characteristics of itself [65]. Overall, aging is associated with multiple cartilage changes which provide the tissue loss itself. Several studies are supported by clinical data in canine patients, which suggests that the long-term outcome for dogs with cranial cruciate ligament disease and secondary osteoarthritis is not as good in older dogs [66].

Body weight.

Data on the effects of body weight on OA are still confused and need to be clarified as well. Body weight appears to increase the risk of some triggers of osteoarthritis, in a particular way with a *secondary* osteoarthritis. In a birth cohort study of Boxers, Van Hagen et.al found that large body weight at birth was associated with increased risk of the development of clinical signs of canine hip dysplasia [67]. In addition, the risk of cranial cruciate ligament rupture increased in dogs with larger body weigh [68].

Overweight and Obesity.

Obesity is another key risk factor for the development and progression of osteoarthritis [69]. Canine populations have been shown to be useful in defining the contribution of dietary restriction in improving the appearance and progression of osteoarthritis. Lawler et al. with the aim of evaluating the effect on restrictions diet in dogs, enrolled 48 patients at high risk for canine hip dysplasia developing and obesity, Labrador Retrievers (n = 48), and then paired at age of 6 weeks by sex and body weight within each of seven litters. Afterwards they were randomly assigned within two groups, a "control fed " group (*ad libitum*) and a "25% restriction diet" group [70]. From the age of 8 weeks, each "control-fed" dog was given the dry and extruded diet *ad libitum*, while each coupled-mate in the "restriction diet" group was given 75% of the amount of food that their companion fed had consumed the previous day. Each feeding group was given the same diet, with only the quantity offered differed. When the dogs were 3.25 years old, the "control fed" group's rations were held constant at a daily dietary energy level consistent with an ideal body weight for that breed. Among "control fed" dogs at age 2 years, 42% had radiographic [71] evidence of hip osteoarthritis, compared with 4% hip osteoarthritis among "diet restriction" dogs. By 5 years of age, 52% of "control fed" dogs had radiographic evidence of hip osteoarthritis, compared with 13% of "restriction diet" dogs. Body weight at 5 years old correlated moderately with severity of hip osteoarthritis, suggesting that body weight alone might not be the primary driving force for development of hip osteoarthritis in the dogs. Radiographic hip osteoarthritis in the whole group of 48 dogs had increased in linear fashion during the 14.5-year period of feeding and data collection, from a prevalence of 15% at age 2 years to 67% by age 14 years. By the end of the study, 83% of "control fed" dogs had developed radiographic hip osteoarthritis, compared with 50% of the "diet restriction" group, which resulted in having a longer median life span [72].

Diet restriction also resulted in lower prevalence and severity of OA in the shoulder and elbow joints [73-74] .

Overweight and obesity may be an etiologic factor for osteoarthritis by causing increased load bearing on the joint, as has been demonstrated in human subjects and also can alter the joint alignmen, causing focal overload of the joint tissue [75-76]. Over the years, the scientific community has recognized diet as one of the most important environmental factors that can affect health and disease. Genetics are key to determining disease predilection, and it is also recognized that nutrition can modify and modulate the extent to which different genes are expressed. New genomic technologies, called '-omics tools', are elucidating the basis of the associations between diet and health. These technologies monitor the activity of multiple genes simultaneously at the level of ribonucleic acid (RNA) by transcriptomics, the level of proteins by proteomics, and, ultimately, the level of metabolites by metabolomics. The science of nutrigenomics uses all of these tools to elucidate how nutrients affect health and disease by altering the expression of an individual's genetic makeup [77-78]. Traditionally the mechanical stress of over-weight has been thought to be a primary perpetrator of the progression of OA. However, recent studies have documented metabolic activity in adipose tissue that may be of equal or greater importance. Adipocytes secrete several hormones including leptin and adiponectin, and produce a diverse range of proteins termed adipokines. Among the currently recognized adipokines is a growing list of mediators of inflammation: tumor necrosis factor (TNF)-α, IL-6, IL-8, and IL-10 , which are already documented in both human and canine species [79-81]. Production of these proteins is increased in obesity, suggesting that obesity is a state of chronic low-grade inflammation. This might explain why relatively small reductions in body weight can result in significant improvement in clinical signs; this overproduction of inflammatory mediators in obese individuals is associated with changes in the genome. These changes may enhance the phenotypic expression of OA compared to genetically similar dogs that remain lean their entire lives [82]
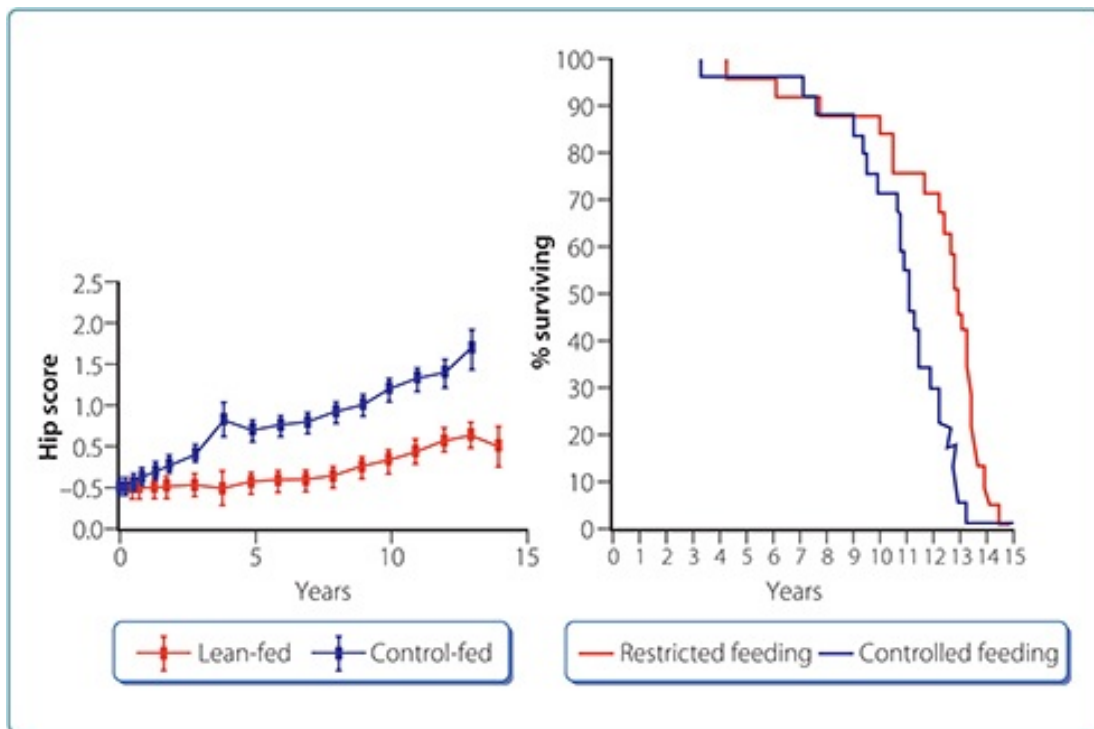
fig.1.3 *Dogs on a diet of restricted caloric intake not only demonstrate a significant reduction in progression of osteoarthritis hip scores but also live longer [71].*

Understanding the relationship between genes, nutrients, and health is the central tenet of nutrigenomics. As this emerging field matures it is reasonable to envision an era where dietary intervention, based on knowledge of nutritional requirements, nutritional status, and genotype can be used to prevent or cure chronic disease

Gender, Environment Factors.

The association between gender and osteoarthritis in dogs and cats has not been extensively studied, although there are many publications regarding gender predisposition with the orthopedic disease, which may be the underlying cause of OA [83-86]. Environmental factors that may contribute to the risk or progression of canine osteoarthritis include variables such as nutrition, exercise, and housing conditions.

1.4 PATHOGENESIS

Osteoarthritis appears to be mechanically driven but chemically mediated, with endogenous attempts at aberrant repair. Although clinically apparent, the vicious catabolic/anabolic cycle of OA is not yet comprehensively understood; however, recent evidence suggests inflammation may be at the genesis of this degradative process. (fig.1.4)
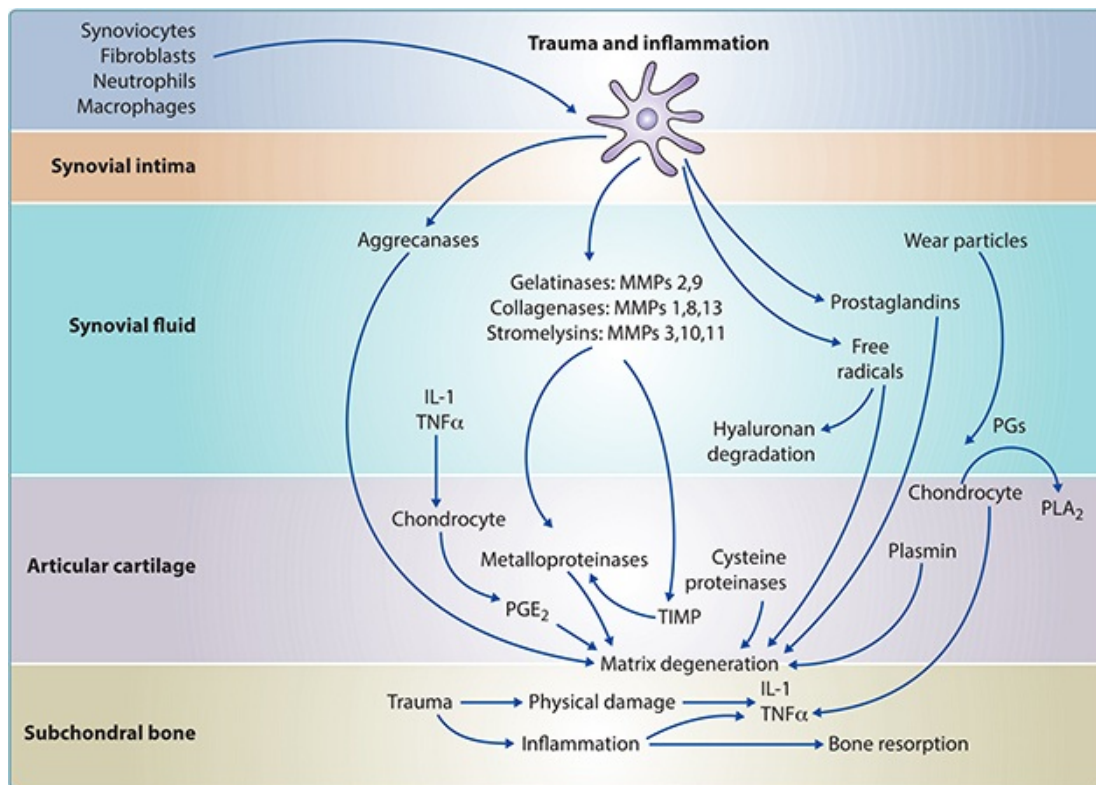

Figure 1.4: Schematic representation of the principal pathological processes in OA.

The joint can be considered an "organ" in which each of its components is affected by the pathogenetic process of osteoarthritis. OA is associated with the destruction and loss of cartilage tissue, radical changes in the metabolism and architecture of the subchondral bone, the formation of osteophytes and entesophytes, synovial inflammation, and fibrosis. Currently, the evidence increasingly shows a cross-talk between the various tissues of the joint, in particular synovium and cartilage.

Instead of being a pure cartilage disorder, considered a key point on progression and pathogenetic process of OA, degeneration joint disease is now considered as a whole-joint disease that affects various anatomical structures in and around the joint capsule. These include muscle, ligaments, entheses, synovial tissue and the subchondral bone. Furthermore, the changes in the central nervous system caused by this chronic condition can lead to the phenomenon of pain sensitization.

Typical features of OA include degeneration or progressive loss of structure and functionality of articular cartilage. As the disease process progresses, cartilage tissue is lost, and erosion and ulceration ensue. Hyaline cartilage in a high water content tissue (approximately 70%), forms the load-bearing surface of the joint and provides a low-friction, and moderate resistance to the compressive load. Proteoglycans comprise most of the extracellular matrix (ECM) that is not collagen (50%), and make-up 22–38% of the dry weight of adult articular cartilage [98] .

Articular cartilage is structured in IV layers *(fig 1. 5)* with different histologic structure, and biomechanics function*;* surface layer forms a pre-compressed protective diaphragm resistant to wear and tension inside of the joint surface plane, while the fibrils in the middle and deep areas are organized to provide greater capacity to resist the compressive load [99]. The concentration of proteoglycan increases with increasing depth from the articular surface, and therefore the collagen fibrils are more concentrated at the surface. The increased concentration of proteoglycan in the deeper cartilage is associated with resistance to compression.

Subchondral bone is a thin layer of bone, which joins hyaline cartilage with cancellous bone; whose wavy nature of the osteochondral junction allows for the conversion of shear stresses into compressive forces reducing the risk of overload on cartilage. The subchondral/cancellous region has been found to be approximately 10 times more deformable than cortical bone and plays a major role in the distribution of forces across a joint [100]. Compliance of subchondral bone to applied joint forces allows congruity of joint surfaces for increasing the contact area of load distribution, thereby reducing peak loading and potential damage to cartilage [101].
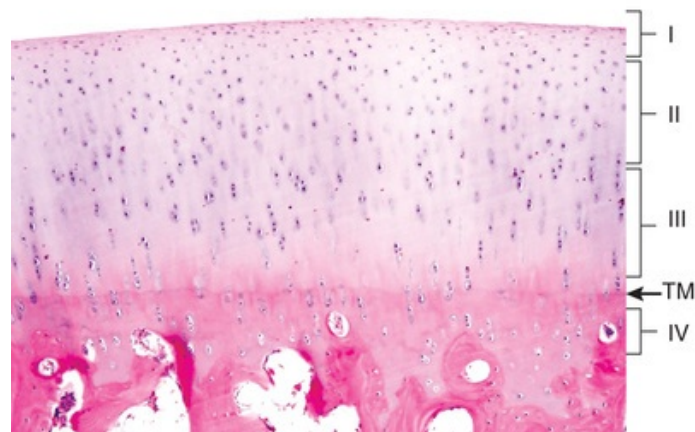
Figure.1.5: Articular cartilage from an adult dog with labeling of the cartilage zones and the tidemark [40].

Loads applied to the articular surface are shared between various components of the cartilage matrix; the collagen fibrils dominate its tensile behavior, whereas the osmotic properties of the proteoglycans provide its resistance to volumetric compression. The relatively low modulus of elasticity of articular cartilage allows tissue deformation that increases the congruity of opposing articular cartilage surfaces. This increase in surface area, improves joint stability, and decreases surface stresses. Additionally, this deformation redistributes fluid away from the compressed region into adjacent stretched regions, a process that is facilitated by the low permeability and the high internal swelling pressure of cartilage. Both properties reflect the cartilage's ability to maintain hydration under pressure, obtained from the low hydraulic permeability and high osmotic pressure of the constituent proteoglycans, which contributes up to 50% to the compressive rigidity of the cartilage [102-103]. Glycosaminoglycans account for approximately 75% of the osmotic pressure of the proteoglycans [41-102]. The swelling pressure of cartilage is balanced by the tensile resistance of the collagen network. The magnitude of the swelling pressure varies with the density, distribution, and molecular conformation of charged groups on the proteoglycans.

The magnitude of the resisting tensile force depends on the structural organization, tensile stiffness, and strength of the collagen network [42]. Articular cartilage receives its nutrients and clears its waste products by movement of synovial fluid under the influence of weight bearing.

During the pathophysiologic process of osteoarthritis, the cartilage tissue undergoes a radical change that can be divided into three overlapping phases. In the beginning, the extracellular matrix degrades at the molecular level, the water content increases, the size of the aggrecan molecules within the tissue decreases, and the structure of the collagen network is damaged, resulting in reduced rigidity of the cartilage [87]. At the second phases , chondrocytes try to compensate for the damage through enhanced proliferation and metabolic activity cell clusters, formed by cloning, appear surrounded by newly synthesized matrix molecules. This condition can remain for several months to years. In the third stage, the chondrocytes are unable to maintain their repair activity resulting in complete loss of cartilage tissue. During the osteoarthritis disease, the cartilage has an evident imbalance between anabolic and catabolic processes, with both degradation and synthesis up-regulated. Throughout life there is a constant turnover of cartilage matrix. This fragile homeostatic process is under the control of the matrix metalloproteinases (MMPs) and their inhibitors (the inhibitors of the matrix metalloproteinases or TIMPs (*fig.1.4)*. In OA the activity of the MMPs and the aggrecanases is increased substantially, fewer TIMPs and cytokine inhibitors are produced and more cytokine receptors are expressed on articular cells, which leads in homeostasis lost, with consequent a net loss of cartilage matrix, and progression and perpetuation of the joint disease.

The increase in enzymatic activity is linked to the release of inflammatory cytokines, such as interleukin (IL) -1, tumor necrosis factor-α, and IL-6, which stimulate the release of MMP and aggrecanases from the cells synovial and from the same chondrocytes [88-90-94].

In the short to medium term an increase in cartilage thickness occurs, which is associated with tissue swelling and an anabolic response that produces more cells and

more extracellular matrix [91-92]. However, as disease progresses, cartilage tissue is lost and end-stage disease involves ulceration of cartilage and eburnation of sub-chondral bone [93]. Degradation of the components of the extracellular matrix of articular cartilage and cell death are the key processes of the osteoarthritis. Considering the potential role of inflammatory prostaglandins, and the use of non-steroidal anti-inflammatory drugs for the treatment of osteoarthritis, the role of COX expression has been extensively investigated, and evidence suggests that COX inhibition may provide beneficial effects in cartilage. [95]

Chondrocytes from human osteoarthritic cartilage explants express COX-2 and spontaneously produce prostaglandin E 2(PGE 2) [96]. In addition, it is also reported that PGE2, as well as nitric oxide (NO) releases, produced by osteoarthritic cartilage explants decreased proteoglycan synthesis and enhanced the degradation of both aggrecan and type II collagen. These effects are associated with down-regulation of MMP-1, together with up-regulation of MMP-13 (collagenase 3), which degradate ECM with preferentially effect on deep zone of cartilage (perpendicular zone) [136], and disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5) [97]. Among other inflammatory mediators of interest in the pathogenesis of osteoarthritis are both oxygen- and nitrogen-derived free radicals. Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, promote chondrocyte apoptosis, most likely via mitochondrial dysfunction.

Nitric oxide (NO), produced by the inducible isoform of nitric oxide synthase (iNOS), appears to be another major catabolic factor produced by chondrocytes in response to cytokines such as IL-1β and TNF-α. Considerable evidence indicates that overproduction of NO by chondrocytes plays a role in the progression of cartilage loss in osteoarthritis, promoting cartilage degradation with inhibition of matrix synthesis, activation of matrix metalloproteinases, and apoptosis. NO is generated through the activation of inducible nitric oxide synthase (iNOS), which is calcium-independent, and thereby long-lasting, which generates large amounts of NO over an extended period of time. NO inhibits the synthesis of proteoglycans and collagen in cartilage culture and up- regulates the synthesis of MMPs that are typically kept in

check by tissue-inhibiting metalloproteinases (TIMPs). Although normal cartilage does not express iNOS nor produce NO without stimulation by inflammatory cytokines, osteoarthritic cartilage explants spontaneously produce considerable quantities of NO.

However, NO and its derivatives may also play protective roles in that protease activity and proteoglycan degradation, which is enhanced when the NO production is stopped. Degradation of aggrecan appears to be a very early event in canine osteoarthritis and is followed by disruption of the collagen network [104]. Aggrecan can be degraded by matrix metalloproteinases, such as MMP-13, but the "aggrecanase" enzymes appear to be particularly important. The aggrecanases, also known as *ADAMTS-4* and *ADAMTS-5*, cleave the aggrecan protein core in the inter-globular domain between G1 and G2 [105-106]; this action releases most of the molecule, including the negatively charged sugar side chains, from the matrix [107].

It is currently thought that ADAMTS-5 is up-regulated in osteoarthritis, and studies indicate that this enzyme may be critical for disease progression [108-109-110]. However, the understood of which of these enzymes is the most important in canine and feline osteoarthritis remains unknown. The intact triple helix of type II collagen can be degraded only by MMP-1 and MMP-13, and possibly MMP-8 and MMP-14 [111-112-113]. The enzymes are secreted as pro-forms that are activated by partial proteolysis. Furthermore, the action of these enzymes is controlled by natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), produced in such a way that the balance between proteolytic activity and inhibitors is fundamental [111].

Cartilage oligomeric matrix protein (COMP) is the most abundant non-collagenous protein of articular cartilage [114-115]. Its putative role is seen in the assembly of collagen fibrils, and it has been extensively studied in human OA as biomarker of human disease progression [116-117-118]. Data are limited on the role of cartilage oligometrix matrix protein in canine OA, but they suggest increased COMP catabolism in early osteoarthritis [119-120].

Synthesis of cartilage matrix molecules can be stimulated by various growth factors such as insulin- like growth factor (IGF-1 and IGF-2) , and transforming growth factor-β (TGF-β). Both IGF and TGF-β can stimulate aggrecan and collagen synthesis. The availability of IGF is controlled by circulating and locally produced binding proteins (IGFBPs). Disturbance to the IGF-IGFBP "system" in canine osteoarthritis reveals that the availability of IGF may be decreased [121]. TGF-β expression is reduced in osteoarthritis [122].

Table 1.1.: Major inflammatory mediators of osteoarthritis

Inflammatory Mediator	Activity
COX-2	Cyclo-oxygenase-2 gives rise to destructive and inflammatory eicosanoids
PGE2	Prostaglandin E2 is a major enzyme in the arachidonic acid pathway leading to the pathologic features of pain and inflammation
IL-1β	Interleukin-1β induces COX-2 with resultant central nervous system hypersensitivity. Both IL-1α and IL-1β possess strong proinflammatory effects
MMP-13	Matrix metalloproteinase-13 (collagenase 3) cleaves type II collagen
iNO	Inducible nitric oxide (NO) synthase leads to increased synthesis of NO, associated with cartilage degradation, inhibition of matrix synthesis, and chondrocyte apoptosis
TNF-α	Tumor necrosis factor-α stimulates prostaglandin secretion and increases activity of matrix- degrading proteinases as well as regulating immune cells
ADAMTS-4 (Aggrecanases)	Degradation of aggrecan in ECM and critical for perpetuation and progression of joint disease

The synovial joint capsule plays one of the major role in early OA, as changes in the synovium precede changes in the articular cartilage [125]. The joint capsule can be divided into three strata, given various nomenclature, but the most commonly accepted refer to the synovium as the synovial lining (intima), subsynovial layers, and, with the term joint capsule referring to the fibrous tissue surrounding the joint. Within the synovial intima (usually only one to two cell layers thick) reside type A sy-

noviocytes (macrophage-like in function) and type B synoviocytes (producing hyaluronan; also capable of producing degradative enzymes). The sub-synovial (sub-intimal) layer is vascular, neural, and allows independent movement of the synovial membrane from the fibrous joint capsule. Depending on the joint-specific biomechanics, the sub-intima can be composed of different types of connective tissue: fibrous, fatty or areolar, loose and highly viscoelastic connective tissue which permits stretching or folding. The tough fibrous layer contributes to physical stability of the joint. Synovial tissue separates the joint capsule from the joint cavity. Apart from cartilage nutrition and lubrication, the main function of synovial tissue is to prevent adherence of the capsule with cartilage. By the production of hyaluronan and plasminogen activator, the synovium preserves articular mobility [137]. Hyaluronan is also responsible for ensuring constant synovial fluid (SF) volume during exercise [138]. Synoviocytes do not possess a basal layer and lack cell-cell junctions, which facilitate the exchange between SF with blood or lymphatic vessels.

Osteoarthritis involves variable synovitis and capsular fibrosis, and indeed interest in this aspect of the disease process is increasing [123-124]. Articular cartilage receives its nutrients and clears its waste products by movement of fluid under the influence of weight bearing. Synovial lining macrophages phagocytize proteoglycans and collagen fragments released from diseased cartilage into the synovial fluid. This stimulates the synoviocytes to produce cytokines and MMPs, which, under the influence of weight bearing, are forced back into the cartilage matrix to further perpetuate the process of degradation [126]. As a species the dog seems particularly prone to development of synovitis during the process of osteoarthritis, particularly during the early stages. Most available information has been gathered by evaluating change in the stifle joint associated with, or following, cranial cruciate ligament transection. Synovial histologic changes include synovial hypertrophy and often hyperplasia with an increased number of lining cells, often accompanied by marked infiltration of the sub-lining tissue with foci of lymphocytes in chronic condition [127-128-129] .

Some observations indicate that the grade of macrophage infiltration can be more pronounced in early stages of OA [139]. Vascular endothelial growth factor produc-

tion by synovial macrophages has been postulated as a possible mechanism that exacerbates synovial angiogenesis and inflammation in OA [140]. Lymphoid cell aggregates are rarely seen in early OA but do occur in up to one-third of synovial samples from patients with severe OA [141]. Mast cell numbers in the synovial tissue are high in OA, despite a lower synovitis score. A trend towards correlation between mast cells and radiographic OA severity, independent of synovitis, has been also postulated [142]. Mast cells are a substantial source of preformed cytokines such as TNF. Interestingly, mechanical loading promotes mast cell degranulation [143]. Therefore synovial mast cells are potentially involved in mechano-inflammation in OA. Synovial surface fibrin deposition is commonly observed in endstage OA [144]. Cartilage breakdown products, derived from the articular surface as a result of mechanical or enzymatic destruction of the cartilage, can provoke the [113] release of collagenase and other hydrolytic enzymes from synovial cells and macrophages. Indeed, the macrophage is likely to be a key cell in driving synovial control of cartilage metabolism through the release of catabolic cytokines such as IL-1β and TNF-α, which are probable contributors to the degradative cascade.
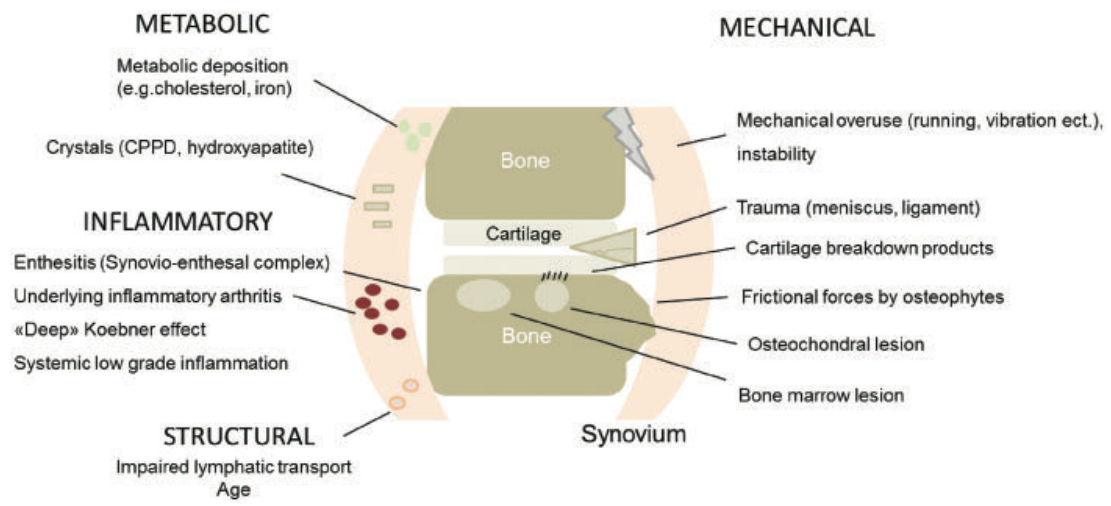

Figure.1.6: Illustration of pathogenic stimuli during OA.[144]

Osteophyte formation and subchondral bone sclerosis are another key features of osteoarthritis. The articular cartilage and subchondral bone form a functional osteo-chondral unit in order to optimize shock ab- sorption and load distribution. A minera-lized or calcified cartilage layer forms a junction between bone and non- calcified cartilage tissues. The interface between calcified and non-calcified cartilage is called the tidemark. Osteoarthritic cartilage displays multiplication of the tide- mark repre-senting enhanced calcification of the deep cartilage zone [145].

The genuine osteophyte, or osteochondrophyte, arises in the periosteum overlying the bone at the junction between cartilage and bone [130]. Osteophytes can contribu-te both to the functional properties of affected joints and to clinically relevant symp-toms. Osteophyte formation is highly associated with cartilage damage, but osteo-phytes can develop without explicit cartilage damage. Mesenchymal stem cells pre-sent in the periosteum or synovial lining are thought to be the precursors of osteo-phytes. In murine experimental osteoarthritis, osteophytes originate primarily from the periosteum covering the bone at the cartilage-bone junction. However, cell popu-lations from the synovium can be triggered to form cartilage in vitro, and synovium-derived mesenchymal stem cells have been shown to be even more efficient in carti-lage formation than bone marrow–derived mesenchymal stem cells. Growth factors of the TGF-β superfamily appear to play a crucial role in the induction of osteophy-tosis. TGF-β, when introduced into the joint in experimental animals, induces osteo-phyte formation, and TGF-β expression is observed in osteophytes in human patients and experimental animals with osteoarthritis [130-131]. Several studies have evalua-ted the subchondral bone changes associated with canine osteoarthritis. The canine cranial cruciate transection model of osteoarthritis thinning and increased porosity of the subchondral bone plate is followed by sclerosis [132-133]. Data suggest that sub-chondral bone plate thinning is associated with cartilage damage, but the co-localiza-tion of pathologies in these adjacent tissues does not elucidate cause and effect [134] . Data from gene knockout mouse models of osteoarthritis, induced by destabi-lization of the medial meniscus, suggest that pathology in articular cartilage and that in subchondral bone are progressive over time but are likely independent of each

other [135].

Given the closely associated changes in subchondral bone and articular cartilage, molecular targets that alter osteoclast and/or osteoblast function may represent opportunities to modulate pathologic subchondral changes in osteoarthritis; they are therefore under consideration in efforts to develop disease-modifying treatments.

1.5 THE "PAIN PATHWAY" IN OSTEOARTHRITIS

Pain is the result of a complex signaling network. The cognition of pain, like cognition in general, is controlled by neurologic system. The physiologic component of pain is termed nociception, which consists of the processes of transduction, transmission, and modulation of neural signals generated in response to a noxious stimulus. When carried to completion in the conscious animal, nociception results in pain. Basically, the pain track can be understood as a chain of three neurons; the first-order neuron originating from the periphery and projecting towards the spinal cord, the second-order neuron ascending the spinal cord, and the third-order neuron projecting to the cerebral cortex. Furthermore, the trace involves a network of branches and communications with other sensory neurons and inhibitory neurons descending from the midbrain that modulate the afferent transmission of noxious stimuli. The process begins with the conversion by nociceptors of mechanical, chemical, or thermal energy into electrical impulses. These nociceptors exist as free nerve endings of primary afferent neurons, and have considerably higher stimulus thresholds for activation than thermoreceptors or low-threshold mechanoreceptors active under ambient conditions. Within the dorsal horn, the communication of afferent nociceptive information between various neurons occurs via chemical signaling, mediated by excitatory and inhibitory amino acids and neuropeptides that are produced, stored, and released in the terminals of afferent nerve fibers and dorsal horn neurons. It is here in the dorsal horn that the afferent nociceptive impulse lives or dies, and is modulated by various integrative influences. [146]

The descending modulatory system works on four tiers, the cortical and thalamic structures, the periaqueductal gray matter of the midbrain, the rostral medulla and pons of the brainstem, and the medullary and spinal cord dorsal horn. Again, the spinal cord is the site of most active modulation. Dense concentrations of γ- aminobutyric acid, glycine, serotonin, norepinephrine, and the endogenous opioid peptides (enkephalins, endorphins, and dynorphins) have been identified in dorsal horn neurons, and all produce inhibitory effects on nociceptive transmission. It is now apparent that a single neuron may be influenced by many neurotransmitters, that each neurotransmitter may have numerous actions in a given region, and that multiple neurotransmitters may exist within a single neuron. It is important to note that the pain response is not confined to the nervous system. Pain induces both segmental and suprasegmental reflex responses which result in: increased sympathetic tone; vasoconstriction; increased system vascular resistance; increased cardiac output through increases in both stroke volume and heart rate; increased myocardial work through increases in metabolic rate and oxygen consumption, decreased gastrointestinal and urinary tone; and increased skeletal muscle tone. Endocrine responses include increased secretion of adrenocorticotrophic hormone, cortisol, antidiuretic hormone, growth hormone, cyclic adenosine monophosphate, catecholamines, renin, angiotensin II, aldosterone, glucagons, and IL-1, with concomitant decreases in insulin and testosterone secretion, i.e. the classic stress response. [146]

Our current understanding of joint pain is poor, and osteoarthritis provides additional challenges with respect to understanding the relationship between joint pathology and pain, which apparently is not linear and is not predictable. Literature information on nociception in the joint derives from human studies or animal models. Pain in the joint is often dull and aching, and it is poorly localized in contrast to cutaneous pain. To date, the neuronal organization of joint pain has not been fully elucidated, but most available information on this topic describes the innervation of joints. Joint nerves contain Aβ-, Aδ-, and C-fibers. Corpuscular endings of Aβ-fibers are found in ligaments and in the fibrous joint capsule, and free nerve endings are present in all structures of the joint except normal articular cartilage. From all joint structures in-

cluding ligaments, fibrous capsule, adipose tissue, meniscus, periosteum, and synovial layer, but not cartilage, conscious sensations can be evoked. In human beings who are awake, direct stimulation of fibrous structures with innocuous mechanical stimuli evokes pressure sensations. However, pain is elicited when noxious mechanical, thermal, and chemical stimuli are applied to fibrous structures such as ligaments and fibrous capsule [147]. No pain is elucidate by stimulation of cartilage, nor eventual stimulation of normal synovial tissue [148]. In general, the pain sensation in a normal joint is most commonly elicited by twisting and contusion of the joint. Movement in physiologic range of motion is normally not painful, and palpation of normal joint does not hurt. A large group of mainly C-fibers are so-called *silent nociceptors* because they do not respond even to noxious mechanical stimuli of the normal joint.They begin to respond to mechanical stimulation during inflammation of the joint. During inflammation, numerous silent nociceptors develop sensitivity for mechanical stimulation of the inflamed joint. This recruitment of fibers significantly increases input into the spinal cord and neuronal changes provide a plausible explanation for the occurrence of mechanical hyperalgesia or pain in the inflamed joint [149-151].

Whether they also evoke other sensations such as pressure or stiffness is unknown. In summary, the joint is equipped with a large number of nerve fibers that are suitable to encode painful mechanical stimuli [152-153].

It is thought that sensation of primary afferent fiber is produced by inflammatory mediator, including TNF-α, IL-6, bradykinin, PGE_2, PGI_2, serotonin, substance P, galanin, and so on [154]. Inglis and colleagues in a study of induced arthritis in mice, founds that neutralization of TNF-α reduced both mechanics hyperalgesia and inflammatory process [155]. This demonstrate that inflammatory mediators play a significant role in adapting the responses of nociceptors within the articular tissues. Evidence also indicates that joint pain results in the development of central sensitization, which is one of the mechanisms leading to increased pain [156-157]. Furthermore, it has been demonstrated that COX enzymes play a role in central sen-

sitization and that COX inhibitors can prevent the establishment of central sensitiza-

tion [158-159]. Central sensitization can actually drive the progression of OA patho-

logy and that downward modulation of central sensitization can result in decreased

joint pathology [160-161]. In addition, a direct effect on NSAIDs at the level of the

joint may result in a reduction in disease progression [162-163].

One such mechanism is seen in the prevention of NO-induced cel death. Several

studies evidences that osteoarthritis cartilage has higher number of apoptotic chon-

drocytes compared with normal cartilage [164-165]. The production of NO may re-

present an important component in the pathogenesis of OA, produced in large

amounts by chondrocytes upon pro inflammatory cytokine stimulation. Selective in-

hibition of COX-2 significantly inhibits NO-induced cell death [166].

2 DIAGNOSTIC EVIDENCE AND STAGING OF OSTEOARTH-RITIS

The diagnosis of osteoarthritis in small animals is often not sufficiently accurate, as it

is more frequently secondary, due to some other primary joint abnormality, despite

the presence of idiopathic primary forms of OA (foot and manus in the small joint).

The clinical history often reports very mild clinical signs, which are highlighted

more in the case of owners of sporting dogs.

Basically the owner may complain that their dogs show reluctance to exercise, exer-

cise intolerance, stiffness from inactivity, and often report intermittent sign.

The main clinical sign reported by the owner is pain. Identifying, assessing and sco-

ring pain is one of the clinician's most challenging challenges, especially in chronic

diseases. Various acute pain assessment measures have been used by researchers to

quantify pain. These include verbal rating scales (VRS), simple descriptive scales

(SDS), numeric rating scales (NRS), and visual analog scales (VAS), all of which

have their limitations. Historical limitations of scales used to assess pain have inclu-

ded assessment of pain on intensity alone. The Glasgow Pain Scale is such a multi-dimensional scheme, and although it is detailed, its on-going refinement may result in greater utilization [167]. Currently, there are no validated 'scales' to assess chronic pain for the clinicians. For that reason several studies have sought to develop the means to evaluate pain via the accrual of information from owners of dogs with osteoarthritis. This interest has undoubtedly arisen because osteoarthritis is usually a chronic and insidious disease, and it is difficult for the veterinarian to stage disease severity in the consultation room. In such a situation, information from the owner becomes critical, but general nervousness is associated with relying too heavily on information from untrained parties. Nevertheless, several attempts have been made to design and validate owner questionnaires for canine osteoarthritis [168-171]. The competition of these questionnaires may help the clinician track disease progression. Some questionnaires , called clinical metrology instrument, are very feasible for re-search as a validate strategy for assessment the long term response to treatment[168,169]. The Canine Orthopedic Index (COI) and the Canine Brief Pain Inventory Index are two example of these questionnaire.

2.1 CLINICAL SIGN OF OSTEOARTHRITIS

All of the clinical sign are referable to the morphological change of the joint affected by osteoarthritis. OA in dogs is associated with a variety of clinical signs and physi-cal examination findings. The main examination finding are muscle atrophy, joint swelling, capsular and extracapsular fibrosis, joint effusion, reduced range of motion, crepitus, and pain on joint manipulation. Clinical signs are variable depending on multiple factors, including the breed and demeanor of the patient, the stage of the disease, and the particular joint affected. For instance, more proximal joints are less palpable and it is not possible to feel effusion or capsular fibrosis, whereas distal joints may demonstrate such changes. All these clinical signs become silent and dif-

ficult for the clinician to perceive if the patient is a cat, where the most important tests are often the owner's medical history and imaging findings.

2.2 IMAGING MODALITIES TO EVALUATE OA

Radiography is probably the most common diagnostic test to evaluate orthopedic disease, including osteoarthritis. Most often the degenerative joint disease is secondary, and the x-ray evaluation can be useful to assess joint pathology, such as dysplasia or articular fracture.

Radiographic assessment of osteoarthritis is not the ideal method of disease assessment, because can provide only information on bony changes, such as osteophytosis, enthesophytosis, narrowing of the joint (in stance), joint effusion, soft tissue swelling, subchondral sclerosis, interarticular mineralization, and subchondral cyst; these changes could be considered a limitation, especially in the initial phase of OA, by reducing information on soft tissue, synovial membrane and cartilage.

However, osteophytosis is certainly a useful marker through which to diagnose osteoarthritis, even if it is not pathognomonic of OA, because other arthritis may induce their formation. The value of osteophytosis for *staging* the severity of osteoarthritis is controversial. Studies on the post-surgical cruciate-deficient stifle joint indicate that osteophytosis often continues to progress after surgery [172-174]. One prospective study with three sampling points indicated that ostheophyte growth may be more active in the first 7 months following surgery, compared with the period between 7 and 13 months [172].

Another limit to consider could be that radiographs of dogs and cats are generally obtained while the patient is not weight bearing, and therefore is not generally possible to infer anything meaningful on the status of articular cartilage from the interbone distance on radiographs. Some authors suggest evaluating the interbonic distance at the narrowest point of the patellofemoral joint space during flexion, because in that case we can simulate in a loaded patellofemoral space [91].

The femoropatellar joint space had a strong independent correlation with body weight (larger in larger dogs) and with stifle joint flexion angle (decreased joint

space with greater flexion). Evidence also suggests a weaker, negative, independent effect of age. This latter finding is interesting in that it suggests that older dogs have less cartilage, or have a less anabolic response in the cartilage, compared to younger dogs. In the temporal analysis of femoropatellar joint space over a 13-month period, femoropatellar joint space width increased significantly in the first 7 months of this prospective study. Results indicate a moderate to strong positive relationship between body weight at baseline and change in femoropatellar joint space from entry to 7 months (greater body weight tends toward a greater increase in femoropatellar joint space). To a lesser extent, body weight also related positively to femoropatellar joint space change from 7 to 13 months.

Advanced imaging modalities such as magnetic resonance imaging (MRI) and computed tomography (CT) are used increasingly to image joints. Of these two modalities, MRI is generally more appropriate because it can provide information on soft tissues such as cartilage, ligaments, menisci, and synovium, as well as bone. However, canine cartilage is so thin that even the more powerful magnets available in some centers do not have sufficient signal-to-noise ratio to allow accurate delineation of canine articular cartilage. Nevertheless, MRI can provide useful information on structures such as menisci, ligaments, and tendon [175;176].

The signal intensity of gadolinium-enhanced MRI has been demonstrated to detect differences in glycosaminoglycan content in articular cartilage. This holds promise for the use of such studies to detect changes in cartilage over time [177]. Computed tomography provides excellent information on bony changes but is less useful for soft tissue pathology. Therefore, CT has received much less attention as an imaging modality for diagnosis and staging of osteoarthritis. However, positive-contrast CT has been investigated for imaging the canine stifle joint to assess structures such as menisci [178;179]. CT can be useful for imaging joint with more complex anatomy, such as elbows, carpi, and tarsi.he cross-sectional nature of the images avoids superimposition that can mask osteophytes on plain radiography. In summary, CT is not a routine modality for assessing osteoarthritis per se, but it is often used in the elbow joint, carpus, and tarsus to investigate the initiating cause of osteoarthritis. Scintigra-

phy using technetium99m linked to a diphosphonate carrier is an imaging modality that can provide infor mation on bone remodeling. The diphosphonate binds to hydroxyapatite crystals, and recently formed bone has large crystals for the carrier to bind. Scintigraphy has been used as a clinical research tool to assess bone turnover in canine OA [180;181]. In human beings, some predictive value in osteoarthritis in terms of progression of cartilage loss , but such studies have not been performed in dogs or cats [182;183]. Arthroscopy has become commonplace in small-animal surgery and probably represents the most valuable and cost-effective means for the veterinary clinician to gain an assessment of the stage of osteoarthritis in a canine joint. The technique can be used to evaluate cartilage status degree of synovial change, and status of other intra-articular structures. Chondropathy can be graded using a discontinuous ordinal scale (modified Outerbridge scale) to facilitate clinical record keeping and comparison between studies [184-185]. Arthroscopy is minimally invasive and, in trained hands, is a quick and effective method to assess articular pathology.

2.4 SYNOVIAL FLUID ANALYSIS

Synovial fluid analysis is recommended in the diagnostic work-up for joint diseases and is useful in categorizing the type of arthritic process that is present. If there is any doubt as to the underlying disease process in a joint, synovial fluid should be sampled and analyzed for total and differential cell counts. Synovial fluid in osteoarthritis typically shows evidence of *mild* inflammatory change with mild to moderate increases in mononuclear cell numbers (fig 2.1)
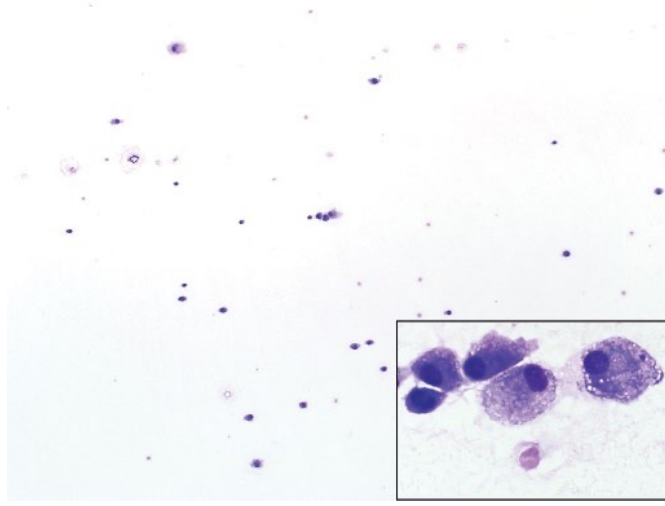
Figure.2.1: citological analysis of synovial fluid [187].

 In dogs and cats, commonly sampled joints include shoulder, stifle, elbow, carpus, and tarsus. Knowledge of the specific joint anatomy and approach is required in order to properly collect the best diagnostic sample while minimizing patient discomfort [187]. Generally it is easier to collect sufficient sample required to perform a complete fluid analysis from the shoulder and stifle joints, as these spaces typically have the largest potential space for fluid accumulation. The synovial fluid analysis can help the clinician to understand and track the disease during the treatment. Synovial fluid should be clear and colorless, xanthochromia suggests prior hemorrhage, while turbidity is consistent with presence of increased erythrocytes and /or nucleated cells [187]. Synovial fluid should be highly viscous due to presence of hyaluronic acid. If viscosity is decreased and the sample seems watery, inflammation, hemorrhage, or other cause for effusion is likely. The mucin clot test is a semiquantitative assessment of viscosity [188]. The mucin clot test is best performed on a sample that is collected into a plain or heparinized tube as EDTA can degrade hyaluronic acid [187].

A rigorous assessment of the additional diagnostic value of the mucin clot test cannot be found. Cellularity of normal synovial fluid in dogs and cats is generally low ($<3.0 \times 10^9$/L for dogs and $<1.0 \times 10^9$/L for cats). Cells consist predominantly of mononuclear cells, which are mostly lymphocytes, macrophages, and synovial cells. Neutrophils are typically less than 5–10% of cells. In a study evaluating 126 synovial fluid

samples from cats, the mean cell count was 0.091×10^9/L with 96.4% mononu- clear

cells and 3.6% neutrophils. The highest cell count in this study was 1.134×10^9/L.

Normal protein values are generally accepted to be less than 25–30g/L. Protein con-

centration is better performed using a quantitative biochemical assay. Protein measu-

re- ment by refractometer often is not performed because of sample viscosity. In one

study, a control group of 10 dogs had a mean protein concentration of 14.6g/L with

0.22 standard deviation, by the Bradford protein method.

In the future, more detailed protein assessment may be available using a proteomics

approach [187].Cytological examination provides the physician with an assessment

of the qualitative state of the joint health. In recent few years , the scientific commu-

nity have tried to obtain more information from synovial fluid for predictive and

especially prognostic purposes . Several biomarker, such as serum C-reactive protein,

resistin, or synovial cytokine analysis where used at this scopes [189-192]. Allen et

al shows the correlation between synovial fluid cytokine in dogs with and without

OA, and concludes that Monocyte chemoattractant protein 1 was the most sensitive

biomarker to differentiate normal from OA dogs [190]. This evidence were confir-

med to Klein and colleague which shows lack of correlation between synovial fluid

IL-8, MCP-1 or IL-6 and radiographic osteoarthritis severity or joint pain and dy-

sfunction [191]. The predictive value of these biomarkers has not yet been clearly

understood, but they appear to be important for comparing the effect of intra-articular

therapies.

3 MANAGEMENT OF OSTEOARTHRITIS

The management of osteoarthritis is often conservative and multimodal, consisting of medical and non-medical aspects *(fig.3.1)*.
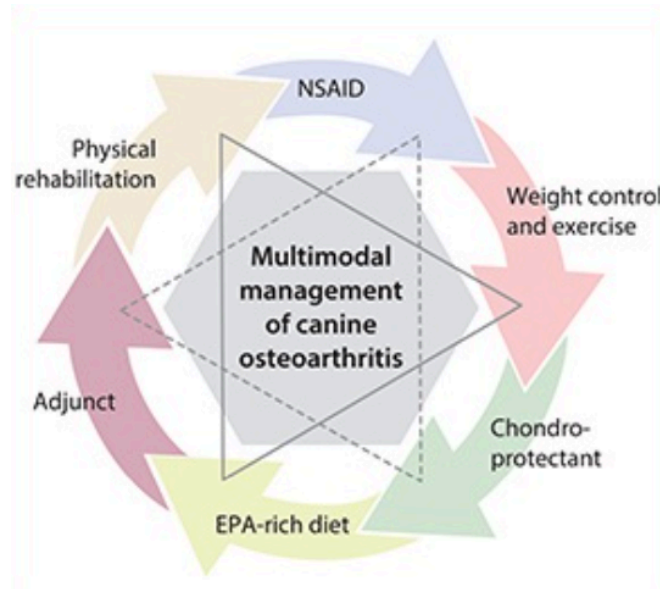
Figure.3.1: multimodal approach of OA: medical management treatment [216]

For many years, the pain has been managed by the administration of a single pharmacological agent, and often only when "proven" by clinical examination. Within the past 10–20 years advancements in the understanding of pain physiological mechanism, the introduction of more efficacious and safer drugs, and the maturation of ethics toward animals have considerably improved the management of pain that our veterinary patients need. Multimodal management was initially intended as the administration and combination of different drugs, which provided different and non-competitive mechanisms of action. At present day the concept is wider and includes a non-pharmacological management, which is basically the basis of the therapeutic protocol (*fig.3.2).*
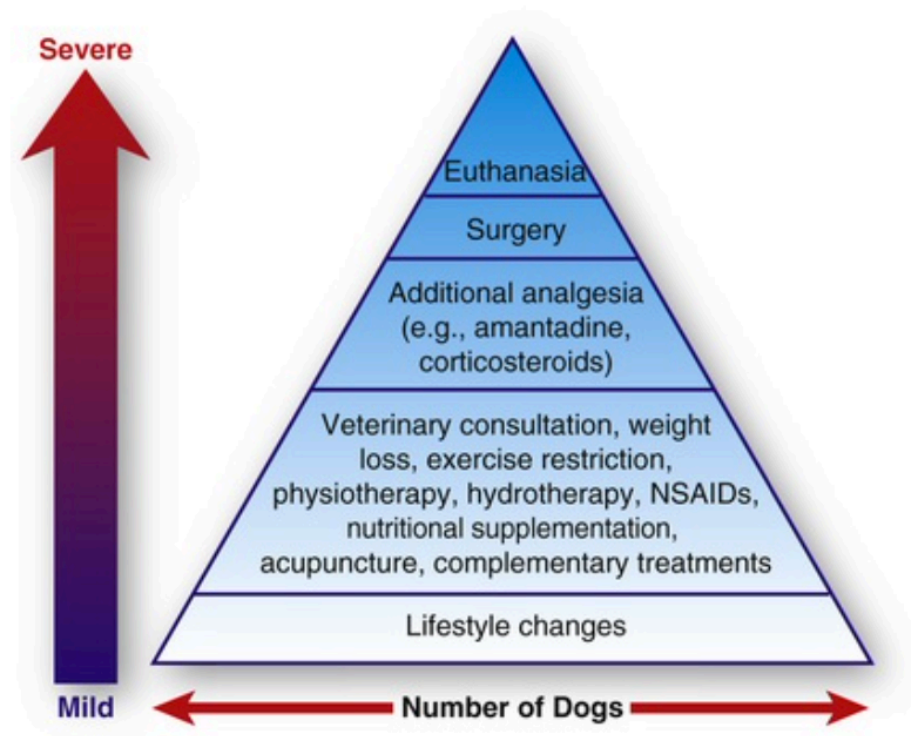
Figure 3.2: Schematic summarizing the current management of canine osteoarthritis.[40]

3.1 NON-MEDICAL CONSERVATIVE MANAGEMENT

Non-medical management consists of three principal aspects, which are diet, weight control, and physical rehabilitation.

-*Diet* is arguably one of the most important environmental factors influencing health and disease. Although genes are critical for determining predilections, nutrition modifies the extent to which different genes are expressed and thereby modulates whether individuals fully express the promise established by their genetic background. The application of nutrigenomics to specific veterinary conditions is opening new avenues of disease prevention and therapy [78]. The role of n-3 fatty acids in canine OA is one example of application of nutrigenomics principles to clinically important conditions in veterinary medicine. Arachidonic acid (AA) and eicosapentaenoic acid

(EPA) act as precursors for the synthesis of these inflammatory cytokines, including PGs and leukotrienes, which are also known as eicosanoids. The quantities and types of eicosanoids synthesized are determined by the availability of the precursor of fatty acids and by the activities of the enzymatic systems that synthesize them. In most cases, the main precursor of these compounds is AA, although the EPA competes with it for the same enzyme systems.

The eicosanoids produced from arachidonic acid are pro-inflammatory. In contrast, eicosanoids derived from eicosapentaenoic acid provide very little or no inflammatory activity. Ingestion of oils containing n-3 fatty acids results in a decrease in membrane AA levels. This produces an accompanying decrease in the capacity to synthesize eicosanoids from AA. Studies have documented that levels of inflammatory eicosanoids produced from arachidonic acid are depressed when dogs consume foods with relatively high levels of n-3 fatty acids [194].

Reducing the production of pro-inflammatory mediators is only one mechanism by which n- 3 fatty acids promote reduced inflammation and the return to homeostasis. Clinical trial results from feeding EPA-rich diets have demonstrated increased serum EPA concentrations, improved clinical performance as assessed by both the veterinarian and pet owner, improved weight bearing as measured by force plate gait analysis, and have shown effective NSAID dose reduction [195].

Based on these studies, a food designed to aid in the management of OA in dogs should provide levels of total omega-3 fatty acids of 3.5–4.5% (dry matter) and specifically 0.41– 1.1% (dry matter) EPA. The n-6 to n-3 fatty acid ratio should be less than 1:1. In summary, eicosapentaenoic acid diets have two principal modes of action: 1) by providing an alternative substrate for COX and lipoxygenase metabolism, the resultant prostanoids are less inflammatory; and 2) help suppress the degradative enzymes associated with cartilage destruction. This helps maintain the integrity of hyaline cartilage, and subsequently its function (*fig.3.3*)
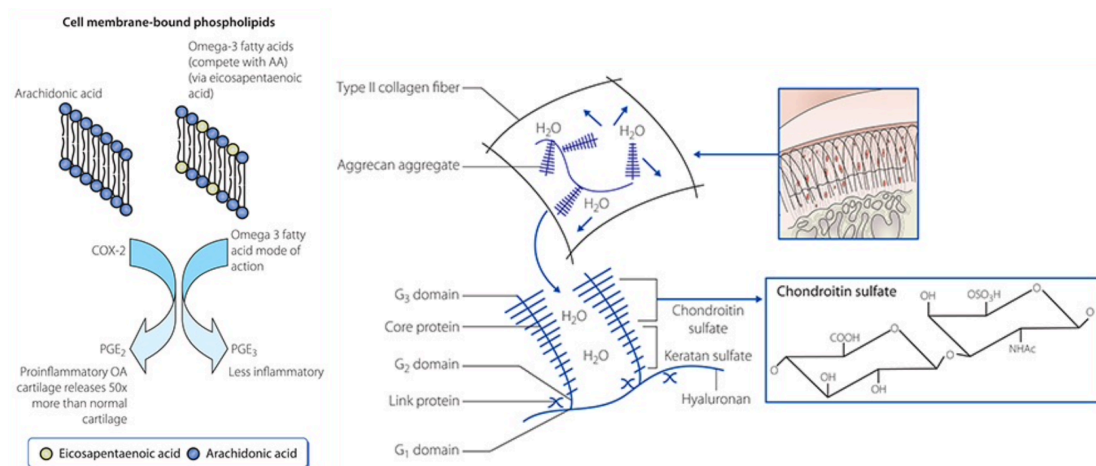
Figure.3.3: (1)*Comparison of eicosanoid end-products as influenced by substrate.* (2)*Dietary eicosapentaenoic acid (EPA) suppresses the up-regulation of the aggrecanase enzyme, thereby sparing the integrity of the aggrecan aggregate, and sparing the function of articular cartilage. [216]*

On the diet category should be advanced some information about nutraceutical issue

-*Weight control:* overweight and obesity have already been discussed as risk factors for the development and progression of osteoarthritis. Therefore, management of obesity also has an important role to play in treating osteoarthritis. Evidence indicates that reduction in obesity of dogs with clinical signs of osteoarthritis can lead to

improvement in clinical signs such as lameness,[196,82] and multiple studies in human beings show that treatment of overweight and obesity can improve symptoms [197,198].

Fundamentals of weight loss programs include client education, nutritional management (e.g., prescription diets to reduce energy intake), and exercise management (aimed at increasing energy consumption). Dogs on a restricted diet showed a significant reduction in progression of OA hip scores and lived longer [71,193].

In his study Kealy and colleagues demonstrate that over the life-span of investigated dogs, the mean age at which 50% of the dogs required long-term treatment for clini-

cal signs attributable to OA was significantly earlier (10.3 years, p<0.01) in the overweight dogs as compared to the dogs with normal body condition scores (13.3 years) [71]. Traditionally the mechanical stress of excess weight has been thought to be a primary perpetrator of the pathophysiology and progression of OA. However, recent studies have documented metabolic activity in adipose tissue that may be of equal or greater importance. Adipocytes secrete several hormones including leptin and adiponectin, and produce a diverse range of proteins termed adipokines. Among the currently recognized adipokines is a growing list of mediators of inflammation: tumor necrosis factor (TNF)-α, IL-6, IL-8, and IL-10 .These adipokines have been documented in both human and canine adipocytes [80,81]. Production of these proteins is increased in obesity, suggesting that obesity is a state of chronic low-grade inflammation. The presence of low-grade inflammation may contribute to the pathophysiology of a number of diseases commonly associated with obesity, including OA. This might explain why relatively small reductions in body weight can result in significant improvement in clinical signs [82]. The overproduction of inflammatory mediators in obese individuals is associated with changes in the genome. These changes may enhance the phenotypic expression of OA compared to genetically similar dogs that remain lean their entire lives.

However, in the osteoarthritic patient, it may be challenging to increase exercise without exacerbating the clinical signs. For this reason, a relatively recent development is the use of weight loss drugs, two of which are currently available and licensed for

dogs. Mitratapide is a microsomal triglyceride transfer protein inhibitor [199,200].

Dirlotapide is also a microsomal triglyceride transfer protein inhibitor, but it also appears to suppress appetite in dogs [201].Both drugs are designed to be used as part of a weight management program, but no published studies have investigated their use in dogs with osteoarthritis. So far, these agents are likely to be useful in weight management in arthritic patients, despite the lack of clinical evidence.

-*Physical rehabilitation* is considered an important component of a multimodal approach to treating OA. The effects of exercise on osteoarthritis in dogs are largely unexplored. One study reports that a short period of exercise (trotting for 1.2 km) in dogs with osteoarthritis in a pelvic limb was associated with a reduction in peak vertical force in the index limb [202]. This study indicates how, in the short term, exercise can exacerbate pain and clinical signs in dogs with osteoarthritis. Anecdotally, and extrapolating from human medicine, it seems that regular, moderate, controlled exercise may be beneficial for osteoarthritis patients.

There are many techniques used in physical rehabilitation, which may be appropriate for the different debilitations of patients. Some modalities are handled very easily by the pet owner, such as walking on a leash and some proprioceptive exercises. On the other hand, some modalities are complex, sophisticated techniques, requiring professional personnel sufficiently skills and trained with inclusive knowledge of risks and precautions for these modalities. Physical rehabilitation focuses on the patient's inability to exercise, providing a consequent "freedom of movement" which can become a good palliative for disease progression. Frequently, physical rehabilitation together with weight control can be as effective as, or more effective than pharmacologic intervention in human and veterinary medicine.

3.2 MEDICAL MANAGEMENT

Medical treatment of the osteoarthritis can be classified as symptom modifying agents or structure-modifying agents [203,204]. Basically the first category includes agents designed to treat pain associated with osteoarthritis, while the second category includes agents designed to delay, stop or reverse the perpetuating pathological changes, which occur during joint degeneration disease.

Many symptom-modifying agents are available for use in canine osteoarthritis; the most important one is represented by nonsteroidal anti-inflammatory drugs. The criteria for validating a drug as a structure-modifying agent are rigorous and include

demonstrating efficacy in delaying or halting cartilage erosion in clinical cohorts of patients, which might not currently be as predictable as expected due to the lack of methods fully validated to assess that change in cats and dogs. MRI and arthroscopy can be used for this task in veterinary medicine with some arguable limitations in small animals.

SYMPTOM-MODIFYING AGENTS.
-Nonsteroidal Antiinflammatory Drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs are one of the most investigated classes of pharmaceuticals for OA in veterinary practice. Generally, this pharmaceutical class works by inhibiting one or more steps in the metabolism of the arachidonic acid cascade. Despite the mechanism of action of some of these drugs is not completely explained by the effect on arachidonic acid metabolism, the main role of NSAIDs is the inhibition of the cyclooxygenase pathway.

NSAIDs can be classified, based on their chemical structure, as salicylate or carboxylic acid derivatives, including indoles (indomethacin), propionic acids (carprofen), fenamates (e.g., mefenamic acid), oxicams (e.g., meloxicam), pyrazolones or enolic acids (e.g., phenylbutazone), and, more recently, coxibs (e.g., deracoxib, firocoxib). Osteoarthritis is a complex disease process that affects all tissues of the synovial joint. Central to this condition is the increased degradation of the extracellular matrix of the articular cartilage with the subsequent processing of inflammatory mediators and the activation of degradative enzymes, which leads to further degenerative and inflammatory modifications. Strong evidence indicates that pro-inflammatory cytokines, such as IL-1, TNF, and IL-6, play a key role in this process. Synovitis also appears to be a key element in the pathology of osteoarthritis and the synovium is the

key tissue of nociception in the affected joints [205] . In late-stage disease, when the cartilage is eroded, a greater rationale is seen for the role of subchondral bone in pain pathways. NSAIDs can relieve the clinical signs of pain. This is achieved by suppression of prostaglandins (PGs), primarily PGE_2, produced from the substrate ara-

chidonic acid within the prostanoid cascade, mediated by cyclooxygenase.

PGE2 plays a number of roles during the OA process, such as lowering the activation threshold of nociceptors, promoting joint synovitis, enhancing the formation of degradative metalloproteinases (MMPs), and depressing the synthesis of the cartilage matrix. At the same time, PGs are involved in positive metabolic roles such as enhancing platelet aggregation, maintaining the integrity of the gastrointestinal tract, and facilitating renal function. These different activities depends on the target tissues. The main goal of NSAID management is to inhibit the formation of prostaglandins that contribute to the clinical signs of osteoarthritis, limiting the reduction of PGs associated with beneficial physiological functions [206]. Therefore, maintaining an optimal balance of PGs production in the body is considered the real challenge for this pharmaceutical class.

Advances in the pharmacodynamic study of NSAIDs in the early 1990s have shown the presence of two isoforms of cyclooxygenase enzymes, which have different activities and different trigger pathways [207]. COX-1 is considered to be the "constitutive" form and associated with the production of "constitutive " prostaglandins, which are important for physiologic function; on the other hand, COX-2 is considered an "inducible" isoform, whose expression is strictly controlled and down-regulated in basal conditions, but dramatically induced during inflammation. Pro-inflammatory cytokines, such as TNF and IL-1, up-regulate the expression of COX-2 in many cells, such as synovial cells, endothelial cells, chondrocytes, osteoblasts, monocytes, and macrophages. Data confirmed that synovial and subchondral bone tissue from canine hips with osteoarthritis have increased COX-2 expression compared to healthy controls [208].

The inhibitory action of cyclooxygenase by NSAIDs is both dose and drug-dependent, and its main therapeutic and toxic effects have been largely correlated with this mechanism. This concept of COX-1 "good" and COX-2 "bad" greatly oversimplifies a very complex situation. Interestingly, COX-2 may be constitutively expressed in the kidney and brain and also associated with a cytoprotective effect in the damaged or inflamed gastrointestinal mucosa. No clear delineation between beneficial and in-

flammatory prostaglandins and their respective pathways is evident so far. However, much of the current literature is based on the hypothesis that a selective COX-2 inhibitor NSAIDs may allow analgesia without the common side effects of COX-1 inhibition. Methods of establishing COX-1 and COX-2 activity have relied on in vitro exposure of cell systems to increasing concentrations of the nonsteroidal antiinflammatory drug, and subsequent measurement of the levels of enzyme activity. The amount of drug necessary to inhibit 50% (IC50) of activity of each enzyme is recorded and expressed as a ratio of COX-1:COX-2 . Care is required to not over interpret these ratios, because the use of different cell systems precludes direct comparison of the data obtained in various studies. In the metabolism of arachidonic acid, the lipoxygenase pathway, which is not under regulation of NSAIDs, may play a big role in cells inflammation.

The lipoxygenase pathway can synthesize numerous products, in particular 5-hydroperoxy eicosatetraenoic acid (5-HPETE), an intermediate compound that is metabolized into leukotriene (LT) A_4, and converted into different forms (LT B_4, C_4, D_4, E_4) whose result is a powerful chemotactic agent that attracts neutrophils and inflammatory cells, with subsequent degranulation and enhancement of the inflammatory response.

COMMON NSAIDs USED IN COMPANION ANIMAL						
Drug	**Class**	**Indication**	**Dose**	**Mechanism of action**	**Bio-availability**	**Interaction**
Carprofen	proprionic acid	relief pain in OA and post operative pain	4.4 mg/Kg die	Inhibition of COX enzyme; *in vitro* selective against COX-2	>90%	other anti-inflammatory drugs
Deracoxib	coxib	Control pain and inflammation in OA and post operative	3.4 mg/Kg post op 1-2 mg/kg die	uniquely targets COX-2 while sparing COX-1	>90%	other anti-inflammatory drugs
Firocoxib	coxib	Control of pain and inflammation associated with OA	5mg/Kg die	Inhibition of COX activity; *in vitro* studies show it to be highly selective for COX-2 in canine blood	Nearly 100%	other anti-inflammatory drugs

COMMON NSAIDs USED IN COMPANION ANIMAL						
Drug	**Class**	**Indication**	**Dose**	**Mechanism of action**	**Bio-availability**	**Interaction**
Mavacoxib	coxib	treatment pain and inflammation in god aged 12 months or more in OA	MONTHLY TREATMENT: 2mg/Kg with food repeated 14 day later and then monthly dosing for up to max 7 doses	uniquely targets COX-2, while sparing COX-1	50% -fasted; 90% -fed conditions	other anti-inflammatory drugs
Robenacoxib	coxib	acute pain and inflammation and also chronic OA	1mg/Kg SID max 6 days 2mg/kg SID (SC) max 3 days	uniquely targets COX-2, while sparing COX-1	62%- fed condition 84% fasted	other anti-inflammatory drugs. not safe for < 2.5kg and 3 months animal
Ketoprofene	proprionic acid- carbo-xylic acid	relief pain and inflammation in OA	0,25mg/Kg die max 30 days	COX-1 selective	-	other anti-inflammatory drugs. limited informa-tion in peer-review for use in dog
Meloxicam	oxicam	Control pain and inflammation in OA and post operative treatment.	0.2 mg/Kg die once 0.1 mg/Kg die	COX-2 selective	Nearly 100%	potentially Nepro-toxic

During the NSAID-mediated anti-inflammatory action, a potential unintended con-sequence of cyclooxygenase blockade is the increased production of leukotrienes, which would otherwise have been metabolized to prostaglandin products. This me-chanism may partially explain the incomplete relief provided by nonsteroidal antiin-flammatory drugs. New dual COX / LOX inhibitors are being developed to prevent this problem.

Another interesting mechanism of non-steroidal anti-inflammatory drugs is to alter cellular and humoral immune responses, and suppress inflammatory mediators other than prostaglandins and leukotrienes [209]. This appear to be the result of interferen-ce with protein interactions within plasma membranes and the disruption of the re-sponse of the inflammatory cells to extracellular signals by affecting signal transduc-tion proteins.

The peripheral antiinflammatory activity of NSAIDs rarely appears to be correlated with the analgesia which they provide, and this has led to the research for other me-chanism of action. A central mechanism of the analgesic effect achieved by intrathe-

cal administration of extremely low doses of NSAIDs has been proposed and suppor-

ted [210-211]. These suggested mechanisms can be explained by including inhibition

of prostaglandin synthesis, interaction with a central opioid mechanism, interaction

with central serotonin activity, and interference with an excitatory amino acid (e.g.

Glutamine) in the system central nervous. Acetaminophen, a drug with potent anal-

gesic and antipyretic activity but with little peripheral anti-inflammatory effect, is

believed to work, to achieve an analgesic effect through the central nervous system,

by indirect activation of cannabinoid receptors (CBI). In brain and spinal cord this

drug, following deacetylation to its primary amine (p-aminophenol), is conjugated

with arachidonic acid to form N-arachidonoylphenolamine, a compound already

known as an endogenous cannabinoid [212].

For chronic inflammation, such as osteoarthritis, non-steroidal anti-inflammatory

drugs could be used for an extended period, therefore, interest and concern has arisen

about the effects of this pharmaceutical class on metabolism and joint tissue. Althou-

gh these effects are poor, over a long period of treatment, they can become cumulati-

ve. Experimental studies in dogs have shown inhibition of ex vivo proteoglycan syn-

thesis following extended treatment with aspirin for example [213]. On the other

hand, all nonsteroidal anti-inflammatory drugs can provide some risk and induce un-

desirable and potentially life-threatening adverse events, which can be exacerbated in

prolonged treatment. A systematic review of long-term use of nonsteroidal anti-in-

flammatory drugs, 28 days or longer, reported experimental event rates, ranging from

0 to 0.31, few of these rates are available to refer to these figures [214]. Another re-

view with the same goal, limiting the eligibility assessment for the blinded placebo-

controlled studies, reported no significant differences in the incidence of adverse

events between treated and control dogs [215]. Typically the clinical sign of toxicosis

in this pharmaceutical class has been a loss of appetite, vomiting, and diarrhea. Ho-

wever, the true incidence of gastrointestinal toxicity in dogs treated with non-steroi-

dal anti-inflammatory drugs is still unknown.

NSAIDs can induce gastric damage through local and systemic effects, which are

commonest with aging. Furthermore, nearly all non-steroidal anti-inflammatory drugs are capable of altering platelet activity and may be associated with bone marrow dyscrasias. This effect can create a bleeding problem, where gastric bleeding is the most common, partly linked to the antiplatelet activity and partly added to the ulcerogenic properties of these drugs. In the kidney, prostaglandins are protective, ensuring that medullary vasodilation and urine production continue during states of renal arterial vasoconstriction. PGE2 and PGI2 have important roles in maintaining renal blood flow and ion transport within the nephron. Any loss of this protective function becomes important in patients with compromised renal function. Predisposing factors associated with "analgesic nephropathy" include cardiac, renal, or liver disease in geriatric patients; hypovolemic states, including shock and dehydration; and the use of nephrotoxic (e.g., aminoglycosides) or nephroactive (e.g., diuretics) drugs. Despite the potential toxicity and unclear metabolic effect of NSAIDs, this pharmaceutical class is now considered the default "standard of care" for osteoarthritis, also used to compare the "medicinal" efficacy of other drugs [216-217]. NSAIDs currently are licensed for use in the United States and Europe for treatment of canine osteoarthritis.

-Other Analgesics for Osteoarthritis

Primarily emerging therapies for the treatment of moderate to severe pain are achieved through the reformulation of existing pain medications combined with new delivery technologies, which may offer improvements in efficacy and safety.
Presently, the greatest need in the treatment of chronic pain is for agents that surmount the disadvantages of NSAIDs and opioid analgesics. Although great progress has been made in terms of efficacy of NSAIDs over the past decades, safety is still an important concerning area. OA is typically both chronic and acute disease, with intermittent flare-ups that may render an NSAID ineffective.
As reported in the section on the pain pathway, when pain signals are generated repeatedly, the neural pathways undergo physicochemical changes that make them hypersensitive, and resistant to antinociceptive inputs. Several receptors and neurotran-

smitters interact and play an important role in the pain path (e.g. glutamate). For example, the discovery of the active role of the N-methyl-D-aspartatae (NMDA) receptor in chronic pain has generated a strong interest in NMDA antagonist drugs, which are occasionally administered in combination with ion channel modulators, such as gabapentanoids, as adjuvants in multimodal OA protocols.The receptors and pathways involved in perception and transmission of noxious stimuli provide multiple sites for potential new analgesic drug development.

Amantadine: Amantadine is an *N*-methyl-D-aspartate (NMDA) receptor antagonist. When activated, the NMDA receptor site allows a massive intracellular influx of Ca^+, and subsequent neuronal release of neurogenic transmitters. First recognized as an antiviral agent and later considered useful in the treatment of Parkinson's disease, amantadine was initially thought to act on the dopaminergic system, while it is now evident that its main action is linked to the inhibition of NMDA responses.
The analgesic effects of amantadine in dogs with chronic osteoarthritis pain were studied and compared with NSAIDs, improving lameness outcome and clinical questionnaire aspects after 42 days [218]. To date, this represents the only study undertaken to explore the use of amantadine in the treatment of canine osteoarthritis.

Gabapentin: Gabapentin is a gamma-aminobutyric acid (GABA) analogue. It was originally developed for the treatment of epilepsy and currently is widely used in human and veterinary patients to relieve pain, particularly neuropathic pain. Gabapentin is not licensed for use in dogs or cats so far and its mechanism of action is still unclear, although its therapeutic action on neuropathic pain is believed to involve voltage-gated N-type calcium ion channels. Adverse effects are not absent, of which the most common in adult humans include dizziness, somnolence and peripheral edema, although these mainly occur at higher doses and in elderly patients. Gabapentin is excreted by the kidneys, and patients with renal insufficiency may require less frequent dosing because of slower elimination. Although rare, several cases of human hepatotoxicity have been reported. A wide dose range for gabapentin is used

(dosage range, 5 to 25 mg/kg); anyway, it should be given to at a lowest effective dose. Recommendation dose start with 10 mg/kg administered orally every 8 hours in dogs, and 5 mg/kg administered orally in cats, followed by ramping up or tapering down to effect [219]. No peer-reviewed studies on the use of gabapentin in dogs and cats have been published, but some positive data have been derived from anecdotal reports of use in dogs and cats [219-220].

*Corticosteroids :*The use of corticosteroids for the treatment of osteoarthritis is controversial [221]. For a local disease such as osteoarthritis, corticosteroids are generally used as intra-articular agents, systemic use of this pharmaceutical class should be judiciously considered and normally not recommended.

Tramadol: This molecule is widely used in dogs, it is a synthetic analogue of codeine, and carries out about 40% of its activity at the mu receptor level. Tramadol is a norepinephrine reuptake inhibitor and is also a serotonin reuptake inhibitor (SRI), in 40-20% proportion respectively. Since tramadol has SRI features, its use can be associated with increased bleeding, especially when used in combination with an NSAID; however, it can be used as an adjunct to an opioid or an NSAID [222]. In humans tramadol is able to reduce the amount of substance P in synovial fluid, as well as IL-6, which seems to be correlate with the stage of OA [223]. The American College of Rheumatology and the American Medical Director 'Association argue that adding tramadol to an NSAID for chronic pain management in humans may have beneficial effects [224]. Despite this support, a meta-analysis study of tramadol concluded that its effect is very small and adverse events, although reversible and not life-threatening, often prompted participants to stop taking the drug, which could limit its usefulness [225].

Piprant drug class: Prostaglandin E2 is the principal pro-inflammatory prostanoid of the arachidonic acid cascade resulting one of the key points of inflammation, and pain hypersensitivity.PGE2 exerts its cellular effects through four different G pro-

tein-coupled receptors, which are called EP receptors (EP1, EP2, EP3, and EP4). Of these four, EP4 is the main receptor in mediating pain associated with both rheumatoid arthritis and OA, so the antagonist EP4 has been shown to be as effective as a COX-2 inhibitor in suppressing joint inflammation [226-227].

Selective EP4 antagonists play a different role than NSAIDs and COX-2 inhibitors, as they do not inhibit the AA cascade, but their effect is mediated by the direct blockade of PGE_2 *(figure 3.4).* In an experiment on dorsal root ganglion cultures pretreated with $EP3_C$ and EP4 antagonists, the increased release from PGE_2 of substance P and the calcitonin gene-related peptide is abolished, therefore unlike COX-2 inhibitors, this pharmaceutical class does not suppress PGI_2, which possess potent vasodilatory and antithrombic activities, and can be cardioprotective [228]. The World Health Organization (WHO) in 2013 recognized a new pharmaceutical class whose action is the antagonism of EP receptors; drugs belonging to this class take the suffix of "piprant", in which grapiprant is the progenitor, and was approved in the United States in March 2016 for the control of osteoarthritis pain and inflammation in dogs. The European homologation was obtained in 2017 and in September 2018 this piprant molecule has been introduced for the Italian trade.
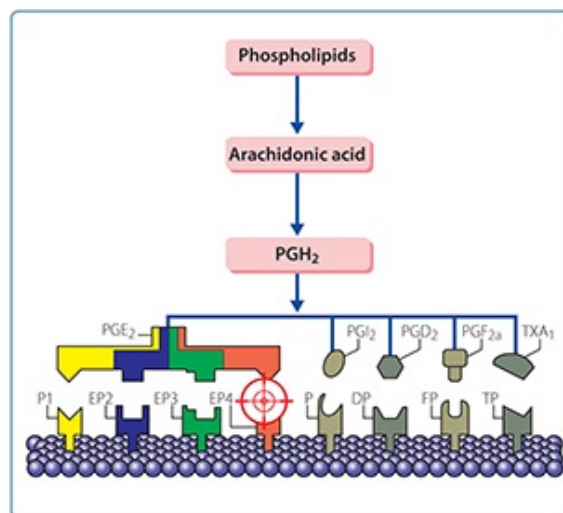
STRUCTURE-MODIFYING AGENTS

-Polysulfated glycosaminoglycan

Polysulfated glycosaminoglycan is licensed for the treatment of osteoarthritis in dogs in some countries, such as United States and Europe. The in vivo mechanism of action is still unknown, but an in vitro study has shown the efficacy of this molecule on proteoglycan metabolism and DNA content in explants of canine articular cartilage [229]. The design study envisaged comparing incubated cartilage explants with and without polysulfated glycosaminoglycan and concluded that this treatment helps to modify the progression of osteoarthritis in joint cartilage by maintaining chondrocyte viability or stimulating chondrocyte division, as well as protecting against degradation of the extracellular matrix. Another in vivo study in dogs affected by OA evaluated the effects of polysulfated glycosaminoglycan on a range of biomarkers, including cartilage oligomeric matrix protein (COMP), C-reactive protein, and serum activity of matrix metalloproteinases-2 and -9 [230]. Authors concluded that intramuscular administration of glycosaminoglycan polysulfate may inhibit the degradation of cartilage oligomeric matrix protein with an improvement in lameness, which may likely cause decreased serum concentrations of cartilage oligomeric matrix protein. Unpublished clinical data from Millis et al. (2005) had shown an improvement of the comfortable angle of extension and lameness scores following the administration of PSGAG at both 4 and 8 weeks following cranial cruciate ligament transection (CCLT), while the concentration of neutral MMP was reduced relative to transected controls [231]. However, two systematic reviews of treatment for canine osteoarthritis concluded that there was a mild to moderate level of comfort in the use of polysulfated glycosaminoglycan, which should be kept in mind during the clinical indication [232-233].

-Pentosan polysulfate

Pentosan polysulfate is a semi-synthetic glycosaminoglycan, with structures similar to heparin, which gives it anticoagulant properties [234]. The rational use of this molecule is associated with in vitro properties, in fact, it has been shown that it delays the degradation of articular cartilage and stimulates the synthesis of hyaluronan by the synovial cells and of proteoglycan by the chondrocytes [235-236]. Clinical trials report very mixed and confusing results in dogs with osteoarthritis [237-238].

Between medical therapy and dietary management or "functional food", there is a category of supplements that takes the name of nutraceuticals, whose main objectives are similar to structure-modifying agents, which promise an effect of prevention and treatment of the disease. This class of molecules is considered a hot topic in the treatment of many chronic diseases, such as OA, and are critically evaluated just like pharmaceuticals. The most investigated and commonly used nutraceuticals are chondroitin sulfate, glucosamine sulfate/hydrochloride.

Chondroitine sulfate

This molecule shows approximately 5% of bioavailability in dogs after a single dose [239]. Pharmacokinetic studies for orally administered chondroitin sulfate in dogs did not agree with the fate of this molecule, demonstrating no cartilage distribution after their administration. The implication of these findings is that oral chondroitin sulfate may not reach intact joint cartilage [239,240]. However, in vitro studies have investigated the effects of chondroitin sulfate on tissues and cells, showing antichemotactic activity on human neutrophils, reduced phagocytic activity, reduced release of lysosomal enzymes and reduced damage to membranes [241].
Chondroitin sulfate has been shown to have anti-inflammatory effects in experimental animals; in particular, it has been shown to reduce edema formation in a rat carrageenan model, although less potent than NSAID administration [241].
An important finding is provided by a systematic review in which no published study was found on the use of chondroitin sulfate alone for the treatment of canine OA [233]. Additionally, a systematic review of the management of human osteoarthritis concluded that the effect size for chondroitin sulfate pain relief had decreased from

the previous review, showing greater heterogeneity of results and noting further evidence of publication bias [242]. In summary, there is still no evidence to recommend the use of chondroitin sulfate for the management of canine osteoarthritis.

Glucosamine sulfate and glucosamine hydrochloride
Unlike chondroitin sulfate, glucosamine sulfate is 90% orally absorbed and diffusely distributed in the joint space [243,244]. The in vitro effect is linked to the influences of the metabolism of chondrocytes, which acts by increasing the synthesis of glycosaminoglycans, and of the mRNA of the core aggrecan protein, stimulating the production of monomeric proteoglycans capable of assembling large aggregates of proteoglycans [245]. Nevertheless, the study of canine chondrocytes in three-dimensional cultures showed the damaging effect of glucosamine on cell viability and glycosaminoglycan production [246]. This molecule has also been demonstrated to have a weekly anti-inflammatory effect in several animal models [247].

Several studies have examined the efficacy of glucosamine in the relief of OA-associated pain and disability, most of them in human patients, with variable results and often associated with criticism related to study design and patient group size.

A meta-analysis study in human patients likely suggests that symptom modification could be associated with the use of glucosamine sulfate, although there is a lack of evidence for therapeutic effect and strong concern over publication bias towards positive outcomes [248]. The lack of a well-designed clinical trial in veterinary medicine and the conclusion of many reviews of a strong publication bias on the effect of glucosamine, place this molecule on the same recommendation line as chondroitin sulfate in the decision-making process of the management of OA [233,242].

INTRA-ARTICULAR THERAPIES

Combined with the systemic administration of medications, to relieve pain and discomfort of patients associated to osteoarthritis, there is a broad category of local intra-articular therapies on which clinicians may rely on. According to the same criteria, these therapies can be divided into agents that modify symptoms or structure, as well as any other medications for osteoarthritis. Importantly, treatment options may be limited by complications resulting from comorbidities and long-term systemic

medications, which contribute to a growing, incompletely met, clinical need for additional treatment modalities, which more often rely on local therapies.

Various intra-articular therapeutic techniques, for the treatment of canine osteoarthritis, are reported in veterinary literature, from the most classic use of corticosteroids, achieving a pure anti-inflammatory effect, to the entire category of regenerative therapies, such as hyaluronic acid and other biological therapies, until other novel biomolecular technologies, for example the radiosynoviorthesis.

-IA Corticosteroids:

Rapid and pronounced suppression of local joint inflammation is the main action of this molecule. Severe adverse metabolic effects have been reported for the use of this pharmaceutical class which emphasize judicious use for systemic and even local therapy [221]. Although some evidence suggests that corticosteroids may protect articular cartilage in experimental canine osteoarthritis providing significant relief of clinical signs , can also have deleterious effects on the joint tissues, more serious with

repeated use, caused by the cartilage matrix suppression [249,250]. Despite its strong

effect, it is recommended to avoid repeated corticosteroid injections into the same joint ,with general indication of no more than three or four injections in a single year in humans [251]. Base on many human reviews recommendation it would seem prudent to adopt similar guidelines for small animals. Before intra-articular corticosteroid injections are given, the indications and contraindications should always be considered, in particular, articular infections should be ruled out, and strict aseptic technique shall have been respected. The two most commonly used corticosteroids as intra-articular therapy to manage OA are methylprednisolone acetate and triamcinolone acetonide, where the latter would appear to have less deleterious effects on cartilage and synovial viability resulting in the molecule suggested in the literature for IA infiltration [252].

-IA Hyaluronic Acid:

Hyaluronic acid (HA) occurs naturally as a constitutive molecule of many tissues and fluids, but most abundantly in joint cartilage and synovial fluid (SF). It is a non-sulfated, non-protein glycosaminoglycan (GAG), with distinct physicochemical properties, produced by synoviocytes (synoviocyte B), fibroblasts and chondrocytes. HA has an important role in the biomechanics of normal synovial fluid, where it is partially responsible for lubrication and viscoelasticity of itself. Hyaluronic acid concentration, such as its molecular weight (MW), decline with aging, as well as with progression of osteoarthritis. For that reason, HA has been used for more than four decades in the treatment of DJD in dogs, horses and humans.

Basically, the main mechanisms proposed for the beneficial clinical effects detected by hyaluronic acid therapy are partly related to its anti-inflammatory and analgesic properties [173-176], and partly related to its regenerative properties, associated with the restoration of elastic and viscous properties (viscosupplementation), and the bio-synthetic- chondroprotective effect on joint cells, thanks to the induction by hyalurons of the endogenous synthesis of HA and the inhibition of cartilage degradation [253-254].

Generally HA acts as an aggregating factor between the collagen, and cartilage structural network as a whole. Intra-articular injection of HA, called viscosupplementation, has demonstrated significant improvement of symptoms in patients with OA [254]. Several commercial forms are available, which differ in treatment regimens, total dosage and average molecular weight. Due to its molecular weight, it is important to note that HA is poorly absorbed from the gastrointestinal tract (5% in the dog) and therefore must be administered locally into the joint [255]. After intra-articular injection, the HA reaches the synovial membranes by simple diffusion, the extracellular matrix of cartilage and subchondral bone through the lymphatic flow [256]. Under physiological conditions, in the extracellular matrix the HA appears mainly high molecular weight (HMW-HA) (> 500 kDa). This feature promotes cellular quiescence and tissue integrity by binding to receptors for pro-inflammatory signaling pathways [257]. Despite the clear improvement in DJD symptoms, the sustained

beneficial effects of HA therapy are unlikely to come from temporary restoration of synovial fluid lubrication and viscoelasticity [253]. HA therapy is more likely to have disease-modifying biological activity and an impact on OA progression, which is not fully understood so far. Currently, the mechanism and doses by which HA produces beneficial effects remains controversial. The hyaluronic acid is classified based on the molecular weight in low molecular weight ($0.5-1 \times 106Da$), intermediate molecular weight ($1-1,8 \times 106Da$), and high molecular weight (6×106 Da); some controversial advantage is suggested in vivo using different the molecular weight [258]. Evidence in the literature reports an increase in efficacy in prolonging the duration of intra-articular infiltration with hyaluronic acid, associated with an increase in molecular weight [259]. One study compared efficacy on low molecular weight HA infiltration frequencies, obtaining non-significant statistical outcome among patients receiving weekly treatment for 3 weeks and weekly treatment for 6 weeks [260]. Another study in dogs with osteoarthritis-related to hip dysplasia, result in low pain scores, and better clinical outcome with a single intra-articular injection of hyaluronic acid (molecular weight 500–730 kDa) compared to intra-articular saline injection in combination with oral nutraceutical and carprofen [261]. Additionally, Hellström and colleagues showed better results in a canine OA study with two intra-articular injections of high molecular weight sodium hyaluronate applied at a 3-week interval compared to using oral anti-inflammatory therapy, noted a clinical improvement for over 6 months following administration [262]. The current recommendation for intra-articular HA therapy for OA is considered to administer HA once weekly for 3 weeks with very mild complication associated to its use.

-Radiosynovioorthesis

Radiosynovioorthesis (RSO) is an injection into the synovial space of a radioisotope to treat joint inflammation and mitigate chondromalacia. It is used when systemic therapies or other traditional therapies fail to produce a satisfactory response, with the main goal of reducing synovitis which is characterized by pain and synovial hypertrophy. Its use in veterinary medicine is under investigation and associated with a great interest in the scientific community [263,264]

4 REGENERATIVE MEDICINE

In recent decades, the scientific community has sought adequate cures for major chronic diseases, where definitive cures are lacking, turning more and more frequently to regenerative medicine. Regenerative medicine is defined as an interdisciplinary field of clinical research and therapy, focused on the repair, replacement, or regeneration of cells, tissues, and organs, with the aim of restoring the structure and function of those affected [265]. Regenerative medicine encompasses a wide variety of treatment strategies, including stem cell therapy, tissue engineering, genetic therapy, and the use of autologous biological products, such as platelet-rich plasma (PRP) or autologous conditioned sera (ACS). Basically, the principal goal for these therapies is to reduce pain and inflammation, improving the healing process [265].

4.1 PRP

Platelet-rich plasma (PRP) is an autogenous fluid concentrate composed primarily of platelets and free growth factors. The main important rationale to use this therapies reside in the presence of alpha granules , which contain hundreds of growth factors, cytokines, vasoactive peptides, and extracellular matrix proteins, that are critically important in all the physiologic phases wound healing, from inflammation to the maturation phase [266-267]. Initially, the first clinical applications of PRP were limited to dentistry and maxillofacial surgery to improve bone healing. However, currently has much broader clinical applications, extending to orthopedic surgery and sports medicine. Multiple formulations of PRP have been developed and studied. The platelet concentration is not the only important component of a PRP production; the inclusion or exclusion of mononuclear cells, neutrophils, and red blood cells (RBC) are responsible of different clinical efficacy of the product, and play major roles affecting the inflammatory responses after PRP injection [268-272].
Generally, it is believed that RBC and WBC should be reduced for intra-articular injection because they may provide an inflammatory effect, despite the effect of mononuclear cells remains largely unknown [271]. A recent study had shown the pro-in-

flammatory effect of red blood cells, demonstrating that synoviocytes die significantly more, when compared to the use of leukocyte-poor PRP (LP-PRP) [271]. The effect of intra-articular PRP infiltration in canine osteoarthritis was investigated. *Fahie* and colleagues compared a single infiltration of leucocyte-poor platelet-rich plasma with saline infiltration (control group) in 20 client-owned dogs with OA, concluding that a single intra-articular injection of PRP resulted in led to clinical improvement for up to 3 months after treatment, which may suggest the potential useful of this molecule [273]. Nowadays PRP therapy is largely used for the treatment of OA and is often performed as a series of 1-3 injections with 2 weeks between each injection. Despite the routine clinical uses, there are still several limitations on the scientific data, which can generate some skepticism.

4.2 AUTOLOGOUS CONDITIONED SERA

The main product on this class of biologics therapeutics reside on the Interleukin-1 receptor antagonist (IL-1RA) , better known as interleukin-1 receptor antagonist protein (IRAP). The IRAP is an endogenous protein that inhibits interleukin-1 activity by binding to the IL-1 receptor, preventing its ligand-receptor interactions and signaling events, which play a key role in the progression of OA-associated inflammation [274]. The use of this molecule has been investigated in veterinary medicine, as well as in humans medicine [274-278]. Nowadays, numerous system have become available for veterinary use to produce IRAP*,* which can be administered intra-articularly to reduce IL-1 signaling that potentiates inflammatory pathways associated with OA. These systems are designed to increase the expression of leukocyte-derived IL-1RA by incubating coagulated whole blood within a chamber containing borosilicate beads. Normally the serum is then collected, filtered, and administered intra-articularly. Recent studies have evaluated and validated the use of these systems and have shown a large increase in IL-1RA and other anti-inflammatory proteins occur [279]. In a recent report, dogs with unilateral OA of the elbow or knee who received a single intra-articular injection of IRAP had significantly improved lameness scores, pain scores, and peak vertical strength at 12 weeks post-injection, compared to pretreatment values [278].

4.3 STEM CELL

- Backgroud

 Medical advances in the 20th century have radically revolutionized the knowledge and understanding of many physiological and pathological mechanisms on various chronic diseases in humans and veterinary field. The study of stem cells goes back a long time ago , from the first use of the term "stem cell", coined by Ernst Hackel to describe fertilized eggs in 1868, to the hypothesis that a set of "stem cells" in the bone marrow, which could differentiate into red and white blood cells, theorized in 1909 by Alexander Maximow. Since that time, the entire scientific community has undertaken extensive research until 1960, when McCulloch and Till define the key properties of stem cells by discovering the hemopoietic stem cell in mice. Other discoveries in this field were observed in 1981 when Martin Evans (Nobel prize) and Matt Kauffman successfully identified, isolated, and cultured embryonic stem (ES) cells using mouse blastocysts, which were isolated in humans in the late 1990s by James Thomson [280]. Recently, a significant milestone on stem cells was discovered in 2006 by Shina Yamanaka and colleagues, who thanks to their experiment on murine fibroblasts, were able to achieve cell reprogramming by inducing adult cells into pluripotent stem cells, generating a new class of cells, today known as induced pluripotent stem cells (iPSCs) [281].

-Definition and Classification

Stem cells are present in every mammalian tissue and are responsible for growth, maintenance, and repair at every developmental stage, including adulthood.
By definition, stem cells are undifferentiated cells capable of self-renewal and transformation into different specialized cells [282]. They are classified by their source as embryonic (ESC), perinatal (PSC), adult (ASC) , and induced pluripotent stem cells (IPSC) [282]. Considering their phase of development and differentiation, or their plastic capacity, they are further classified as totipotent, pluripotent, or multipotent cells [283]. Totipotent stem cells are present only in a very early embryo pha-

se during the morula stage before gastrulation starts. They are capable of developing into all embryonic and extra-embryonic tissues, such as placenta and other annexes. Subsequent cell divisions during early embryonic development lead to the appearance of the blastocyst, which has pluripotent ESC activity. ESC can give rise to all tissue cells in the body, with the exception of extra-embryonic tissues and germ cells [282-284]. With further cell development, pluripotent ESC gradually lose their pluripotency and become multipotent. The multipotent stage is characterized by the ability of cells to differentiate into limited types of specific cells, often depending on their germ layer origin. During adulthood the cells slowly become more differentiated until they reach a state of unipotence, in order to regulate and maintain the homologous tissue. Despite adult stem cells are generally multipotent or unipotent, rare populations of these cells maintain pluripotent quality, and have been identified in some studies [285,286]. Each type of stem cell has its own strengths and weaknesses with respect to sample acquisition, expansion in culture, potential applications, and even more important to note, ethical considerations.
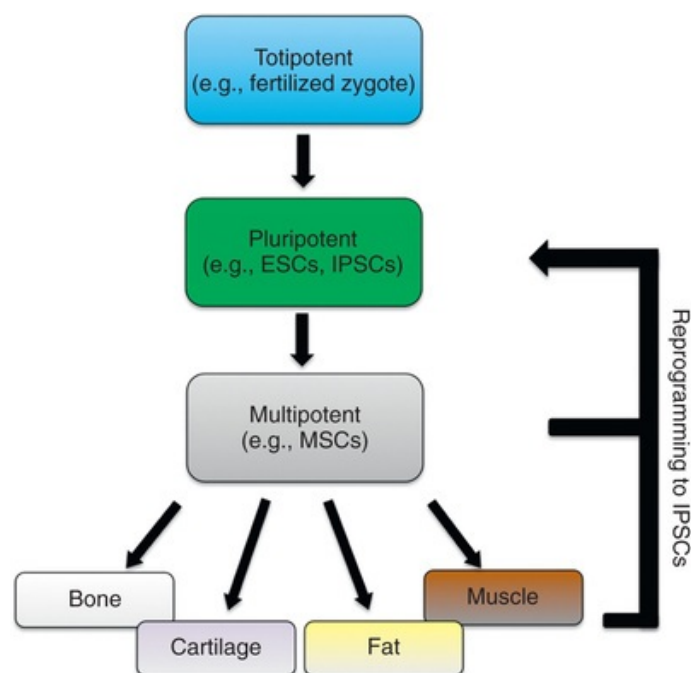
Figure 4.1: Hierarchical nature of stem cell differentiation. [40]

-*Adult Stem Cells*

An important alternative to embryonic stem cells are represented by adult stem cells. These cells are likely present in every tissue and organ system of adult mammals, existing as tiny populations of progenitors, considered multipotent progenitor cells (MAPCs), which reside in a defined microenvironment, better known as a "stem niche" [287,288]. This tissue-resident stem cell population, is believed to be the source of replacement cells, which guide tissue maintenance and repair.
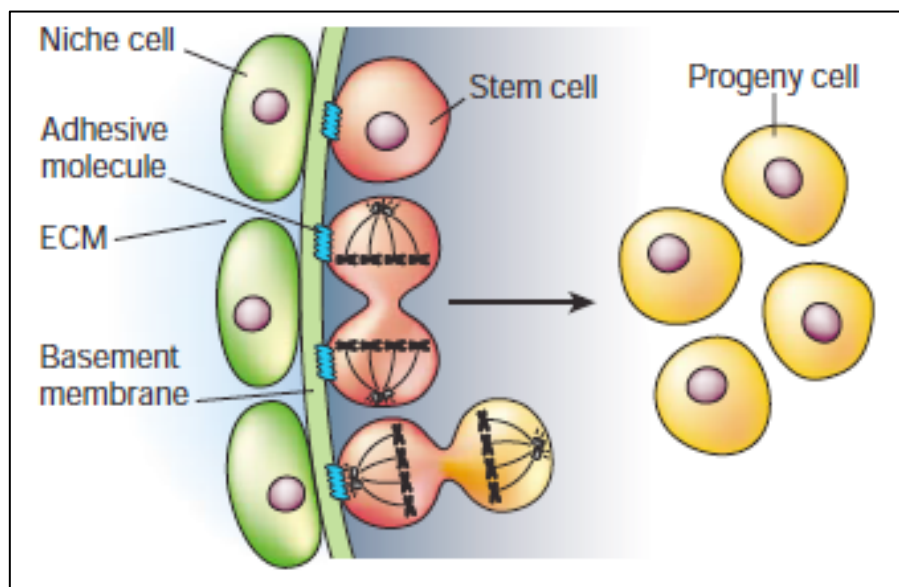
Figure 4.2 : Graphic rappresentation of the microenvironmental stem niche. [287]

Adult stem cells can be divided, due to their phenotypic background, into hematopoietic stem cells (HSC) and not hemopoietic stem cells, even know as mesenchymal stem cells (MSC) and neural stem cells (NSC) [289].
Interestingly, it has been suggested by some authors that bone marrow-derived stem cells may be the original source of these tissue-resident adult stem cells because in

this tissue reside the phenotypical background of whole hemopoietic and not hemopoietic source [289-291]. While HSCs can differentiate into different cells of the immune system, erythrocytes and platelets, MSCs are able to differentiate into cells of bone, cartilage, ligaments, tendons, fat, skin, muscle, and connective tissue. The first mention of adult multipotent progenitors cells/MSC dates to 1968 when the osteogenic population of cells with fibroblast-like morphology was isolated from the bone marrow [292]. Early studies showed that multipotent stem cells are capable of differentiating into osteoblasts, chondroblasts, and adipocytes [293]. This leads to the belief that MSCs show their therapeutic potential through differentiation into tissue cells [294,295]. However, numerous subsequent studies have questioned this, and today it is believed that the primary mechanism of MSC regenerative abilities , reside in their immunomodulatory and tissue repair mechanisms. It is presumed that perivascular localization of MSC in various tissues plays an essential role in enabling these cells to detect local or distant tissue damage and respond to it by directed migration to the site of injury and participation in the healing process [296]. Based on this, Caplan proposed in 2017 that the term "mesenchymal stem cells" should be changed into "medicinal signaling cell" (MSC) [297].

Compared to other types, MSCs are recognized as the most promising stem cell type for therapies, due to the simple procedures needed for their harvest, isolation, high cell yield upon their harvesting, and the lack of ethical concerns restraint when in use.

-*Mesenchymal stem cells*

Mesenchymal stem cells stem cells have been isolated from many tissue in small animal, such as bone marrow, adipose tissue, synovium, synovial fluid, synovial membrane, infra-patellar fat pad, umbilical cord, muscle, periosteum, and so on [298-303]. In their native state, these cells reside in a perivascular location, in which they are in active communication with endothelial cell and the resident somatic cell population. This perivascular arrangement constitutes the peri-cellular niche responsible for many of the characteristics of these cells. Because of their perivascular loca-

tion, some authors have suggested that mesenchymal stem cells are a unique subset of pericytes-specialized vascular smooth muscle cells that, in conjunction with endothelial cells, are responsible for production and maintenance of the vascular basement membrane during normal tissue homeostasis as well as after vascular disruption [290,304]. The fact that mesenchymal stem cells reside in a perivascular location likely explains also the reason why these cells can be successfully isolated from many adult tissues. The morphologic and functional properties of mesenchymal stem cells are affected by a number of variables, depending on the individual characteristic (e.g. species of origin, tissue source, donor age), tissue isolation (adipose tissue, bone marrow), technique of isolation (enzymatic or mechanic) and culture replication (oxygen tension, the number of passages) [305-307]. *Prockop et al.* describe the presence of two different cells during MSC replication, small rapidly renewing cells, also called RS cells, and larger, slower replicating cells [291]. The functional properties of these two cells are different, in general rapidly self-renew cells , the smaller, are superior to lineage-specific differentiation, while larger cells, which replicate slowly, do not differentiate as readily as the small one, although both cell types appear to have the ability to secrete immunomodulatory and trophic agents [291]. To clarify this issue, according to the publication of minimal criteria of MSC by *Dominici et al.* in 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed a set of standards to define human MSC for both laboratory-based scientific investigations and pre-clinical studies [308].

In order, mesenchymal stem cells are currently defined as cells that adhere to plastic flasks or culture exhibiting the typical spindle-shaped appearance, form colonies of cells from single parent cells when cultured in low- density "clonal" cultures without media exchange, express a specific set of cell surface marker proteins, that exclude the cells from hematopoietic lineages, and possess the ability to differentiate into osteoblast, adipocytes, and chondrocytes (i.e., trilineage differentiation) using defined in vitro differentiation assays (*figure 4.3*). In addition to these in vitro features, it has been suggested that MSC should require an in vivo production of cartilage, bone,

and adipose tissue using classic transplantation, as well as an immunomodulation effect, which should be considered as an additional property of mesenchymal stem cells.
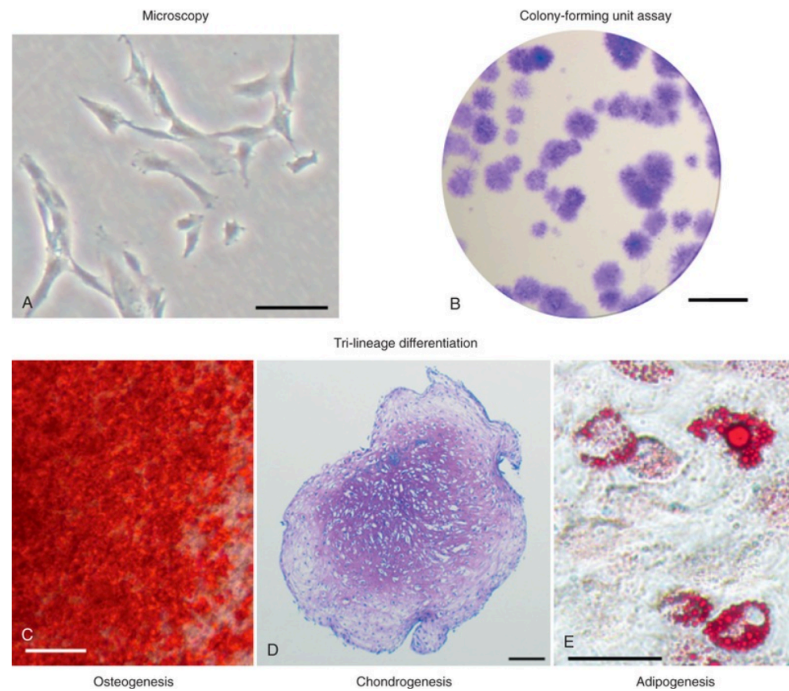
Figure 4.3: exemple of bone marrow derived stem cells which have the common characteristic of MSC; (a) adhere in plastic culture showing typical spindle-shaped appearance, (b) forming colony, (c,d,e) and showing the trilineage diffe-rentiation ability. [40]

Despite these cells are easily isolated from many different tissue, currently the most commonly used sources of MSC for stem cell therapies are bone marrow and adipose tissue. Among these two, adipose tissue is considered an attractive source of MSC due to the minimally invasive procedure required to obtain a high concentration of cells. Although MSCs isolated from bone marrow and adipose tissue have similar surface immunophenotyping and trilineage differentiation, there are important diffe-rences in terms of proliferation and differentiation capacity, and their secretory profi-les [298,299,309]. In some studies, canine adipose tissue derived MSC (ADMSC) were shown to have higher proliferative potential, whereas bone marrow derived

MSC (BMMSC) exhibited a higher secretory production of soluble factors and exosomes [299,309,310]. Canine ADMSCs were reported to have superior chondrogenic and osteogenic potential in comparison to BMMSCs [298,299]. Another potential source of high chondrogenic potential stem cells could be synovium-derived stem cells, which appear to have higher potential than ADMSC and BMMSC in dogs [298,299]. This could be explained by the ability of these progenitor cells to maintain the homologous tissue, which in this case is the "joint organ". When choosing adipose tissue as a source of MSC, the anatomical site of sampling is also important. Several articles in the literature compare the effect of the anatomical site on stem cell characteristics, however, despite the quality and quantity of cells yielded with visceral fat appears to be better, according to Bahamondes and colleagues, the subcutaneous site is currently preferred for ease of collection and proliferative features [311-313]. Since these different properties of MSCs could lead to different successful therapy scenarios, it will be necessary to explore them more closely in the next future. Currently, there is no evidence to generally suggest the preferential tissue source of MSC, although some authors suggest the importance of their use in homologous tissues. Furthermore, while all animal MSCs show plastic adherence and differentiation potential, not all express the same panel of surface antigens that has been described for human MSCs [314].

-*Role of stem cells and therapeutic potential use.*
Although it was initially thought that the efficacy of stem cells was linked to the ability of these cells to differentiate and replace damaged or diseased tissues, it has become even more evident that the therapeutic properties of MSCs are mainly achieved through their immunomodulatory functions, which operate in interaction with the cells of the immune system. The complex immunomodulation activity of MSCs, including their paracrine action, extracellular vesicle (ECV) secretion, apoptosis-mediated immunomodulation, and mitochondrial and other organelle transfer appear to be critical to their features. The purported "mesogenic process" of stem cells, that is, the process by which these cells are able to terminal differentiate into various somatic cells, is no longer considered the main effect. Currently, mounting evidence sug-

gests that the primary mechanism of action of MSCs is based on paracrine signaling, which results in functional changes in immune cells [315-317]. Several factors have been reported to contribute to the immunomodulatory effects of MSC. Among them are well-established effectors such as transforming growth factor-beta (TGF-β), indolamine-2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), interleukin 10 (IL-10), and tumor necrosis factor- (TNF) stimulated gene-6 (TSG-6). TGF-β is involved in many different biological process, stimulating proliferation and differentiation of cells, enhance wound healing, and angiogenesis [318]. It is responsible, together with stromal cell derived factor 1 (SDF-1) and other chemokines released by damaged tissue, for the migration or homing of these cells, a very interesting mechanism for many therapeutical uses [318,318]. Futhermore TGF-β was shown to induce a switch from inflammatory (M1) to antiinflammatory/regulatory (M2) state of macrophages and, in association with indolamine2,3- dioxigenase (IDO), actively participates in the modulation of the induction of the regulatory T-cells (T-regs) [320-323].This modulation lead to T and B cells cycle arrest , associated with inhibition of T-cell, B-cells and natural killer (NK cells) proliferation and activation [324]. Apporting of inteleukine 10 is translate as antiinflammatory effect, which guide the immunomodulation effect arouse with the other chemokine [325]. These paracrine effect is not the solely limited to the secretion of soluble factor, since these cells have the ability of transfer various molecules through the extracellular vesicles (ECV). The ECVs are responsible of miRNA, mRNA, protein, and mitochondria transfer, which is very important activity of stem cells and the main activity of stromal vascular fraction too [326,327]. The effects of these vesicles seem to be similar to those exhibited by MSC themcells, by enhancing and up regulate and mitigate the immunomodulation and antinfiammatori effect [328,329]. MSC derived ECVs were reported to be beneficial in many different treatment, such as osteoarthritis [330]. ECVs represent the potential to exploit MSC effects in a cell-free manner, with the main advantage being the avoidance of possible MSC side effects such as immune response and pulmonary embolism upon intravenous (IV) application of MSC [327]. The modulation of apoptosis by MSCs plays an important role in the immunomodulatory

effect. During the phagocytic clearance of dying cells we have the resolution of the inflammation and the restoration of the function of the damaged tissue but also a consequent adaptive and immune response in the inflamed tissues [331]. A recent study, conducted by Luk et al, suggests that the immune response after MSC administration does not depend on their active immunomodulatory activity but provided by other cells, triggered by the presence of MSC [332]. Other evidence also shows that innate immune cells are instrumental in mediating the MSC effect [333]. In addition to their complex immunomodulation mechanisms, one of the main advantages of MSC-based therapies is their ability to home damaged tissue. This homing propriety is closely related to chemical factors such as chemokines, cytokines and growth factors. One of the major chemical factors involved in MSC migration is stromal cell-derived factor 1, a chemokine released from damaged tissue, which sends chemo-attractive signals to cells expressing specific membrane receptors [334]. Upon activation, MSCs can easily express and translocate these receptor molecules to the cell surface, allowing them to follow the migration cues [335,336]. These mechanisms provide the ability to use stem cells in a systemic way such as intravenouse , intrarterios, intreaperitoneal and so on. IA application of MSCs by administering autologous BMMSCs via the hepatic artery in a canine model of hepatic fibrosis [337]. Intraperitoneal (IP) administration of MSC is rarely used, but potentially allows reaching intra-abdominal sites, appearing relatively safe [338]. IP administration of MSC has also been shown to be useful in the treatment of bladder detrusor deterioration in rats and in inflammatory bowel disease in mice [339,340]. Additionally, the IP approach was recently used to inject Neo-islets, ADMSC aggregates, and pancreatic islet cells, in an FDA-led pilot study in insulin-dependent diabetes mellitus in companion dogs. The results showed good grafting, redifferentiation, insulin production, resulting in a reduction in clinical signs in the treated dogs, without triggering an auto or alloimmune response [341].

MSCs are currently considered capable of altering the course and consequences of many chronic diseases, through the activation, enhancement and modulation of many different tissue pathways (Fig. 4.4).
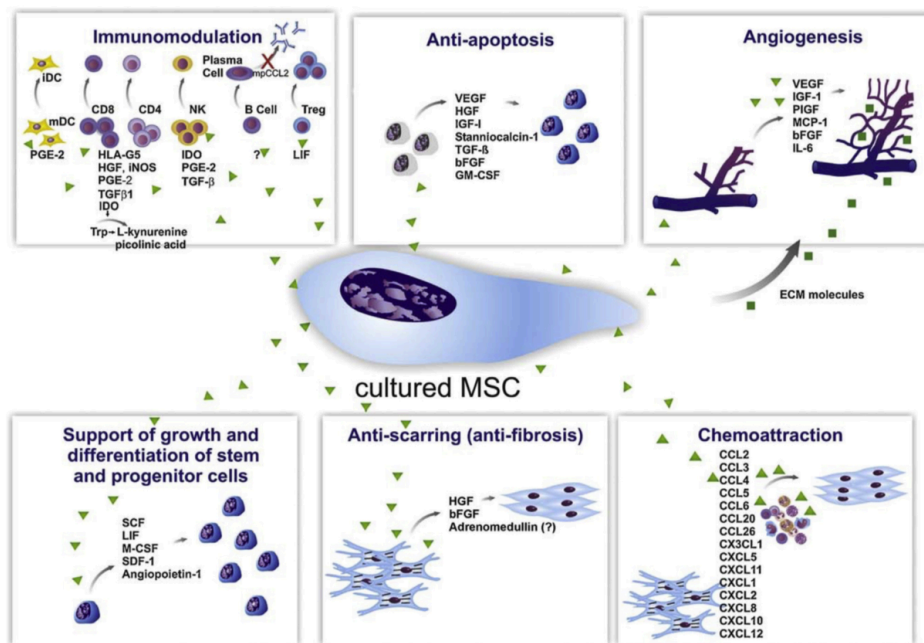
Figure 4.4: raffigurato of the main modulation effect of cultured mesenchymal stem cells [346]

However, it is important to note that many patients treated with these cells suffer from acute or chronic inflammatory diseases, which means that an inflammatory environment is likely to be present *in vivo* when administered. Some authors suggest that this eviroment may hamper the viability and efficacy of MSCs [342,343, 363]. To deceive this issue, the proposed solution has been the preconditioning of cells with pro-inflammatory cytokines during culture. Priming MSCs with IFN-γ before treatment was proposed and might enhance the interaction between MSCs and B-cells. Luk and colleagues demonstrated that MSCs preconditioned under pro-inflammatory cytokines significantly reduced B-cell proliferation through induction of indolamine 2,3-dioxygenase (IDO) activity. , while MSCs grown under normal conditions increased the percentage of B-regs, without influencing their proliferation. Even preconditioning of ECVs has also been shown to be beneficial for their therapeutic effectiveness. Recently it was reported that ECVs from canine MSCs, preconditioned with antiinflammatory cytokines, enhanced macrophage polarization and generation of Tregs in murine colitis [344]. Despite this scenario, pretreatment with IFN-γ may also significantly regulates expression although of genes involved in

apoptosis, reflecting in severe negative influence on MSC [345]. Neverless a recent study demonstrated that equine ADMSCs are compromised in an inflammatory environment, altought the preconditioning increased ADMSCs proliferation potential and osteogenic differentiation, can negatively affected cells' viability, engraftment, chondrogenic and adipogenic differentiation potential [342]. Conflicting results from various MSC preconditioning studies suggest that beneficial effects should be carefully considered, underlining the importance of further studies to elucidate the potential positive effects of such preconditioning.

- *Clinical use in canine OA*

Although there are thousands of experimental publications on this topic, fewer are representative of spontaneous diseases in small animals, and much smaller if look for owned-patients. Most publications on dogs are directed to the therapeutic use of these stem cells in cases of induced or spontaneous cranial cruciate ligament rupture [347-350]. This rupture is normally associated with varying degrees of osteoarthritis and is considered the most common cause of lameness in adult dogs, which explains the amount of publication on this topic [119,173,351]. Currently, the recommended therapy is a surgical correction, although the latter normally cannot stop the perpetuation of degenerative joint disease at all [173,352]. Positive treatment results from several studies highlighted the value of MSC use in this condition. Taroni and colleagues, in a recent pilot study, demonstrated that the level of lameness and postoperative pain after a single intra-articular injection of allogeneic BMMSC could be a viable alternative to the 1-month course of oral administration of non-steroidal anti-inflammatory drugs (NSAIDs) in dogs previously undergoing to tibial plateau leveling osteotomy (TPLO) [350]. Another clinical research by Miur and colleagues (2016) concluded that intraarticular or intravenous post-operative injection of autologous MSCs in dogs with the same ligamentous condition resulted in a decrease in CD8 + T cell level, decrease in serum and synovial CPR, and synovial decrease IFN-γ levels that normally persisted for 8 weeks after BMMSC injection [348]. Some authors suggest promising results in nonsurgical partial cruciate ligament ruptures by

associating autologous BMMSC treatment with PRP, which appears to provide prevention of progression of further degenerative joint changes [349].

Another broad publication topic on orthopedic disease in which mesenchymal stem cell therapy has been primarily evaluated to date, is the spontaneous canine osteoarthritis [354-358]. Several studies in dogs demonstrated that MSC administration into the OA joints decrease the patients' discomfort and increase their functional ability. In 2007 Black and colleagues evaluate the effect of single injection of stem cells in 18 dogs with osteoarthritis of the hip. Despite limited information on the cell processing methods was provided, it was reported a significant improvements in lameness and limb function [353]. In a follow-up study the same authors evaluated the effect of a single intra-articular ADSCs in 14 dogs with chronic osteoarthritis of the elbow joint. This multicenter case series did not utilize a control group and followed dogs subjectively for 6 months after treatmen [354]. As with the prior publication, scant information was provided regarding the cells. Authors concluded that clinical outcomes after therapy improved 30% to 40% compared to baseline. Although these results are encouraging, they should be interpreted with caution given the small patient numbers, omission of cell data, lack of control group in one study, and the use of subjective assessment methods alone to assess efficacy. Significant improvement of MSC therapy for treating osteoarthritis has also been shown with the use of allogeneic ADMSCs in 74 dogs in a prospective, randomized, masked, and placebo-controlled study, no adverse effects were reported, and efficacy in reducing clinical signs was shown in comparison to the placebo group [355]. Another extensive study performed on 203 dogs with severe osteoarthritis, causing severe chronic pain, and lameness, results showed excellent improvement in 90% of young dogs and good improvement in 60% of older dogs 10 weeks after the treatment [356]. Olsen and colleagues in a 2019 pilot study of canine elbow OA evaluated the efficacy and safety of intravenously administered ADMSCs suggesting that this therapy represents a safe treatment with significant patient pain relief. The association between adipose-derived stem cells and PRP in the treatment of osteoarthritis was also evaluated in a recent controlled group study of 17 dogs, resulting in statistically better pain control

without no statistical differences on gait analysis. The authors suggest resetting the PVF baseline cut-off as a limitation for a new prospective study [359]. Currently, the know-how of the global literature on the use of stem cells in osteoarthritis suggests in veterinary medicine, as well as in humans, a significant statistical improvement in pain control assessed by the owner or by personal questionnaire or clinical evaluation, although it wasn't showed a greater effect on cartilage restoration (MRI or arthroscopic evaluation) or other improvement of objective gait analysis has been shown [360].

Current Concerning and Limitation

There are currently several limitations regarding the use of mesenchymal stem cells, including gaps in basic scientific knowledge, inherent variability in these cells, risk of contamination, with regard to long-term safety, optimal dose and time of administration, quality control and production (e.g. promotion of some oncogenic effects), effect of manipulation and preconditioning, and the lack of strong evidence which support the efficacy of mesenchymal stem cell therapy in animals with properties associated with the statistical outcome limit in pain control, which can be partially representative of a placebo effect whose result was between 39.7% and 79% in dogs in some clinical veterinary studies [361,362].

4.4 Stromal Vascular Fraction

Since its first clinical use we have witnessed an exponential increase in the literature on Mesenchymal Stromal Cells (MSCs) in the last twenty years, as briefly reported in the previous chapter. In parallel with the advancement of knowledge about these cells, numerous studies have been initiated to evaluate the characteristics and properties of crude tissue isolates, as well as its clinical uses, for example, bone marrow concentrate, derived from bone marrow aspiration, and stromal vascular fraction derived from adipose tissue [364-367]. The latter tissue, due to the minimally invasive procedure necessary to obtain it and the high quality characteristics demonstrated, is considered one of the best sources of stem cells for culture or crude tissue use

[17,298,299,364,367,386] . The Stromal Vascular Fraction is often considered synonymous with the perivascular niche, responsible for regeneration, secretion of growth factors, inflammatory modulation and maintenance of damaged tissues in adulthood [369,370]. Therefore it could be considered as the matrix or tissue component of adult mesenchymal stem cells [370].

Considered by many Authors as the "niche matrix" of many tissues, it is often used to define specifically the adipose tissue. Autologous adipose tissue has been used as intact lipo-aspirate (lipofilling), enzymatically derived Stromal Vascular Fraction (SVF), and mechanically Micro Fragmented Adipose Tissue (MFAT). The differential use of these three tissue preparations, has demonstrated quite different "regenerative properties" in the literature [371,372]. Vezzani and collegues, has demonstrated, for example, that MFAT preparations are significantly more productive in the release of grow factor and cytokines concomitant with notably superior effects on repairing tissues, induction and modification of immunomodulatory activity and in supporting vascular angiogenesis [372]. The current literature also provides evidence that tissue or cellular treatments beyond a 'minimal' mechanical manipulation of adipose tissue, such as enzymatic dissociation, leads to a different gene expression pattern and exosome content of the MSCs [378-380].

Since its first use, describe by Coleman in 1990, as the ideal filler in plastic surgery , the crude lipoaspirate or lipograft has been used for many other disease [373-374]. The rationale for the Coleman technique has been attributed to the importance of maintaining the perivascular niche environment. These pericytes and other perivascular cells play the key role in the regenerative effect, providing immune and anti-inflammatory modulation to restore and repair damaged tissue [375]. Lipostructure grafting, reported as the Coleman method, requires adipose aspiration and subsequently centrifugation, which allows to discharge the oil, and obtain the purified lipoaspirate. This technique has the advantage of rapid and inexpensive isolation with good viability (50-70%) of cells. On the other hands, it needs an elevate standard of procedures (SOP), with moderate risk of contamination, minimizing the damage to the perivascular environment, and maximize the regenerative effect [376].

Intact centrifuged lipoaspirate was recently used for a clinical study that included 22 client-owned dogs affected by spontaneous osteoarthritis [377]. The Authors evaluated the efficacy of a single intra-articular infiltration of lipoaspirate graft with 180 days of follow-up, concluding a statistical improvement in pain control whithin the group, and suggesting a better clinical outcome when the elbow joints were treated [377]. It is crucial to note that these evidence must be taken with care, due to the lack of a control group, the small cohort of patient , and the different size of the treated dogs, which can vary the vertical impulse during gait analysis.

Advances in biomaterials and new technologies provide a novel system that enables mechanical fragmentation of tissue to be achieved with minimal manipulation, supporting cell viability. Today, many different devices are available on the human market for mechanical fragmentation of adipose tissue and subsequent clinical application. One of the most investigated devices in human and veterinary literature is undoubtedly the Lipogems® adipose micro fragmentation system. Lipogems® system provide to obtain rapidly small groups of "adipose" cluster which maintain an intact perivascular environment by means of mechanical shock induced by ball bearings. The efficacy of Lipogems® has been studied for numerous spontaneous or experimentally induced clinical pathologies. In 2018, a multicenter study conducted by Zeira and colleagues on 130 dogs with OA, reported the efficacy and feasibility of Lipogem®, validating this system in canine species. Although the conclusion suggests safe, easy and good pain control over a 6-month follow-up, and the big sample size of dogs, this study sadly lacks a control group and relies solely on poorly objective clinical evidences [381]. Despite this, it is certainly interesting to further explore this system in the canine species in the next future.

Another interesting device introduced in recent years is represented by the "Rigenera® micro-grafting technology" (Human Brain Wave LLC, Turin, Italy) [36]. Rigenera® technology embraces the tissue skin micrograft theory conceived by Cicero Parker Meek at the University of South Carolina Aiken in 1958 [382].

The original micrograft proposed by Meek, is based on increasing the surface of a skin graft fragmenting it into smaller parts, providing the possibility of covering a

larger wound than the original donor site. This technique, despite some limitations, is currently used in cases of burning or severe skin loss [383]. The main objective introduced with Rigenera® system is to mechanically disaggregate tissue with a calibrated size of 80 mm, collecting autologous micrografts enriched in progenitor cells, growth factors, and particles of extracellular matrix derived from the patient's own tissue [36, 386]. The real strength of this technology is the great flexibility of use, as it might potentially rapidly disaggregate any type of soft tissue in a single surgical time, in order to obtain a micrograft rich in progenitor cells and growth factors, which can be rapidly uses. Clinically, these micrografts have been applied in many different fields of human medicine such as dentistry, dermatology, orthopedics and especially wound care [387-394]. This latter field is currently the one in which Rigenera® technology has been extensively studied with very interesting results.

Given the flexibility of its tissue source, many authors have suggested the use of Rigenera® system to deliver the micro-fragmented adipose tissue graft (MFAT) [17, 385,386]. Considering the possibility of obtaining the stromal vascular fraction from adipose tissue, a recent publication in 2019 compared the mechanical methods Rigenera® and Lipogems® by comparing them with the enzymatic gold standard in an in vitro model. The Authors evaluated the differential cell cluster (CD) gene expression and the trilineage differentiation capacity after 72 H, concluding that Rigenera® demonstrated superior differentiation compared to Lipogem®, with a better immuno-modulatory effect if compare to enzymatic SVF.[17]

Historically, interest in small animal stem cell therapy has mainly been driven by commercial suppliers and pet owners, which in some cases has led to unrealistic expectations regarding treatment outcomes. However, the amount of interesting clinical evidence reported in recent years and the potential of Rigenera® technology, generate a deep curiosity for a new experimental evaluation for various chronic diseases, even in small animals.

The following research aims to validate Rigenera® system for the disintegration of adipose tissue in the canine species, in order to evaluate the effectiveness of intra-articular injection of micro-fragmented adipose tissue (MFAT) for the treatment of spontaneous osteoarthritis by comparing the use of hyaluronic acid, currently consi-dered standard of care for intra-articular the treatment of OA.

EXPERIMENTAL RESEARCH

The aim of the study

The study that will be discussed below consists of two related objectives. The first part of the research was aimed to evaluate the efficacy and validate, through a *in vi-tro* study, the use of the Rigenera technology in the canine species, given the lack of scientific evidence on this novel system. This preliminary study was followed by a prospective, randomized, controlled, blinded *in vivo* clinical study, which evaluated the efficacy of intra-articular infiltration of adipose SVF in the treatment of sponta-neous osteoarthritis in dogs. Specifically evaluating clinically pain and lameness, the radiographic progression of osteoarthritis and the synovial fluid inflammation.

PRELIMINARY *IN VITRO* RESEARCH

Materials and Methods

-Canine enrollment and tissue sampling

Six adult dogs, subjected to euthanasia for non-research causes, were provided and selected by the intensive care service of the Veterinary Teaching Hospital of the University of Camerino. Each dog was selected based on inclusion and exclusion criteria, which comprehended subjects aged 1 to 15 years, weighing 5 to 60 kg, with no race or sex restriction, with adeguate adipose tissue coverage, whose cause of death was unrelated to cancer, and without an history of oncological diseases.
Two samples of approximately 10 grams of adipose tissue were taken sterile from

each dog. Sampling was performed at two different anatomical sites, one from the thigh region, distal to the greater femoral trochanter, and one from the paralumbar region, basically on the lumbar midline, above the V lumbar vertebrae (fig.1). Each sample was taken using a lipoaspiration technique.
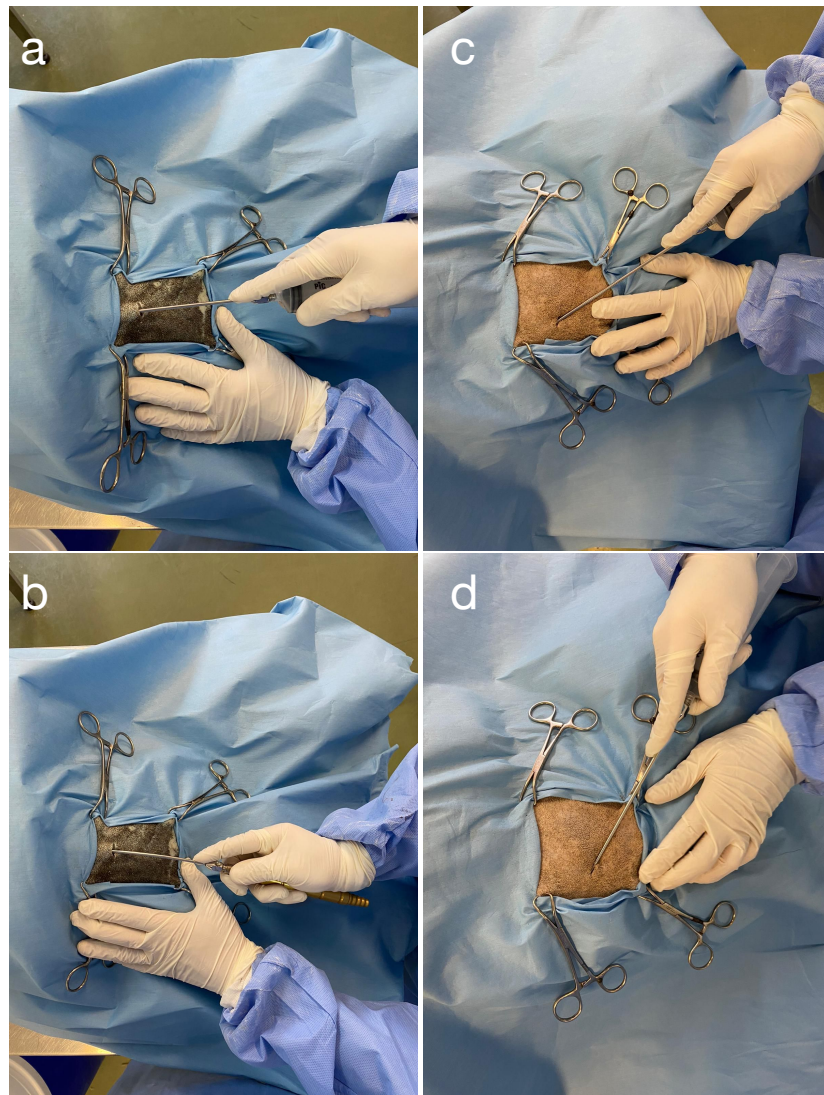
Figure 1 : (a; b) imagine of adipose tissue sampling from thigh region, and from lumbar region (c; d)

-Lipoaspiration technique:

This technique is routinely used in plastic surgery for aesthetic roles and provides isolation and collection of adipose tissue at the body's storage site. This technique requires two surgical steps. The first step allows for tissue preparation and facilitates tissue isolation by inoculating liposuction solution. The latter is called Klein's solution, which is a solution of crystalloid fluid (NaCl or Ringer) enriched with lidocaine and adrenaline, that have an analgesic and hemostatic effect. The usual concentration of tumescent solution is 0.05% lidocaine and c 1: 1,000,000–1.5: 1,000,000 for the adrenaline. Surgical skin preparation is mandatory, so an incision was made with a 15 scalpel blade. After the latter, the solution was inoculated with a 4 mm blunt infiltration cannula. Subsequently, aspiration was performed using the appropriate liposuction cannula connected with a 50 mL Luer-lock syringe with negative pressure (fig.2).
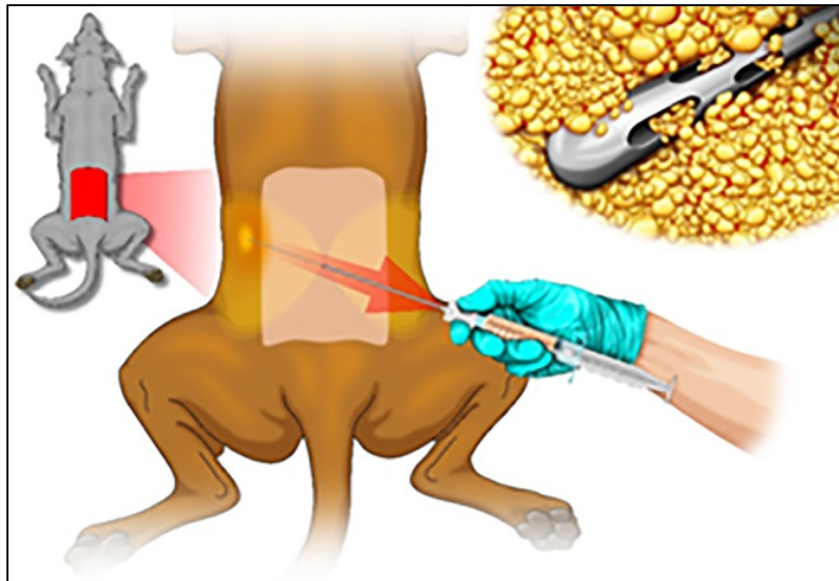
Figure 2 : lipoaspiration technique using lipoastiration blunt cannula [381].

-<u>Rigenera® technology</u>

The Rigenera® mechanical disintegration system (CE Class I certified, Human Brain Wave, Turin, Italy) is a medical device that allows to obtain in a standardized way, with high reproducibility and minimal manipulation, micro-grafts starting from different tissues readily available for clinical practice.The micro-grafts obtained are characterized by a size of about 50-70 microns, which allows them high cell viability and cell nutrition by simple interstitial diffusion. The Rigenera® system consists of a motorized apparatus that generates a rotation of the blade inside of the proper sterile capsule (Rigeneracons®), which allows the tissue mechanical disintegration (fig.3 a,b). Each Rigeneracons® consists internally of a helical blade managed by the electric motor, which makes it rotate at 80 rpm, allowing precise, uniform, and constant tissue fragmentation.

Furthermore, at the end of the helix, there is a metal filter containing 100 holes about 50μ, each equipped with 6 micro-scalpel blades. The disintegrated and filtered tissue is collected at the bottom of this capsule, where, thanks to a syringe fitting, it is possible to aspirate the micro-graft preparation for the use. The main study report dermal and adipose tissue micro-grafting isolation using this system in literature. Basically the tissue inserted inside the capsule, must be previously manually sectioned by the surgeon to obtain a size of about 2 mm (dermal tissue), or it must be aspirated and inserted as it is, which happens for the adipose tissue after lipoaspiration technique.
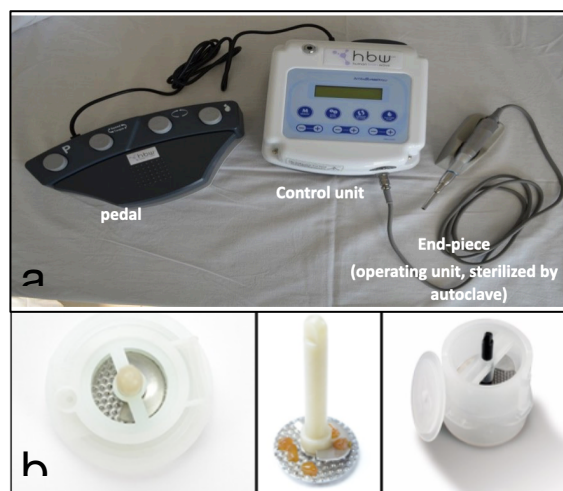
Figure 3: (a) motorized apparatus of Rigenera® system;(b) Rigeneracons® sterile capsule.

-Processing of adipose tissue and culture collection:

The lipoaspirate sample was divided into two portions: 4 gr were treated with the Rigenera® method, 4 gr with enzymatic digestion using collagenase.

In the Rigenera® capsules, was inserted 4 ml of lipoaspirate and 4 ml of complete culture medium Dulbecco Minimum Essential Medium (DMEM) (Sigma-Aldrich, Italy). This medium contains 10% fetal bovine serum (FBS), 1% of a mixture of penicillin/streptomycin, and 0.5% amphotericin B.

The Rigenera® device was used for 60 seconds, which is the recommended time for this use in human medicine, a time which provides the entire processing of the tissue inserted in the capsule. The cell pellet collected at the bottom of the capsule was aspirated with a syringe, filtered through 70 μm pores, and subsequently centrifuged at 3000 rpm for 7 minutes. The supernatant obtained was discarded and the cell pellet resuspended in 6 ml of complete medium, seeded in a 25 cm^2 cell culture plate, and incubated at 37 ° C and 5% CO_2 for 45 minutes. The second portion of lipoaspirate was treated with enzymatic digestion, using collagenase. The 4 ml of the sample were digested with 1 mg/ml of type I collagenase, resuspended in saline solution (HBSS) and 2% bovine serum albumin (BSA) and subsequently incubated at 37 ° C for 45 minutes. The enzymatic action was neutralized by adding complete medium, then the sample was centrifuged at 3000 rpm for 7 minutes. The supernatant was discarded and the cell pellet was incubated with 3 mL of 160 mM NH_4Cl at room temperature for 10 minutes for erythrocyte lysis. After centrifugation, the cells were resuspended in 6 ml of complete medium, filtered through 70 μm pores, seeded in a 25 cm^2 cell culture flask with complete culture medium and incubated at 37 ° C and 5% CO_2.

The medium was renewed for the first time after 72 hours (cell passage 0 = p0) and, subsequently, every 48 hours (cell passage 1 = p1; cell passage 2 = p2).

-Morphological analysis, Growth curve and Clonogenic capacity:

The morphological analysis of the preparation obtained with the Rigenera® and Enzymatic systems was performed by observing the cells under an optical microscope (Optika Microscopes, Italy). Cell viability was assessed by the Trypan Blu test. To obtain the cell growth curve, $2x10^4$ cells were seeded (in T25 flasks) for each treatment and the days required to reach plate confluence were evaluated. Upon reaching confluence, the cells were detached from the dish by incubation with trypsin-EDTA 1% (GIBCO Life Technology, USA) at 37 ° C for 5 minutes and subsequently resown in 75 cm^2 cell culture flasks.

To evaluate clonogenic capacity, 5,000 cells were seeded in Petri dishes and cultured with a complete medium. After 10 days, the total number of cell colonies was detected and counted, highlighted by the blue toluidine stain. Groups of at least 50 adherent and fibroblast-like cells were considered as colony-forming units (CFUs).

 - Immunophenotypic Characterization:

After isolation, the cells were counted and $2x10^5$ cells were placed in a tube for flow cytometric analysis.The pellet was washed with 1 mL of PBS and then labeled with antibody conjugated with fluorescent dye in a final volume of 100 μL. The sample was then incubated for 30 minutes on ice. The antibodies examined were: CD90 conjugated with APC (dilution 1: 5), CD73 conjugated with BV421 (dilution 1:20); CD44 conjugate BV785 (dilution 1:20), CD34 conjugate PE (dilution 1: 5).

After incubation, the pellet was rinsed, resuspended in 300 μl of PBS, and transferred to flow cytometry tubes. Immunophenotyping was performed through a FACS II chant (BD, Becton Dickinson, Italy).

Results

We noticed differences between the isolation of the technique by liposuction; in the lumbar site, the adipose tissue was, in fact, easily isolated, providing a good amount of tissue in a single surgical step, while in the thigh region the isolation of the tissue was very demanding and required more than one surgical approach.

The number of cells obtained by the Rigenera® treatment was $2.29\text{x}10^5 \pm 7.86$ for the thigh area and $2.23\text{x}10^4 \pm 1.2$ cells/ml for the lumbar area, while the number of cells obtained after enzymatic digestion was $5.51\text{x}10^5 \pm 1.47$ for the thigh and $9.33\text{x}10^4 \pm 3.3$ cells/ml for the lumbar region(fig.4).

SEDE DI PRELIEVO DEL TESSUTO ADIPOSO	RESA CELLULARE (%) (RIGENERA vs DIGESTIONE ENZIMATICA)
LOMBARE	23,93 ± 2,5
COSCIA	41,62 ± 1,3
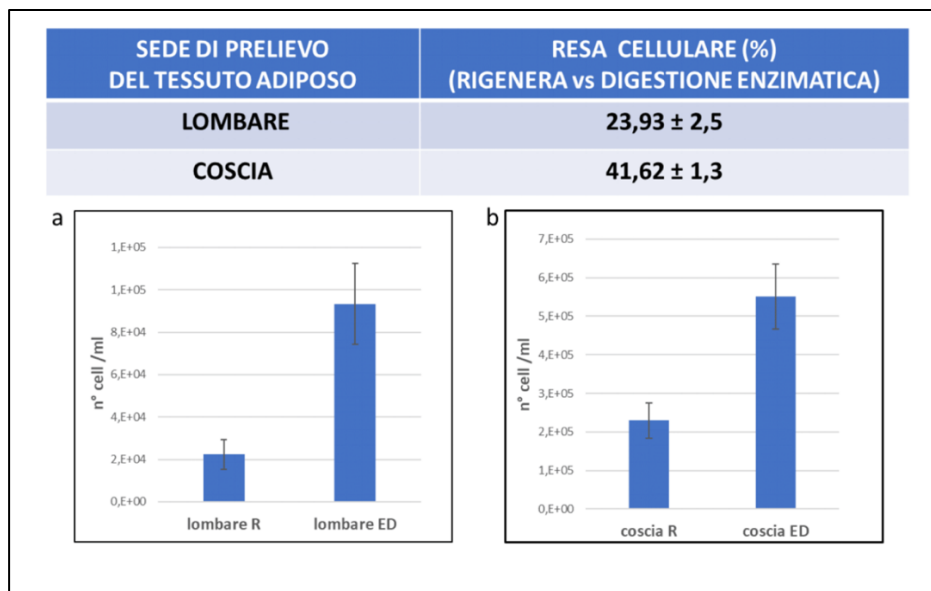
Figure 4 :Comparison of cell yield expressed in% between Rigenera® and collagenase. a) Comparison between the number of cells obtained from lumbar samples treated with Rigenera® and the enzymatic method. b) Comparison between the number of cells found in the thigh area after treatment with the Rigenera® and enzymatic method.

Considering the cell yield, obtained from enzymatic digestion, as 100%, the cell yield obtained by mechanical disaggregation of the adipose tissue with Rigenera®, was about 41% for the thigh, and about the 24% for the lumbar region. By comparing the two isolation sites, the cell yield of the lumbar area was resulted 9.7% and 17% lower than the thigh, for the mechanical and enzymatic treatment respectively(fig.5)
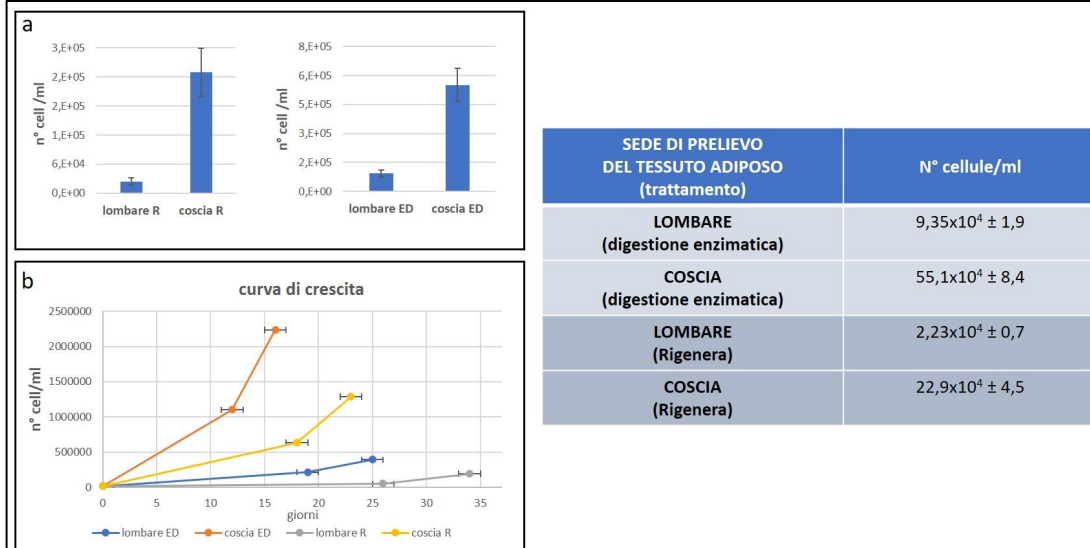
Figure 5: Cell replication rate from the lower back and thigh. The cells obtained from the thigh have a major factor.

The first dish confluence was reached, for the thigh tissue, at 8 ± 1 for Rigenera® and 7 ± 1.3 for Enzyme Digestion respectively, before the cells extracted from the lumbar area (Figure 6b). Through the microscopic study of cell replication, we noticed a difference, already in the first cell passage, between adipose tissue of the lumbar region and of the thigh, showing a greater replication rate for the latter.
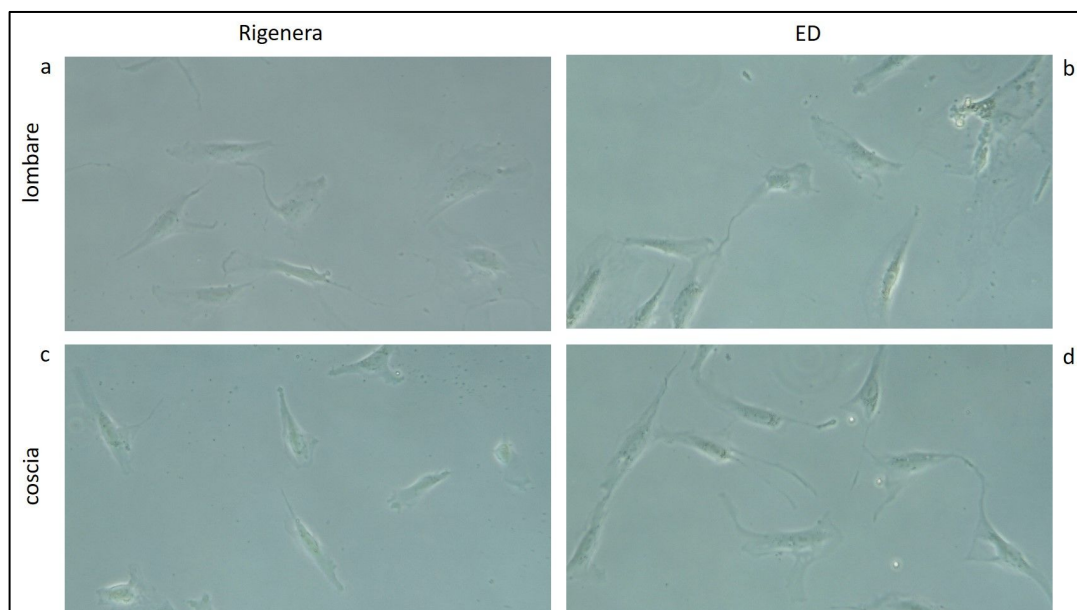
Figure 6 : Cell morphology. a-c) lumbar and thigh cells obtained with Rigenera®; b-d) lumbar and thigh cells obtained with enzymatic digestion.

Figure 6 shows the cell morphology under the microscope after the first renewal of the medium (p0). In these images it is possible to see that the Rigenera® treatment (a and c) did not affect the cell morphology, while the cells obtained from enzymatic

digestion (b, d), show a homogeneous fibroblastic-like morphology. It is also important to note that no signs of suffering of the cellular structures were observed. Despite the mechanical disaggregation in fact the membranes and nuclei were well preserved both in the cells extracted from the lumbar area and from the thigh.

For each sample processed, good foramant colony units (CFU) were also highlighted, expressing the clonogenicity and plastic capacity of these cells.

Figure 9 represents the CFU detected by toluidine blue staining after 10 days from treatment with Rigenera® (Figure 18a thigh, 18b lumbar) compared to enzymatic digestion (Figure 7c thigh, 7d lumbar). The images show that the cells obtained with Rigenera®, isolated from both the thigh and the lumbar area, are able to grow forming clusters and these colonies are likely comparable to those obtained by enzymatic digestion.
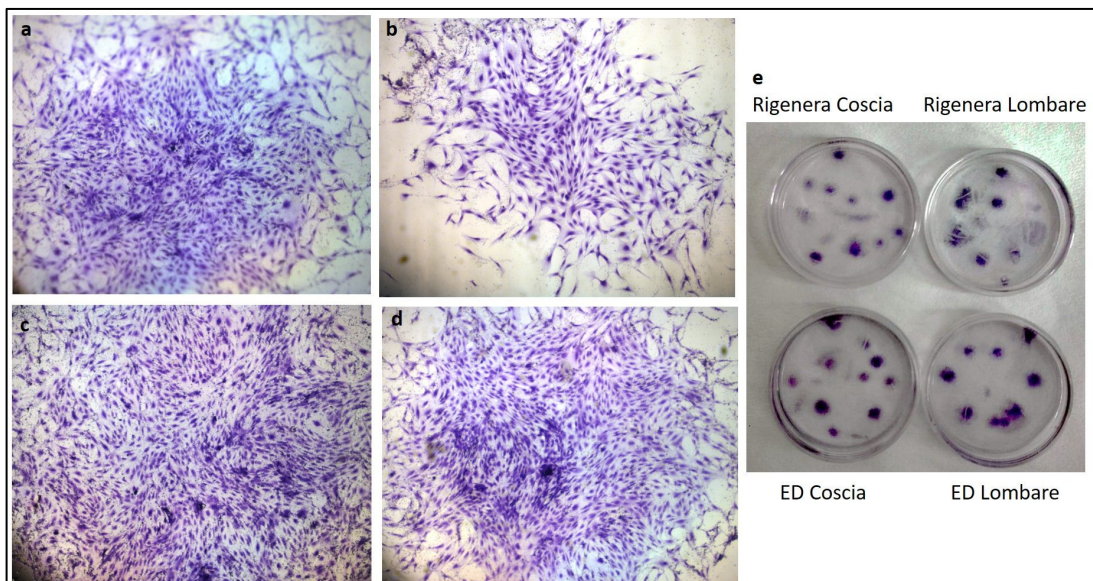
Figure 7. Cell micrograph 10 days after treatment with Rigenera® from the thigh (a) and lumbar (b). Cell micrograph of collagenase-treated cells from the thigh (c) and lumbar (d). (e) Arrangement of cell colonies on a petri dish.

Colony-forming unit (CFU) assays were performed on cells obtained from Rigenera® treatment and enzymatic digestion.

Finally, the presence of Mesenchymal Stem Cells in the product was detected through a p2 immunophenotypic test. Based on the size, shape, and internal structure of the cells, MSCs, such as those presented in the scatter diagram, can be selected, which is used as a method for identifying their present in the analyzed samples (Fig. 19). With the Rigenera® treatment, about 87% and 83% of the cells present as MSC in the thigh and lumbar area respectively were selected (Figure 19 a, b), data comparable to those obtained from enzymatic digestion (Figure 8 c, d).
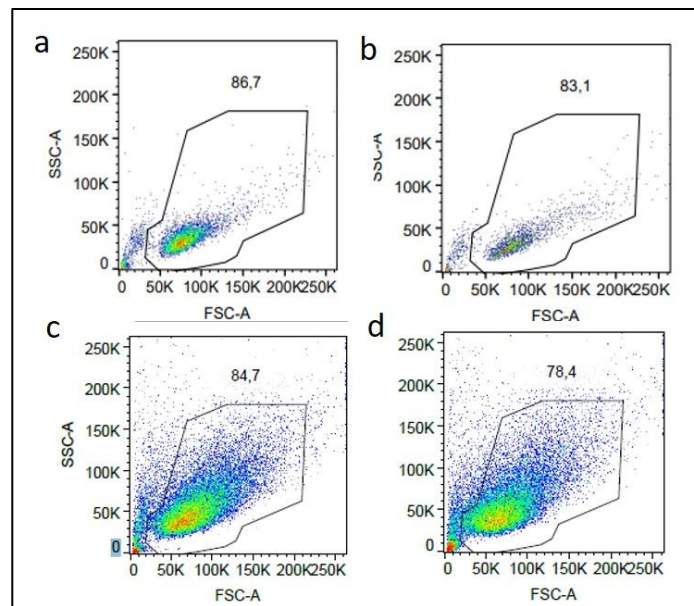
Figure 8. Scatter diagram of MSCs. a-b) thigh and lumbar cells received from the Rigenera® treatment; c-d) thigh and lumbar cells with collagenase.

Subsequently, specific antigens were tested on the previously selected cells. In p2, the cells isolated with the Rigenera® mechanical method show a medium / high expression of the surface markers specific for MSC CD90, CD73, CD44, while the hematopoietic marker CD34 was poorly expressed (Figure 9 a, b). Furthermore, no differences in marker expression were found between the thigh and lumbar regions.
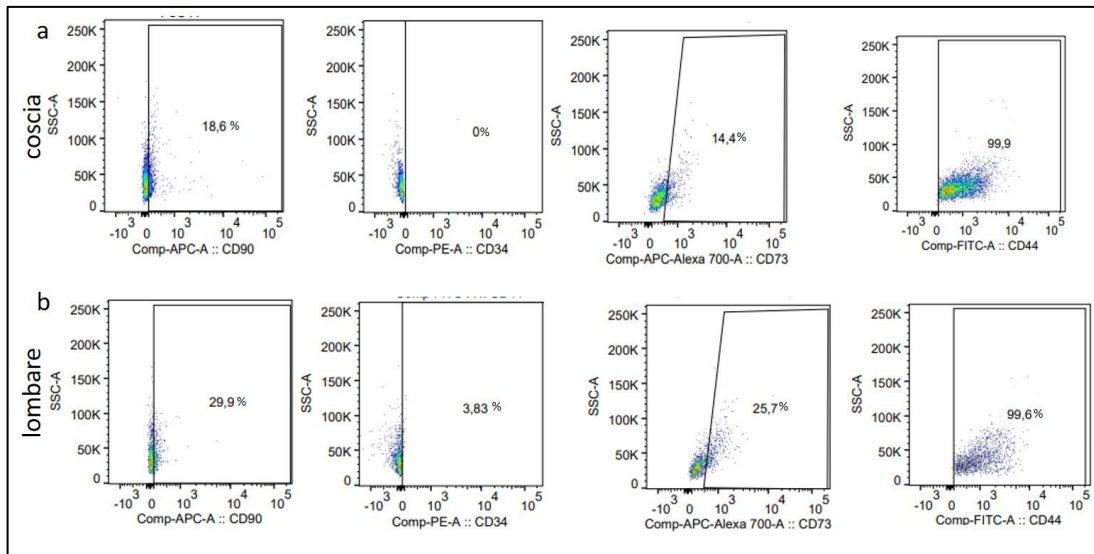
Figure. 9. Expression of CD surface markers of mesenchymal stem cells.

Discussion and Conclusion

Results of our study were found to be totally overlapping with the evidence of the study performed on a human model [386]. Both isolation sites assessed in the dog were found to be equivalent, concerning the enzymatic method, in terms of conservation of cell morphology, the ability of the elements present to grow and form cell clusters, and expression of cell-specific MSCs surface markers. However, the adipose tissue isolate from the thigh region has higher cell yield, faster replication rate, and more mesenchymal stem cells in dogs, as in humans being. While there are, undoubtedly, some peculiarities which leads to prefer the tight region for isolation, it is to be noted that this surgical site brings many problems during lipoaspiration and might be very challenging, due to the reduced fat reserve in dogs. In fact, with the same adipose tissue, more than one surgical approach was required, and this led the Author to prefer the lumbar site for the subsequent *in vivo* study. It is important to note that specific dog breeds (Amstaff, Pitbull, Bull Terrier) were characterized by a marked muscular tropism of the thigh, which generates a very poor availability of adipose tissue; even in subjects with a low Body Condition Score (BCS), adipose tissue in the thigh region was found to be absent or insufficient to perform the procedure, unlike humans in whom the sub-trochanteric area is also a source of tissue adipose pre-

sent in underweight patients. In addition, the thigh area, in the canines, is more prone to trauma or slowed healing than the lumbar, due to the greater strain stress in which the tissue is subjected during movement, and to the lateral decubitus position which might easily create morbidity for this procedure.

Despite preliminary *in vitro* evidences of the successful isolation, preservation, plasticity features and stem capacity of adipose graft, there are lacks of knowledge about the concentration of growth factor, interleukins, and other cytokines, which are responsible for paracrine effect, likely consider the main role of these therapies [379,380,388,389,390,394]. Undoubtedly this aspect will certainly have to be deepened and implemented in a second phase. In conclusion, the Rigenera® system proved to be an excellent system compared to the enzymatic method to obtain a Stromal Vascular Fraction ready for use in the canine species.

IN VIVO RESEARCH : Treatment of spontaneous ostheoartritis with micro-fragmented adipose tissue obtained with Rigenera® in dog

Introduction and Study Design

The following research is a prospective study which aims to evaluate the effectiveness of intra-articular injection of micro-fragmented adipose tissue (MFAT) provides with the Rigenera® system for the treatment of spontaneous osteoarthritis, by comparing the use of hyaluronic acid (control group), currently considered an intra-articular standard of procedure for OA.

The designed study was approved by the ethics committee responsible for animal welfare of the University of Camerino (O.P.B.A. protocol 1D580.18A).

A list consisting of 40 lines, randomly divided into the *RIGENERA* group and the *HYALURONIC* group, was created using QuickCalcs, a computerized random number generator (http://www.graphpad.com/quickcals/randomN1.cfm).

To avoid research bias, the list was not consulted by the radiologist and clinical pathologist who examined the cytology and processed the synovial fluid for quantitative analysis of cytokines. This list was among other things consulted by the surgeon,

who used to perform the liposuction, and by the clinical examiner, such as the owner, who could easily saw the trichotomy of the surgical procedure in the *RIGENERA group.* Therefore the clinical project developed was designed to obtain a prospective, randomized, controlled, blinded *in vivo* study.

Material and Methods

-Eligibility Criteria :

Owned-dogs suffering from osteoarthritis lameness referred at the Veterinary Teaching Hospital of the University of Camerino were selected and enrolled for the following prospective research. Eligibility criteria included dogs aged 1 to 15 years, with no weight and sex restriction, belonging to the ASA 1-2 anesthetic risk class, in the absence of comorbidity, pregnancy or lactation, affected by osteoarthritis lameness manifesting in one joint, in the absence of osteoarthritis in other joints of the same limb, belonging to radiographic grade OA between 1 and 4 (according to a modified Kellgren -Lawrence scale), and who had not received anti-inflammatory drugs nor nutraceuticals in the 15 days prior to treatment and during the whole study time.

-Clinical Trial Procedures:

Dogs were randomly divided into equal groups, a study group (Rigenera® group) and a control group (Hyaluronic group), which differ only for the intra-articular therapy. The clinical trial was started (T0) into a first part, carried out by a graduate student, in which anamnestic data and the owner's perception of pain were collected, providing and asking them to fill out the Canine Brief Pain Inventory questionnaire. General and orthopedic specialist visit were carried out by a specialist clinician later, who filled out a clinical card, assessing the degree of ambulatory and stance lameness, arthralgia and the visual analogue score of pain (VAS).
After clinical evaluation in awake patients, the radiographic examination was carried out under anesthesia to confirm and grading the osteoarthritis; at the same time, clinical measurement of the circumference of the affected limb and the range of motion

(ROM) of the joint was performed. During sedation, a trichotomy of the affected joint was executed, and synovial fluid was collected by arthrocentesis. The synovial fluid was divided into two aliquots, a part useful for qualitative cytological analyzes, and a part preserved to quantify the concentration in synovial cytokines. Each patient followed the same anesthesia protocol for radiographic, intra-articular therapy, and synovial evaluation, reported in the proper paragraph. The clinical procedure, radio-graph, and synovial fluid assessment were performed and repeated for a short term follow-up, after 30 (T1) and 60 (T2) days in both groups. At time 0 the entire study population was treated with a single intra-articular injection, using hyaluronic acid or Rigenera micro fragmented adipose tissue graft according to the designated group. After 180 days (T3) the cohort of patients was re-examined for a long term follow-up to collect data relating to the perception of pain by the owner, through the CBPI, and a subsequent last orthopedic specialist visit, without performing sedation, useful for the evaluation of the synovial fluid and radiographic study. This designed procedure were reported graphically in the following figure (fig.10).

TRIAL PROCEDURE	T0 (start trial)	T1 (30 days)	T2 (60 days)	T3 (180 days)
Canine Brief Pain Inventory	x	x	x	x
Specialistic Clinician Assessment	x	x	x	x
Radiographic Examination	x	x	x	
Synovial Fluid Examination	x	x	x	
Synovial Assay Test	x	x	x	
Intra-articular Injection Control group	x			
Intra-articular Injection Study group	x			

Figure 10 :Summary and timing of the clinical trial procedures

Anesthesiological Protocol :

Patients were sedated with dexmedetomidine 3μg/kg and metadone 0.2 mg/kg IM and then anesthetized by propofol 2–3 mg/kg IV ad effect until tracheal intubation was achieved. Anesthesia was maintained by isofluorane 1.2% for the necessary time of the procedure.

Control Group Therapy:

The treatment dedicated to the control group involved a single injection (T0) of high molecular weight (650 KDa) hyaluronic acid, using Hyalgan® 20 mg / 2mL (fig.11). The dosage of the product has been optimized concerning the affected joint and the patient's build, so as not to over-extend the joint capsule, causing pain in the days following the treatment. The procedure was performed while maintaining generally a single administration of the 1- 1.5 mL of the intra-articular product as the gold standard.

Figure 11 :Hyaluronic acid used for the control group .

Study Group Therapy:

For the patients of the Rigenera® study group, after anesthesia, a trichotomy was performed in the region of the lumbar spine, indicatively taking the fifth lumbar vertebra

as anatomical reference. Each dog was placed in sternal recumbency, and an antiseptic skin preparation at the surgical site was performed. After a normal sterile dressing, the surgeon performed an incision with a 15 scalpel blade, which allowed the use of the 2,8 mm blunt infiltration cannula (fig.12 a). The preparatory infiltration of the adipose tissue was performed using a surgical fanning technique on different planes, taking care to obtain a homogeneous infiltration of the tumescent Klein's solution into the underlying fat. After few minutes a 3.8 mm blunt liposuction cannula was used, equipped with a 50 mL Luer-Lock syringe under suction (fig.12 b); the same surgical fanning technique was performed during lipoaspiration (fig.13).
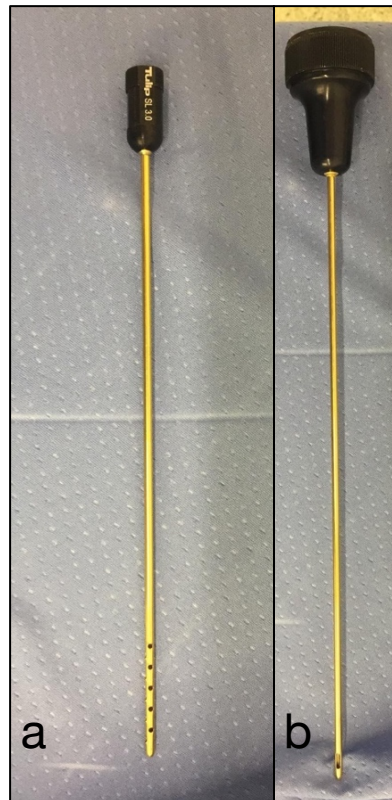
Figure 12 :(a) 2.8 blunt infiltration cannula, and (b) 3.8 blunt liposuction cannula.

Figure 13 : Surgical lipoaspiration procedure.

Once the adipose tissue was obtained, it was homogenized and mixed between two syringes connected via a three-way stopcock. The subsequent procedure required a resting phase of the tissue, in order to achieve a separation of the liquid part from the adipose part, which was brought into suspension. The liquid part was subsequently discharged and the homogenized adipose tissue was inserted into the proper Rigene-raCons® (fig.14).

Figure 14: homogenized adipose tissue before micro-fragmentation.

At this point the capsule was activated by the surgical motor for 60 seconds, until reaching power of 80 RPM, allowing the processing of the entire tissue through the Rigenera® micro-blades located in the sterile capsule. Once the procedures were performed, the syringe was connected to the proper fitting port of the RigeneraCons® obtaining a micro-fragmented adipose tissue graft (MFAT) (fig.15). MFAT was subsequently used intra-articularly in the patient's pathological joint; approximately 1-1.5 mL of MFAT was used as the gold standard for each joint.
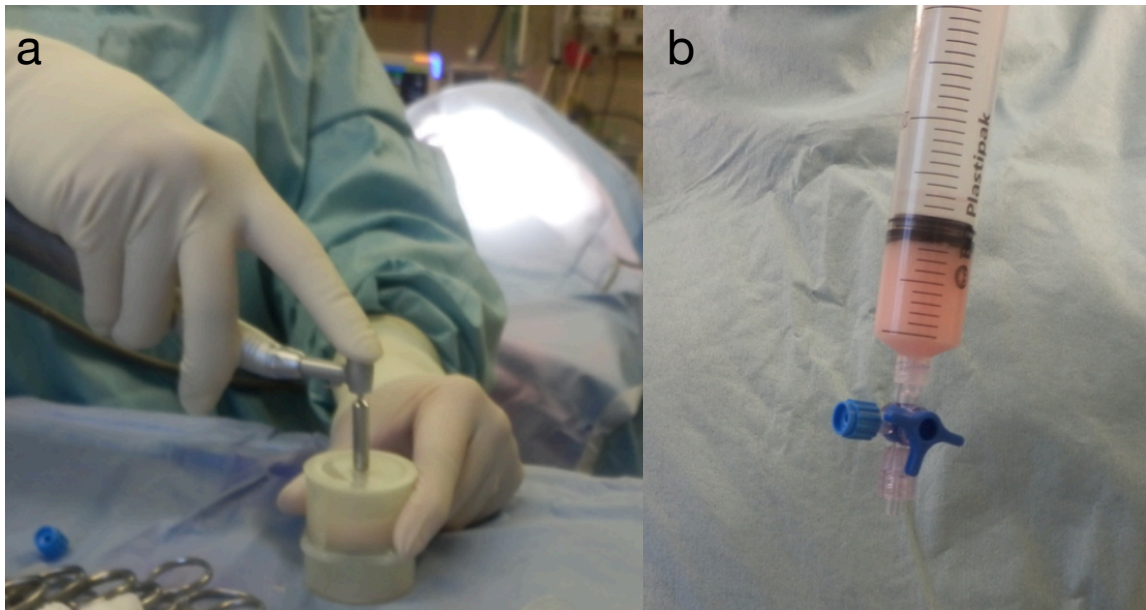
Figure 15: (a) Microfragmentation of adipose tissue through Rigeneracons®, (b) MFAT obtained ready to use.

Canine Brief Pain Inventory :

The Canine Brief Pain Inventory (Canine BPI) allows owners to rate the severity of their dog's pain and the degree to which that pain interferes with function. Initially developed to assess pain related to osteoarthritis, the Canine BPI has been shown to be an appropriate measure for pain caused by bone cancer as well. This questionnaire contains four items relating to the severity of the dog's pain and six items describing how that pain interferes with the dog's daily activities, for a total of 10 items which the owner had to answer. Each Canine Brief Pain Inventory pain item is presented with numerical rating scales from 0 to 10, basically where "0" represents no pain, while "10" represents extreme pain. In the same way for the interference items "0" represents no interference and "10" represents completely interferes (fig.16).
In this clinical study, the CBPI was used as an outcome, submitted and completed by the owner at any time during the trial, at time 0, before treatment, and 30, 60, and 180 days after treatment, respectively for the short and long term outcome.

Canine Brief Pain Inventory

Description of pain:
Rate your dog's pain:

1. Fill in the oval next to the one number that best describes the pain at its **worst** in the last 7days.

0 1 2 3 4 5 6 7 8 9 10
No pain   Extreme pain

2. Fill in the oval next to the one number that best describes the pain at its **least** in the last 7 days

0 1 2 3 4 5 6 7 8 9 10
No pain   Extreme pain

3. Fill in the oval next to the one number that best describes the pain at its **average** in the last 7 days.

0 1 2 3 4 5 6 7 8 9 10
No pain   Extreme pain

4. Fill in the oval next to the one number that best describes the pain as it is **right now**.

0 1 2 3 4 5 6 7 8 9 10
No pain   Extreme pain

Description of function:
Fill in the oval next to the one number that best describes how during the last 7 days **pain has interfered** with your dog's:

5. General Activity
0 1 2 3 4 5 6 7 8 9 10
Does not interfere   Completely interferes

6. Enjoyment of Life
0 1 2 3 4 5 6 7 8 9 10
Does not interfere   Completely interferes

7. Ability to Rise to Standing From Lying Down
0 1 2 3 4 5 6 7 8 9 10
Does not interfere   Completely interferes

8. Ability to Walk
0 1 2 3 4 5 6 7 8 9 10
Does not interfere   Completely interferes

9. Ability to Run
0 1 2 3 4 5 6 7 8 9 10
Does not interfere   Completely interferes

10. Ability to Climb Stairs, Curbs, Doorsteps, etc.
0 1 2 3 4 5 6 7 8 9 10
Does not interfere   Completely interferes

Overall impression:
11. Fill in the oval next to the one number that best describes your dog's overall quality of life over the last 7 days.

Poor   Fair   Good   Very Good   Excellent

Figure 16: the Canine Brief pain inventory questionary

Clinical examination:

This part of the examination was performed by the Head Orthopedic Surgeon of the University of Camerino, where the completion of a specific clinical form was requested (Vesseur, Horstman modifies the lameness classification system, fig.17).

WALK/ TROT	CLINICAL SIGNS
I	No lamness noted at work or trot
II	No lamenss at a walk, mild lameness at a trot
III	Mild lameness at walk , significant lameness at a trot
IV	Significant lameness at a walk, non-weight bearing at trot
V	Non-weight bearing lameness at a walk and a trot
STANDING	
I	Normal weight bearing at a stance
II	Mild decrease in weight bearing at a stance
III	Significant decrease in weight bearing at a stance
IV	Occasional toe-touching at a stance
V	Holds limb off the ground at a stance
CONTROLATERAL LIMB	
I	Readily accepts contralateral limb being held up and bears full weight on affected limb
II	Offer resistance to elevation of contralateral limb but bears full weight on affected limb for more than 1 minute after contralateral limb is elevated
III	Offer moderate resistance to elevation of contralateral limb and replaces it after 30 seconds
IV	Offer resistance to elevation of contralateral limb and replaces it after 10 seconds
V	Refuses to rise contralateral limb
PAIN ON PALPATION	
I	No sign of pain during palpation of affected joint/bone
II	Sign of mild pain during palpation of affected joint/bone; dogs turn head in recognition
III	Sign of moderate pain during palpation of affected joint/bone; dogs pulls limb away
IV	Sign of severe pain during palpation of affected joint/bone; dogs vocalizes or becomes aggressive
V	Dog will not allow examiner to palpate joint

Figure 17: Modified Lameness Grading System

This clinical card contained several fields to be filled in using a numerical scale from 1 (no clinical evidence of lameness) to 5 (non-weight bearing lameness). The fields were divided into lameness during the walking phase, in stance, pain on palpation and evaluation of the contralateral limb. For each dog, the specialist filled out the patient's pain sensation during a preliminary inspection using the visual analogue pain (VAS) scale, which has the same numerical severity scale as the CBPI, from 0 to 10 (fig 18).

UNIVERSITÀ DICAMERINO
UNICAM
Università di Camerino
1336

Esaminatore:_____

Proprietario Sig._____-via:_____   data: _____
dell'animale da compagnia _____

Esame clinico:
Grado di zoppia (Vsseur et al; Horstman)   arto _____ distretto anatomico_____

PASSO	1	2	3	4	5
TROTTO	1	2	3	4	5
STAZIONE	1	2	3	4	5
DOLORE PALPAZIONE	1	2	3	4	5
ARTO CONTROLATERALE	1	2	3	4	5

VAS   1   2   3   4   5   6   7   8   9   10

ROM   flessione _____   estensione _____

Test specifici

CAMPBEL	positivo	negativo	_____
TIBIAL THRUST	positivo	negativo	_____
DRAWER TEST	positivo	negativo	_____
SIT TEST	positivo	negativo	_____
BICIPITAL	positivo	negativo	_____
INWARD (valgus)	positivo	negativo	_____
OUTWARD (varus)	positivo	negativo	_____
STAND TEST	positivo	negativo	_____
ORTOLANI	positivo	negativo	_____
ANGOLO DI RIDUZIONE	destra _____	sinistra _____	
ANGOLO DI SUBLUSSAZIONE	destra _____	sinistra _____	

Figure 18: Specialistic Orthopedic Clinical card.

 The clinical examination card include also a measurement of range of motion of affected joint and limb circumference. This evaluation was used as clinical outcome, and performed by the head orthopedic surgeon, at time 0 (before treatment) and after 30, 60 and 180 days after treatment for each group.

Radiography Assesment:

The radiographic examination provided to confirm the diagnosis of OA and allowed to obtain orthogonal projections of the joint involved in the pathological process.
The study was reported by the Head of Radiologist of the Veterinary Teaching Hospital of the University of Camerino, whose attributed blindly for each patient, a degree of osteoarthritis according to a modified Kellgren Lawrence scale, considering different radiographic signs, including the presence of osteophytes, bone sclerosis, joint

narrowing and/or incongruence, and the presence of capsular ectasia(fig.19). The degree of OA was attributed by a mean of a numerical scale from 0 (absence of osteoarthritis) to 4 (highest degree of OA), established on the basis of the interval determined by the calculation of the total score.This radiographic scoring system created a degree of osteoarthritis for each patient at T0 allowing to evaluate its evolution in the times following the treatment after 30 and 60 days (T1 and T2).

RADIOGRAPHIC SIGN	0	1	2	3	4
Osteophytes	Absence	< 1mm	1-2 mm	2-3 mm	>3 mm
Bone sclerosis	Absence	Localized	Pervasive	-	-
Joint narrowing and/or Incongruence	Absence	Mild <25%	Moderate 25%-50%	Serious >50%	Joint deformity
Capsular ectasia	Absence	Evident	-	-	-
FINAL SCORE	0	1-3	4-6	7-9	>10
OA GRADE	0	1	2	3	4

Figure 19: Radiographic modified scale of OA.

Synovial Fluid Examination:

At the time of treatment (T0), after 30 (T1) and 60 (T2) days, an aliquot of a sample of synovial fluid was taken for cytological examination. In the Pathology Department of the Veterinary Teaching Hospital of the University of Camerino, after cytological assessment, a degree of synovial fluid sign of OA was established, considering the severity of the cytological alterations present, and cellular modifications concerning the characteristics and physiological concentrations of the fluid. In order to generate a cytological grade, various parameters were considered, including the presence of inflammatory cells, synovial cells, cartilage fragments, blood contamination, and matrix. The synovial parameters above were assigned a numerical severity score from 1 to 3, and the final score was extrapolated from these grading (Fig.20)

Through the evaluation range indicated in the table, a final score was assigned to each cytology, expressed on a scale of values from 1 to 4.

Each value reflects the description of the synovial fluid evaluated according to the following interpretation:

1 = Paraphysiological synovial fluid;

2 = mild inflammation of the synovial fluid;

3 = medium inflammation of the synovial fluid;

4 = severe inflammation of the synovial fluid;

This system allowed the pathologist to blindly establish for each patient of each group an initial degree of inflammation of the synovial fluid at T0, and to evaluate its evolution during the clinical trial at T1 and T2.

CYTOLOGIC SIGN	1	2	3
Inflammatory cells	Absence	Average prevalence	High prevalence
Synovial cells	Absence	Average prevalence	High prevalence
Cartilage fragments	Absence	Average prevalence	High prevalence
Blood contamination	Absence	Average prevalence	High prevalence
Matrix	Absence	Average prevalence	High prevalence

FINAL SCORE	1-2	3-5	6-9	>10
SYNOVIAL SCORE OF OA	1 Paraphysiological synovial fluid	2 Mild inflammation of the synovial fluid	3 Medium inflammation of the synovial fluid	4 Severe inflammation of the synovial fluid

Figure 20: Cytological score of OA.

Synovial Cytokine Assay :

Concentration of three biomolecules (TNFa, IL-6 IL-1b) were measured using Nori® Canine ELISA Synovial Fluid Cytokine Kits (Genorise).

The biomarker analysis was ranked based on the volume required to perform the test and previous literature supporting biomarkers related to OA and its severity. In this case the synovial fluid aliquot was used first for TNF-α and subsequently for IL-6 and IL-1b. For this reason, some samples had insufficient synovial fluid volume to include all enzyme-linked immunosorbent assay tests. These ELISA tests were used at time 0, before treatment and repeated after 30 (T1) and 60 (T2) days for each group.

-*Statistical Analysis:*

Data from the whole evaluations performed during the study were pooled and reported with the arithmetic mean and standard deviation. Ordinal variables were analyzed and compared between the two groups with the Mann-Whitney test.

Friedman's test followed by Dunn's posthoc test was also used to compare times within each group. Values of $P < 0.05$ are considered statistically significant.

All data were analyzed with Prism 8 for MacOS software, version 8.2.1 (GraphPad software Inc., San Diego, California, USA).

Results

<u>*Enrolled Patient:*</u>

Forty owned dogs , belonging to different breeds *(American stafforshire 1, Australian shepherd 1, Border Collie 1, Boxer 1, Chow-Chow 1 , Dalmatian 1, Duchshound 1, Fox Terrier 1, German Shepherd 2, Golden Retriever 3, Labrador Retriever 6, Maremma Shepherd 2, Mixed Breed 16 , Parson Terrier 1, Pitt Bull 1, Samoyed 1)* affected by OA, met the eligibility criteria and were randomly divided into two groups, Rigenera Group (group A) and a Hyaluronic group (group B) *(tab.xx.).* Dogs selected for each group were aged between 4 and 12 years with a mean of 7.5 for group A, and from 3 to 13 with a mean of 8 for the group B; the mean weight was 29.1 Kg, associated to a body condition score (BCS) of 5.2 for group B, and 26.1 Kg, associated to BCS of 5,8 for the A, respectively. No restrictions related to sex nor to the affected joint were used. The obtained gender division result in 6 males (1 neutered) and 14 females (9 neutered) for the group A, and 10 males (2 neutered) and 10 females (5 neutered) for the group B. The affected joints were respectively, 7 shoulders, 6 elbows, 4 hips, and 3 stifles for the Rigenera group, and 6 shoulders, 4 elbows, 3 hips, 6 stifles, and 1 tarsus, for the hyaluronic group (tab.1). No statistically significant difference between groups was present for the inclusion criteria of weight, BCS, age, and sex.

Patient	QuickCalcks *random generator*	Breed	weight Kg	Sex	Age	BCS	Joint affected by OA
1	A	Mixed breed	17	F/OE	7	7	left shoulder
2	B	Pit Bull	27	M	4	5	right shoulder
3	A	Mixed breed	21	F/OE	6	8	right shoulder
4	B	Mixed breed	18	M/N	13	6	left elbow
5	A	Amstaff	25	F/OE	6	5	left stifle
6	B	Maremma Shepherd	47	M	6	7	right tarsus
7	A	Mixed breed	31	F/OE	5	8	right stifle
8	B	German Shepherd	32	F	10	5	left elbow
9	A	Labrador Retriever	41	F	7	8	right shoulder
10	B	Mixed breed	26	M	11	3	left stifle
11	A	Mixed breed	10	M	12	8	right hip
12	B	Labrador Retriever	39	M	12	8	right shoulder
13	B	Mixed breed	15	F/OE	10	5	Left stifle
14	B	Golden Retriever	36	F/OE	3	6	Left hip
15	A	Mixed breed	12	F	7	8	left hip
16	B	Golden Retriever	40	M	5	7	right hip
17	B	Labrador Retriever	36	F	8	8	right shoulder
18	B	Mixed breed	27	F	7	6	right stifle
19	A	Chow-Chow	31	F/OE	9	8	right elbow
20	A	Samoyed	37	M	10	8	left elbow
21	B	Mixed breed	31	M	10	6	right stifle
22	A	Labrador Retriever	41	M/N	12	8	left stifle
23	B	Parson Terrier	9	F/OE	7	7	left stifle
24	B	Golden Retriever	27	F	5	5	right elbow
25	A	Labrador Retriever	38	F/OE	5	6	right elbow
26	A	Mixed breed	33	M	6	6	left elbow
27	A	Fox Terrier	10	M	8	6	right shoulder
28	B	Labrador Retriever	25	M	7	3	right elbow
29	B	Mixed breed	35	F/OE	11	3	right shoulder
30	A	Duchshound	7	F/OE	8	6	left shoulder
31	A	Mixed breed	31	F	9	3	right elbow
32	A	Mixed breed	35	M	7	3	left elbow
33	B	Boxer	27	F	8	3	left hip
34	B	Australian shepherd	18	M/N	7	3	right shoulder
35	A	Dalmatian	23	F	5	3	right shoulder
36	A	German Shepherd	24	F/OE	9	2	right hip
37	B	Maremma Shepherd	42	M	8	5	left shoulder
38	A	Border collie	15	F	4	2	left hip
39	B	Mixed breed	25	F/OE	8	3	left stifle
40	A	Mixed breed	40	F/OE	8	3	right shoulder

Table 1: *List of enrolled patient; complete signalment data and randomization*

The mean value of the 40 patients related to Clinical examination, the Canine Brief Pain Inventory Index, and the Radiographic score at time 0 (before treatment) is reported in the following table 2. There were no statistically significant differences between the groups concerning clinical examination, CBPI, and radiographic evaluation.

A Rigenera Group	mean	min value	max value	SD
Weight	26,1	7	41	11,2992780393495
Age	7,5	4	12	2,2124052165246
BCS	5,8	2	8	2,30788123383195

Sex	Number
Male	5
Neutered Male	1
Female	5
Neutered Female	9

Breed	Number
American Stafforshire	1
Chow-Chow	1
German shepherd	1
Labrador Retriever	3
Duchshound	1
Mixed breed	9
Fox terrier	1
Border Collie	1
Dalmatian	1
Samoyed	1

Joint	Number
Shoulder	7
Elbow	6
Hip	4
Stifle	3

B Hyal Group	mean	min value	max value	SD
weight	29,1	9	47	9,61303939010912
age	8	3	13	2,69502465568255
BCS	5,2	3	8	1,73508683234859

Sex	Number
Male	8
Neutered Male	2
Female	5
Neutered Female	5

Breed	Number
Pit Bull	1
Maremma shepherd	2
German shepherd	1
Labrador Retriever	3
Golden Retriever	3
Mixed breed	7
Parson Terrier	1
Boxer	1
Australian shepherd	1

Joint	Number
Shoulder	6
Elbow	4
Hip	3
Stifle	6
Tarsus	1

	Mean value	Group A (Rigenera)	SD	Group B (Hyaluronic)	SD
Orthopedic Clinician	Amblatory Lameness	2,85	0,670820393249937	2,95	0,759154654516248
	Stence Lameness	1,75	0,716350399411379	2	0,794719414239026
	Pain on joint palpation	2,8	0,951453182187509	2,75	0,850696309223401
	Pain value during inspection (VAS)	6,5	1,50437957136384	6,45	1,66938375014948
Owner	Pain Severity (Canine Brief Pain Inventory)	12,75	5,70203103242492	13,55	7,87049787765077
	Pain Interference (Canine Brief Pain Inventory)	28	12,8963479122457	28,2	15,7834023415005
Radiologist	Radiographic score	2,15	0,812727700887249	2,25	0,966545666958261

Table 2: Mean data of enrolled patient.

Arthrocentesis produced an adequate volume of synovial fluid for cytological evaluation in 35 of 40 dogs at time 0, while the volume was very challenging for the cytokines assay evaluation, resulting adequate in only 11, 6, and 3 of 40 patients, respectively for TNFα, IL- 6, and IL -1β. The resulting mean value of the cytology assessment score was 2.277 + - 0.894 for the Rigenera group, associated with 203.90 pg / μl of TNFα, 201.41 pg / μl of IL-6 and 180 pg / μl for the IL - 1b; for the hyaluronic group the average values are 2.41 + - 1.12, associated with 108.056 pg / μL of TNFα, 187.51 pg / μL of IL-6 and 300 pg / μL pg / μL of IL-1β (tab.3). No statistically significant differences were detected for the cytological score at time 0 between the groups, while the sample size for the cytokine assay was not adequate for statistical analysis

Mean value	Group A (Rigenera)	Standard Deviation	Group B (Hyaluronic)	Standard Deviation
Cytological score	2,27777777777778	0,894792486988599	2,41176470588235	1,1213175023946
TNFa	203,90625	42,3	208,056666666667	94,7366666666667
IL-6	201,416666666667	28,6970526941938	187,513333333333	91,805
IL-1b	180,0	nd	300,0	59,395

Table 3: Mean data of enrolled patient.

Short term outcome :

-Clinical Examination:

We identified 35 out of 40 patients (87.5%) who completed the short evaluation period after 60 days at time 2, respectively 19 out of 20 (95%) for the Rigenera group and 16 out of 20 (80%) for the Hyaluronic group. The excluded patients (1 for group A and 4 for group B) required the use of systemic NSAIDs already after thirty days due to the aggravation of pain.

The raw data released to the clinical card are report in the following table (tab.4).

	Ambulatory Lameness					
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	2	1	1	3	2	2
2	3	2	1	3	3	3
3	3	2	2	4	3	3
4	4	2	2	3	3	3
5	4	2	2	3	3	3
6	2	2	1	4	3	2
7	3	3	3	4	3	3
8	3	4	4	3	3	3
9	3	2	2	2	2	3
10	3	2	2	3	2	2
11	2	1	1	4	4	4
12	3	3	2	2	2	1
13	3	2	2	3	2	2
14	2	1	1	3	2	3
15	3	2	2	2	2	2
16	3	2	2	2	1	2
17	3	2	1	3	-	-
18	2	2	2	2	-	-
19	2	1	1	4	-	-
20	4	-	-	2	-	-
Mean	2,85	2	1,78947368421053	2,95	2,5	2,5625

	Stance Lameness					
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	1	1	1	2	1	1
2	1	1	1	2	2	2
3	2	2	1	3	2	2
4	2	1	1	2	2	2
5	3	2	1	2	2	2

	Stance Lameness					
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
6	1	1	1	2	2	1
7	2	1	1	4	3	2
8	3	3	3	2	2	2
9	2	2	2	2	2	2
10	1	1	1	2	1	1
11	1	1	1	3	3	3
12	2	1	1	2	2	1
13	2	1	1	3	1	1
14	1	1	1	2	2	2
15	2	1	1	1	1	1
16	1	1	1	1	1	1
17	2	1	1	1	-	-
18	2	2	1	1	-	-
19	1	1	1	2	-	-
20	3	-	-	1	-	-
Mean	1,75	1,31578947368421	1,15789473684211	2	1,8125	1,625

	Pain on Palpation					
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	2	1	1	3	3	3
2	2	1	1	4	4	4
3	3	2	2	3	2	2
4	4	3	3	2	2	2
5	4	2	2	2	2	2
6	1	1	1	3	2	2
7	1	1	1	3	2	2
8	4	5	5	3	3	3
9	3	3	3	4	3	4
10	2	1	1	3	2	2
11	3	3	2	4	4	4
12	3	1	1	1	1	1
13	4	4	3	3	2	3
14	3	4	2	3	3	3
15	3	2	1	3	2	3
16	2	1	1	2	2	2
17	2	1	1	3	-	-
18	4	3	3	2	-	-
19	3	3	3	3	-	-
20	3	-	-	1	-	-
Mean	2,8	2,21052631578947	1,94736842105263	2,75	2,4375	2,625

Visual Analogue Scale of Pain					
PATIENT	Rigenera Group (A)			Hyaluronic Group (B)		
	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	4	3	2	4	4	4
2	6	4	2	10	10	10
3	8	6	3	8	7	6
4	6	6	5	4	4	4
5	8	4	2	7	7	6
6	6	5	3	5	3	2
7	8	4	4	8	7	6
8	8	10	10	7	8	8
9	7	7	6	7	7	9
10	5	3	2	5	3	3
11	8	5	5	7	7	7
12	6	5	3	6	4	4
13	9	10	8	9	5	7
14	7	9	5	7	5	7
15	7	5	4	6	4	6
16	5	2	2	4	4	4
17	4	1	1	5	-	-
18	8	6	4	6	-	-
19	5	5	5	8	-	-
20	5	-	-	6	-	-
Mean	6,5	5,26315789473684	4	6,45	5,5625	5,8125

Body Condition Score					
PATIENT	Rigenera Group (A)			Hyaluronic Group (B)		
	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	5	5	5	5	4	4
2	7	5	5	6	5	4
3	8	8	6	7	7	6
4	8	8	6	5	5	5
5	8	8	8	3	3	3
6	8	6	6	8	6	6
7	8	6	6	5	4	3
8	8	8	8	6	6	6
9	8	8	8	7	7	7
10	8	8	8	8	8	8
11	6	6	6	6	5	5
12	6	6	6	6	6	6
13	6	6	6	7	7	6
14	6	6	6	5	4	3
15	3	2	2	3	3	2
16	3	2	2	3	1	2
17	3	2	2	3	-	-
18	2	2	2	3	-	-
19	2	2	2	5	-	-
20	3	-	-	3	-	-
Mean	5,8	5,47368421052632	5,26315789473684	5,2	5,0625	4,75

Range Of Motion

PATIENT	Rigenera Group (A)			Hyaluronic Group (B)		
	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	103	105	105	78	78	82
2	95	101	102	44	46	45
3	112	118	118	61	58	60
4	118	118	118	93	87	90
5	93	97	97	92	92	94
6	100	97	112	103	108	107
7	90	88	100	102	101	103
8	69	70	70	82	80	80
9	53	55	53	92	90	90
10	88	88	85	108	110	110
11	112	112	112	82	84	82
12	99	112	112	115	118	116
13	70	72	70	81	80	85
14	62	54	58	72	76	68
15	103	106	105	121	121	121
16	95	94	97	109	105	109
17	103	105	104	91	-	-
18	70	66	71	72	-	-
19	102	105	104	85	-	-
20	88	-	-	88	-	-
Mean	91,25	92,7894736842105	94,3684210526316	88,55	89,625	90,125

Circumference

PATIENT	Rigenera Group (A)			Hyaluronic Group (B)		
	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	18	20	20	23	23,4	25
2	20	21	20	19	19	18
3	31	31	33	18	18.5	19
4	32	33	33	30	30	30
5	28	27	28	32	32	32
6	16	15	16	22	24	24
7	16	16	16	27	29	30
8	27	26,5	27	25	26	26
9	27	27	27	31	32	30
10	37	37	37,5	24	27	27
11	24	24	25	38	35	35
12	20	20	22	37	37	38
13	11	11	10	18	20	20
14	8	7	8	22	21	21
15	22	23	23	25	26	26
16	25	24	24	22	22	23
17	22	23	23	25	-	-
18	32	30	31	21	-	-
19	28	28	27	28	-	-
20	32	-	-	30	-	-
Mean	23,8	23,3421052631579	23,7105263157895	25,85	26,36875	26,5

Table 4: raw data of clinical card; (a) ambulatory lameness, (b) stance lameness,(c) pain on palpation, (d) VAS,

*(e) BCS, (f) ROM, (g) circumference.,*

Statistical analysis of the 35 patient for the walking lameness showed significant differences between the Rigenera and the Hyaluronic group after 30 days (P-value 0.0338 *), and after 60 days (P-value 0.0037 **). Friedman's test, within the groups, showed a statistical difference for the Hyaluronic group only between T0 and T1 (P-value 0.0077 *), while for the Rigenera group, the Friedman test noticed an important statistically significant difference between T0 and T1 (P-value 0.0027 **), and between T0 and T2 (P-value 0.0001 ***).
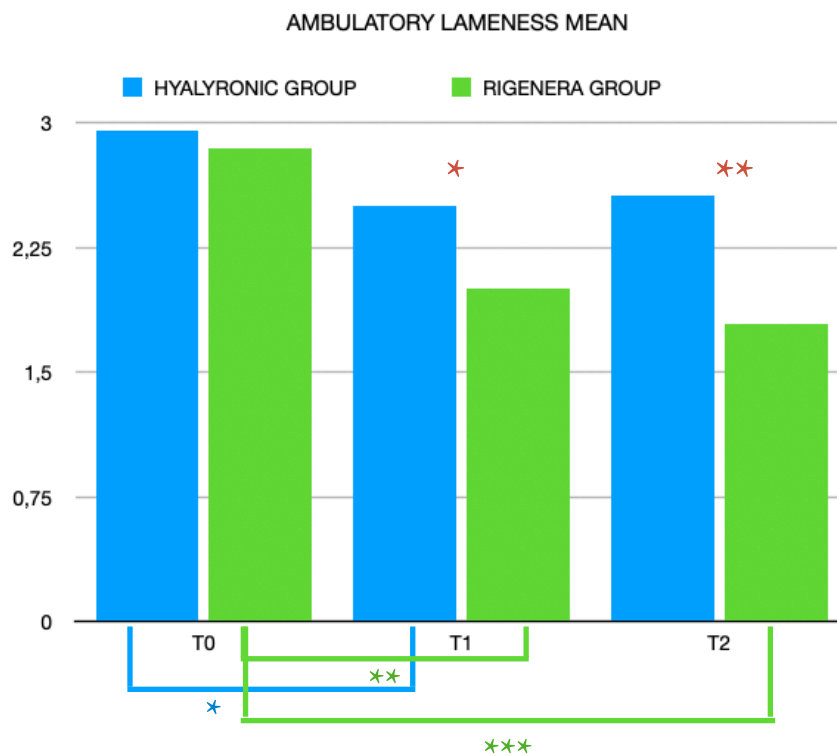
For the stance lameness statistical analysis showed significant differences between the Rigenera group and the Hyaluronic group after 30 days (P-value 0.0247 *), and after 60 days (P-value 0.0087 **). Statistical analysis within the groups showed significant difference only for the Rigenera group (P-value 0.0006 ***) between T0 and T2.

STANCE LAMENESS MEAN
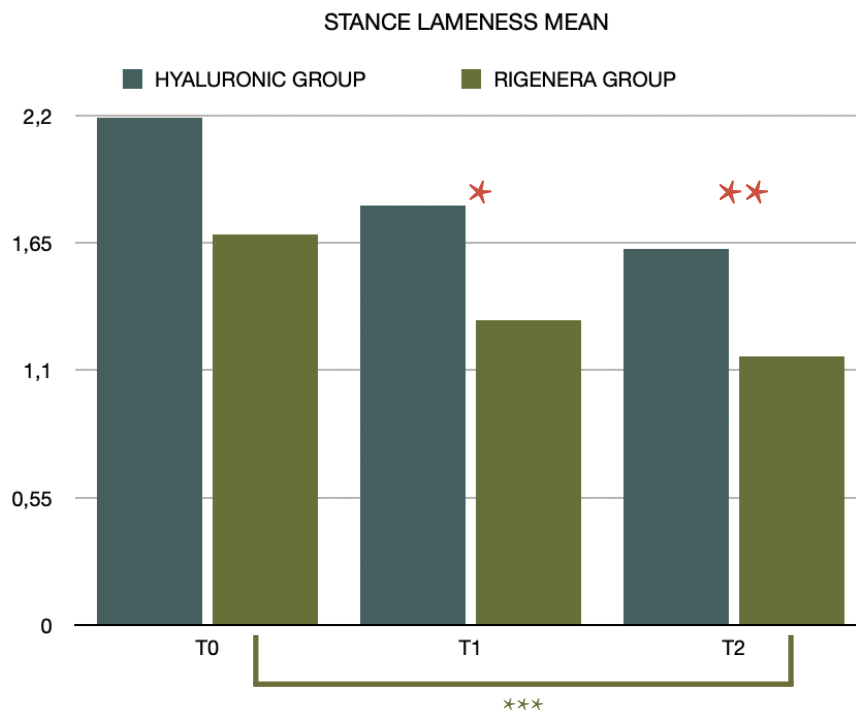
Mann-Whitney statistical test for the pain on palpation showed mild significant diffe-rences between Rigenera and Hyaluronic after 60 days (P-value 0,0372 *), while it was showed a strong statistical difference (P-value 0,0002***) within the Rigenera group between time 0 and time 2.

PAIN ON PALPATION MEAN
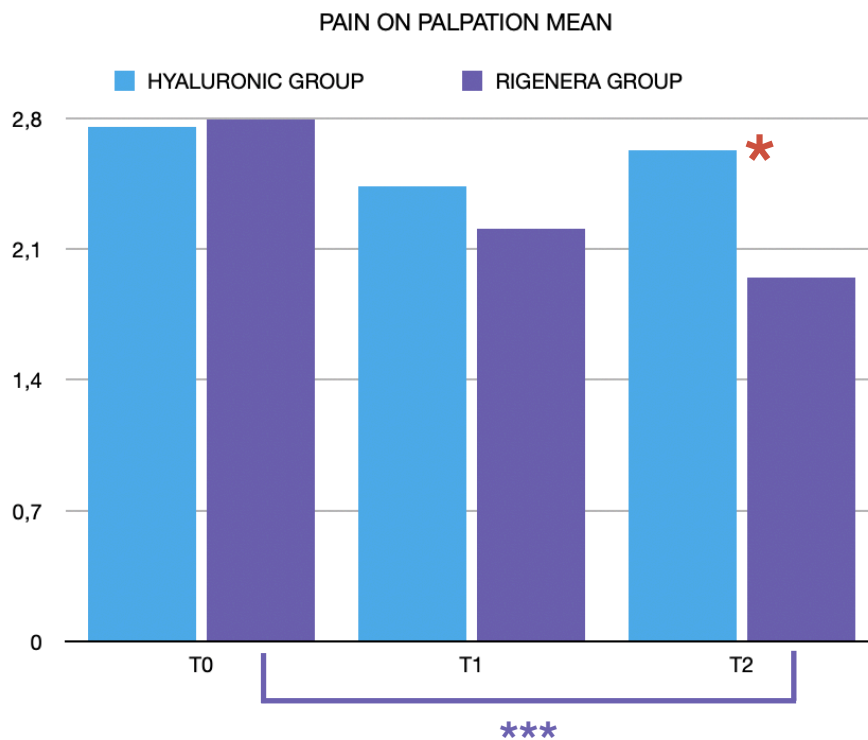
The statistical analysis for visual analogue score of pain proved significant different between the groups at time 2 (P-value 0,0149 *) associated to a strong significancy within the Rigenera group between time 0 and time 2 (P- value < 0,0001 ****) and time 1 and time 2 (P-value 0,0094 **)

VISUAL ANALOGUE SCORE OF PAIN MEAN
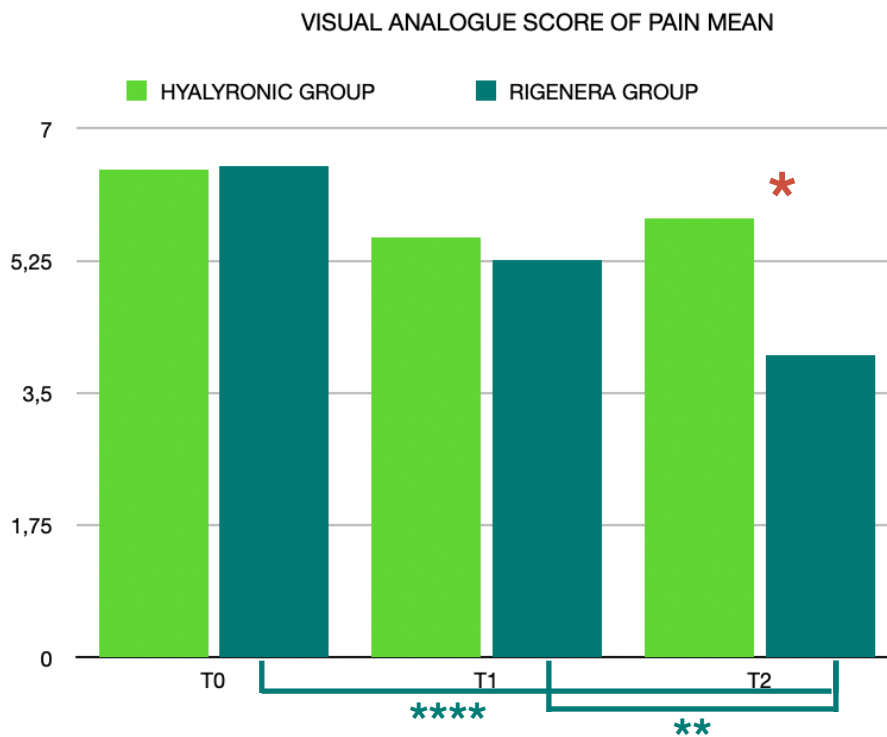
No statistically significant differences were noted between groups for body condition score with only one difference within the hyaluronic group (P value 0.0047**) between time 0 and time 2.

BCS MEAN
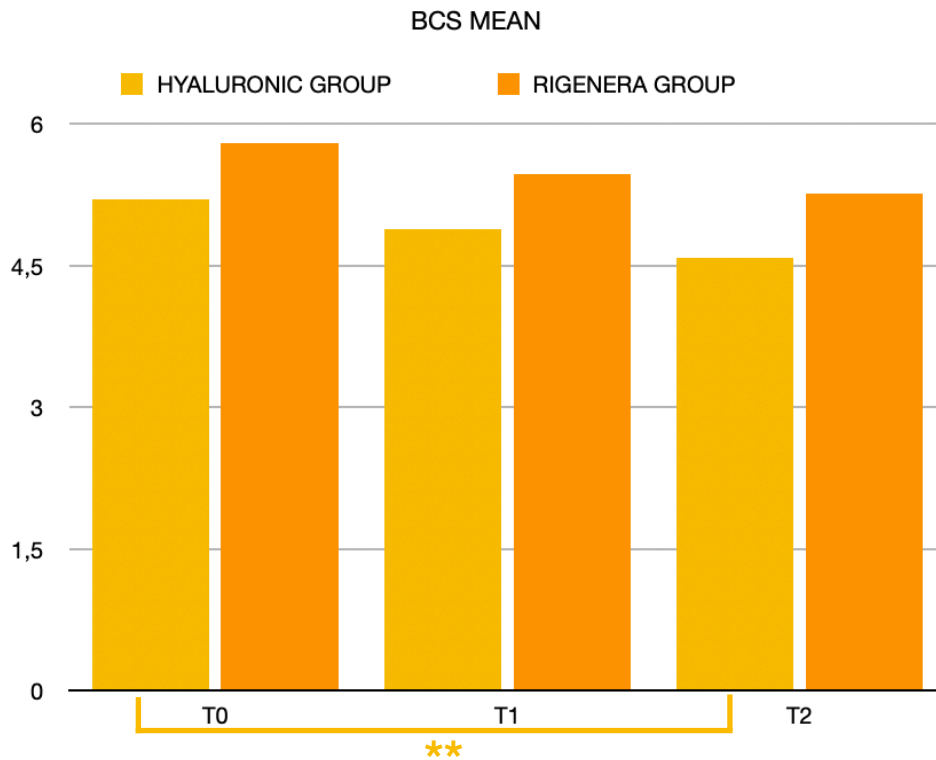
For the statistical analysis of circumference and range of motion, as deriving from different joints, the raw data at time 0 were normalized by calculating the absolute variations taking as reference point T0 (Var T1-T0 and T2-T0) and transforming the variations between times in relative variations (% variation between times,% Var). No statistical differences in relative variation were reported either between or within groups.

-Canine Brief Pain Inventory Index:

In addition to the clinical examination, 35 out of 40 patients completed the short term follow-up period for CBPI, 16 for the Hyaluronic group and 19 for the Rigenera group respectively. The resulting data derived from the questionnaire were divided into two domains, the pain severity domain (PSI) and the pain interference domain (PII), as is usually used for the Canine Brief Pain Inventory index.

The table below represents raw data from the entire patient cohort before treatment up to 60 days (tab.5).

	CBPI Severity Pain Domain					
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 *(pre-treat)*	Time 1 *(30days)*	Time 2 *(60 days)*	Time 0 *(pre-treat)*	Time 1 *(30days)*	Time 2 *(60 days)*
1	7	5	2	8	5	7
2	11	3	0	28	22	25
3	20	12	7	22	18	13
4	9	7	5	12	8	11
5	15	11	2	18	15	19
6	11	6	5	32	27	27
7	15	6	10	7	5	6
8	5	24	28	18	19	25
9	11	8	8	5	7	7
10	4	4	4	18	6	13
11	18	12	5	17	12	13
12	7	5	3	5	8	5
13	21	15	6	11	8	9
14	15	5	4	13	16	18
15	8	5	5	5	6	5
16	15	7	5	18	22	20
17	8	3	3	8	-	-
18	25	12	4	6	-	-
19	12	5	5	15	-	-
20	18	-	-	5	-	-
Mean	*12,75*	*8,15789473684211*	*5,84210526315789*	*13,55*	*12,75*	*13,9375*

	CBPI Interference Pain Domain					
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 *(pre-treat)*	Time 1 *(30days)*	Time 2 *(60 days)*	Time 0 *(pre-treat)*	Time 1 *(30days)*	Time 2 *(60 days)*
1	13	7	0	14	15	12
2	18	7	4	40	38	30
3	26	20	11	31	32	30
4	22	21	17	23	24	23
5	23	9	1	22	25	21
6	15	9	9	32	27	27
7	21	8	15	46	42	39
8	26	42	61	25	30	26
9	17	18	17	46	46	38
10	17	14	11	15	9	12
11	31	29	13	28	15	18
12	12	11	8	26	12	15
13	39	31	35	47	21	25
14	25	13	21	53	46	51
15	47	26	24	18	12	13
16	51	21	16	58	42	60
17	24	25	22	21	-	-
18	56	43	14	11	-	-

CBPI Interference Pain Domain						
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 *(pre-treat)*	Time 1 *(30days)*	Time 2 *(60 days)*	Time 0 *(pre-treat)*	Time 1 *(30days)*	Time 2 *(60 days)*
19	39	34	32	5	-	-
20	38	-	-	3	-	-
Mean	28	20,4210526315789	17,4210526315789	28,2	27,25	27,5

Table 5: raw data of CBPI (a) Pain Severity Index, (b) Pain Interference Index,

Statistical analysis for the Pain Severity Domain proved a mild significant difference between the Rigenera group and the Hyaluronic group after 30 days (P-value 0.0236*), and a strong significant difference after 60 days (P-value <0.0001****). Friedman's test, didn't showed a significancy in any time for the Hyaluronic group, while within the Rigenera group noted an important significant difference between between T0 and T1(P-value 0.0021**), and between T0 and T2 (P-value <0.0001****).

CBPI (PAIN SEVERITY) MEAN
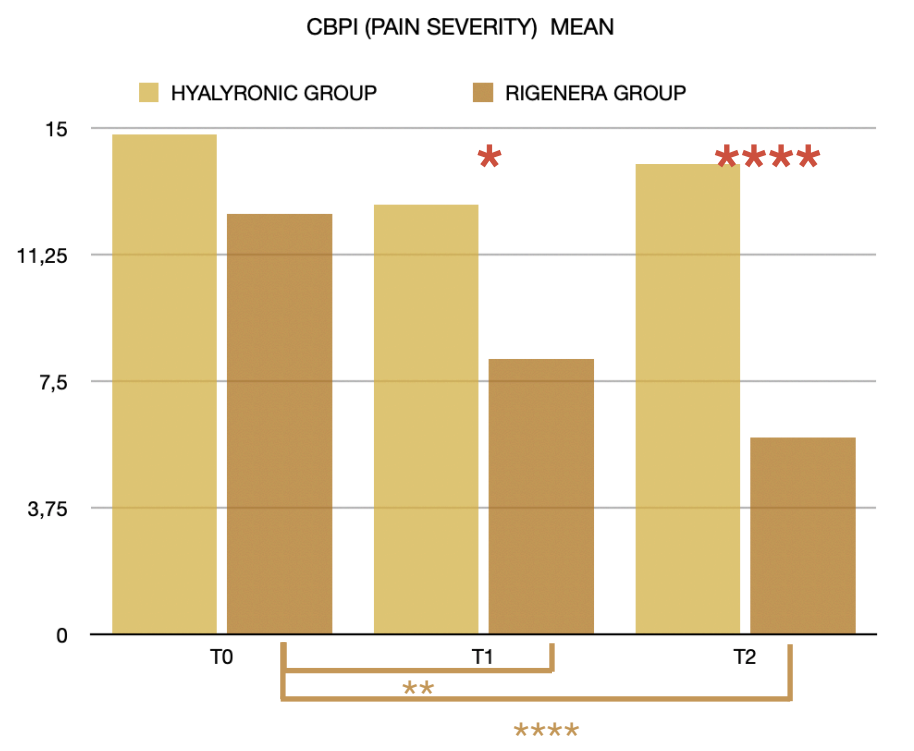
For the Pain Interference Domain Mann-Whitney test between Rigenera and Hyalu-ronic group showed a significant difference (P-value 0,0154*) after 60 days; signifi-cant different was detected within time 0 and time 2 for the Hyaluronic group (P-va-lue 0,0133 *) and between time 0 and time 1 (P-value 0,0094*), and between T0 and T2 (P-value <0,0001****) within the Rigenera group.
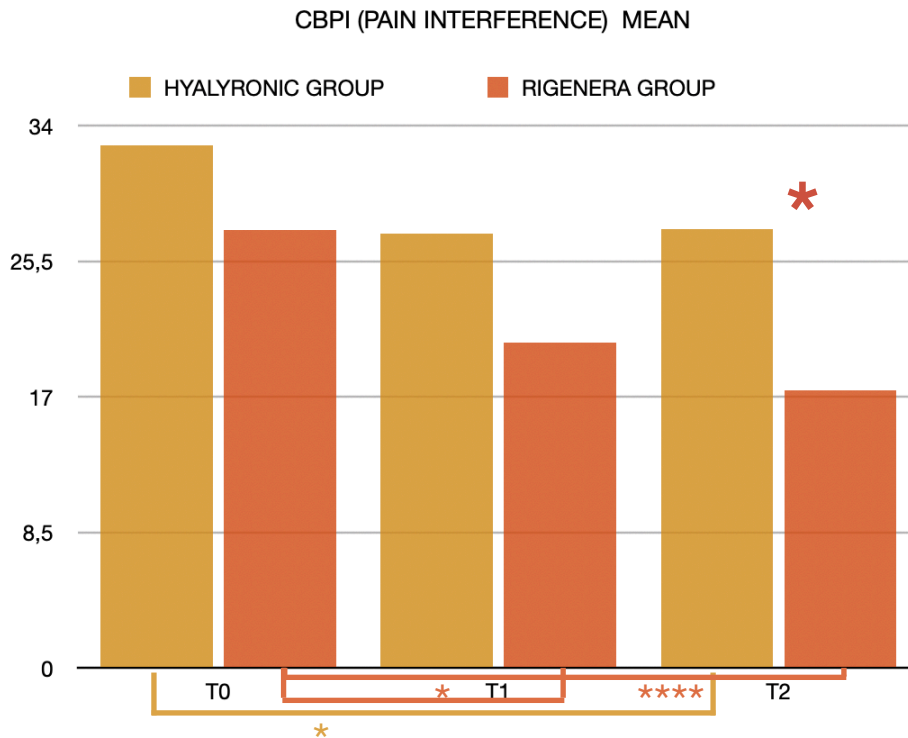
-Radiographic Assessment :

Such as for the clinical examination and the Canine Brief Pain Inventory index, we collected radiographic data on 35 out of 40 patients. The radiological score of osteoarthritis up to sixty days is shown in the table below. (tab.6.)

PATIENT	Rigenera Group (A)			Hyaluronic Group (B)		
	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	1	1	1	2	2	2
2	1	1	1	4	4	4
3	1	1	1	3	3	3
4	2	2	2	2	2	2
5	1	1	1	2	2	2
6	2	2	2	2	2	2
7	2	2	2	1	1	1
8	3	3	3	3	4	4
9	3	3	3	2	3	3
10	3	2	3	1	1	2
11	2	2	2	3	3	3
12	2	2	2	1	1	1
13	2	1	1	4	4	4
14	2	1	1	3	3	3
15	2	3	2	1	1	1
16	2	2	2	1	2	2
17	2	3	2	2	-	-
18	3	2	2	3	-	-
19	3	2	2	2	-	-
20	4	-	-	3	-	-
Mean	2,15	1,89473684210526	1,84210526315789	2,25	2,375	2,4375

Table 6: raw data of radiographic OA score.

Statistical analysis did not reveal any significant differences between the group and within the group.

-*Synovial assenssment:*

In 35 of the 40 patients enrolled in the study (87.5%), it was possible to obtain an adequate amount of synovial fluid before treatment (T0), to evaluate cytologically and classify the inflammatory state of the joint.; of these, only 31 patients (88.57%) completed the short time follow-up (T2), rispectively 14 for Hyaluronic group and 17 for the Rigenera group. The raw data for each patient of the group are shown in the table below (tab.7)

	Cytologic synovial score					
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	1	1	1	3	2	2
2	2	1	1	3	3	2
3	3	3	2	-	-	-
4	2	1	1	4	3	3
5	3	2	1	3	3	2
6	2	1	1	1	1	1
7	4	1	3	3	2	2
8	2	1	1	1	2	3
9	2	1	1	4	3	1
10	2	1	1	2	2	2
11	1	3	1	4	2	4
12	2	3	1	3	3	3
13	2	2	1	-	-	-
14	3	3	1	1	1	2
15	4	1	2	2	2	2
16	1	1	1	3	2	1
17	2	1	1	-	-	-
18	-	-	-	1	-	-
19	-	-	-	1	-	-
20	4	-	-	2	-	-
Mean	2,33333333333333	1,58823529411765	1,23529411764706	2,41176470588235	2,21428571428571	2,14285714285714

Table 7: raw data of Cytological synovial score.

The statistical analysis of the 31 patient for the cytological synovial score has shown a mild significant difference between the group after 30 days (P-value 0,0331*), and a strong significant different after 60 days (P-value 0,0008 ***). No statistically significant differences were reported within the Hyaluronic group, while was showed for Rigenera group between time 0 and time 1 (P-value 0,0258 *), and between time 0 and time 2 (P-value 0,0006 ***).

SYNOVIAL CYTOLOGICAL GRADE MEAN
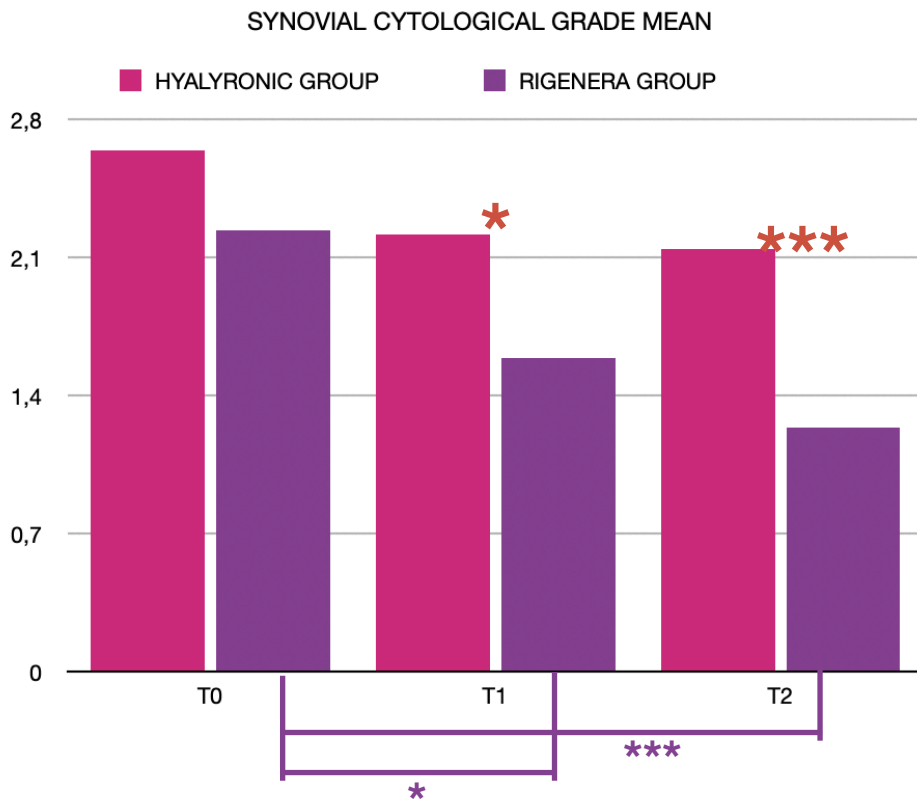
Evaluation of synovial cytokine assay resulted hard to achieve an adequate sample volume. The raw data obtained by the ELISA tests were not sufficient to perform statistical analyzes neither for TNFα, nor for IL-6, nor IL-β (tab.8).

	TNFa					
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1						
2						
3	175,83	145,0	167,50			
4	196,67	226,25	184,17	119,17	150,03	186,25
5	260	250,83	166,25	-	-	-
6				151,25	177,50	220,0
7						
8	165					
9	195					
10	+					

TNFa						
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
11	158,75					
12	260					
13				353,75	360,25	380,16
14						-
15						184,17
16						151,25
17	220	184,17	151,25			
18						
19						
20						
Mean	203,90625	207,36	172,64	208,056666666667	229,26	224,366

IL-6						
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	-	-	-			
2	-	-	-			
3	208	170,0	165			
4	226,25	196,67	132,0	76,67	100,02	115,34
5		-	-			
6				235,0	246,67	330,0
7						
8						
9						
10						
11	170,0					
12						
13				250,87	274	286,67
14						-
15						
16						
17						
18	-	-	-			
19	-	-	-			
20	-	-	-			
Mean	201,416666666667	183,335	148,5	187,513333333333	206,896666666667	244,003333333333

| IL-1b						
	Rigenera Group (A)			Hyaluronic Group (B)		
PATIENT	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
1	-	-	-			
2	-	-	-			
3	-	-	-			
4	-	-	-	400,0	-	-
5	-	-	-	-	-	-

PATIENT	Rigenera Group (A)			Hyaluronic Group (B)		
	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)	Time 0 (pre-treat)	Time 1 (30days)	Time 2 (60 days)
6	-	-	-	200,0	210,0	223
7	-	-	-			
8	-	-	-			
9	-	-	-			
10	-	-	-			
11	180,0					
12						
13						
14						
15						
16						
17						
18	-	-	-			
19	-	-	-			
20	-	-	-			
Mean	180			300	210	223

IL-1b

Table 8: raw data of synovial cytokine assay.

We collected useful data during the short follow-up for seven out of forty patients for tumor necrosis factor α (17.5%), respectively four for the Rigenera group and three for the hyaluronic group, 5 out of 40 for interleukin - 6 (12.5%), respectively two for the Rigenera group and three for the hyaluronic group, and only one patient for interleukin 1β (2.5%), belonging to the hyaluronic group. Despite no statistical analysis, the ELISA tests suggested a mild decrease in terms of cytokine concentration in the Rigenera group, while showed a mild increase of cytokine concentration in the Hyaluronic group, as the graphic below shows.

Rigenera group

TNFa

IL-6

Hyaluronic group

TNFa

IL-6
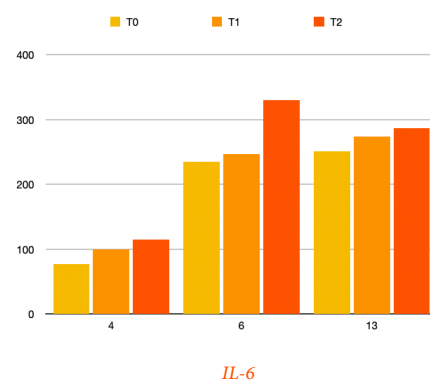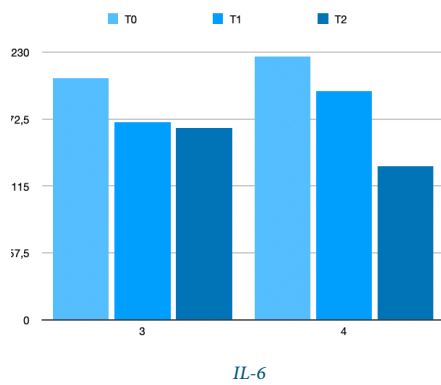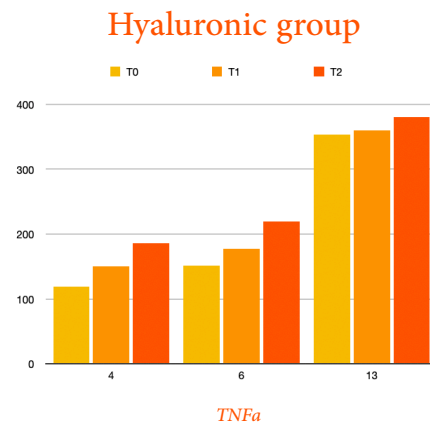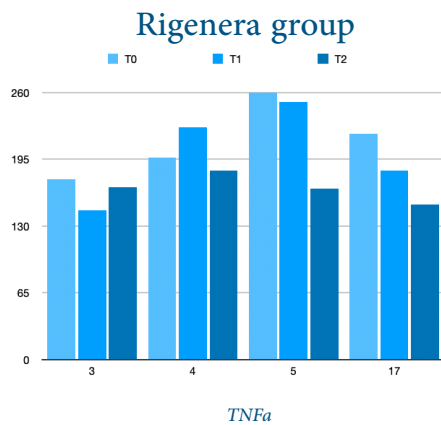
Long term outcome

<u>-Clinical examination:</u>

Only 25 patients, out of 35 (71.43%) who completed the short term follow-up, concluded the study with long-term follow-up after 180 days of treatment (T3), respectively 15 of 19 (78, 94%) for the Rigenera group and 10 out of 16 (62.5%) for the hyaluronic group. These patients (4 for group A and 6 for group B) required the use of systemic NSAIDs between 60 and 180 days, due to the aggravation of the joint pain. The raw data resulting from the clinical examination after six months are shown in the following table (tab 9).

Ambulatory Lameness		
	Rigenera Group (A)	Hyaluronic Group (B)
PATIENT	Time 3 (180 days)	Time 3 (180 days)
1	1	-
2	2	3
3	3	3
4	3	3
5	3	3
6	-	-
7	3	3
8	-	-
9	3	-
10	2	3
11	1	4
12	3	2
13	2	3
14	2	-
15	2	2
16	-	-
17	2	-
18	2	-
19	-	-
20	-	-

Stance Lameness		
	Rigenera Group (A)	Hyaluronic Group (B)
PATIENT	Time 3 (180 days)	Time 3 (180 days)
1	1	-
2	1	2
3	1	2
4	1	2
5	1	2
6	-	-
7	2	3
8	-	-
9	2	-
10	1	1
11	1	3
12	1	1
13	1	2
14	1	-
15	1	1
16	-	-
17	1	-
18	1	-
19	-	-
20	-	-

Pain on Palpation		
	Rigenera Group (A)	Hyaluronic Group (B)
PATIENT	Time 3 (180 days)	Time 3 (180 days)
1	2	-
2	2	4
3	3	2
4	3	2
5	2	2
6	-	-
7	1	2
8	-	-
9	3	-
10	2	2
11	3	4
12	1	1
13	3	3
14	3	-
15	2	3
16	-	-
17	1	-
18	3	-
19	-	-
20	-	-
Mean	2,26666666666667	2,5

Visual Analogue Scale of pain		
	Rigenera Group (A)	Hyaluronic Group (B)
PATIENT	Time 3 (180 days)	Time 3 (180 days)
1	2	-
2	4	10
3	5	6
4	6	4
5	4	5
6	-	-
7	8	6
8	-	-
9	6	-
10	5	3
11	5	7
12	3	5
13	8	7
14	5	-
15	5	6
16	-	-
17	2	-
18	3	-
19	-	-
20	-	-
Mean	4,73333333333333	5,9

	Body Condition Score	
	Rigenera Group (A)	Hyaluronic Group (B)
PATIENT	Time 3 (180 days)	Time 3 (180 days)
1	5	-
2	5	4
3	5	5
4	6	5
5	8	4
6	-	-
7	7	5
8	-	-
9	8	-
10	8	6
11	6	5
12	5	6
13	8	7
14	6	-
15	3	3
16	-	-
17	2	-
18	3	-
19	-	-
20	-	-
Mean	5,66666666666667	5

Table 9: raw data of clinical card after 180 days; (a) ambulatory lameness, (b) stance lameness,(c) pain on palpation, (d) VAS, (e) BCS.

Statistical analysis refers to clinical examination data relating to the short-term follow-up associated with 6-month follow-up data of the 25 patients, both between and within groups. Mann Whitney's test for ambulatory lameness showed a statistically significant difference between groups at 30 days (P value 0.0118 *), after 60 days (P value 0.0094 **), which persisted at 180 days (P - value 0.0413 *); no statistical significancy are noticed within Hyaluronic group, while were significant in Rigenera group between time 0 and 30 days (P-value0,0007 ***), T0 and 60 days (P-value<0,0001 ****), time 0 and 180 days (P-value 0,0339*), and between 60 and 180 days (P-value 0,0477 *).

AMBULATORY LAMENESS MEAN
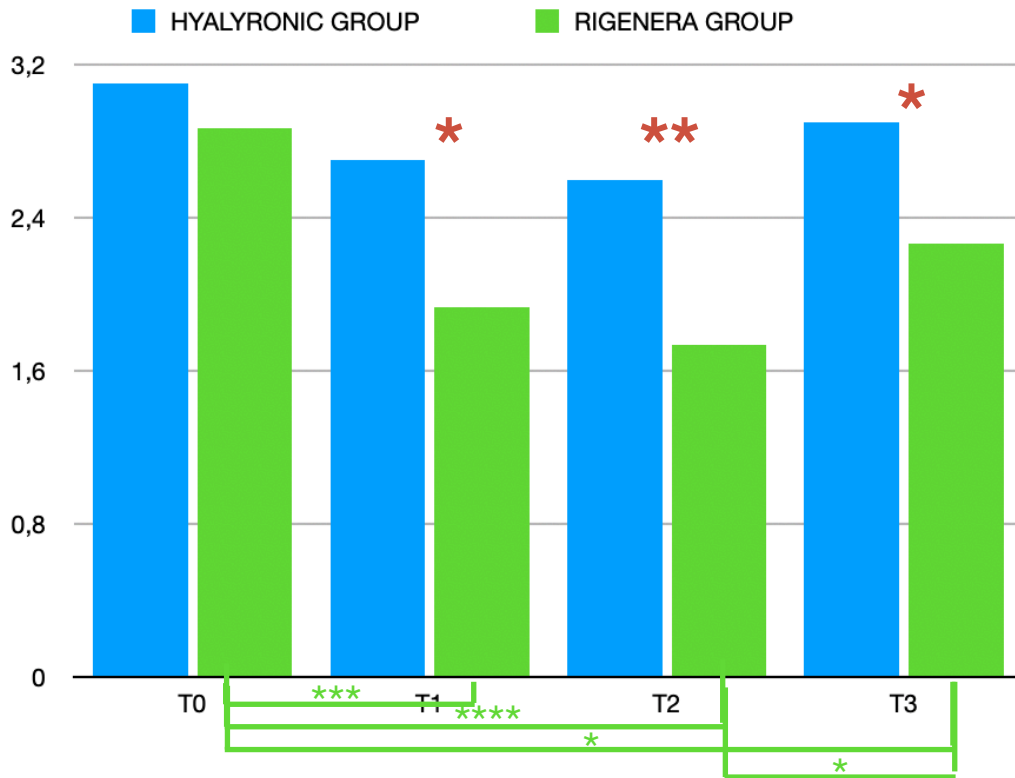
Investigation of stance lameness showed a significant difference between group at time 1 (P-value 0,0259 *), time 2 (P-value 0,0059 **) and time 3 (P-value 0,0064 **); the statistical difference were noticed within Hyaluronic group between time 0 and time 2 (P-value 0,0464 *), while were showed in the Rigenera group between time 0 and time 2 (P-value 0,0089 **), and still between time 0 and time 3 (P-value

0,0196 *).

STANCE LAMENESS MEAN
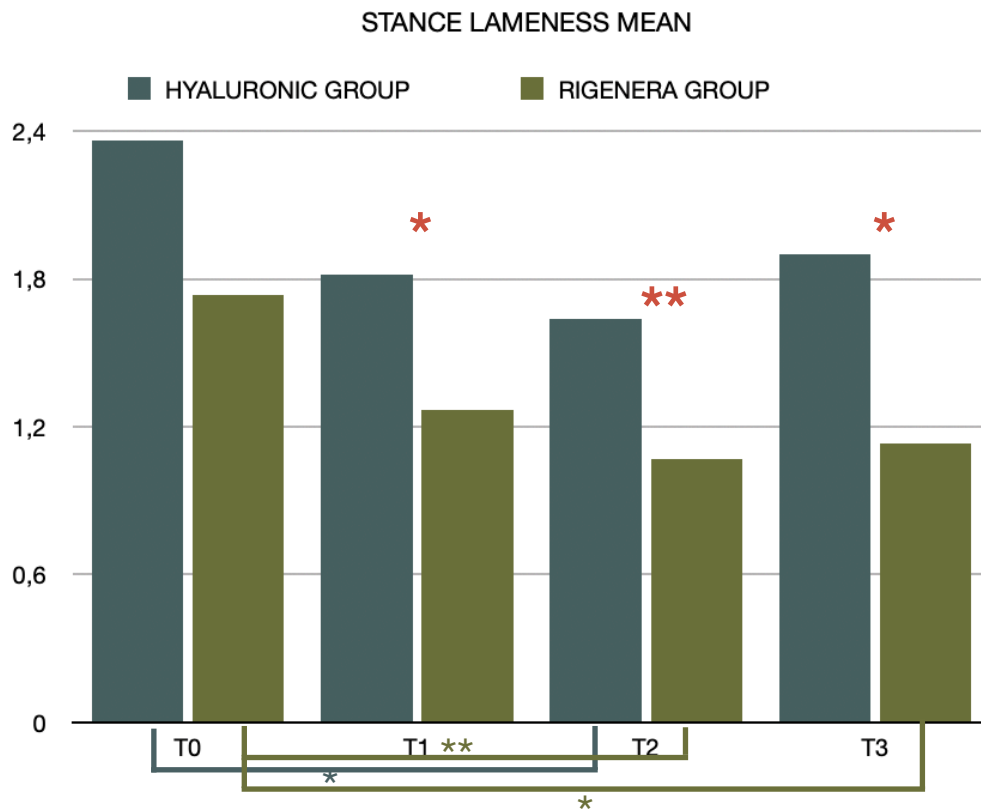
Mann-Whitney test didn't prove any significancy between the group considering pain on palpation, which were noticed only within the Rigenera group between time 0 and 30 days (P-value 0,0109 *) and between time 0 and 60 days (P-value 0,0002 ***)

JOINT PAIN MEAN
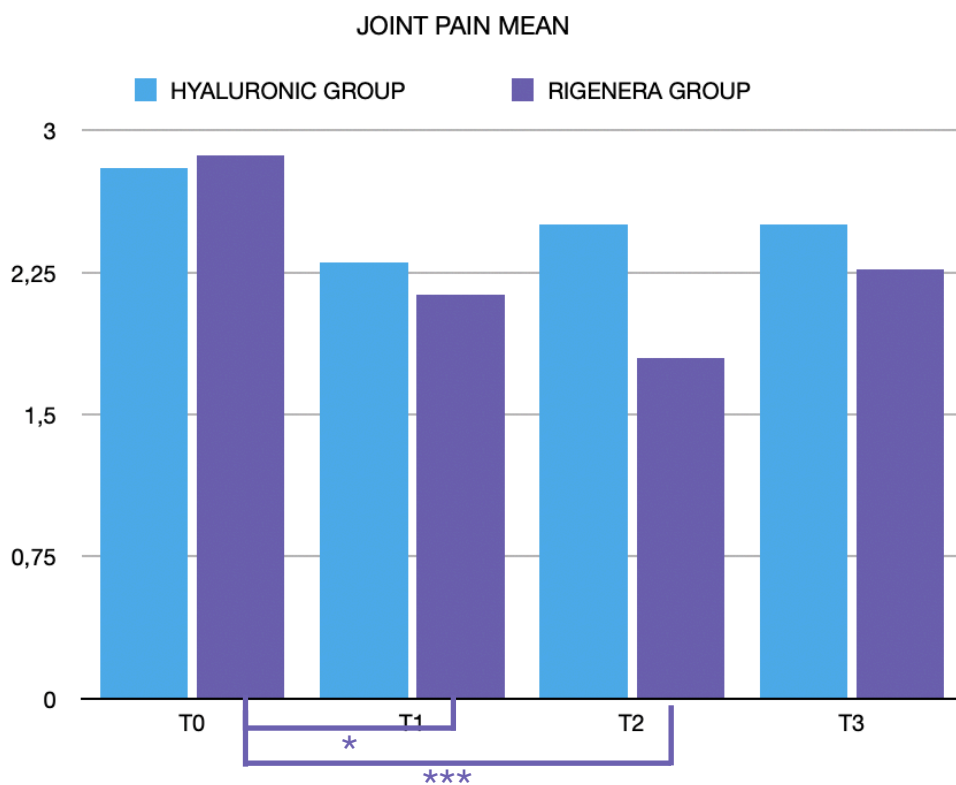
Statistical analysis between Rigenera and Hyaluronic group proved a significant difference for the visual analogue scale of Pain (VAS) only after 60 days from treatment (P-value 0,0100 *); statistical test had shown a significancy within the groups between day 0 and any other follow up in both group (Hyal: T0- T1 P-value 0,0464 *, T0-T2 P-value 0,0304 *, and T0-T3 P value 0,0377 *; Rig.: T0-T1 P-value 0,0404 *, T0-T2 P-value <0,0001 ****, T0-T3 P-value 0,0015 **.), and within Rigenera group between 30 and 60 days (P-value 0,0037 **).

VISUAL ANALOGUE SCORE OF PAIN MEAN
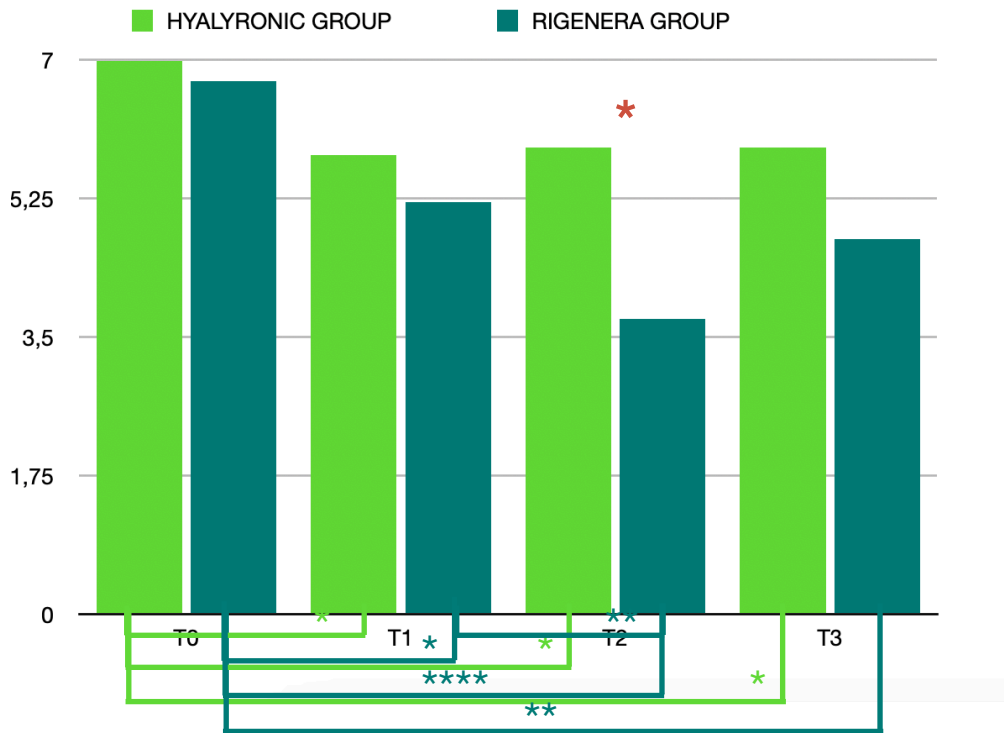
No statistical evidence was found between groups for the body condition score of 25 patients at any time, while a significant difference was noted with respect to time 0 and time 2 within both groups (Hyal: P-value 0,0050**; Rig: 0,0381 *).

BCS MEAN
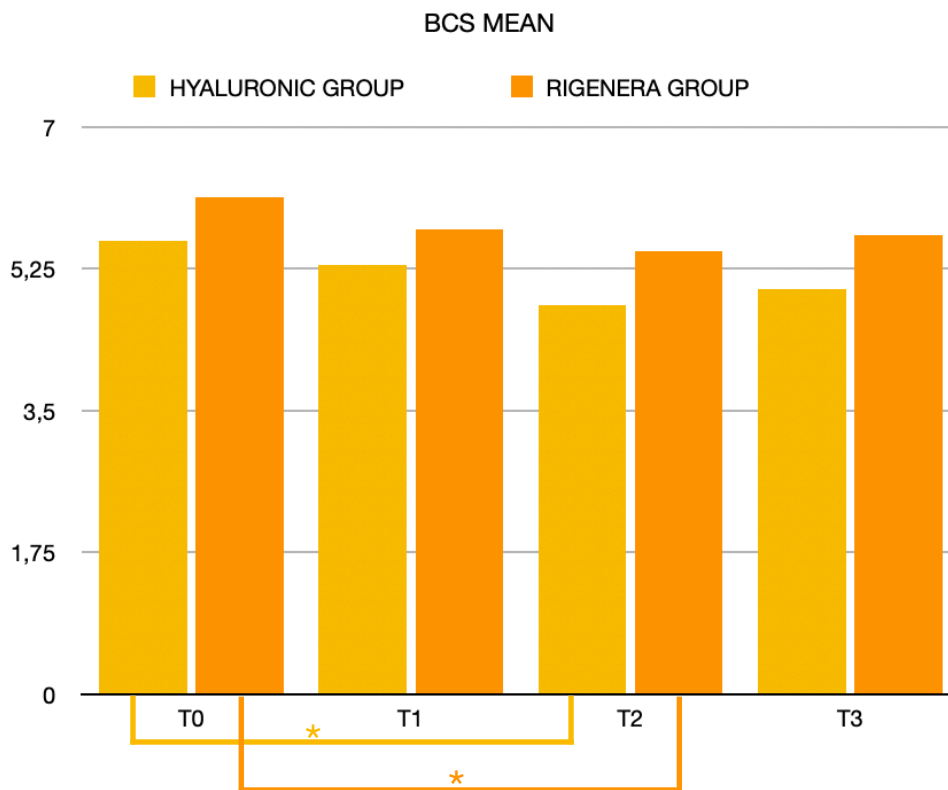
-*Canine Brief Pain Inventory Index:*

Another long-term imported result comes from the questionnaire completed by the owners. Similarly for the clinical evaluation, we collected the results of 25 of the 35 patients who completed the short study period. The summary score of the CBPI divided into the two domains is shown in the following table.

Pain Severity Domain (CBPI)			Pain Interference Domain (CBPI)		
	Rigenera Group (A)	Hyaluronic Group (B)		Rigenera Group (A)	Hyaluronic Group (B)
PATIENT	Time 3 (180 days)	Time 3 (180 days)	PATIENT	Time 3 (180 days)	Time 3 (180 days)
1	2		1	1	
2	1	23	2	1	36
3	10	18	3	15	31
4	7	10	4	13	21
5	5	20	5	2	19
6			6		
7	15	5	7	20	35
8			8		
9	6		9	10	
10	4	8	10	8	15
11	5	15	11	15	25
12	3	7	12	5	22
13	6	15	13	20	31
14	5		14	13	
15	6	5	15	18	21
16			16		
17	5		17	18	
18	4		18	12	
19			19		
20			20		
Mean	_5,6_	_12,6_	**Mean**	_11,4_	_25,6_

Table 10: raw data of CBPI after 180 days, (a) Pain Severity Index, (b) Pain Interference Index,

Statistical analysis resulting from the pain severity domain between groups has shown significant difference after 60 (P-value 0,0004 ***) and 180 days (P-value 0,0025 **), associated to a significant difference within Rigenera group between day 0 and any other days (T1 P-value 0,0281 *, T2 P-value <0,0001 ****, T3 P-value 0,0011 **).

CBPI (PAIN SEVERITY) MEAN
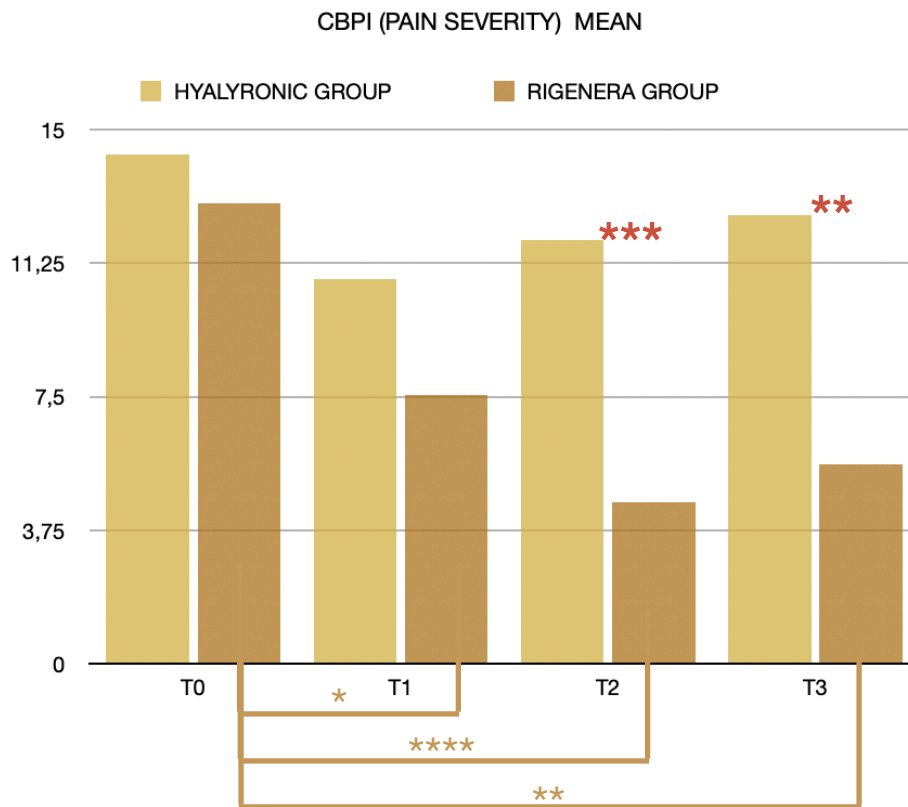
Pain interfence score resulted statistically significant different between groups at time 2 (P-value 0,0317 *) and 3 (P-value <0,0001 ****), whilst it showed a significancy within the Hyaluronic group between day 0 and 60 (P-value 0,0335 *), and within the Rigenera group between time 0 and time 2 (P-value 0,0002 ***), time 0 and time 3 (P-value <0,0001 ****), and between time 1 and time 3 (P-value 0,0179 *).

CBPI (PAIN INTERFERENCE) MEAN
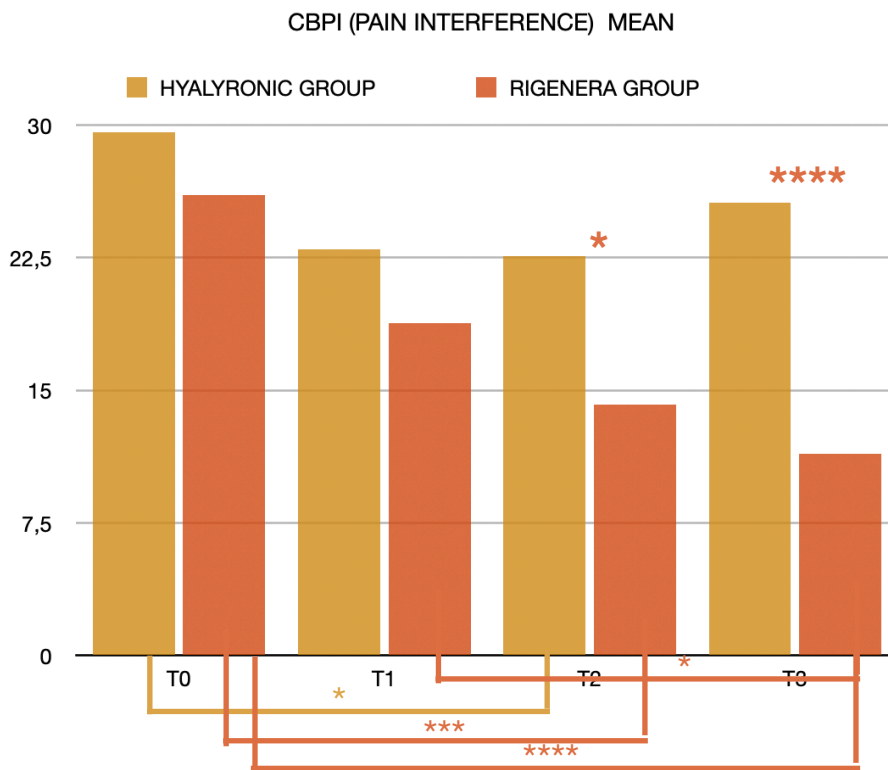
Excluded patient:

Some patients were lost during the research period, in particular, 5/40 (12.5%) in the short-term outcome (10% of the hyaluronic group and 2,5% of the Rigenera group), which rises to 15/40 (37.5%) at the long-term outcome, rispectively 25% for the hyaluronic group and 12,5% for the Rigenera group, respectively. Considering the percentage of patients who abandoned the current research due to inadequate pain control by intra-articular therapy, the Authors wanted to evaluate the presence of statistical differences in the frequency of administration of anti-inflammatories at different follow-up terms. Despite the Author's hypothesis, advanced highlighted the raw data, Fisher's exact test found no statistically significant differences between the groups.

PRE-TREATMENT PATIENT SAMPLE

LONG TERM OUTCOME PATIENT SAMPLE

SHORT TERM OUTCOME PATIENT SAMPLE
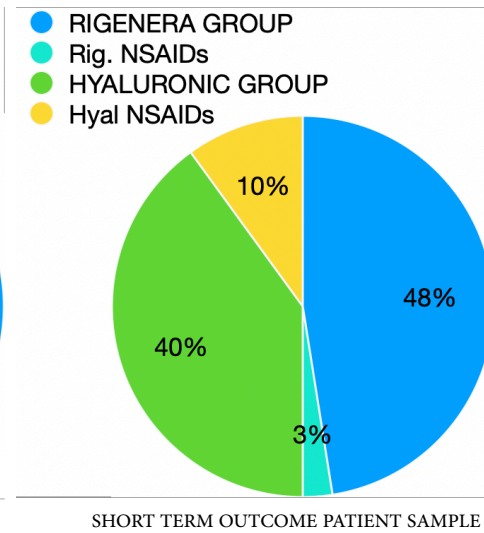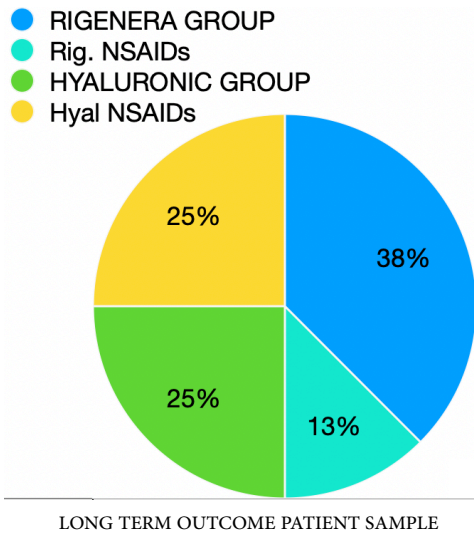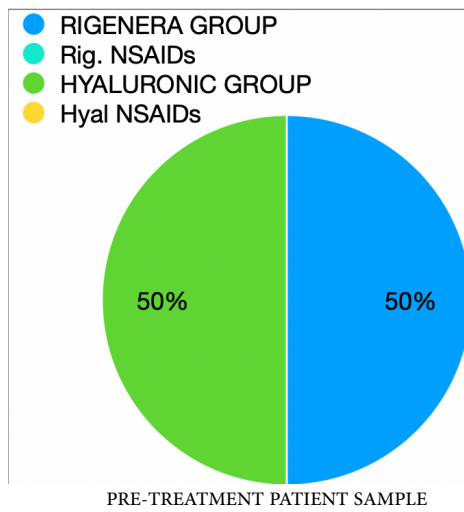
Discussion

Extensive research has been conducted in recent decades to treat the OA process, or at least to slow its progression. Degenerative joint disease is currently considered one of the World's most significant challenges for health systems. This condition places OA among the most investigated for mutual co-study of animals and humans.

The foundation of this concept is introduced on the One Health Medicine, where Human Health recognizes a close connection with Veterinary Health.

In particular, the link with the animal model becomes very close with the canine species, given the large population of these companion animals, associated with the incidence of this pathology, and the duration of the dog's life, which is equivalent in stages to that of humans, allowing a longitudinal assessment of disease and therapy in spontaneous OA. The exponential Literature increase on the Regenerative Medicine, associated with a particular focus on advances regarding stem cells, and the evaluation of the characteristics and uses of crude isolated tissue, such as bone marrow concentrate, or Stromal Vascular Fraction from adipose tissue, has recently shown an acceptable therapeutic range for many diseases, as well as for orthopedic conditions. Many Human clinical case reports have demonstrated the efficacy of intra-articular administration of purified micro-fragmented adipose tissue (MFAT) for spontaneous osteoarthritis in different joints [395-400]. As in Humans, the recent Veterinary Literature suggests evidence of clinical pain control over 180 days due to intra-articular administration of autologous purified and micro-fragmented adipose tissue to treat osteoarthritis in dogs [381;377].

The establishment of the concept of "niche matrix" and minimal mechanical manipulation of the tissue to support the gene expression pattern and the efficacy of mitigating the inflammatory cascade is likely responsible for the clinical success in the treatment with MFAT. In addition, this notion offers new frontiers for biomedical engineering, which increasingly introduces new devices that provide automated tissue disaggregation systems.[371,372,378,379,381,36]. The Rigenera® micro-grafting technology has recently been seen as a promising system for rapidly harvesting various disaggregated tissues, as a source of SVF, to be used for many different patho-

logic processes [36,17,383,385,386]. The principal use of this technology was inve-stigated on plastic surgery with an increasing trend focus on the orthopedic field. The in vitro validation of the Rigenera® system for MFAT in the canine species was obtained previously in the Ph.D research period, allowing the consequent prospective clinical evaluation in vivo.

Our study aimed to evaluate the safety, feasibility, and efficacy of the treatment of a single intra-articular infiltration of adipose micro-graft obtained with Rigenera tech-nology in the canine species, comparing, as a control group, with the performance of the single administration of hyaluronic acid, currently considered a standard of the procedure (SOP) for the management of osteoarthritis.

The results obtained from our study showed an interesting statistically significant difference in evaluated parameters, both for the short and long-term outcome (180 days), connected with a symptomatic improvement, which persevered longer for the Rigenera® treated group.

The six clinical items evaluated during each visit showed superior efficacy for the treatment with adipose micro-grafts within the patient and in the comparison bet-ween groups over 180 days. The graphical representation of the short-term clinical outcome shows a good decrease in the parameters evaluated after 30 days in both therapies, while it shows differences between treatments at the evaluation after 60 days. The clinical improvement continues and persists better for the MFAT injection; similar trends were observed between groups for long-term follow-up after 180 days, although connected to gradual increases of lameness and pain in both.

The long-term clinical outcome shows less importance in the difference between the groups, which persist only for lameness score, while shows more significant diffe-rences within the Rigenera patient. Clinical efficacy loss over time was supposed in the control group. This scenario is widely supported by Veterinary Literature, due to the temporary anti-inflammatory action of viscosupplementation, especially after a single infiltration of hyaluronic acid. Repeated intra-articular infiltrations of hyaluro-nic acid are therefore recommended by most of the authors [260-262]. However, using a single administration of HA in our study was necessary to compare the diffe-

rence between these intra-articular treatments correctly.

There were no statistical differences in circumference, range of motion, body condition score over two months for both groups. The slight significance highlighted after six months on the body condition score item in both groups between 0 and 60 days (P-value <0.05) might be probably related to the patients' inhomogeneity, altering the data.

Owners reported an improvement for their dogs on the severity of pain and its interference with quality of life in both groups, although data shows greater significance on subjects treated with purified micro-fragmented adipose tissue. Crucial differences were noticed between groups on the short-term outcome, detected on pain severity at time 2 (P<0,0001) with solid evidence of reduction of pain interference within the Rigenera group (T0-T2, P<0,0001). Similarly, the long-term outcome shows a substantial decrease in pain severity and interference on the Rigenera group supported by statistical analysis and a statistical significance for pain interference after 180 days (P<0,0001). One of the Author's main concerns was correlated to the analysis of these data, linked to the impracticality of making the owner and clinician blind for the treatment group to which the patients belong. This condition may undoubtedly have influenced the pet owners' perception of pain as a placebo effect after treatment. The same issue was encountered for the clinician's assessment, due to the lack of an analytical evaluator of lameness, as a force plate system for gait analysis. At the same time, other criticalities are related to the patient's pain assessment, which remains personal information. This effect, known as the Placebo effect, was broadly investigated in the veterinary literature and represented a considerable chunk of therapy efficacy for the dog's owner, whose ranging mid 39.7% and 79% [361,362]. Conzemius et al. report a caregiver placebo effect as expected in evaluating response to treatment for OA in both pet owners and veterinarians. This effect appears to be enhanced with time and represents 57% for the pet owner and among 40-45% for clinicians.[362].

The control group should be considered mandatory in the clinical evaluation of new therapies to mitigate the bias effect, yielding more representative results, even when

subjective evaluation data have been used. Another strategy adopted during the research period was to have the same person, both veterinarian and pet owner, complete the clinical form.

Our study's most interesting data was obtained from the cytological synovial fluid analysis. The resulting score indicates an improvement of inflammation grade for both groups, showing statistically significant differences in support of the MFAT group respect the control group at 30, strengthening after 60 days. Furthermore, the statistical analysis showed an exclusive significance within the group treated with adipose micro-grafts, enhanced after 60 days. In particular, 76.5% of patients treated with Rigenera technology improved the cytological grade at T2.

To the best of our knowledge, this is the first study appraising synovial fluid after administration of micro-fragmented adipose tissue in dogs. Cytological analysis of synovial fluid provides the qualitative state of joint health, mainly associated with prognostic purposes [189-192].

In addition to the short-term cytological evaluation, the concentration of inflammatory cytokines in the synovial fluid was evaluated. In this case, the enzyme-linked immunosorbent assay was performed on seven patients (four Rigenera and three hyaluronic) for TNFa and five patients (two Rigenera and three hyaluronic) for IL-6 who completed the analysis over 60 days. Although no statistical analysis, the data indicated a reduction in cytokine concentration in patients treated with MFAT while increasing the control group. Allen et al. show the correlation between synovial fluid cytokine increasing and OA in dogs, even without any statistical correlation of force plate outcome on lameness [190]. Thus, the predictive value of these biomarkers has not yet been clearly understood. However, it appears to be essential to compare the effect of intra-articular therapies, mainly to try to correlate and explain the clinical outcome of this treatment. The same operator carried out the cytological analysis and the ELISA test, who operated blindly, not knowing the group to which the sample belonged. This condition made it possible to essentially decrease the presence of bias in the analysis of intra-articular treatment. Radiographic assessment showed no differences between the group nor within group over the follow up. This evaluation was

in agreement with the veterinary literature on the intra-articular treatment using MFAT [377].

The entire cohort of patients, who concluded the study, had to follow a management therapy, limiting uncontrolled activities, favoring leashed walks, and avoiding nutraceuticals or NSAIDs. In addition, the patients that required administered anti-inflammatories were excluded from the research, causing inconsistencies between groups in both short and long-term outcomes. These inconsistencies have been accentuated over time. The number of excluded patients resulted very differently between treatment groups; in fact, 50% of patients in the control group needed to use NSAIDs within six months, compared to 12.5% in the MFAT group. Although there is no statistical evidence, the data obtained proved to be very interesting to support the treatment with Rigenera better. No statistically significant differences were appraised between the groups at time 0, albeit the randomization of patients, considering different sex, age, weight, and joints affected by osteoarthritis. In our study was highlighted some limitations related to the eligibility criteria. Various dog sizes and joints influenced the clinical parameters assessed by the veterinarian and the pet owner. For example, with the same osteoarthritis grade, we observed higher pain severity scores (PSS) and interference scores (PIS) in patients who demonstrated reduced weight-bearing at the station, such as those with stifles versus elbow arthropathy. These differences highlighted in our study agree with Pavarotti and colleagues. Despite having observed the control of pain and lameness in patients treated with MFAT, they failed to demonstrate significance in the kinetics gait analysis. In contrast, in their study, statistical significance was obtained exclusively by dividing the investigation by the affected joints [377].

The absence of complications related to the use of adipose micro-grafts obtained with the Rigenera technology, although considering the small sample size, is undoubtedly a promising result and in harmony with the literature [377,381, 395-400]. Besides, Zeira et al. showed very high feasibility and safety in the context of these therapies on a much more extensive sample, using intra-articular MFAT in 130 dogs affected by OA [381]. Nonetheless, further studies are needed to determine both the rege-

nerative properties and the maximum duration of positive effects. Furthermore, the actual regenerative capacity of MFATs, although demonstrated in vitro, has never been proven in vivo [370,371,372,376,378,380]. In any case, the clinical efficacy in pain control has been highlighted in several studies, both in humans and veterinary medicine [377,381, 395-400].

Although some Authors suggest poor correlations concerning inflammatory biomarker concentration and clinical assessments [190,191], cytological examination and principal inflammatory cytokines in synovial fluid, in contrast, provided significant food for thought in our study, corroborating the clinical outcome of the treatment with MFAT. The research focus could undertake future studies in proteomics to understand which regulatory activities these cellular fragments have in an inflammatory environment. Some Authors in medicine suggest using arthroscopic synovectomy combined with fat grafting in the early stages of osteoarthritis, showing very promising efficacy [396]. In recent years, moreover, during the pathogenetic research of the progression of osteoarthritis, increasing importance has been given to the synovial capsule in the joint organ [123-129]; In the next Future, this statement should consider in association with the cultural characteristics of the synovium [297]. Rigenera® technology can obtain micro-fragmented tissue from different sampling sites and, therefore, better than other devices, and it could be helpful in the investigation in this sense.

Conclusion

To the best of our knowledge, this is the first prospective blind-controlled in vivo study which evaluates the efficacy of purified micro fragmented adipose tissue in dogs. In conclusion, our results revealed intra-articular MFAT use in dogs to be safe and feasible in a single infection. Moreover, it has demonstrated superior clinical efficacy compared to the control group in both the short- and long-term outcomes concerning osteoarthritis pain and lameness, considerably reducing intra-articular inflammation. This consideration, therefore, confirms the in vivo validation of the Rigenera system in the canine species, previously evaluated in vitro.

References

1   G.K. Smith, E.R. Paster, M.Y. Powers, D.F. Lawler, D.N., Biery, F.S. Shofer, P.J. McKelvie & R.D. Kealy, "Lifelong diet restriction and radiographic evidence of osteoarthritis of the hip joint in dogs". *Journal of American Veterinary Medical Association*, 2006, 690–693.

2   M. R Lam, H.B. Lee, M.S Kim & N.S. Kim, "Surgical model of osteoarthritis secondary to medial patellar luxation in dogs". *Veterinary Medicine,* 2011, 123–130.

3   A.M. McCoy, "Animal Models of Osteoarthritis: Comparisons and Key Considerations". *Veterinary Pathology,* 2015, Vol. 52, 803-818.

4   Murphy, L. & Helmick, C. G. The impact of osteoarthritis in the United States: a population-health perspective: a population-based review of the fourth most common cause of hospitalization in U.S. adults. Orthop. Nurs. 31, 85–91 (2012).

5   March L. et al. Osteoarthritis: a serious disease. OARSI.org https://www.oarsi.org/sites/default/files/docs/2016/oarsi_white_paper_oa_serious_disease_121416_1.pd f (2016).

6   C.L. Aragon, E.H. Hofmeister & S.C. Budsberg, "Systematic review of clinical trials of treatments for osteoarthritis in dogs". *Journal of the American Veterinary Medical Association,* 2007, 514–521.

7   Hardie EM, Roe SC, Martin FR. Radiographic evidence of degenerative joint disease in geriatric cats: 100 cases (1994-1997). *J Am Vet Med Assoc.* 2002;220:628.

8   Gregory, M. H. et al. A review of translational animal models for knee osteoarthritis. Arthritis 2012, 764621 (2012).

9   Meeson RL, Todhunter RJ, Blunn G, Nuki G, Pitsillides AA. Spontaneous dog osteoarthritis - a One Medicine vision. *Nat Rev Rheumatol.* 2019;15(5):273-287. doi:10.1038/s41584-019-0202-1.

10   Cassidy, A. in Investigating Interdisciplinary Collaboration: Theory and Practice Across Disciplines (eds Frickel, S., Albert, M. & Prainsack, B.) 213–236 (Rutgers Univ. Press, 2016).

11   J.K. Rychel, "Diagnosis and treatment of osteoarthritis". *Top Companion Animal Medicine,* 2010, 20–25.

12   P.F. Lachiewicz & E.S. Soileau, "Fifteen-year survival and osteolysis associated with a modular posterior stabilized knee replacement. A concise follow-up of a previous report". *Journal of Bone and Joint Surgery of America,* 2009, 19–23.

13   W. Zhang, H. Ouyang, C.R. Dess, J. Xu, "Current research on pharmacologic and regenerative therapies for osteoarthritis". *Bone Research,* 2016, n° 15040.

14   Lee MI, Kim JH, Kwak HH, et al. A placebo-controlled study comparing the efficacy of intra-articular injections of hyaluronic acid and a novel hyaluronic acid-platelet-rich plasma conjugate in a canine model of osteoarthritis. *J Orthop Surg Res.* 2019;14(1):314.

15   Voga M, Adamic N, Vengust M, Majdic G. Stem Cells in Veterinary Medicine-Current State and Treatment Options. *Front Vet Sci.* 2020;7:278.

16   D Lodi, T. Iannitti, & B. Palmieri, "Stem cells in clinical practice: applications and warnings".*Journal of Experimental & Clinical Cancer Research,* 2011, 30-39.

17   L. Senesi, F. De Francesco, L. Farinelli, S. Manzotti, G. Gagliardi, G.F. Papalia, M. Riccio, A. Gigante, "Mechanical and Enzymatic procedures to isolate the stromal vascular fraction from adipose tissue: preliminary results". *Frontiers in cell and developmental biology,* 2019, 1-9.

18   Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol. 1966;16: 381–390.

19   Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–147.

20   Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7: 211–228.

21   Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 2004;22:625–634.

22   Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol. 2000;109:235–242.

23 Goodwin HS, Bicknese AR, Chien SN, et al. Multilineage differenti- ation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. Biol Blood Marrow Transplant. 2001; 7:581–588.

24 Kogler G, Sensken S, Airey JA, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differ- entiation po- tential. J Exp Med. 2004;200:123–135.

25 Zvaifler NJ, Marinova-Mutafchieva L, Adams G, et al. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res. 2000; 2:477–488.

26 Kuznetsov SA, Mankani MH, Gronthos S, et al. Circulating skeletal stem cells. J Cell Biol. 2001;153:1133–1140.

27 Jiang Y, Vaessen B, Lenvik T, et al. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol. 2002;30:896–904.

28 C.D. Marshall, E.A. Brett, A.L. Moore, D.C. Wan & M.T. Longaker, "In Vitro and In Vivo Osteogenic Differentiation of Human Adipose-Derived Stromal Cells. Bone Morphogenetic Proteins".*New York, NY: Humana Press,* 2019, 1-7.

29 Wagner W, Wein F, Seckinger A, et al. Comparative characteristics of mesenchymal stem cells from human bone marrow, adi- pose tissue, and umbilical cord blood. *Exp Hematol.* 2005;33(11):1402-1416.

30 P.A. Zuk, M.I.N. Zhu, H. Mizuno, J. Huang, J.W. Futrell & A.J. Katz, "Multilineage cells from human adipose tissue: implications for cell-based therapies".*Advances in Tissue Engineering,* 2001, 211–228.

31 P. Bora & A.S. Majumdar, "Adipose tissue-derived stromal vascular fraction in regenerative medicine: a brief review on biology and translation". *Stem Cell Research & Therapy,* 2017, n° 145.

32 J.A. Aronowitz, R.A. Lockhart, C.S. Hakakian, "Mechanical versus enzymatic isolation of stromal vascular fraction cells from adipose tissue", *Springer Plus* 2015, n° 713.

33 Vinatier, C.; Guicheux, J. Cartilage tissue engineering: From biomaterials and stem cells to osteoarthritis treatments. *Ann. Phys. Rehabil. Med.* 2016, *59,* 139–144.

34 Hurley, E.T.; Yasui, Y.; Gianakos, A.L.; Seow, D.; Shimozono, Y.; Kerkhoffs, G.; Kennedy, J.G. Limited evidence for adipose-deri- ved stem cell therapy on the treatment of osteoarthritis. *Knee Surg. Sports Traumatol. Arthrosc.* 2018.

35 Zeira O, Scaccia S, Pettinari L, et al. Intra-Articular Administration of Autologous Micro-Fragmented Adipose Tissue in Dogs with Spontaneous Osteoarthritis: Safety, Feasibility, and Clinical Outcomes. *Stem Cells Transl Med.* 2018.

36 L. Trovato, M. Monti, C. del Fante et al., "A new medical device Rigeneracons allows to obtain viable micro-grafts from mechan- ical disaggregation of human tissues," Journal of Cellu- lar Physiology, vol. 230, no. 10, pp. 2299–2303, 2015

37 F. Svolacchia, F. De Francesco, L. Trovato, A. Graziano, and G. A. Ferraro, "An innovative regenerative treatment of scars with dermal micrografts," Journal of Cosmetic Dermatology, vol. 15, no. 3, pp. 245–253, 2016.

38 Tremolada C, Colombo V, Ventura C. Adipose Tissue and Mesenchymal Stem Cells: State of the Art and Lipogems® Technology Development. *Curr Stem Cell Rep.* 2016.

39 Johnston S.A., "Osteoarthritis: Joint anatomy, physiology and pathobiology". *Vet Clin North Am Small Practice,* 1997, 699-723.

40 Tobias K. & Johnston S., "Veterinary Surgery: small animal".2th ed., Copyright © 2018 by Elsevier, Inc.

41 Yuan GH, Masukp-Hongo K, Kato T, *et al.* Immunologic intervention in the pathogenesis of osteoarthritis. *Arthritis Rheumatol* 2003;48:602–11.

42 Kee CC. Osteoarthritis: manageable scourge of aging. *Nurs Clin North Am* 2000;35:199–208.

43 Pfizer Animal Health proprietary market research; survey of 200 veterinarians, 1996.

44 Rimadyl A&U Study, USA: 039 DRIM 197,1999.

45 B.S. Beale, "Arthritis of dog and cat: Making the diagnosis". *Banfield Journal,* 2010, 7-15.

46 Dieppe PA, Lohmander LS. Pathogenesis and management of pain in osteoarthritis. *Lancet.* 2005;365(9463):965-973.

47 Mateescu RG, Burton-Wurster NI, Tsai K, et al. Identification of quantitative trait loci for osteoarthritis of hip joints in dogs. *Am J Vet Res.* 2008;69:1294.

48 Wilke VL, Zhang S, Evans RB, et al. Identification of chromosomal regions associated with cranial cruciate ligament rupture in a population of Newfoundlands. *Am J Vet Res*. 2009;70:1013.

49 Zhu L, Zhang Z, Feng F, et al. Single nucleotide polymorphisms refine QTL intervals for hip joint laxity in dogs. *Anim Genet*. 2008;39:141.

50 Zhu L, Zhang ZW, Friedenberg S, et al. The long (and winding) road to gene discovery for canine hip dysplasia. *Vet J*. 2009;181:97.

51 Kellgren JH, Bier F, Lawrence JS. Genetic factors in generalized osteoarthrosis. *Ann Rheum Dis*. 1963;22:237.

52 Valdes AM, Doherty M, Spector TD. The additive effect of individual genes in predicting risk of knee osteoarthritis. *Ann Rheum Dis*. 2008;67:124.

53 Holderbaum D, Haqqi TM, Moskowitz RW. Genetics and osteoarthritis: exposing the iceberg. Arthritis Rheum. 1999;42:397.

54 Evangelou E, Chapman K, Meulenbelt I, et al. Large-scale analysis of association between GDF5 and FRZB variants and osteoarthritis of the hip, knee, and hand. *Arthritis Rheum*. 2009;60:1710.

55 Vaes RBA, Rivadeneira F, Kerkhof JM, et al. Genetic variation in the GDF5 region is associated with osteoarthritis, height, hip axis length and fracture risk: The Rotterdam study. *Ann Rheum Dis*. 2009;68:1754.

56 Valdes AM, Spector TD, Doherty S, et al. Association of the DVWA and GDF5 polymorphisms with osteoarthritis in UK populations. *Ann Rheum Dis*. 2009;68:1916.

57 Sandell, L. J. Etiology of osteoarthritis: genetics and synovial joint development. *Nat. Rev. Rheumatol.* 8, 77–89 (2012).

58 Sánchez-Molano, E. et al. Quantitative trait loci mapping for canine hip dysplasia and its related traits in UK Labrador Retrievers. *BMC Genomics* 15, 833 (2014).

59 Feldman, G. J., Parvizi, J., Sawan, H., Erickson, J. A. & Peters, C. L. Linkage mapping and whole exome sequencing identify a shared variant in CX3CR1 in a large multi-generation family. *J. Arthroplasty* 29, 238–241 (2014).

60 Loeser RF, Shanker G, Carlson CS, et al. Reduction in the chondrocyte response to insulin-like growth factor 1 in aging and osteoarthritis: Studies in a non-human primate model of naturally occurring disease. *Arthritis Rheum*. 2000;43:2110.

61 Martin JA, Buckwalter JA. Aging, articular cartilage chondrocyte senescence and osteoarthritis. Biogerontology. 2002;3:257.

62 Martin JA, Buckwalter JA. The role of chondrocyte senescence in the pathogenesis of osteoarthritis and in limiting cartilage repair. *J Bone Joint Surg Am*. 2003;85:106

63 DeGroot J, Verzijl N, Wenting-Van Wijk MJG, et al. Age-related decrease in susceptibility of human articular cartilage to matrix metalloproteinase-mediated degradation: The role of advanced glycation end products. *Arthritis Rheum*. 2001;44:2562.

64 Verzijl N, DeGroot J, Thorpe SR, et al. Effect of collagen turnover on the accumulation of advanced glycation end products. *J Biol Chem*. 2000;275:39027.

65 Innes J, Sharif M, Barr A. Relations between biochemical markers of osteoarthritis and other disease parameters in a population of dogs with naturally acquired osteoarthritis of the genual joint. *Am J Vet Res*. 1998;59:1530.

66 Innes JF, Bacon D, Lynch C, et al. Long-term outcome of surgery for dogs with cranial cruciate ligament deficiency. *Vet Rec*. 2000;147:325.

67 Van Hagen MAE, Ducro BJ, van den Broek J, et al. Incidence, risk factors, and heritability estimates of hind limb lameness caused by hip dysplasia in a birth cohort of Boxers. *Am J Vet Res*. 2005;66:307.

68 Whitehair JG, Vasseur PB, Willits NH. Epidemiology of cranial cruciate ligament rupture in dogs. J Am Vet Med Assoc. 1993;203:1016.

69 Lauten SD. Nutritional risks to large-breed dogs: From weaning to the geriatric years. *Vet Clin North Am Small Anim Pract*. 2006;36:1345.

70 Lawler DF, Larson BT, Ballam JM, et al. Diet restriction and ageing in the dog: Major observations over two decades. *Br J Nutr*. 2008;99:793.

71 Kealy RD, Lawler DF, Ballam JM, et al. Five-year longitudinal study on limited food consumption and development of osteoarthritis in coxofemoral joints of dogs. *J Am Vet Med Assoc*. 1997;210:222.

72 Smith GK, Paster ER, Powers MY, et al. Lifelong diet restriction and radiographic evidence of osteoarthritis of the hip joint in dogs. *J Am Vet Med Assoc*. 2006;229:690.

73 Runge JJ, Biery DN, Lawler DF, et al. The effects of lifetime food restriction on the development of osteoarthritis in the canine shoulder. *Vet Surg*. 2008;37:102.

74 Huck JL, Biery DN, Lawler DF, et al. A longitudinal study of the influence of lifetime food restriction on development of osteoarthritis in the canine elbow. *Vet Surg*. 2009;38:192.

75 Felson DT, Goggins J, Niu JB, et al. The effect of body weight on progression of knee osteoarthritis is dependent on alignment. *Arthritis Rheum*. 2004;50:3904.

76 Sharma J, Lou C, Cahue S, et al. The mechanism of the effect of obesity in knee osteoarthritis: The mediating role of malalignment. *Arthritis Rheum*. 2000;43:568.

77 Muller M, Kersten S. Nutrigenomics: goals and strategies. *Nature Rev* 2003;4:315–22.

78 Vester BM, Swanson K. Nutrient-gene interactions: application to pet nutrition and health. *Vet Focus* 2007;17:25– 32.

79 German AJ, Hervera A, Hunter L, et al. Improvement in insulin resistance and reduction in plasma inflammatory adipokines after weight loss in obese dogs. *Domest Anim Endocrinol*. 2009;37:214.

80 Eisele I, Wood IS, German AJ, *et al.* Adipokine gene expression in dog adipose tissues and dog white adipocytes differentiated in primary culture. *Horm Metabol Res* 2005;37:474–81.

81 Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutrition* 2004;92:347–55.

82 Impellizeri JA, Tetrick MA, Muir P. Effect of weight reduction on clinical signs of lameness in dogs with hip osteoarthritis. *JAVMA* 2000;216(7):1089–91.

83 Lang J, Busato A, Baumgartner D, et al. Comparison of two classification protocols in the evaluation of elbow dysplasia in the dog. *J Small Anim Pract*. 1998;39:169.

84 Ohlerth S, Busato A, Gaillard C, et al. Epidemiologic and genetic study of canine hip dysplasia in a colony of Labrador Retrievers. *Dtsch Tierarztl Wochenschr*. 1998;105:378.

85 Slauterbeck JR, Pankratz K, Xu KT, et al. Canine ovariohysterectomy and orchiectomy increases the prevalence of ACL injury. *Clin Orthop Relat Res*. 2004;429:301.

86 Whitehair JG, Vasseur PB, Willits NH. Epidemiology of cranial cruciate ligament rupture in dogs. J Am Vet Med Assoc. 1993;203:1016.

87 Lark MW, Bayne EK, Flanagan J, et al. Aggrecan degra dation in human cartilage: Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. *J Clin Invest*. 1997;100:93.

88 Hegemann N, Wondimu A, Kohn B, et al. Cytokine profile in canine immune-based polyarthritis and osteoarthritis. Vet Comp Orthop Traumatol. 2005;18:67-72.

89 Lorenz H, Richter W. Osteoarthritis: Cellular and molecular changes in degenerating cartilage. *Prog Histochem Cytochem*. 2006;40:135.

90 Maccoux LJ, Salway F, Day PJR, et al. Expression profiling of select cytokines in canine osteoarthritis tissues. *Vet Immunol Immunopathol*. 2007;118:59.

91 Innes JF, Shepstone L, Holder J, et al. Changes in the canine femoropatellar joint space in the postsurgical, cruciate-deficient stifle joint. *Vet Radiol Ultrasound*. 2002;43:241.

92 Adams ME, Brandt KD. Hypertrophic repair of canine articular cartilage in osteoarthritis after anterior cruciate ligament transection. *J Rheumatol*. 1991;18:428.

93 Brandt KD, Braunstein EM, Visco DM, et al. Anterior (cranial) cruciate ligament transection in the dog—A bona-fide model of osteoarthritis, not merely of cartilage injury and repair. *J Rheumatol*. 1991;18:436.

94 Fujita Y, Hara Y, Nezu Y, et al. Proinflammatory cytokine activities, matrix metalloproteinase-3 activity, and sulfated glycosaminoglycan content in synovial fluid of dogs with naturally acquired cranial cruciate ligament rupture. *Vet Surg*. 2006;35:369.

95 de Boer TN, Huisman AM, Polak AA, et al. The chondroprotective effect of selective COX-2 inhibition in osteoarthritis: Ex vivo evaluation of human cartilage tissue after in vivo treatment. *Osteoarthritis Cartilage*. 2009;17:482.

96   Shimpo H, Sakai T, Kondo S, et al. Regulation of prostaglandin E_2 synthesis in cells derived from chondrocytes of patients with osteoarthritis. *J Orthop Sci*. 2009;14:611.

97   Attur M, Al-Mussawir HE, Patel J, et al. Prostaglandin E-2 exerts catabolic effects in osteoarthritis cartilage: Evidence for signaling via the EP4 receptor. *J Immunol*. 2008;181:5082.

98   Mankin HJ, Brandt KD. Pathogenesis of osteoarthritis. In: Kelly WN, Harris ED, Ruddy S, Sledge CB (eds). *Textbook of Rheumatology*, edn 5. WB Saunders, Philadelphia, 1997, p.1369.

99   Setton LA, Elliott DM, Mow VC. Altered mechanics of cartilage with osteoarthritis: Human osteoarthritis and an experimental model of joint degeneration. *Osteoarthritis Cartilage*. 1999;7:2–14.

100   Radin EL, Paul IL, Lowry M. A comparison of the dynamic force transmitting properties of subchondral bone and articular cartilage. *J Bone Joint Surg Am* 1970;52:444–56.

101   Radin EL, Paul IL. Does cartilage reduce skeletal impact loads? The relative force-attenuating properties of articular cartilage, synovial fluid, periarticular soft tissues, and bone. *Arthritis Rheumatol* 1970;13:139–44.

102   Henrotin Y, Labasse A, Jaspar JM, *et al.* Effects of three avocado/soybean unsaponifiable mixtures on metalloproteinases, cytokines and prostaglandin E2 production by human articular chondrocytes. *Clin Rheumatol* 1998;17:31–9.

103   Hickery MS, Palmer RM, Charles IG, *et al.* The role of nitric oxide in IL-1 and TNF alpha-induced inhibition of proteoglycan synthesis in human articular cartilage. *Trans Orthop Res Soc* 1994;19:77.

104   Webb AA, Taylor SM, Muir GD. Steroid-responsive meningitis arteritis in dogs with noninfectious, nonerosive, idiopathic, immune-mediated polyarthritis. *J Vet Intern Med*. 2002;16:269.

105   Tortorella MD, Malfait AM, Deccico C, et al. The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. *Osteoarthritis Cartilage*. 2001;9:539.

106   Hardingham TE, Fosang AJ. The structure of aggrecan and its turnover in cartilage. *J Rheumatol*. 1995;22:86.

107   Innes JF, Little CB, Hughes CE, et al. Products resulting from cleavage of the interglobular domain of aggrecan in samples of synovial fluid collected from dogs with early- and late-stage osteoarthritis. *Am J Vet Res*. 2005;66:1679.

108   Fosang AJ, Little CB. Drug insight: Aggrecanases as therapeutic targets for osteoarthritis. *Nat* Clin Pract Rheumatol. 2008;4:420.

109   Glasson SS, Askew R, Sheppard B, et al. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature*. 2005;434:644.

110   Stanton H, Rogerson FM, East CJ, et al. ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. *Nature*. 2005;434:648.

111   Murphy G, Nagase H. Progress in matrix metalloproteinase research. *Mol Aspects Med*. 2008;29:290.

112   Billinghurst RC, Wu W, Ionescu M, et al. Comparison of the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1 and the selective inhibition of type II collagen cleavage by collagenase. *Arthritis Rheum*. 2000;43:664.

113   Davidson RK, Waters JG, Kevorkian L, et al. Expression profiling of metalloproteinases and their inhibitors in synovium and cartilage. *Arthritis Res Ther*. 2006;8:R124.

114   Posey KL, Hecht JT. The role of cartilage oligomeric matrix protein (COMP) in skeletal disease. Curr Drug Targets. 2008;9:869.

115   Halasz K, Kassner A, Morgelin M, et al. COMP acts as a catalyst in collagen fibrillogenesis. *J Biol Chem*. 2007;282:31166.

116   Saxne T, Heinegard D. Cartilage oligomeric matrix protein: A novel marker of cartilage turnover detectable in synovial fluid and blood. *Br J Rheumatol*. 1992;31:583.

117   Sharif M, Saxne T, Shepstone L, et al. Relationship between serum cartilage oligomeric matrix protein levels and disease progression in osteoarthritis of the knee joint. *Br J Rheumatol*. 1995;34:306.

118   Carlson CS, Guilak F, Vail TP, et al. Synovial fluid biomarker levels predict articular cartilage damage following complete medial meniscectomy in the canine knee. *J Orthop Res*. 2002;20:92.

119   Girling SL, Bell SC, Whitelock RG, et al. Use of biochemical markers of osteoarthritis to investigate the potential disease-modifying effect of tibial plateau levelling osteotomy. *J Small Anim Pract*. 2006;47:708.

120 Misumi K, Vilim V, Carter SD, et al. Concentrations of cartilage oligomeric matrix protein in dogs with naturally developing and experimentally induced arthropathy. *Am J Vet Res*. 2002;63:598.

121 Fernihough JK, Innes JF, Billingham MEJ, et al. Changes in the local regulation of insulin-like growth factors I and II and insulin-like growth factor-binding proteins in osteoarthritis of the canine stifle joint secondary to cruciate ligament rupture. *Vet Surg*. 2003;32:313.

122 Davidson ENB, van der Kraan PM, van den Berg WB. TGF-beta and osteoarthritis. *Osteoarthritis Cartilage*. 2007;15:597.

123 Bondeson J. Activated synovial macrophages as targets for osteoarthritis drug therapy. *Curr Drug Targets*. 2010;11:576.

124 Bondeson J, Lauder S. Osteoarthritis synovial macrophages regulate the production of proinflammatory cytokines and matrix metalloproteinases via interleukin-1 and tumor necrosis factor alpha. *Arthritis Rheum*. 2004;50:S279.

125 Lust G, Summers BA. Early, asymptomatic stage of degenerative joint disease in canine hip joints. *Am J Vet Res* 1981;42:1849–55.

126 Pelletier JP, Martel-Pelletier J, Ghandur-Mnaymneh L, *et al*. Role of synovial membrane inflammation in cartilage matrix breakdown in the Pond–Nuki dog model of osteoarthritis. *Arthritis Rheumatol* 1985;28:554–61.

127 Brandt KD, Myers SL, Burr D, et al. Osteoarthritic changes in canine articular cartilage, subchondral bone, and synovium fifty-four months after transection of the anterior cruciate ligament. *Arthritis Rheum*. 1991;34:1560.

128 Hewicker-Trautwein M, Carter SD, Bennett D, et al. Immunocytochemical demonstration of lymphocyte subsets and MHC class II antigen expression in synovial membranes from dogs with rheumatoid arthritis and degenerative joint disease. *Vet Immunol Immunopathol*. 1999;67:341.

129 Lemburg AK, Meyer-Lindenberg A, Hewicker-Trautwein M. Immunohistochemical characterization of inflammatory cell populations and adhesion molecule expression in synovial membranes from dogs with spontaneous cranial cruciate ligament rupture. *Vet Immunol Immunopathol*. 2004;97:231.

130 van der Kraan PM, van den Berg WB. Osteophytes: Relevance and biology. *Osteoarthritis Cartilage*. 2007;15:237.

131 Vanbeuningen HM, Vanderkraan PM, Arntz OJ, et al. Transforming growth factor-beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. *Lab Invest*. 1994;71:279.

132 Sniekers YH, Intema F, Lafeber F, et al. A role for subchondral bone changes in the process of osteoarthritis: A micro-CT study of two canine models. *BMC Musculoskelet Disord*. 2008;9:20.

133 Dedrick DK, Goldstein SA, Brandt KD, et al. A longitudinal study of subchondral plate and trabecular bone in cruciate-deficient dogs with osteoarthritis followed up for 54 months. *Arthritis Rheum*. 1993;36:1460.

134 Intema F, Hazewinkel H, Gouwens D, et al. In early OA, thinning of the subchondral plate is directly related to cartilage damage: Results from a canine ACLT-meniscectomy model. Osteoarthritis Cartilage. 2010;18:691.

135 Little C. Update on matrix degradation: Role of matrix degradation in OA. *World Congress on Osteoarthritis*. 2009:S6 [Montreal, Quebec].

136 Moldovan F, Pelletier JP, Hambor J, *et al*. Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor beta. *Arthritis Rheum* 1997;40:1653–61.

137 Busso N, Peclat V, So A, Sappino AP. Plasminogen acti- vation in synovial tissues: differences between normal, osteoarthritis, and rheumatoid arthritis joints. Ann Rheum Dis 1997;56:550-7.

138 Levick JR, McDonald JN. Fluid movement across syno- vium in healthy joints: role of synovial fluid macromol- ecules. Ann Rheum Dis 1995;54:417-23.

139 Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis 2005;64:1263-7.

140 Haywood L, McWilliams DF, Pearson CI et al. Inflammation and angiogenesis in osteoarthritis. Arthritis Rheum 2003;48:2173-7.

141 Smith MD, Triantafillou S, Parker A, Youssef PP, Coleman M. Synovial membrane inflammation and cytokine pro- duction in patients with early osteoarthritis. J Rheumatol 1997;24:365-71.

142 de Lange-Brokaar BJ, Kloppenburg M, Andersen SN et al. Characterization of synovial mast cells in knee osteoarth- ritis: asso- ciation with clinical parameters. Osteoarthritis Cartilage 2016;24:664-71.

143 Fowlkes V, Wilson CG, Carver W, Goldsmith EC. Mechanical loading promotes mast cell degranulation via RGD-integrin dependent pathways. J Biomech 2013;46:788-95.

144 Loeuille D, Chary-Valckenaere I, Champigneulle J, Rat AC, Toussaint F, Pinzano-Watrin A, Goebel JC, Mainard D, Blum A, Pourel J, Netter P, Gillet P. Macroscopic and microscopic features of synovial membrane inflammation in the osteoarthritic knee: correlating magnetic resonance imaging findings with disease severity. Arthritis Rheum. 2005 Nov;52(11):3492-501.

145 Bonde HV, Talman ML, Kofoed H. The area of the tidemark in osteoarthritis--a three-dimensional stereological study in 21 patients. APMIS. 2005 May;113(5):349-52. doi: 10.1111/j.1600-0463.2005.apm_113506.x. PMID: 16011661.

146 Fox SM. Chronic Pain in Small Animal Medicine. Manson Publishing, London, 2009.

147 Dye SF, Vaupel GL, Dye CC. Conscious neurosensory mapping of the internal structures of the human knee without intraarticular anesthesia. *Am J Sports Med*. 1998;26:773.

148 Kellgren JH, Samuel EP. The sensitivity and innervation of the articular capsule. *J Bone Joint Surg Br*. 1950;32:84.

149 Grigg P, Schaible HG, Schmidt RF. Mechanical sensitivity of group III and group IV afferents from posterior articular nerve in normal and inflamed cat knee. *J Neurophysiol*. 1986;55:635.

150 Schaible HG, Schmidt RF. Effects of an experimental arthritis on the sensory properties of fine articular afferent units. *J Neurophysiol*. 1985;54:1109.

151 Schaible HG, Schmidt RF. Time course of mechanosensitivity changes in articular afferents during a developing experimental arthritis. *J Neurophysiol*. 1988;60:2180.

152 Schaible HG, Schmidt RF. Activation of group III and group IV sensory units in medial articular nerve by local mechanical stimulation of knee joint. *J Neurophysiol*. 1983;49:35.

153 Schaible HG, Schmidt RF. Responses of fine medial articular nerve afferents to passive movements of knee joint. *J Neurophysiol*. 1983;49:1118.

154 Schaible HG, Richter F, Ebersberger A, et al. Joint pain. *Exp Brain Res*. 2009;196:153.

155 Inglis JJ, Notley CA, Essex D, et al. Collagen-induced arthritis as a model of hyperalgesia. *Arthritis Rheum*. 2007;56:4015.

156 Menetrey D, Besson JM. Electrophysiological characteristics of dorsal horn cells in rats with cutaneous inflammation resulting from chronic arthritis. *Pain*. 1982;13:343.

157 Neugebauer V, Schaible HG. Evidence for a central component in the sensitization of spinal neurons with joint input during development of acute arthritis in cat's knee. *J Neurophysiol*. 1990;64:299.

158 Samad TA, Moore KA, Sapirstein A, et al. Interleukin-1 beta-mediated induction of COX-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature*. 2001;410:471.

159 Veiga APC, Duarte IDG, Avila MN, et al. Prevention by celecoxib of secondary hyperalgesia induced by formalin in rats. *Life Sci*. 2004;75:2807.

160 Sluka KA, Jordan HH, Westlund KN. Reduction in joint swelling and hyperalgesia following posttreatment with a non-NMDA glutamate receptor antagonist. *Pain*. 1994;59:95.

161 Fiorentino PM, Tallents RH, Miller JNH, et al. Spinal interleukin-1 beta in a mouse model of arthritis and joint pain. *Arthritis Rheum*. 2008;58:3100.

162 Pelletier JP, Boileau C, Brunet J, et al. The inhibition of subchondral bone resorption in the early phase of experimental dog osteoarthritis by licofelone is associated with a reduction of MMP-13 and cathepsin K synthesis. *Ann Rheum Dis*. 2004;63:145

163 Pelletier JP, Lajeunesse D, Hilal G, et al. Carprofen reduces the structural changes and the abnormal subchondral bone metabolism of experimental osteoarthritis. *Osteoarthritis Cartilage*. 1999;7:327.

164 Kouri JB, Aguilera JM, Reyes J, et al. Apoptotic chondrocytes front osteoarthrotic human articular cartilage and abnormal calcification of subchondral bone. *J Rheumatol*. 2000;27:1005.

165 Aigner T, Soeder S, Gebhard PM, et al. Mechanisms of disease: Role of chondrocytes in the pathogenesis of osteoarthritis—Structure, chaos and senescence. *Nat Clin Pract Rheumatol*. 2007;3:391.

166 Jovanovic DV, Mineau F, Notoya K, et al. Nitric oxide induced cell death in human osteoarthritic synoviocytes is mediated by tyrosine kinase activation and hydrogen peroxide and/or superoxide formation. *J Rheumatol*. 2002;29:216

167 Holton L, Reid J, Scott EM, *et al.* Development of a behaviour-based scale to measure acute pain in dogs. *Vet Rec* 2001;28:525–31.

168 Brown DC, Boston RC, Coyne JC, et al. Ability of the Canine Brief Pain Inventory to detect response to treatment in dogs with osteoarthritis. *J Am Vet Med Assoc*. 2008;233:1278.

169 Hercock CA, Pinchbeck G, Giejda A, et al. Validation of a client-based clinical metrology instrument for the evaluation of canine elbow osteoarthritis. *J Small Anim Pract*. 2009;50:266.

170 Hielm-Bjorkman AK, Kuusela E, Liman A, et al. Evaluation of methods for assessment of pain associated with chronic osteoarthritis in dogs. *J Am Vet Med Assoc*. 2003;222:1552.

171 Hudson JT, Slater MR, Taylor L, et al. Assessing repeatability and validity of a visual analogue scale questionnaire for use in assessing pain and lameness in dogs. *Am J Vet Res*. 2004;65:1634.

172 Innes JF, Costello M, Barr FJ, et al. Radiographic progression of osteoarthritis of the canine stifle joint: A prospective study. *Vet Radiol Ultrasound*. 2004;45:143.

173 Rayward RM, Thomson DG, Davies JV, et al. Progression of osteoarthritis following TPLO surgery: A prospective radiographic study of 40 dogs. *J Small Anim Pract*. 2004;45:92.

174 Vasseur PB, Johnson AL, Budsberg SC, et al. Randomized, controlled trial of the efficacy of carprofen, a nonsteroidal anti-inflammatory drug, in the treatment of osteoarthritis in dogs. *J Am Vet Med Assoc*. 1995;206:807.

175 Schaefer SL, Forrest LJ. Magnetic resonance imaging of the canine shoulder: An anatomic study. *Vet Surg*. 2006;35:721.

176 Barrett E, Barr F, Owen M, et al. A retrospective study of the MRI findings in 18 dogs with stifle injuries. *J Small Anim Pract*. 2009;50:448.

177 Naughton JF, Stewart MC, Clobanu L, et al. Contrast magnetic resonance imaging for measurement of cartilage glycosaminoglycan content in dogs: A pilot study. *Vet Comp Orthop Traumatol*. 2013;2:100–104.

178 Tivers MS, Mahone P, Corr SA. Canine stifle positive contrast computed tomography arthrography for assessment of caudal horn meniscal injury: A cadaver study. *Vet Surg*. 2008;37:269.

179 Tivers MS, Mahoney PN, Baines EA, et al. Diagnostic accuracy of positive contrast computed tomography arthrography for the detection of injuries to the medial meniscus in dogs with naturally occurring cranial cruciate ligament insufficiency. *J Small Anim Pract*. 2009;50:324.

180 Geels JJ, Roush JK, Hoskinson JJ, et al. Evaluation of an intracapsular technique for the treatment of cranial cruciate ligament rupture: Clinical, radiographic, scintigraphic and force plate analysis findings in 20 dogs. *Vet Comp Orthop Traumatol*. 2000;13:197.

181 Innes JF, Barr ARS, Patteson MW, et al. Scintigraphy in the evaluation of osteoarthritis of the canine stifle joint: Relationship with clinical, radiographic and sur gical observations. *Vet Comp Orthop Traumatol*. 1996;9:53.

182 McAlindon TE, Watt I, McCrae F, et al. Magnetic resonance imaging in osteoarthritis of the knee: Correlation with radiographic and scintigraphic findings. *Ann Rheum Dis*. 1991;50:14.

183 Dieppe P, Cushnaghan J, Young P, et al. Prediction of the progression of joint space narrowing in osteoarthritis of the knee by bone scintigraphy. *Ann Rheum Dis*. 1993;52:557.

184 Fitzpatrick N, Smith TJ, Evans RB, et al. Radiographic and arthroscopic findings in the elbow joints of 263 dogs with medial coronoid disease. *Vet Surg*. 2009;38:213.

185 Glenn RE, McCarty EC, Potter HG, et al. Comparison of fresh osteochondral autografts and allografts: a canine model. *Am J Sports Med*. 2006;34:1084.

186 Clements, D. (2006). Arthrocentesis and synovial fluid analysis in dogs and cats. *In Pract* 28: 256–262.

187 *Veterinary Cytology*, First Edition. Edited by Leslie C. Sharkey, M. Judith Radin, and Davis Seelig. © 2021 John Wiley & Sons, Inc. Published 2021 by John Wiley & Sons, Inc.

188 MacWilliams, P.S. and Friedrichs, K.R. (2003). Laboratory evaluation and interpretation of synovial fluid. *Vet Clin Small Anim Pract* 33: 153–178.

189   Muir P, Hans EC, Racette M, Volstad N, Sample SJ, Heaton C, Holzman G, Schaefer SL, Bloom DD, Bleedorn JA, Hao Z, Amene E, Suresh M, Hematti P. Autologous Bone Marrow-Derived Mesenchymal Stem Cells Modulate Molecular Markers of Inflammation in Dogs with Cruciate Ligament Rupture. PLoS One. 2016 Aug 30;11(8):e0159095. doi: 10.1371/journal.pone.0159095. PMID: 27575050; PMCID: PMC5005014.

190   Allen PI, Conzemius MG, Evans RB, Kiefer K. Correlation between synovial fluid cytokine concentrations and limb function in normal dogs and in dogs with lameness from spontaneous osteoarthritis. Vet Surg. 2019 Jul;48(5):770-779. doi: 10.1111/vsu.13212. Epub 2019 Apr 29. PMID: 31032990.

191   Kleine SA, Gogal RM, George C, Thaliath M, Budsberg SC. Elevated Synovial Fluid Concentration of Monocyte Chemoattractant Protein-1 and Interleukin-8 in Dogs with Osteoarthritis of the Stifle. Vet Comp Orthop Traumatol. 2020 Mar;33(2):147-150. doi: 10.1055/s-0039-3399525. Epub 2019 Nov 22. PMID: 31756750.

192   Kleine SA, Gogal RM, Krunkosky TM, Sanderson SL, George C, Norton M, Budsberg SC. Resistin concentrations in serum and stifle synovial fluid from normal and cruciate deficient dogs with osteoarthritis. Vet Surg. 2020 May 29. doi: 10.1111/vsu.13450. Epub ahead of print. PMID: 32472596.

193   Kealy RD, Lawler DF, Ballam JM, et al. Evaluation of the effect of limited food consumption on radiographic evidence of osteoarthritis in dogs. JAVMA 2000;217(11):1678–80.

194   Wander RC, Hall JA, Gradin JL, et al. The ratio of dietary (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation, and vitamin E status in aged dogs. J Nutr 1997;127:1198–205.

195   Clinician's UpdateTM, Supplement to NAVC Clinician's Brief®. April 2005.

196   Budsberg SC, Bartges JW. Nutrition and osteoarthritis in dogs: Does it help? Vet Clin North Am Small Anim Pract. 2006;36:1307.

197   Messier SP. Obesity and osteoarthritis: Disease genesis and nonpharmacologic weight management. Rheum Dis Clin North Am. 2008;34:713.

198   Felson DT, Lawrence RC, Hochberg MC, et al. Osteoarthritis—New insights: Part 2. Treatment approaches. Ann Intern Med. 2000;133:726.

199   Dobenecker B, De Bock M, Engelen M, et al. Effect of mitratapide on body composition, body measurements and glucose tolerance in obese Beagles. Vet Res Commun. 2009;33:839

200   Vandaele T. Treatment of obesity in the dog: Mitratapide blocks the resorption of triglycerides. Point Vet. 2007;38:16.

201   Gossellin J, Wren JA, Sunderland SJ. Canine obesity: An overview. J Vet Pharmacol Ther. 2007;30:1.

202   Beraud R, Moreau M, Lussier B. Effect of exercise on kinetic gait analysis of dogs afflicted by osteoarthritis. Vet Comp Orthop Traumatol. 2010;23:87.

203   Roush JK, Dodd CE, Fritsch DA, et al. Multicenter veterinary practice assessment of the effects of omega-3 fatty acids on osteoarthritis in dogs. J Am Vet Med Assoc. 2010;236:59.

204   Hochberg MC, Altman RD, Brandt KD, et al. Design and conduct of clinical trials in osteoarthritis: Preliminary recommendations from a task force of the Osteoarthritis Research Society. J Rheumatol. 1997;24:792.

205   Creamer P, Hunt M, Dieppe P. Pain mechanisms in osteoarthritis of the knee: Effect of intra- articular anaesthetic. Br J Rheumatol. 1995;34(Suppl 2):78.

206   Warner TD, Mitchell JA. Cyclooxygenase-3 (COX-3): filling in the gaps toward a COX continuum? Proc Natl Acad Sci USA 2002;99:13371–3.

207   Xie WL, Chipman JG, Robertson DL, et al. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by messenger-RNA splicing. Proc Natl Acad Sci USA. 1991;88:2692.

208   Lascelles BDX, King S, Roe S, et al. Expression and activity of COX-1 and 2 and 5-LOX in joint tissues from dogs with naturally occurring coxofemoral joint osteoarthritis. J Orthop Res. 2009;27:1204.

209   Garcia Vicuna R, Diaz Gonzalez F, Gonzalez Alvaro I, et al. Prevention of cytokine-induced changes in leukocyte adhesion receptors by nonsteroidal antiinflammatory drugs from the oxicam family. Arthritis Rheum. 1997;40:143.

210   Miranda HF, Prieto JC, Pinardi G. Spinal synergy between nonselective cyclooxygenase inhibitors and morphine antinociception in mice. Brain Res. 2005;1049:165.

211 Southall MD, Michael RL, Vasko MR. Intrathecal NSAIDs attenuate inflammation-induced neuropeptide release from rat spinal cord slices. *Pain*. 1998;78:39.

212 Bertolini A, Ferrari A, Ottani A, et al. Paracetamol: new vistas of an old drug. *CNS Drug Rev*. 2006;12:250.

213 Palmoski M, Brandt K. In vivo effects of aspirin on canine osteoarthritic cartilage. *Arthritis Rheum*. 1983;26:994.

214 Innes J, Clayton J, Lascelles BDX. Review of the safety and efficacy of long-term NSAID use in the treatment of canine osteoarthritis. *Vet Rec*. 2010;166:226.

215 Monteiro-Steagall BP, Steagall PVM, Lascelles BDX. Systematic review of nonsteroidal anti- inflammatory drug-induced adverse effects in dogs. *J Vet Intern Med*. 2013;27:1011–1019.

216 Millis DL. A multimodal approach to treating osteoarthritis. 2006 Western Veterinary Conference Symposium Proceedings (comparing relative efficacy of various NSAIDs by force plate gait analysis in cruciate deficient dogs

217 Millis DL. Dasuquin's efficacy may be similar to that of NSAIDs in dogs. Joint Health: a roundtable discussion. (Nutramax Laboratories Inc.) Veterinary Medicine 2010:p.10

218 Lascelles BDX, Gaynor JS, Smith ES, et al. Amantadine in a multimodal analgesic regimen for alleviation of refractory osteoarthritis pain in dogs. *J Vet Intern Med*. 2008;22:53.

219 Mathews KA. Neuropathic pain in dogs and cats: If only they could tell us if they hurt. *Vet Clin* North Am Small Anim Pract. 2008;38:1365.

220 Gaynor J. Other drugs used to treat pain. Gaynor J, Muir S. *Handbook of veterinary pain management*. Mosby: St Louis; 2008:260.

221 Johnston SA, Budsberg SC. Nonsteroidal anti-inflammatory drugs and corticosteroids for the management of canine osteoarthritis. *Vet Clin North Am Small Anim Pract*. 1997;27:841.

222 McQuay HJ, Moore A. NSAIDs and coxibs: clinical use. In: McMahon SB, Koltzenburg M (eds). *Wall and Melzack's Textbook of Pain*, edn 5. Elsevier Churchill Livingston, Philadelphia, 2006, pp. 471–80.

223 Bianchi M, Broggini M, Balzarini P, *et al*. Effects of tramadol on synovial fluid concentrations of substance P and interleukin-6 in patients with knee osteoarthritis: comparison with paracetamol. *Int Immunopharm* 2003;3(13– 14):1901–8

224 American College of Rheumatology Subcommittee on Osteoarthritis. Recommendations for the medical management of osteoarthritis of the hip and knee. *Arthritis Rheumatol* 2000;43:1905–15.

225 Cepeda MS, Camargo F, Zea C, *et al*. Tramadol for osteoarthritis. *Cochrane Database of Systematic Reviews* 2006, Issue 3. Art. No: CD005522. DOI:10.1002/14651858.CD005522.pub2

226 Clark P, Rowland SE, Denis D, *et al*. MF498 (N-{(4- (5,9-Diethoxy-6-oxo-6,8-dihydro-7H-pyrrolo(3,4-g) quinolin- 7-yl)-3-m ethylbenzyl)sulfonyl)-2- (2-methoxyphenyl)acetamide), a selective E prostanoid receptor 4 antagonist, relieves joint inflammation and pain in rodent models of rheumatoid and osteoarthritis. *J Pharmacol Exp Ther*. 2008 May; 325(2):425–34

227 Chen Q, Muramoto K, Masaaki N, *et al*. A novel antagonist of the prostaglandin E(2) EP(4) receptor inhibits Th1 differentiation and Th17 expansion and is orally active in arthritis models. *Br J Pharmacol* 2010 May;160(2):292– 310

228 Southall MD and Vasko MR. Prostaglandin receptor subtypes, EP3C and EP4, mediate the prostaglandin E2- induced cAMP production and sensitization of sensory neurons. *J Biol Chem* 2001;276:16083–91.

229 Sevalla K, Todhunter RJ, Vernier-Singer M, et al. Effect of polysulfated glycosaminoglycan on DNA content and proteoglycan metabolism in normal and osteoarthritic canine articular cartilage explants. *Vet Surg*. 2000;29:407.

230 Fujiki M, Shineha J, Yamanokuchi K, et al. Effects of treatment with polysulfated glycosaminoglycan on serum cartilage oligo- meric matrix protein and C-reactive protein concentrations, serum matrix metalloproteinase-2 and -9 activities, and lameness in dogs with osteoarthritis. *Am J Vet Res*. 2007;68:827.

231 Steven M.F., "Multimodal Management of Canine Osteoarthritis".2th ed., Copyright © 2017 by Taylor & Francis Group, LLC

232 Aragon CL, Hofmeister EH, Budsberg SC. Systematic review of clinical trials of treatments for osteoarthritis in dogs. *J Am Vet Med Assoc*. 2007;230:514.

233 Sanderson RO, Beata C, Flipo RM, et al. Systematic review of the management of canine osteoarthritis. *Vet Rec*. 2009;164:418.

234 Giedrojc J, Radziwon P, Klimiuk M, et al. Experimental studies on the anticoagulant and antithrombotic effects of sodium and calcium pentosan polysulphate. *J Physiol Pharmacol*. 1999;50:111.

235 Francis DJ, Hutadilok N, Kongtawelert P, et al. Pentosan polysulfate and glycosaminoglycan polysulfate stimulate the synthesis of hyaluronan in vivo. *Rheumatol Int*. 1993;13:61.

236 Francis DJ, Read RA. Pentosan polysulfate as a treatment for osteoarthritis (degenerative joint disease) in dogs. *Aust Vet Pract*. 1993;23:104

237 Budsberg SC, Bergh MS, Reynolds LR, et al. Evaluation of pentosan polysulfate sodium in the postoperative recovery from cranial cruciate injury in dogs: A randomized, placebo-controlled clinical trial. *Vet Surg*. 2007;36:234.

238 Innes JF, Barr ARS, Sharif M. Efficacy of oral calcium pentosan polysulphate for the treatment of osteoarthritis of the canine stifle joint secondary to cranial cruciate ligament deficiency. *Vet Rec*. 2000;146:433

239 Bernard P, Germain C, Bousquet E, et al. Radioactivity distribution in a young dog dosed orally *with (acetyl-1-14C) chondroitin sulphate*. British Small Animal Veterinary Association: Birmingham, AL; 2000:85.

240 Ronca F, Palmieri L, Panicucci P, et al. Anti-inflammatory activity of chondroitin sulfate. Osteoarthritis Cartilage. 1998;6:14.

241 Zhang W, Nuki G, Moskowitz RW, et al. OARSI recommendations for the management of hip and knee osteoarthritis: Part III. Changes in evidence following systematic cumulative update of research published through January 2009. *Osteoarthritis Cartilage*. 2010;18:476.

242 Setnikar I, Palumbo R, Canali S, et al. Pharmacokinetics of glucosamine in man. Arzneimittelforschung. 1993;43:1109.

243 Setnikar I, Zanolo G. Pharmacokinetics of exogenous glucosamine in rats and dogs. *Scand J Rheumatol*. 1986;S60:12.

244 Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage. 1998;6:427.

245 Anderson CC, Cook JL, Kreeger JM, et al. In vitro effects of glucosamine and acetylsalicylate on canine chondrocytes in three-dimensional culture. *Am J Vet Res*. 1999;60:1546.

246 Setnikar I, Pacini MA, Revel L. Antiarthritic effects of glucosamine sulfate studied in animal models. Arzneimittelforschung. 1991;41:542

247 McAlindon TE, LaValley MP, Gulin JP, et al. Glucosamine and chondroitin for treatment of osteoarthritis: A systematic quality assessment and meta-analysis. *JAMA*. 2000;283:1469.

248 Pelletier JP, Mineau F, Raynauld JP, et al. Intraarticular injections with methylprednisolone acetate reduce osteoarthritic lesions in parallel with chondrocyte stromelysin synthesis in experimental osteoarthritis. *Arthritis Rheum*. 1994;37:414.

249 Murphy DJ, Todhunter RJ, Fubini SL, et al. The effects of methylprednisolone on normal and monocyte-conditioned medium-treated articular cartilage from dogs and horses. *Vet Surg*. 2000;29:546.

250 Evans CH, Kraus VB and Setton LA. Progress in intra-articular therapy. *Nat Rev Rheumatol* 2014; 10: 11–22.

251 Sherman SL, Khazai RS, James CH, *et al.* In vitro toxicity of local anesthetics and corticosteroids on chondrocyte and synoviocyte viability and metabolism. *Cartilage* 2015;6(4):233–40.

252 Abate M, Salini V. Hyaluronic Acid in the Treatment of Osteoarthritis: What is New, Osteoarthritis - Diagnosis, *Treatment and Surgery*, Prof. Qian Chen (ed.), inTech, Italy, 2012, pp. 102–114. ISBN 978-953-51-0168-0

253 Wang CT, Lin J, Chang CJ, *et al.* Therapeutic effects of hyaluronic acid on osteoarthritis of the knee. A met- analysis of randomized controlled trials. *J Bone Joint Surg Am* 2004;Mar 86-A(3):538–45.

254 Balogh L, Polyak A, Mathe D, *et al.* Absorption, uptake and tissue affinity of high-molecular-weight hyaluronan after oral administration in rats and dogs. *J Agric Food Chem* 2008;56:10582–93.

255 Liu NF. Trafficking of hyaluronan in the interstitium and its possible implications. *Lymphology* 2004;37:6–14.

256 Campo GM, Avenoso A, D'Ascola A, *et al.* Hyaluronan differently modulates TLR-4 and the inflammatory response in mouse chondrocytes. *Biofactors* 2012;38: 69–76.

257 Rezende UM, Campos GC. Viscosuplementation. *Rev Bras Ortop*. (2012) 47:160–4.

258 Smith GN Jr, Mickler EA, Myers SL, Brandt KD. Effect of intraarticular hyaluronan injection on synovial fluid hyaluronan in the early stage of canine post-traumatic osteoarthritis. *J Rheumatol*. (2001) 28:1341–6.

259   Petrella RJ, Petrella M. A prospective, randomized, double-blind, placebo controlled study to evaluate the efficacy of intraarticular hyaluronic acid for osteoarthritis of the knee. *J Rheumatol* 2006;33(5):951–56.

260   Carapeba GOL, Cavaleti P, Nicácio GM, Brinholi B. Intra-articular hyaluronic acid compared to traditional conservative treatment in dogs with osteoarthritis associated with hip dysplasia. *Evid Based Complement Alternat Med.* (2016) 2016:2076921.

261   Hellström LE, Carlsson C, Boucher JF, Michanek P. Intra-articular injections with high molecular weight sodium hyaluronate as a therapy for canine arthritis. *Vet Rec.* (2003) 153:89–90

262   Lattimer JC, Selting KA, Lunceford JM, Holland JR, Simon J, Stevenson NR, Doerr CA. Intraarticular injection of a Tin-117 m radiosynoviorthesis agent in normal canine elbows causes no adverse effects. Vet Radiol Ultrasound. 2019 Sep;60(5)

263   Wendt RE 3rd, Selting KA, Lattimer JC, Wong J, Simón J, Stevenson NR, Stearns SD. Radiation Safety Considerations in the Treatment of Canine Skeletal Conditions Using 153Sm, 90Y, and 117mSn. Health Phys. 2020 Jun;118(6):702-710.

264   Greenwood HL, Singer PA, Downey GP, et al. Regenerative medicine and the developing world. *PLoS Med.* 2006;3(9):e381.

265   Anitua E, Andia I, Ardanza B, et al. Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemost.* 2004;91(1):4–15.

266   Foster TE, Puskas BL, Mandelbaum BR, et al. Platelet-rich plasma: From basic science to clinical applications. *Am J Sports Med.* 2009;37(11):2259–2272.

267   Dohan Ehrenfest DM, Doglioli P, de Peppo GM, *et al.* Choukroun's platelet-rich fibrin (PRF) stimulates in vitro proliferation and differentiation of human oral bone mesenchymal stem cell in a dose-dependent way. *Arch Oral Biol* 2010;55:185–94

268   McLellan J, Plevin S. Does it matter which platelet-rich plasma we use? *Equine Vet Educ* 2011;23(2):101–4.

269   Patel S, Shillon MS, Aggarwal S, *et al.* Treatment with platelet-rich plasma is more effective than placebo for knee osteoarthritis: a prospective, double-blinded, randomized trial. *Am J Sports Med* 2013;41(2):356–64.

270   Braun HJ, Kim HJ, Chu CR, *et al.* The effect of platelet-rich plasma formulations and blood products on human synoviocytes. *Am J Sports Med* 2014;42(5):1204–10.

271   Stief M, Gottschalk J, Ionita JC, *et al.* Concentration of platelets and growth factors in canine autologous conditioned plasma. *Vet Comp Orthop Traumatol* 2011;24:285–90.

272   Fahie MA, Ortolano GA, Guercio V, et al. A randomized controlled trial of the efficacy of autologous platelet therapy for the treatment of osteoarthritis in dogs. *J Am Vet Med Assoc.* 2013;243(9):1291–1297.

273   Arend WP, Malyak M, Guthridge CJ, et al. Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol* 1998;16:27–55

274   Frisbie DD, Ghivizzani SC, Robbins PD, *et al.* Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. *Gene Therapy* 2002;9:12–20.

275   Baltzer AWA, Ostapczuk MS, Stosch D, *et al.* A new treatment for hip osteoarthritis: clinical evidence for the efficacy of autologous conditioned serum. *Orthop Rev* 2013;5:e13.

276   Bertone AL, Ishihara A, Zekas LJ, *et al.* Evaluation of a single intra-articular injection of autologous protein solution for treatment of osteoarthritis in horses. *Am J Vet Res* 2014;75:141–51.

277   Wanstrath A, Hettlich B, Zekas L, *et al.* Intra-articular injection of autologous protein solution for treatment of canine osteoarthritis: a prospective, randomized, double-blinded, placebo-controlled In Proceedings ACVS Symposium 2015, Nashville, TN, USA.

278   Huggins SS, Suchodolski JS, Bearden RN, *et al.* Serum concentrations of canine interleukin-1 receptor antagonist protein in healthy dogs after incubation using an autologous serum processing system. *Res in Vet Sci* 2015;101:28– 33.

279   Maehle AH. Ambiguous cells: the emergence of the stem cell concept in the nineteenth and twentieth centuries. Notes Rec R Soc Lond. 2011 Dec 20;65(4):359-78. doi: 10.1098/rsnr.2011.0023.

280   Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* (2006) 126:663–76. doi: 10.1016/j.cell.2006.07.024

281   Morrison SJ, Wandycz AM, Hemmati HD, Wright DE, Weissman IL. Identification of a lineage of multipotent hematopoietic progenitors. *Development.* (1997) 124:1929–39.

282   Wagers AJ, Weissman IL. Plasticity of adult stem cells. *Cell.* (2004) 116:639– 48. doi: 10.1016/S0092-8674(04)00208-9

283   O'Donoghue K, Fisk NM. Fetal stem cells. *Best Pract Res Clin Obstet Gynaecol*. 2004;18(6):853–875.

284   Kuroda Y, Kitada M, Wakao S, et al. Unique multipotent cells in adult human mesenchymal cell populations. *Proc Natl Acad Sci USA*. 2010;107(19):8639–8643.

285   Roy S, Gascard P, Dumont N, et al. Rare somatic cells from human breast tissue exhibit extensive lineage plasticity. *Proc Natl Acad Sci USA*. 2013;110(12):4598–4603.

286   Frenette PS, Pinho S, Lucas D, et al. Mesenchymal stem cell: keystone of the hematopoietic stem cell niche and a stepping-stone for regenerative medicine. *Annu Rev Immunol*. 2013;31:285–316.

287   Scadden DT. Nice neighborhood: emerging concepts of the stem cell niche. *Cell*. 2014;157(1):41–50.

288   Caplan, A.I. Are All Adult Stem Cells The Same?. *Regen. Eng. Transl. Med.* 1**,** 4–10 (2015).

289   Bianco P, Cao X, Frenette PS, et al. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med*. 2013;19(1):35–42.

290   Prockop DJ, Gregory CA, Spees JL. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. *Proc Natl Acad Sci USA*. 2003;100(Suppl 1):11917–11923.

291   Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation*. (1968) 6:230–47. doi: 10.1097/00007890-196803000-00009

292   Dennis JE, Merriam A, Awadallah A, Yoo JU, Johnstone B, Caplan AI. A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. *J Bone Miner Res*. (1999) 14:700–9. doi: 10.1359/jbmr.1999.14.5.700

293   Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med*. (2006) 12:459–65. doi: 10.1038/nm1391

294   Quinn C, Flake AW. *In vivo* differentiation potential of mesenchymal stem cells: prenatal and postnatal model systems. *Transfus Med Hemother*. (2008) 35:239–47. doi: 10.1159/000129129

295   Niess H, Thomas MN, Schiergens TS, Kleespies A, Jauch KW, Bruns C, et al. Genetic engineering of mesenchymal stromal cells for cancer therapy: turning partners in crime into Trojan horses. *Innov Surg Sci*. (2016) 1:19– 32. doi: 10.1515/iss-2016-0005

296   Caplan AI. Mesenchymal stem cells: time to change the name! stem cells. *Transl Med*. (2017). 6:1445–51. doi: 10.1002/ sctm.17-0051

297   Sasaki A, Mizuno M, Ozeki N, Katano H, Otabe K, Tsuji K, et al. Canine mesenchymal stem cells from synovium have a higher chondrogenic potential than those from infrapatellar fat pad, adipose tissue, and bone marrow. *PLoS ONE*. (2018) 13:e0202922. doi: 10.1371/journal.pone.0202922

298   Bearden RN, Huggins SS, Cummings KJ, Smith R, Gregory CA, Saunders WB. In-vitro characterization of canine multipotent stromal cells isolated from synovium, bone marrow, and adipose tissue: a donor-matched comparative study. *Stem Cell Res Ther*. (2017) 8:218. doi: 10.1186/s13287-017-0639-6

299   Webb TL, Quimby JM, Dow SW. *In vitro* comparison of feline bone marrow- derived and adipose tissue-derived mesenchymal stem cells. *J Feline Med Surg*. (2012) 14:165–8. doi: 10.1177/1098612X11429224

300   Zhang BY, Wang BY, Li SC, Luo DZ, Zhan X, Chen SF, et al. Evaluation of the curative effect of umbilical cord mesenchymal stem cell therapy for knee arthritis in dogs using imaging technology. *Stem Cells Int*. (2018) 2018:1983025. doi: 10.1155/2018/1983025

301   Denys M, Leon A, Robert C, Saulnier N, Josson-Schramme A, Legrand L, et al. Biosafety evaluation of equine umbilical cord- derived mesenchymal stromal cells (UC-MSCs) by systematic pathogen screening in peripheral maternal blood and paired UC-MSCs. *Biopreserv Biobank*. (2020) 18:73– 81. doi: 10.1089/bio.2019.0071

302   Kisiel AH, McDuffee LA, Masaoud E, Bailey TR, Esparza Gonzalez BP, Nino-Fong R. Isolation, characterization, and *in vitro* proliferation of canine mesenchymal stem cells derived from bone marrow, adipose tissue, muscle, and periosteum. *Am J Vet Res*. (2012) 73:1305–17. doi: 10.2460/ajvr.73.8.1305

303   Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell*. 2008;3(3):301–313.

304   Ksiazek K. A comprehensive review on mesenchymal stem cell growth and senescence. *Rejuv Res*. 2009;12(2):105–116.

146

305   Levi B, Longaker MT. Concise review: adipose-derived stromal cells for skeletal regenerative medicine. *Stem Cells*. 2011;29(4):576–582.

306   Wagner W, Ho AD. Mesenchymal stem cell preparations—comparing apples and oranges. *Stem Cell Rev*. 2007;3(4):239–248.

307   Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy*. (2006) 8:315–7. doi: 10.1080/14653240600855905

308   Russell KA, Chow NH, Dukoff D, Gibson TW, LaMarre J, Betts DH, et al. Characterization and immunomodulatory effects of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells. *PLoS ONE*. (2016) 11:e0167442. doi: 10.1371/journal.pone.01 67442

309   Villatoro AJ, Alcoholado C, Martin-Astorga MC, Fernandez V, Cifuentes M, Becerra J. Comparative analysis and characterization of soluble factors and exosomes from cultured adipose tissue and bone marrow mesenchymal stem cells in canine species. *Vet Immunol Immunopathol*. (2019) 208:6– 15. doi: 10.1016/j.vetimm.2018.12.003

310   Guercio A, Di Bella S, Casella S, Di Marco P, Russo C, Piccione G. Canine mesenchymal stem cells (MSCs): characterization in relation to donor age and adipose tissue-harvesting site. *Cell Biol Int*. (2013) 37:789– 98. doi: 10.1002/cbin.10090

311   Yaneselli KM, Kuhl CP, Terraciano PB, de Oliveira FS, Pizzato SB, Pazza K, et al. Comparison of the characteristics of canine adipose tissue-derived mesenchymal stem cells extracted from different sites and at different passage numbers. *J Vet Sci*. (2018) 19:13–20. doi: 10.4142/jvs.2018.19.1.13

312   Bahamondes F, Flores E, Cattaneo G, Bruna F, Conget P. Omental adipose tissue is a more suitable source of canine mesenchymal stem cells. *BMC Vet Res*. (2017) 13:166. doi: 10.1186/s12917-017-1053-0

313   Boxall SA, Jones E. Markers for characterization of bone marrow multipotential stromal cells. *Stem Cells Int*. (2012) 2012:975871. doi: 10.1155/2012/975871

314   Laing AG, Fanelli G, Ramirez-Valdez A, Lechler RI, Lombardi G, Sharpe PT. Mesenchymal stem cells inhibit T-cell function through conserved induction of cellular stress. *PLoS ONE*. (2019) 14:e0213170. doi: 10.1371/journal.pone.0213170

315   Luk F, Carreras-Planella L, Korevaar SS, de Witte SFH, Borras FE, Betjes MGH, et al. Inflammatory conditions dictate the effect of mesenchymal stem or stromal cells on B cell function. *Front Immunol*. (2017) 8:1042. doi: 10.3389/fimmu.2017.01042

316   Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood*. (2006) 107:1484– 90. doi: 10.1182/blood-2005-07-2775

317   Deng M, Mei T, Hou T, Luo K, Luo F, Yang A, et al. TGFβ3 recruits endogenous mesenchymal stem cells to initiate bone regeneration. *Stem Cell Res Ther*. (2017) 8:258. doi: 10.1186/s13287-017-0693-0

318   Dubon MJ, Yu J, Choi S, Park KS. Transforming growth factor beta induces bone marrow mesenchymal stem cell migration via noncanonical signals and N-cadherin. *J Cell Physiol*. (2018) 233:201–13. doi: 10.1002/jcp.25863

319   LiuF,QiuH,XueM,ZhangS,ZhangX,XuJ,etal.MSC- secreted TGF-β regulates lipopolysaccharide-stimulated macrophage M2- like polarization via the Akt/FoxO1 pathway. *Stem Cell Res Ther*. (2019) 10:345. doi: 10.1186/s13287-019-1447-y

320   Wu R, Liu C, Deng X, Chen L, Hao S, Ma L. Enhanced alleviation of aGVHD by TGF-β1-modified mesenchymal stem cells in mice through shifting MPhi into M2 phenotype and promoting the differentiation of Treg cells. *J Cell Mol Med*. (2020) 24:1684–99. doi: 10.1111/jcmm.14862

321   Gazdic M, Markovic BS, Arsenijevic A, Jovicic N, Acovic A, Harrell CR, et al. Crosstalk between mesenchymal stem cells and T regulatory cells is crucially important for the attenuation of acute liver injury. *Liver Transpl*. (2018) 24:687–702. doi: 10.1002/lt.25049

322   Schmidt A, Zhang XM, Joshi RN, Iqbal S, Wahlund C, Gabrielsson S, et al. Human macrophages induce CD4(+)Foxp3(+) regulatory T cells via binding and re-release of TGF-β. *Immunol Cell Biol*. (2016) 94:747– 62. doi: 10.1038/icb.2016.34

323   Franquesa M, Mensah FK, Huizinga R, Strini T, Boon L, Lombardo E, et al. Human adipose tissue-derived mesenchymal stem cells abrogate plasmablast formation and induce regulatory B cells independently of T helper cells. *Stem Cells*. (2015) 33:880–91. doi: 10.1002/stem.1881

324   Day AJ, Milner CM. TSG-6: a multifunctional protein with anti-

325   inflammatory and tissue-protective properties. *Matrix Biol*. (2019) 78–9:60– 83. doi: 10.1016/j.matbio.2018.01.011

147

326 Abels ER, Breakefield XO. Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. *Cell Mol Neurobiol*. (2016) 36:301–12. doi: 10.1007/s10571-016-0366-z

327 Makela T, Takalo R, Arvola O, Haapanen H, Yannopoulos F, Blanco R, et al. Safety and biodistribution study of bone marrow-derived mesenchymal stromal cells and mononuclear cells and the impact of the administration route in an intact porcine model. *Cytotherapy*. (2015) 17:392– 402. doi: 10.1016/j.jcyt.2014.12.004

328 Hyvarinen K, Holopainen M, Skirdenko V, Ruhanen H, Lehenkari P, Korhonen M, et al. Mesenchymal stromal cells and their extracellular vesicles enhance the anti-inflammatory phenotype of regulatory macrophages by downregulating the production of interleukin (IL)-23 and IL-22. *Front Immunol*. (2018) 9:771. doi: 10.3389/fimmu.2018.00771

329 Park KS, Svennerholm K, Shelke GV, Bandeira E, Lasser C, Jang SC, et al. Mesenchymal stromal cell-derived nanovesicles ameliorate bacterial outer membrane vesicle-induced sepsis via IL-10. *Stem Cell Res Ther*. (2019) 10:231. doi: 10.1186/s13287-019-1352-4

330 Cosenza S, Ruiz M, Toupet K, Jorgensen C, Noel D. Mesenchymal stem cells derived exosomes and microparticles protect cartilage and bone from degradation in osteoarthritis. *Sci Rep*. (2017) 7:16214. doi: 10.1038/s41598-017-15376-8

331 Elliott MR, Koster KM, Murphy PS. Efferocytosis signaling in the regulation of macrophage inflammatory responses. *J Immunol*. (2017) 198:1387– 94. doi: 10.4049/jimmunol.1601520

332 Luk F, de Witte SF, Korevaar SS, Roemeling-van Rhijn M, Franquesa M, Strini T, et al. Inactivated mesenchymal stem cells maintain immunomodulatory capacity. *Stem Cells Dev*. (2016) 25:1342–54. doi: 10.1089/scd.2016.0068

333 Galleu A, Riffo-Vasquez Y, Trento C, Lomas C, Dolcetti L, Cheung TS, et al. Apoptosis in mesenchymal stromal cells induces *in vivo* recipient-mediated immunomodulation. *Sci Transl Med*. (2017) 9:eaam7828. doi: 10.1126/scitranslmed.aam7828

334 Penn MS. SDF-1:CXCR4 axis is fundamental for tissue preservation and repair. *Am J Pathol*. (2010) 177:2166–8. doi: 10.2353/ajpath.2010.100803

335 Zou C, Song G, Luo Q, Yuan L, Yang L. Mesenchymal stem cells require integrin β1 for directed migration induced by osteopontin *in vitro*. *In Vitro Cell Dev Biol Anim*. (2011) 47:241–50. doi: 10.1007/s11626-010-9377-0

336 Wynn RF, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE, et al. A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood*. (2004) 104:2643–5. doi: 10.1182/blood-2004-02-0526

337 Nishimura T, Takami T, Sasaki R, Aibe Y, Matsuda T, Fujisawa K, et al. Liver regeneration therapy through the hepatic artery-infusion of cultured bone marrow cells in a canine liver fibrosis model. *PLoS ONE*. (2019) 14:e0210588. doi: 10.1371/journal.pone.0210588

338 Parys M, Nelson N, Koehl K, Miller R, Kaneene JB, Kruger JM, et al. Safety of intraperitoneal injection of adipose tissue-derived autologous mesenchymal stem cells in cats. *J Vet Intern Med*. (2016) 30:157– 63. doi: 10.1111/jvim.13655

339 Wiafe B, Kadam R, Metcalfe PD. Intraperitoneal administration of mesenchymal stem cells is effective at mitigating detrusor deterioration after pBOO. *Am J Physiol Renal Physiol*. (2020) 318:F549–56. doi: 10.1152/ajprenal.00486.2019

340 Song WJ, Li Q, Ryu MO, Ahn JO, Bhang DH, Jung YC, et al. TSG-6 released from intraperitoneally injected canine adipose tissue- derived mesenchymal stem cells ameliorate inflammatory bowel disease by inducing M2 macrophage switch in mice. *Stem Cell Res Ther*. (2018) 9:91. doi: 10.1186/s13287-018-0841-1

341 Gooch A, Zhang P, Hu Z, Loy Son N, Avila N, Fischer J, et al. Interim report on the effective intraperitoneal therapy of insulin-dependent diabetes mellitus in pet dogs using "Neo-Islets," aggregates of adipose stem and pancreatic islet cells (INAD 012-776). *PLoS ONE*. (2019) 14:e0218688. doi: 10.1371/journal.pone.0218688

342 Brandt L, Schubert S, Scheibe P, Brehm W, Franzen J, Gross C, et al. Tenogenic properties of mesenchymal progenitor cells are compromised in an inflammatory environment. *Int J Mol Sci*. (2018) 19:2549. doi: 10.3390/ijms19092549

343 Kiefer KM, O'Brien TD, Pluhar EG, et al. Canine adipose-derived stromal cell viability following exposure to synovial fluid from osteoarthritic joints. *Vet Rec Open*. 2015;2(1):e000063.

344 An JH, Li Q, Bhang DH, Song WJ, Youn HY. TNF-α and INF-γ primed canine stem cell-derived extracellular vesicles alleviate experimental murine colitis. *Sci Rep*. (2020) 10:2115. doi: 10.1038/s41598-020-58909-4

345 Sivanathan KN, Rojas-Canales D, Grey ST, Gronthos S, Coates PT. Transcriptome profiling of IL-17A preactivated mesenchymal stem cells: a comparative study to unmodified and IFN-γ modified mesenchymal stem cells. *Stem Cells Int*. (2017) 2017:1025820. doi: 10.1155/2017/1025820

346   Ling Kong, Li-Zhen Zheng, Ling Qin, Kevin K.W. Ho. Role of mesenchymal stem cells in osteoarthritis treatment. *Journal of Orthopaedic Translation*,Volume 9 (2017): 89-103 ISSN 2214-031X.

347   Linon E, Spreng D, Rytz U, Forterre S. Engraftment of autologous bone marrow cells into the injured cranial cruciate ligament in dogs. *Vet J*. (2014) 202:448–54. doi: 10.1016/j.tvjl.2014.08.031

348   Muir P, Hans EC, Racette M, Volstad N, Sample SJ, Heaton C, et al. Autologous bone marrow-derived mesenchymal stem cells modulate molecular markers of inflammation in dogs with cruciate ligament rupture. *PLoS ONE*. (2016) 11:e0159095. doi: 10.1371/journal.pone.0159095

349   Canapp SO Jr., Leasure CS, Cox C, Ibrahim V, Carr BJ. Partial cranial cruciate ligament tears treated with stem cell and platelet-rich plasma combination therapy in 36 dogs: a retrospective study. *Front Vet Sci*. (2016) 3:112. doi: 10.3389/fvets.2016.00112

350   Taroni M, Cabon Q, Febre M, Cachon T, Saulnier N, Carozzo C, et al. Evaluation of the effect of a single intra-articular injection of allogeneic neonatal mesenchymal stromal cells compared to oral non- steroidal anti-inflammatory treatment on the postoperative musculoskeletal status and gait of dogs over a 6-month period after tibial plateau leveling osteotomy: a pilot study. *Front Vet Sci*. (2017) 4:83. doi: 10.3389/fvets.2017. 00083

351   Chuang C, Ramaker MA, Kaur S, Csomos RA, Kroner KT, Bleedorn JA, et al. Radiographic risk factors for contralateral rupture in dogs with unilateral cranial cruciate ligament rupture. *PLoS ONE*. (2014) 9:e106389. doi: 10.1371/journal.pone.0106389

352   Molsa SH, Hyytiainen HK, Hielm-Bjorkman AK, Laitinen-Vapaavuori OM. Long-term functional outcome after surgical repair of cranial cruciate ligament disease in dogs. *BMC Vet Res*. (2014) 10:266. doi: 10.1186/s12917-014-0266-8

353   Black LL, Gaynor J, Gahring D, Adams C, Aron D, Harman S, et al. Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter, controlled trial. *Vet Ther*. (2007) 8:272–84

354   Black L, Gaynor J, Adams C, et al. Effect of intraarticular injection of autologous adipose- derived mesenchymal stem and regenerative cells on clinical signs of chronic osteoarthritis of the elbow joint in dogs. *Vet Ther*. 2008;9(3):192–200

355   Harman R, Carlson K, Gaynor J, Gustafson S, Dhupa S, Clement K, et al. A prospective, randomized, masked, and placebo-controlled efficacy study of intraarticular allogeneic adipose stem cells for the treatment of osteoarthritis in dogs. *Front Vet Sci*. (2016) 3:81. doi: 10.3389/fvets.2016.00081

356   Shah K, Drury T, Roic I, Hansen P, Malin M, Boyd R, et al. Outcome of allogeneic adult stem cell therapy in dogs suffering from osteoarthritis and other joint defects. *Stem Cells Int*. (2018) 2018:7309201. doi: 10.1155/2018/7309201

357   Kriston-Pál E., Haracska L., Cooper P., Kiss-Tóth E., Szukacsov V., Monostori E. Regenerative Approach to Canine Osteoarthritis Using Allogeneic, Adipose-Derived Mesenchymal Stem Cells. Safety Results of a Long-Term Follow-Up. *Front Vet Sc*. (2020) Vol.7: 510 doi:10.3389/fvets.2020.00510

358   Olsen, A., Johnson, V., Webb, T., Santangelo, K. S., Dow, S., & Duerr, F. M. Evaluation of intravenously delivered allogeneic mesenchymal stem cells for treatment of elbow osteoarthritis in dogs: a pilot study. *Veterinary and Comparative Orthopaedics and Traumatology*, (2019). *32*(03), 173-181.

359   Upchurch, D. A., Renberg, W. C., Roush, J. K., Milliken, G. A., & Weiss, M. L. (2016). Effects of administration of adipose-derived stromal vascular fraction and platelet-rich plasma to dogs with osteoarthritis of the hip joints. *American journal of veterinary research*, *77*(9), 940-951.

360   Kim, S. H., Djaja, Y. P., Park, Y. B., Park, J. G., Ko, Y. B., & Ha, C. W. (2020). Intra-articular injection of culture-expanded mesenchymal stem cells without adjuvant surgery in knee osteoarthritis: a systematic review and meta-analysis. *The American journal of sports medicine*, *48*(11), 2839-2849.

361   Munana K, Zhang D, Patterson EE. Placebo effect in canine epilepsy trials. *J Vet Intern Med*. 2010;24(1):166–170.

362   Conzemius MG, Evans RB. Caregiver placebo effect for dogs with lameness from osteoarthritis. *J Am Vet Med Assoc*. 2012;241(10):1314–1319.

363   Kiefer KM, O'Brien TD, Pluhar EG, Conzemius M. Canine adipose-derived stromal cell viability following exposure to synovial fluid from osteoarthritic joints. Vet Rec Open 2015;2:e000063. doi:10.1136/vetreco-2014- 000063

364   Canapp SO, Carr BJ, Huang R, *et al*. Canine bone marrow aspirate and stromal vascular fraction: prospective analysis. In Proceedings of ACVS Symposium 2015. Nashville, TN, USA.

365   Hernigou P, Poignard A, Beaujean F, *et al*. Percutaneous autologous bone-marrow grafting for nonunions: influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am* 2005;87A:1430–37

366   Hauser RA, Eteshola E. Rationale for using direct bone marrow aspirate as a proliferant for regenerative injection therapy. *Open Stem Cell J* 2013;4:7–14.

367   Astor DE, Hoelzler MG, Harman R, *et al.* Patient factors influencing the concentration of stromal vascular fraction for adipose-derived stromal cell therapy in dogs. *Can Vet J* 2013;77(3):177–82.

368   Strem BM, Hedrick MH. The growing importance of fat in regenerative medicine. Trends Biotechnol 2005;23:64

369   Y.A. Panina, A.S. Yakimov, Y.K. Komleva, A.V. Morgun, O.L. Lopatina, N.A. Malinovskaya, A.N. Shuvaev, VV. Salmin, T.E. Taranushenko, A.B. Salmina, "Plasticity of adipose tissue derived Stem Cells and Regulation of Angiogenesis". *Frontiers in physiopatology,* 2018, 1-13.

370   M. Sun, Y. He, P. Zhou, J. Gao, F. Lu, "Adipose extracellular matrix/stromal vascular fraction gel secretes angiogenic factors and enhances skin would healing in a murine model". *Biomed Research International,* 2017, 1-11.

371   Si Z, Wang X, Sun C, Kang Y, Xu J, et al. (2019) Adipose-derived stem cells: Sources, potency, and implications for regenerative therapies. Biomed Pharmacother 114: 108765.

372   Vezzani B, Shaw I, Lesme H, Yong L, Khan N, et al. (2018) Higher Pericyte Content and Secretory Activity of Microfragmented Human Adipose Tissue Compared to Enzymatically Derived Stromal Vascular Fraction. Stem Cells Transl Med 7: 876-886.

373   Coleman SR. Structural fat grafts: the ideal filler? Clin Plast Surg 2001;28:111–119

374   Coleman SR. Structural fat grafting: more than a permanent filler. Plast Reconstr Surg 2006;118(3 suppl):108S–120S

375   Murray IR, Peault B (2015) Q&A: Mesenchymal stem cells - where do they come from and is it important? BMC Biol 13: 99.

376   Tremolada C, Ricordi C, Caplan AI, Ventura C (2016) Mesenchymal Stem Cells in Lipogems, a Reverse Story: from Clinical Practice to Basic Sci- ence. Methods Mol Biol 1416: 109-122.

377   Pavarotti, G. S., Hivernaud, V., Brincin, M., Roche, R., Barreau, P., Festy, F., & Gauthier, O. (2020). Evaluation of a Single Intra-Articular Injection of Autologous Adipose Tissue for the Treatment of Osteoarthritis: A Prospective Clinical Study in Dogs. *Veterinary and Comparative Orthopaedics and Traumatology*, *33*(04), 258-266.

378   Van den Brink SC, Sage F, Vértesy Á, Spanjaard B, Peterson-Maduro J, et al. (2017) Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations. Nat Methods 14: 935-936.

379   Garcia-Contreras M, Messaggio F, Jimenez O, Mendez A (2015) Differences in exosome content of human adipose tissue processed by non-enzymatic and enzymatic methods. CellR4 3: 1423.

380   Elahi FM, Farwell DG, Nolta JA, Anderson JD (2019) Concise Review: Preclinical Translation of Exosomes Derived from Mesenchymal Stem/ Stromal Cells. Stem Cells Pg no: 1-10.

381   Zeira, O., Scaccia, S., Pettinari, L., Ghezzi, E., Asiag, N., Martinelli, L., ... & Aralla, M. (2018). Intra-articular administration of autologous micro-fragmented adipose tissue in dogs with spontaneous osteoarthritis: Safety, feasibility, and clinical outcomes. *Stem cells translational medicine*, *7*(11), 819-828.

382   Meek CP. Successful microdermagrafting using the Meek-Wall microdermatome. Am J Surg 1958; 96: 557.

383   Quintero EC, Escrivá Machado JF and Damian Robles RA. Meek micrografting history, indications, technique, physiology and experience: a review article. J Wound Care 2018; 27: S12–S18. DOI: 10.12968/ jowc.2018.27.Sup2.S12

384   Purpura V, Bondioli E, Graziano A, et al. Tissue characterization after a new disaggregation method for skin micro-grafts generation. J Vis Exp 2016; 109: e53579. doi: 10.3791/53579

385   De Francesco F, Mannucci S, Conti G, Dai Prè E, Sbarbati A, Riccio M. A Non-Enzymatic Method to Obtain a Fat Tissue Derivative Highly Enriched in Adipose Stem Cells (ASCs) from Human Lipoaspirates: Preliminary Results. *International Journal of Molecular Sciences*. 2018; 19(7):2061.

386   Dai Prè E, Busato A, Mannucci S, Vurro F, De Francesco F, Riccio V, Solito S, Biswas R, Bernardi P, Riccio M, Sbarbati A. In Vitro Characterization of Adipose Stem Cells Non-Enzymatically Extracted from the Thigh and Abdomen. *International Journal of Molecular Sciences*. 2020; 21(9):3081.

387   Graziano A, Carinci F, Scolaro S, et al. Periodontal tissue generation using autologous dental ligament micro-grafts: case report with 6 months follow-up. Ann Oral Max Surg 2013; 1: 20.

388   Rodriguez Y Baena R, D'Aquino R, et al. Autologous periosteum-derived micrografts and PLGA/HA enhance the bone formation in sinus lift augmentation. Front Cell Dev Biol 2017; 5: 87. doi: 10.3389/fcell. 2017.00087.

389   Marcarelli M, Trovato L, Novarese E, et al. Rigenera protocol in the treatment of surgical wound dehiscence. Int Wound J 2017; 14: 277–281. doi: 10.1111/iwj.12601.

390   Baglioni E, Trovato L, Marcarelli M, et al. Treatment of oncological post-surgical wound dehiscence with autologous skin micrografts. Anticancer Res 2016; 36: 975–980.

391   Olsson M, Jarbrink K, Divakar U, et al. The humanistic and economic burden of chronic wounds: a systematic review. Wound Repair Regen 2019; 27: 114–125. doi: 10.1111/ wrr.12683

392   Xie T, Ye J, Rerkasem K, et al. The venous ulcer continues to be a clinical challenge: an update. Burns Trauma 2018; 6: 18. doi: 10.1186/s41038-018-0119-y

393   Trovato L, Failla G, Serantoni S, et al. Regenerative surgery in the management of the leg ulcers. J Cell Sci Ther 2016; 7: 238. doi: 10.4172/2157-7013.1000238

394   Riccio M, Marchesini A, Zingaretti N, et al. A multicentre study: the use of micrografts in the reconstruction of full-thickness post- traumatic skin defects of the limbs – A whole innovative concept in regenerative surgery. Stem Cell Int 2019; 2019: Article ID 5043518. DOI: 10.1155/2019/5043518

395   Haas EM, Volkmer E, Giunta RE. [Pilot study on the effects and benefits of autologous fat grafting in osteoarthritis of the CMC-1 joint compared to intraarticular cortisone injection: results after 3 months]. Handchir Mikrochir Plast Chir 2017;49(05):288–296

396   Kemper R, Wirth J, Baur EM. Arthroscopic synovectomy combined with autologous fat grafting in early stages of CMC osteoarthritis of the thumb. J Wrist Surg 2018;7(02):165–171

397   Herold C, Rennekampff HO, Groddeck R, Allert S. Autologous fat transfer for thumb carpometacarpal joint osteoarthritis: a prospective study. Plast Reconstr Surg 2017;140(02):327–335

398   Bohr S, Rennekampff HO, Pallua N. Cell-enriched lipoaspirate arthroplasty: a novel approach to first carpometacarpal joint arthritis. Hand Surg 2015;20(03):479–481

399   Erne HC, Cerny MK, Ehrl D, et al. Autologous fat injection versus Lundborg resection arthroplasty for the treatment of trapeziometacarpal joint osteoarthritis. Plast Reconstr Surg 2018;141(01):119–124

400   Cattaneo G, De Caro A, Napoli F, Chiapale D, Trada P, Camera A. Micro-fragmented adipose tissue injection associated with arthroscopic procedures in patients with symptomatic knee osteoarthritis. BMC Musculoskelet Disord 2018;19(01):176