Current advances on the regeneration of musculoskeletal interfaces

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Abstract

The regeneration of the musculoskeletal system has been widely investigated. There is now detailed knowledge about the organs composing this system. Research has also investigated the zones between individual tissues where physical, mechanical and biochemical properties transition. However, the understanding of the regeneration of musculoskeletal interfaces is still lacking behind. Numerous disorders and injuries can degrade or damage tissue interfaces. Their inability to regenerate can delay the repair and regeneration process of tissues, leading to graft instability, high morbidity and pain. Moreover, the knowledge of the mechanism of tissue interface development is not complete. This review presents an overview of the most recent approaches of the regeneration of musculoskeletal interfaces, describing the latest *in vitro*, preclinical and clinical studies.

1. Introduction

The musculoskeletal system provides support and movement of the human body and protects vital inner organs. It consists of hard and soft tissues. The hard tissue is bone while the soft tissues are cartilage, muscles, tendons and ligaments (Figure 1). Bones are held in position by ligaments that provide restricted, specific articulation and movement. Skeletal muscle is the organ that powers the movement of bones. Muscles and bones are connected through tendons. These tissues together form articulating joints [1].

The anatomy and extracellular matrix (ECM) of these tissues are well known, apart for the skeletal muscle. The compartmentation of the muscle ECM is still arbitrary because it is difficult to physically divide the different regions to study them [2]. Moreover, vast knowledge exists about how these individual organs work and how they interact with each other, transfer loads and how ligaments, tendons and antagonistic muscles work together to hold and enable movement of specific joints and parts of the body.

Interfaces are areas collocated between different tissues. These transition zones are crucial to absorb or transfer load between bone, cartilage, tendon, ligament, and muscle. Interfaces have unique biochemical compositions that are distinguishable from both tissues they connect [3]. Several musculoskeletal disorders can affect these interfaces, including osteoarthritis and tendinopathies, injuries or cancers. These disorders, along with risk factors such as ageing and unhealthy lifestyle (bad posture, carrying or moving heavy loads, poor nutrition, fitness and hydration), can lead to local imbalances, misalignments, inflammation, pain and restriction of motion [4]. In recent

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years, interest in these interfaces has increased, due to their reduced rates of repair and regeneration, which can lead to progressive degradation of the joint. This inability of interfaces to regenerate can affect long-term integration and graft stability, and the long-term clinical outcome proves problematic [5]. Currently, to treat diseases and pathology related to the musculoskeletal system, surgical intervention is required involving implanted devices to replace or augment parts or the entire articulating joint. These methods, along with implants/prosthetics, are only temporary [6]. In recent years, tissue engineering and regenerative medicine approaches were applied to treat interface injuries as they seem to be promising approaches for enhancing interface regeneration [5,7]. However, the mechanism behind the development of tissue interfaces is not completely understood yet [5]. This review focuses on the approaches used for studying the regeneration of musculoskeletal system interfaces. The latest *in vitro* studies are reported, and preclinical and ongoing clinical trials are described.

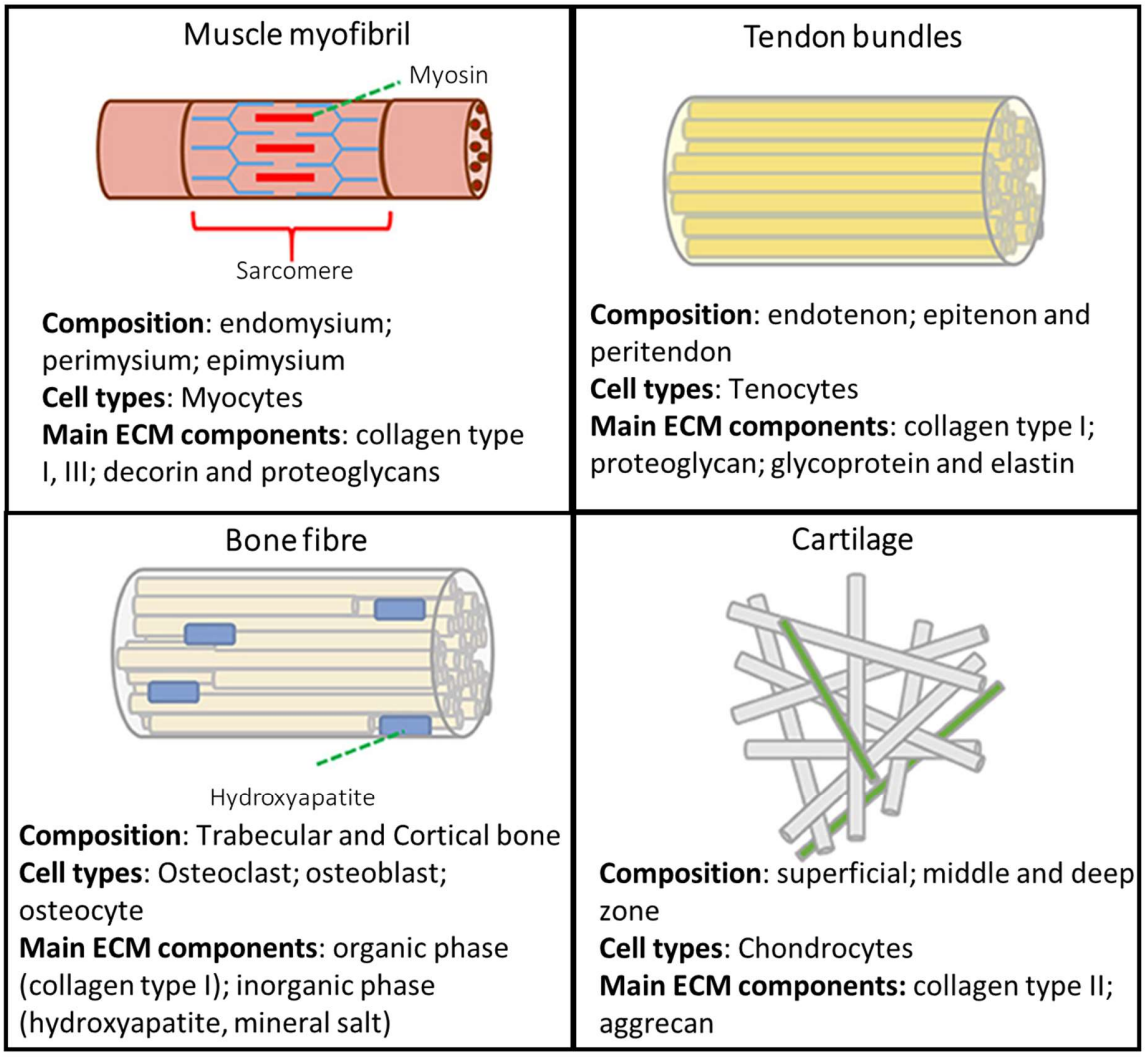

Figure 1: Main components of tissues forming the musculoskeletal system (image adapted from Casanellas I., et al. 2018 [8]).

2. Anatomy of orthopaedic interfaces

Several different interfaces can be found between the tissues of the musculoskeletal system. The orthopaedic interfaces and their structures are described below and represented in Figure 2.

2.1 Osteochondral interface

The osteochondral interface is formed by two main structures, namely the subchondral bone and the articular cartilage. The subchondral bone is composed of the subchondral bone plate and calcified cartilage zone, which is divided into the deep, middle and superficial tangential zones. The subchondral bone maintains the stability of the articular cartilage and contains bone marrow. The calcified zone provides adhesion at the interface between the subchondral bone and articular cartilage [9]. The articular cartilage distributes the load forces to the subchondral bone due to the deposition of type II collagen and proteoglycans such as aggrecan. Structurally, collagen fibres extend from the deep zone to the calcified cartilage through the tidemark, marking calcification that dissipates forces through the vertical orientation of vertical collagen fibres. Calcified cartilage is interdigitated with subchondral bone, but fibres do not extend into the bone [3].

2.2 Enthesis/Osteoligamentous junction

The enthesis is formed of tendon, fibrocartilage, mineralized fibrocartilage and bone. The transition of these four zones occurs over a length of 1 mm [10]. The tendon zone mainly consists of type I collagen fibres. The fibrocartilage zone contains type II and III collagen and a small amount of type I, IX and X collagen as well as proteoglycan aggrecan and decorin. The mineralized fibrocartilage is the boundary between soft and hard tissue. The "bone zone" is composed of 40% type I collagen and 50% hydroxyapatite [11]. There are two different types of osteoligamentous junctions, the fibrous junction (indirect), where Sharpey's collagen fibres connect the ligament and bone-forming acute angles; and the fibrocartilaginous junction (direct), which is a graded transition zone composed of four different tissues. These tissues are the fibrous connective tissue with fibroblast, calcified and uncalcified fibrocartilage and bone tissue with osteocytes [12].

2.3 Myotendinous junction

The myotendinous junction (MTJ) is formed of four domains, namely the internal lamina, the connecting domain, the lamina densa of the external lamina and the ECM. Actin filaments of the terminal sarcomere connect these domains with the collagen fibres of the tendon. The internal lamina consists of actin filaments and associated crosstalk structures. The connecting domain connects the internal lamina to the external lamina. The main components of these regions are collagen IV, glycoproteins and proteoglycans [3,11]. At the MTJ, membranes of myofibers fold to bridge the gap between tendon collagen fibres and muscle fibres, distributing the stress during contraction and improve strength. Adhesion proteins like vinculin, talin and integrin with dystrophin in muscle section; and fibronectin, laminin, tenascin and heparan sulphate in the tendon zone, connect muscle and tendon fibres [3].

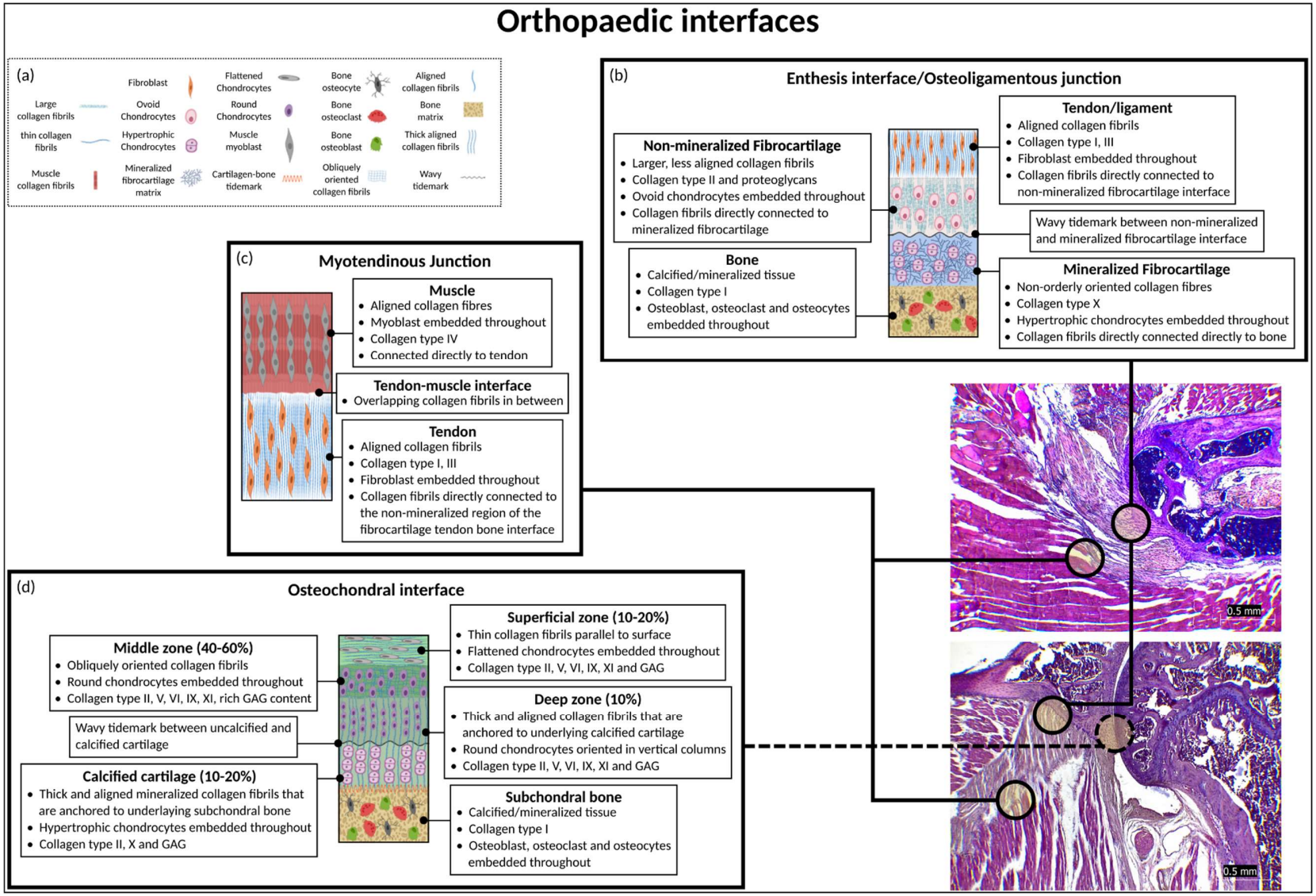

Figure 2: Overview of orthopaedic tissue interfaces. *A description of orthopaedic interfaces as well as their biochemical and cellular compositions is provided. (Histological sections of mouse limbs were kindly provided by Umut Karataş, Images a-d were adapted from Barajaa et al., 2019* [13]*)*

3. Disease and conditions of musculoskeletal interfaces

Joints can become a health and wellbeing problem due to many different issues: several genetic disorders, traumas or deterioration of the surrounding orthopaedic tissues [4,14]. This section describes common conditions and injuries that affect musculoskeletal interfaces.

3.1. Conditions and Injuries affecting bone and cartilage

3.1.1. Injuries due to trauma

Fractures are interruptions of the integrity and anatomic continuity of bone due to trauma. Fractures can be divided into open or compound fractures (penetration of the skin) and closed or simple fractures [15]. Compound fractures can also affect soft tissues due to high impact and/or sports and motor vehicle accidents. In these cases, in addition to the fixation of the fracture, the affected soft tissue needs to regenerate.
However, triage protocols only involve radiography, hence ligament or tendon injuries remain unknown and only bone fractures are treated [16]. Delays in the diagnosis of soft tissue injuries can lead to delays in their healing and ultimately aggravating the effects of injuries, like impaired bone healing, non-unions and pseudarthrosis [17].

3.1.2. Degenerative conditions

Osteoporosis is the most common metabolic bone disorder. It is characterized by an extensive loss of both inorganic and organic bone matter, due to an imbalance between bone formation and bone resorption. The loss of bone matrix results in fragile bones, leading to a higher risk of fractures and increase of spinal deformity (spondylolisthesis, scoliosis and hyperkyphosis). Osteoporosis is more commonly diagnosed in women and elderly [18]. Oestrogen is essential in maintaining the balance in bone remodelling by decrease the number of osteoclasts that are responsible for bone resorption. After menopause, the levels of oestrogen decrease, so the bone resorption occurs more often, reducing the bone mass [19]. The high incidence of osteoporosis in the elderly can be due to a decrease of physical activity, and consequently mechanical loading that leads to an activation of osteoclasts; while a reduction of production of sex steroids that are involved in the activation of bone formation process. Ageing is also correlated to a decrease of vitamin D levels, essential for calcium absorption and bone mineralization [20,21]. Even if osteoporosis is a pathology that affects mainly bones, Calvo et al., 2007 demonstrated that, in a model of osteoarthritis in rabbits, osteoporosis increased the severity of damages in cartilage [22].

Osteoarthritis is the most common degenerative joint disorder that mainly affects hands, knee and hips of elderly people. It is characterized by pain, stiffness and difficulties in mobility, caused by the deterioration of articular cartilage and the inability of chondrocytes to regenerate and form new matrix [23]. Osteoarthritis affects cartilage and subchondral bone, with the formation of osteophytes and subchondral cysts as well as and alterations in the synovial membrane [4,23,24].
The characteristic pain present in osteoarthritis can be due to the high density of nerves present in subchondral bone [24]. Osteoarthritis can also lead to muscle atrophy, due to the disuse of the joint when patients experience pain. Interestingly, muscle weakness has been shown to increase

the risk of development and progression of osteoarthritis [25]. Factors that can promote osteoarthritis are traumas, ageing, obesity, menopause, smoking, as well as developmental and genetic factors [4,26,27].

Other forms of arthritis are gout and rheumatoid arthritis. Gout is due to the formation of uric acid crystals in the joints, leading to inflammation and pain due to swelling of joints [4]. Usually, it occurs in men over the age of 40 and in women over the age of 60. Risk factors associated with gout can be genetic but are mostly due to a diet rich in meat, seafood and alcohol and the use of medication[28].

Rheumatoid arthritis is an autoimmune disease that affects principally small joints. The articular cartilage is eroded and eventually also the subchondral bone. Ultimately the pannus containing synovial lining cells, inflammatory cells, granulation tissue and fibrous connective tissue, can invade the joint and lead to fibrosis and ossification and eventually to ankylosis. The development of the pathology is associated with genetic, hormonal and environmental factors [4,28].

3.2. Conditions effecting tendon and ligaments

The conditions that affect tendon and ligaments are mostly associated with traumas or overuse. Tendinopathies can be classified in tendinosis or tendinitis. In tendinosis, tendon degeneration occurs without inflammation, collagen is degraded, and fibres disoriented. It develops after chronic overuse and reoccurring of mini-traumas and it can lead to pain and eventually, lesions. Risk factors can be ageing, vascular compromise, repetitive loads that cause microtrauma and pre-existing tendon injuries [29–32].
Tendinitis is an inflammation of the tendon that occurs after too heavy and/or too sudden and/or misaligned tensile force is applied to MTJ. Hence, an incomplete structural disruption of the tendon occurs, leading to vascular damage, bleeding and prolonging of the inflammatory healing phase Tendinitis can occur in individuals that perform repetitive motions or practice sports with repetitive loading of the muscle-tendon interface [29,31,32].

Sprains or torsions are injuries of the ligaments. These injuries can be classified according to their severity, namely grade 1, no macroscopic tears or joint laxity; grade 2, partial macroscopic tear with joint laxity; and grade 3, complete rupture with excessive joint laxity.
Sprains occur when ligaments are subjected to forces that exceed their mechanical strength. Continuous overuse injuries can lead to mechanical instability of the ligament and hence ligament trauma. Ligament traumas are characterized by swelling of the adjacent soft tissues [33].

Strains can affect also muscles. They are classified as a disruption in continuity and function of the muscle-tendon unit and can be caused by an acute impact (contusion), overstretching and excessive and/or repetitive load. The muscles mostly involved in this type of damage are usually muscles that cross two joints, like hamstrings, gastrocnemius and rectus femoris. Muscle strain, similar to ligament sprains, can be classified as grade 1, 2 or 3 [32].

Muscular dystrophies are a group of inherited degenerative skeletal muscle diseases that affects male children. They present different symptoms, severity and heritability, but they all lead to muscle fibre necrosis, fibrosis and replacement of muscle with adipose tissue. Duchenne's muscular dystrophy and Becker's muscular dystrophy result from a recessive mutation of the dystrophin gene located on X chromosome resulting in pelvic and limb-girdle weakness. Usually, they manifest in late childhood [34].

Further causes of damage to musculoskeletal interfaces are insurgences of tumours of bone, cartilage or soft tissues (sarcoma), due to the invasiveness of necessary resection of the tumour mass [35,36]. Osteosarcoma and soft tissue sarcoma are commonly treated by surgery, followed by chemotherapy or radiotherapy [37,38]. It is well known that chemotherapies and radiotherapies have severe side effects compromising the health of the other cells [39]. These therapies can induce, for example, chondrocytes senescence and bone differentiation leading to osteoarthritis [40]. Furthermore, numerous other drugs are associated with the development of tendinopathies or muscle rupture [41,42].

4. Choice of Biomaterial and fabrication techniques for orthopaedic interface studies

The *in vivo* environment influences the behaviour of cells and provides mechanical, chemical and physical signals. Hence, it is important to mimic the native ECM *in vitro*. A three-dimensional (3D) scaffold should allow the adhesion of cells and mimics the ECM. It must provide both rigidity and porosity of the physiological matrix. Various biomaterials, such as polymers, ceramics, metals, have been used for this purpose. Biomaterials should not induce inflammation, toxic and allergic reactions in the body. They should be biocompatible, bioactive, bioinert, biodegradable and sterilisable [43,44]. It is crucial that a biomaterial has specific physical, chemical, and mechanical properties required for the proposed application.
These properties are fundamental for cell adhesion [45]. When cells adhere to the biomaterial surface, physical-chemical reactions between cells and biomaterials occur. Such reactions are further influenced by cell behaviour, environmental factors and biomaterial surface properties such as wettability, roughness, softness, and chemical composition [46].
The physical and chemical properties as well as composition further influence cell response, chemical stability and reactivity of the biomaterial. The body's strict requirement to remain within the confines and limits of homeostasis often result in harsh localised environments that may cause corrosion, wear, breakdown or isolation of biomaterials. Therefore, the biomaterials' chemical stability becomes a relevant factor for biocompatibility [47]. For a biomaterial to be utilized for a specific tissue application, its mechanical properties should mimic the native tissue's properties. Hence Young's modulus, ductility, tensile strength, yield strength, compressive strength and fatigue, and wear debris among others, are studied [47]. A wide number of synthetic and natural polymers have been used to fabricate scaffolds for orthopaedic interface studies. The advantages of using synthetic materials are the ability to customize their shapes and the possibility to control their degradation rate by changing the composition of the polymer [48]. However, their bio-inactivity increases the risk of rejection and by-products of their degradation can be toxic [49]. Moreover, the adhesion of cells is often not promoted [50]. Using materials with natural origin can resolve some of these problems. These polymers are biologically active, and most of them can improve cell adhesion and

growth. One exception is alginate, that does not have the adhesion molecules that promote cell attachment [51]. Thanks to the biodegradable nature of natural polymers, the new extracellular matrix that is produced by cells will replace the scaffold as it degrades with time. The big disadvantage of using natural polymers is the difficulty to handle them, because of their poor mechanical properties [48]. In fact, natural polymers are often combined with other materials, such as synthetic polymers [52–54] or ceramics [55–57] to form composite scaffolds and enhance their physical properties. A detailed description of the properties of these biomaterials can be found in Catoira et al., 2019 [58], Gonzales-Fernandez et al., 2019 [59], Ha et al., 2013 [60], Hunt et al., 2014 [61], Li et al., 2018 [62], Qazi et al., 2015 [63], Samavedi et al., 2017 [64] and Stratton et al., 2016 [65].

To mimic the physical and structural properties of the musculoskeletal ECM, several scaffold fabrication techniques have been developed over the years. By combining the right materials and fabrication technique, it is possible to obtain a 3D scaffold resembling the structural and physical properties of native tissues. For bone, for example, it is necessary to have a physically, mechanically supportive porous scaffold. For the fabrication of porous scaffolds, solvent casting-particulate leaching, freeze-drying, gas foaming and additive manufacturing techniques can be used. As previously mentioned in this section, it is necessary to add materials to provide stiffness to improve the mechanical properties of materials. Therefore, several studies described the addition of glass [66]or ceramics [67–69] to natural and synthetic materials. As described in section 3.1.2., cartilage does not regenerate well, and its deterioration can lead to arthritis and consequently, pain. Hence, there is widespread interest in developing treatments for cartilage regeneration. The most commonly used material for *in vitro* cartilage models is collagen [70–72], the main component of native cartilage. To obtain a stable construct, often other materials are added [71,72] and techniques including freeze-drying [53,70–73] and 3D printing [74,75] are employed for the scaffold fabrication. For mimicking tendon, it is important to reproduce its elasticity. Therefore, hydrogels [76–78] and polymers such as polycaprolactone (PCL) [79,80] are used. Additive manufacturing can be used also for the development of fibrous scaffolds, together with electrospinning, phase separation, freeze- drying and self-assembly [81]. For the muscle, it is often preferred to use materials derived from decellularized organs [82–85] to recreate the muscle cell environment. Hydrogels containing ECM components are widely used [86–88]. Fabrication techniques are widely described in Domingues-Goncalves et al. 2020 [49], Jose et al., 2016 [89], Lanza et al. 2020 [90] and Yadegari et al., 2018 [81]. In Eltom et al., 2019 [91], advantages and disadvantages of fabrication techniques are described and a comparison of conventional and modern technique applied in tissue engineering is reported.

The regeneration of orthopaedic tissue interfaces is of growing interest. Numerous approaches have been presented to study tissue and interface regeneration [12,92–97] as well as the differentiation of mesenchymal stem cells into different musculoskeletal cell types [12,46,92,98]. As the tissues have different compositions and features, constructs with compound gradients [93–95] and suitable mechanical and physical properties [12,92,99–101] have been designed An overview of advanced techniques used for fabricating gradient scaffolds can be found in Li et al., 2020 [102]. Li et al., focused on the advantages and disadvantages of using additive manufacturing, component redistribution, controlled phase changes and post-modifications for fabricating scaffolds with gradients of cells, composition, architecture and mechanical properties. The advantages and disadvantages, but also

application of each approach, are discussed in table 1. Some of the studies on musculoskeletal interfaces are summarized in table 2 and fully described in the sections 5 and 6.

Several materials, deriving both from natural and synthetic origin, can be used for studying the osteochondral interface. Looking at sections 5.1 and 6.1.1 and table 2, the most used material was alginate. In these studies, alginate was used alone only as sacrificial material to provide support to chondrocyte to form new matrix [103]. Otherwise, it was mixed with other materials, to create a gradient. The materials added can be of synthetic origin, like poly(vinylic acid), applying chemical precipitation technique [93]; or natural, like chitosan [104] or hydroxyapatite [105]. Another polymer wide used is polyethylene glycol (PEG). It can be found with poly(lactid acid) (PLA) using melting mixing technique [100] or poly(caprolactone) (PCL) and hydroxyapatite using additive manufacturing technique [54]. As said before in this section, collagen is wide used for cartilage 3D models. In section 5.1. it can be found in a composite gel with chitosan for regenerate the osteochondral interface [106]. Finally, articular cartilage was decellularized to make a scaffold for osteochondral regeneration [56].

In sections 5.2. and 6.1.2. it is possible to notice that PLA is wide used to develop scaffolds for studying the enthesis/osteoligamentous. Nanofibers of PLA were fabricated with electrospinning technique [52,98]. PLA can also be mixed with PEG, to form PLGA and it was used to form nanofiber with electrospinning as well [107,108]. Additive manufacturing was also chosen for studying enthesis or osteoligamentous interfaces. PCL was mixed to PLGA [12] or Gelatin methacrylate (GelMa) [109] for the fabrication of scaffolds. Also natural polymers were used, like alginate [110] or composite hydrogel like collagen and silk [111]. They were employed to develop porous scaffold by freeze-drying technique. Decellularized tissues, like Achille's joint [112] or fibrocartilage [113] , were tested to study bone-tendon/ligament interface.

As mentioned before, studies on muscle and myotendinous junction often involve materials of natural origin. Sections 5.3. and 6.1.3. and table 2 report that collagen was mixed with PLLA to fabricate nanofibers by electrospinning [92]. Using 3D printing techniques, scaffolds made of GelMa and PEG were fabricated [114]. Also PCL and polyurethane were 3D printed [97]. As for osteochondral and enthesis interfaces, decellularized tissues can be used, like myotendinous junction [115]. Self-assembled tendons were analysed as well [116].

Table 1: Summary of advantages and disadvantages of fabrication strategies for gradient scaffolds for musculoskeletal interface studies *(adapted from Li et al., 2020 [102])*

Technique	Advantages	Disadvantages	Application	Material used	Reference
	Additive manufacturing				
Layering	Rapid and simple protocol. No specialist equipment	Restricted to stepped transitions. Risk of delamination	Repair of osteochondral defects *in vivo*	Poly(lactide-*co*-glycolide), 45S5 Bioglass® and medical grade calcium sulphate	Niederauer, G. G., et al., 2000 [117]
				Collagen type I and hydroxyapatite	Parisi C. et al., 2020 [118]
				Collagen type I, II and hydroxyapatite	Levingstone T. et al., 2016 [119]
3D printing	Free-form control over the material architecture. Can form continuous gradients. Can form a range of gradients	Requires printable materials. Requires specialist equipment and significant user expertise	Osteoligamentous junction reconstruction *in vitro*	Poly(caprolactone)	Lui, H. et al., 2019 [120]
			Osteochondral regeneration *in vitro*	Poly (ethylene glycol)/Poly (ethylene glycol)- diacrylate and hydroxyapatite	Nowicki, M. A., et al., 2016 [121]
			Myotendinous junction tissue engineering *in vitro*	Gelatin-methacrylate and Poly (ethylene glycol)-methacrylate	Laternser S., et al., 2018 [114]
Fluid mixing	Rapid and simple protocol. Can form continuous gradients. Can form a range of gradients.	Restricted to single gradients	Osteochondral regeneration *in vitro*	Alginate/gelatin methacrylamide/vinyl moieties-chondroitin sulphate hydrogel - Alginate/gelatin methacrylamide/vinyl moieties- chondroitin sulphate/ methacrylate hyaluronic acid hydrogel	Idaszek J., et al., 2019 [122]
Electrospinning	Rapid and simple protocol. Can form continuous gradients. Can form a range of gradients	Restricted to thin scaffolds. Challenging with live cells	Enthesis interface healing *in vivo*	Hydroxyapatite-doped polycaprolactone	Han F., et al., 2015 [123]
			Osteochondral interface tissue engineering *in vitro*	Poly (ethylene glycol)/Poly(caprolactone)	Horner, C. B., et al., 2019 [124]
	Component redistribution				

Convection	Rapid and simple protocol. Can form continuous gradients	Requires certain geometry and convective conditions. Restricted to single gradients	Osteochondral interface tissue engineering *in vitro*	Methacrylate Gelatin and gellan gum and hydroxyapatite microparticles	Canadas, R. F., et al., 2018 [125]
Buoyancy	Rapid and simple protocol. Can form continuous gradients. Can form a range of gradients	Requires a density difference. Restricted to single gradients	Osteochondral interface regeneration *in vitro*	Gelatin methacryloyl and heparin methacryloyl	Li, C., et al., 2019 [126]
Magnetic fields	Rapid and simple protocol. Can form continuous gradients	Requires magnetic particles. Risk of particle cytotoxicity	Osteochondral interface tissue engineering	Growth factor loaded glycosylated superparamagnetic iron oxide nanoparticles, agarose and hydroxyapatite	Li, C., et al., 2018 [127]
Electric fields	Can form continuous gradients	Requires field responsivity. Risk of electrical cytotoxicity	Osteochondral interface regeneration *in vitro*	Silk fibroin nanofibers and silk fibroin hydrogels	Xu, G., et al., 2020 [128]
	Controlled phase changes				
Heat-induced	Rapid and simple protocol. Can form continuous gradients	Requires thermoresponsivity at suitable temperatures	Enthesis interface regeneration in vitro	Collagen-GAG and calcium phosphate	Caliari, S. R., et al., 2015 [129]
	Post-modification				
Dipping or filling	Rapid and simple protocol. Can form continuous gradients	Requires relatively rapid binding kinetics	Bone-soft tissues engineering in vitro and in vivo	Collagen and lentiviral vector with Runx2/Cbfa1 plasmid	Phillips, J. E., et al., 2008 [130]
			Enthesis interface regeneration *in vitro*	Poly(lactic-co-glycolic) acid with NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaH_2PO_4{\cdot}H_2O$ and $NaHCO_3$	Liu, W., et al., 2014 [7]
Diffusion	Simple protocol. Can form continuous gradients	Requires optimized mass transport conditions Slow fabrication process	Osteochondral interface regeneration *in vitro*	Poly(caprolactone) with bone morphogenic protein-2 and transforming growth factor β-3	Di Luca, A., et al., 2017 [131]

Table 2: In vitro studies on musculoskeletal interfaces

Material used	Fabrication technique	Study outcome	Limitations of the approach	Reference
Osteochondral interface				
Poly (Lactic acid/ Polyethylene glycol) (PLA/PEG))	Melt mixing	Multi-layered constructs with pore size gradient improved interfaces formation without fractures; stress response was enhanced; cells proliferation and organisation were promoted	Time of culture should be improved. Lack of studies with human cells. The behaviour of cells at the interface was not evaluated.	Scaffaro R., et al., 2016 [100]
Strontium phosphosilicate (Sr5(PO4)2SiO4 (SPS)) bioactive ceramic	Additive manufacturing	Chondrocyte proliferation and maturation and preservation them from osteoarthritis. Osteochondral formation.	Strontium ranelate leads to osteophyte overgrowth, reduce subchondral bone remodelling. Lack of studies with human cells	Deng, C., et al., 2018 [94]
Poly (ethylene oxide terephthalate)/poly (butylene terephthalate) (PEOT/PBT)	Additive manufacturing	Scaffolds with porous gradient allowed cell adhesion and chondrogenic differentiation.	The behaviour of cells at the interface was not evaluated.	Di Luca, A., et al., 2016 [12]
Alginate/ hydroxyapatite (HA)	Molding	Alginate and hydroxyapatite scaffolds can promote formation of a calcified cartilage-like matrix, with higher mechanical properties than ceramic-free alginate scaffolds.	Lack of studies with human cells.	Khanarian, N., et al., 2012 [105]
Enthesis/Osteoligamentous junction				
Gelatin-grafted poly(lactic-l-acid) (PLLA)	Electrospinning	Gelatin-PLLA membranes biocompatible and biodegradable. In in vivo, increased area of glycosaminoglycan in the tendon-bone interface, improved collagen organization.	The single load to failure biomechanical test does not replicate the clinical setting.	Zhao, S., et al., 2015 [52]
Alginate and transforming growth factor β1 (TGF β1)	Freeze-drying	Alginate nontoxic scaffold, with continuous TGFβ1 release, improved mechanical and histological outcomes	Possible scar formation due to TGF-β1 effect on collagen III production. Some animals presented infection.	Yoon, J. P., et al., 2018 [110]
Poly (lactic – co – glycolic acid) (PLGA)	Electrospinning	Constructs with mineral gradients with modified-simulated body fluid	Biocompatibility was not tested.	Lipner, J., et al., 2014 [107]

Collagen	Gelation	Scaffolds with immobilized Runx2 gradient allowed a spatial pattern of transcription factors expression, osteoblastic differentiation of fibroblasts and mineralized matrix deposition. Graded distribution of mineral deposition and mechanical properties was maintained in vivo.	Intermediate fibrocartilage zone was not visualised.	Phillips, J. E., et al., 2008 [130]
Myotendinous junction regeneration				
3D skeletal muscle constructs co-cultured with self-assembled tendon constructs	Self-assembling	3D muscle–tendon constructs with viable MTJs structural features and protein expression patterns	The average tangent modulus of the constructs was one quarter of a young adult rat soleus muscle modulus.	Larkin, L.M., et al., 2006 [116]
Decellularized tissue	Decellularization	Scaffolds made with porcine preserved native biphasic structure, biological composition and mechanical properties. Cells adhered, proliferated and infiltrated into the scaffolds. Cell differentiation was observed. Minimal immunological reaction was observed in vivo.	Long-term study on regeneration effects of D-MTJ scaffolds is needed. In clinic, use of animal-derived products might cause transmission of pathogens to patients.	Zhao, C., et al., 2018 [77]

5. I*n vitro* studies on musculoskeletal interfaces

5.1 Formation of osteochondral interfaces *in vitro*

For the regeneration of osteochondral interfaces, natural and synthetic materials to develop scaffolds with graded material composition to recreate bone and cartilage cell environment. Radhakrishnan et al. 2018, prepared a scaffold using alginate and (polyvinyl acid) (PVA) hydrogels by wet chemical precipitation to build a construct with a hydroxyapatite gradient, to mimic the inorganic matrix of bone, and chondroitin sulphate gradient, to mimic the cartilage environment, as it is a glycosaminoglycan widely present in this tissue (Figure 3A). Primary rat osteoblasts were seeded together with primary chondrocytes on these scaffolds. Higher proliferation rate and alkaline phosphate (ALP) activity was observed compared to cells seeded on cell-culture plastic plates. Also, the proliferation of chondrocytes seeded on composite scaffolds was higher. Both cell types migrated towards the interface zone [93]. In section 6.1.1. the results of the scaffold tested *in vivo* are reported. A collagen/chitosan triphasic scaffold was designed by Korpayev et al. 2020, who tried to replicate the different phases of the osteochondral interface by developing a porous chitosan/collagen I/hydroxyapatite scaffold for bone; a chitosan/collagen II/hydroxyapatite hydrogel scaffold for calcified cartilage; and a gel matrix of chitosan/collagen II hydrogel for the cartilage layer. Mouse pre-osteoblasts (bone layer) and mouse chondrocytes (calcified cartilage and cartilage layer) were incapsulated in the individual scaffolds and cultured independently for 7 days, then scaffolds were assembled, and co-culture medium was added. After 21 days in culture, metabolic activity and DNA content increased. Glycosaminoglycan (GAG) and collagen content increased. Moreover, a high level of ALP activity was measured in the first days of co-culture in bone and calcified zone; chondrocytes showed a hypertrophic phenotype in the calcified cartilage structure indicating a differentiation of both cell lines [106]. Behaviour of cells in the interfaces was not evaluated.

Another chitosan-based bilayered and porous scaffold was designed by Erickson et al. 2019. Chitosan was mixed with alginate and hydroxyapatite to fabricate the bone layer, while it was mixed with hyaluronic acid for the cartilage layer. MG-63 (bone) and human chondrocytes or mesenchymal stem cells (MSCs) (cartilage) were seeded onto the scaffold layers (Figure 3B). All cell types proliferated after 10 days in culture and migrated to the interface area. Moreover, the osteogenic marker, osteocalcin, and the chondrogenic marker, collagen II, were expressed [104]. Scaffolds can also be used for the release of growth factors, as described by Dong et al., 2020 [132] and Rowland et al., 2018 [56]. Here, a triphasic scaffold was created with a porous structure of silk and hydroxyapatite for bone and silk and ECM proteins for the cartilage [132]. For the bone tissue, microspheres containing bone morphogenic protein-2 (BMP-2) were added, while for the cartilage tissue, transforming growth factor-β containing (TGF-β) microspheres were added. With this approach, human umbilical MSCs seeded on top of these scaffold were viable and proliferated up to 21 days in culture. Furthermore, the content of GAG and collagen II increased and chondrogenic genes were expressed in the cartilage layer; while collagen I and osteogenic genes were expressed in the bone layer [132].

In another study, decellularized porcine articular cartilage was used, using a mould, to fabricate biphasic scaffolds, formed by a shell and a core, with immobilized lentivirus vectors containing

TGF-β (shell), BMP-2 (core), responsible of cartilage and bone formation, respectively; or interleukin-1 (IL-1), to control the degradation of scaffolds (Figure 3C). After 28 days in culture, new matrix was produced and human MSC differentiated in osteoblasts (core) or chondrocytes (shell) [56]. The matrix deposited in the interface was made of collagen I and II and GAG.

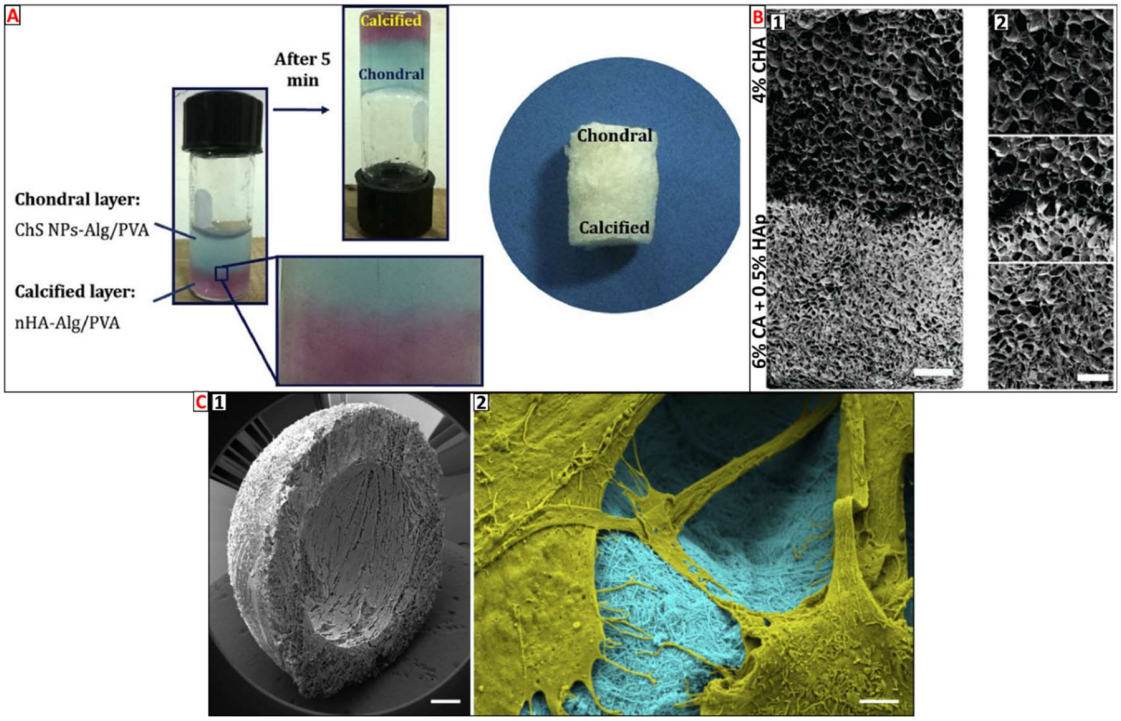

Figure 3: Scaffolds prepared for osteochondral interface studies. *A) Alginate/PVA hydrogel scaffolds with chondroitin sulphate nanoparticles for cartilage and hydroxyapatite nanoparticles for bone (adapted from Radhakrishnan et al., 2018 [93]); B) High magnification (B1) and low magnification (B2) of a bilayered and porous scaffold made of chitosan and hyaluronic acid for cartilage and chitosan and hydroxyapatite for bone (scale bar 1mm) (B1). B2 shows the scaffold section with higher magnification (scale bar 500 µm) (adapted from Erickson et al., 2019 [104]); C) low magnification (C1) and high magnification (C2) of biphasic scaffold made of decellularized porcine articular cartilage and BMP-2 for bone (core) and TGF-ß for cartilage (shell). C1 scale bar: 1 mm; scale bar C2: 2 µm (adapted from Rowland et al., 2018 [56]).*

5.2. Formation of enthesis/osteoligamentouous junction *in vitro*

Criscenti et al., 2016 fabricated a triphasic scaffold for bone-ligament regeneration. The scaffold consisted of a 3D printed poly(caprolactone) (PCL) region for bone and an electrospun poly (lactic-co-glycolic acid) (PLGA) fibrous region for the ligament (Figure 4A). The PLGA section was fabricated using the bone section as the template. Human MSCs were seeded on the scaffold. After 14 days in culture, cells increased their metabolic activity. Cells on the PCL section expressed ALP; while an increase of GAG was evident throughout the entire scaffold [12]. Cells were homogeneously distributed in the scaffold and interface, however the behaviour of cells at the interface was not investigated. A more recent approach for bone-ligament osteointegration was developed by Jiang et al., 2020. Here, an electrospun PLGA fibrous scaffold with a gradient of TGF-β (ligament); and BMP-2 and hydroxyapatite (bone) was fabricated. Rat bone marrow MSC (rBMSC) were seeded on the scaffolds (Figure 4B). The fibres in the bone section were randomly oriented, whereas the fibres in the ligament section were aligned. After 7 days in culture, rBMSC were viable and showed

different morphologies on different scaffold sections. On random fibres the cytoskeleton was disordered, while on the aligned fibres elongated cells aligned themselves along the fibres. Cells expressed more ALP in the bone section compared to the ligament section, while the GAG content was higher in ligament section. Furthermore, the gene expression of bone markers was higher in the bone section than the ligament and interface regions. Sox9 (ligament marker) expression was lower in cells on random fibres and on interface section; however, the difference was not statistically different [108]. For studying the bone-tendon interface development, scaffolds with a platelet-derived growth factor (PDGF) gradient were developed by Perikamana et al., 2018, using aligned electrospun poly(lactic-l-acid) (PLLA) fibres. Human adipose-derived stem cells (hADSC) were seeded on the scaffolds and cultured for 14 days. Results showed greater cell proliferation compared to randomly aligned fibres. Cells differentiated into tenocytes across the scaffold, however differentiation was higher on the scaffold region with the higher concentration of PDGF. Moreover, cells aligned along the fibres of the treated scaffolds [98]. The formation of the interface was not investigated. Another approach was developed by Su et al. 2019, where the bone-fibrocartilage-tendon areas of porcine Achilles joint was decellularized, obtaining a triphasic scaffold. Subsequently, rBMSC were seeded on the acellular matrix and cultured 14 days. The structure maintained the physical and mechanical properties of the native tissue, also at the interface level, and after 7 days in culture metabolic activity and viability of cells did not decrease; and cells differentiated into bone and tendon cells [112]. How cells differentiate at the interfaces was not analysed. The results of in vivo tests are reported in section 6.1.2.

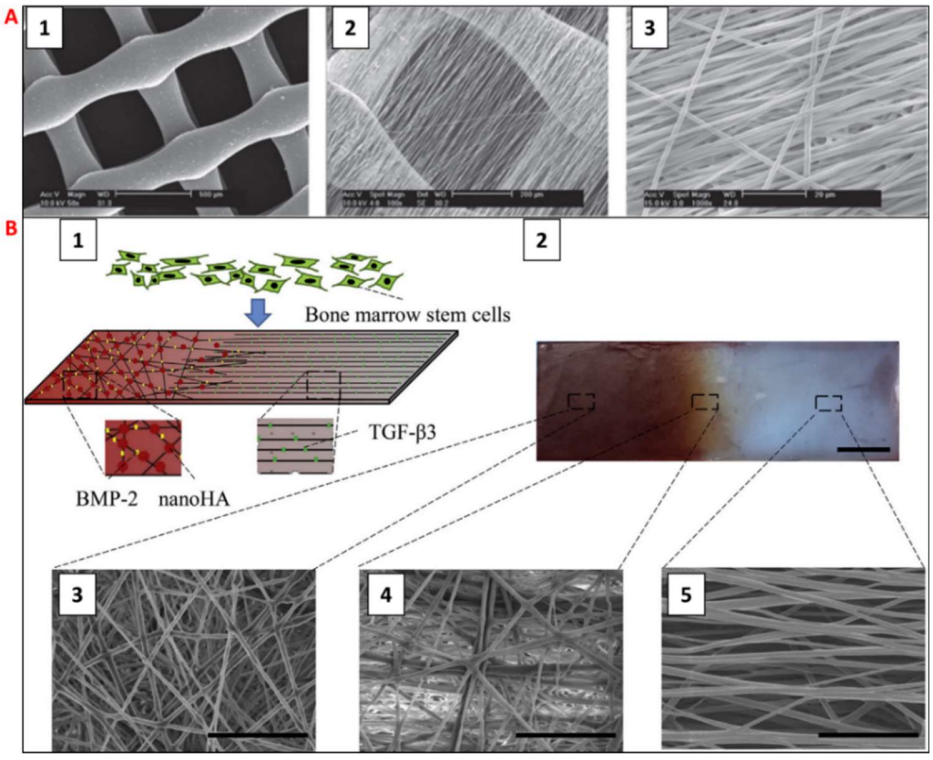

Figure 4: Scaffolds for enthesis interface studies. *A) Triphasic scaffold made of 3D fibre deposited PCL structure for bone (A1), a mixed region (A2) and a PLGA electrospun structure for tendon (A3) (adapted from Criscenti et al., 2016 [12]); B) Schematic of Electrospun scaffold made of PLGA and TGF-β for ligament; and PLGA and hydroxyapatite and BMP-2 for bone (B1). B2 shows hydroxyapatite stained with alizarin red (scale bar: 1cm); higher magnifications of bone (B1), interface (B2) and ligament (B3) regions (scale bar: 10 µm) (adapted from Jiang et al., 2020 [108]).*

5.3. Formation of the myotendinuous junction *in vitro*

There is a scarcity of studies on myotendinous junction in literature. The most recent study was published in 2018 by Laternser et al., where Gelatin methacrylate based (GelMA) bioinks were used to make a 3D printed scaffold of GelMA/Polyethylene glycol dimethacrylate (GelMA/PEGDMA) or pure GelMA (Figure 5A). A 24-well plate was designed with round postholders embedded in agarose at the bottom. Layers of bioinks and layers of cells (rat tenocytes and human myoblasts) were printed on the agarose. Tenocytes and GelMA/GEGDMA were printed around the posts, myoblasts and GelMA were printed in the middle part of the structure, leaving a gap between the cell types. Adding differentiation media, cells differentiated in tendon-like tissues and myofibers. Myofibers aligned on the scaffolds and interacted with tenocytes at the interface [114].

Another interesting use of 3D printing was described by Merceron et al., 2015. Here a triphasic construct with a thickness gradient was developed to create a myotendinous interface using thermoplastic polyurethane (PU), PCL and hydrogel bio-ink by fusion deposition modelling (Figure 5B). Mouse skeletal muscle cells and C2C12, were seeded on the PU side of the construct to mimic muscle and mice fibroblasts, NIH3T3, were seeded on the PCL side to mimic tendon. Scaffolds had characteristics resembling the native tissues and cells were alive after 7 days post-printing. Cells also migrated to the interface and expressed myotendinous junction-specific genetic markers [97].

Ladd et al., 2010 developed a dual electrospun scaffold made of PLLA/collagen (tendon) and PCL/collagen (muscle) (Figure 5C). NIH3T3 were seeded on the tendon section and C2C12 were seeded on the muscle section. The sections had characteristics that resembled the native tissues and myoblasts were able to differentiate into myotubes [92].

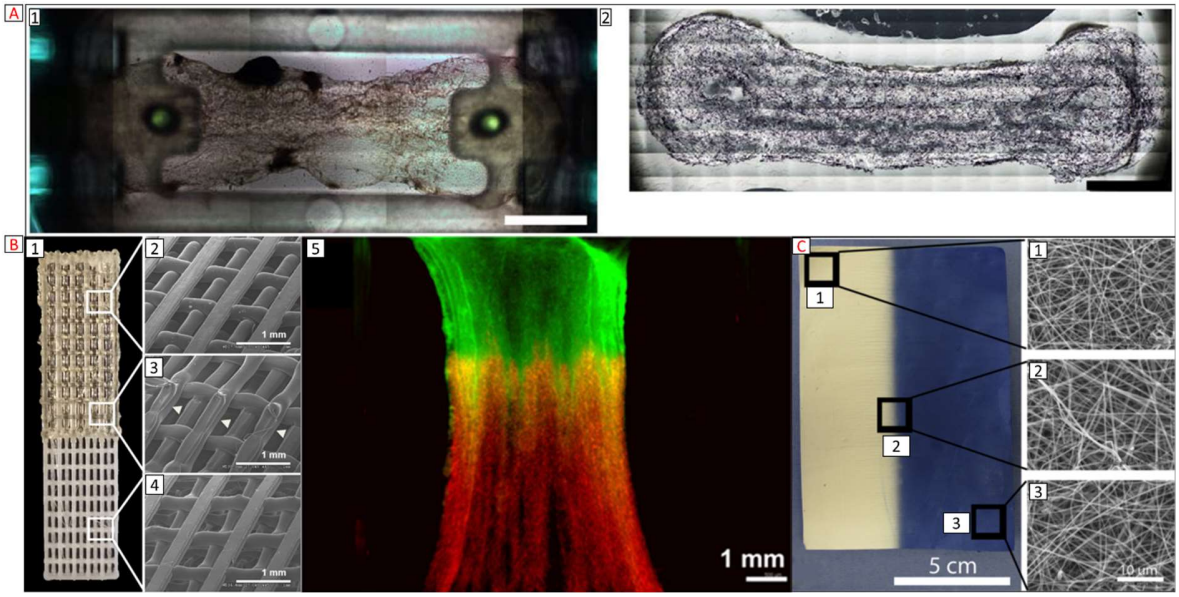

Figure 5: Scaffolds for myotendinous junction studies. *3D printed scaffold made pure GelMA for muscle (A1) or GelMA/PEGDMA for tendon (A2) (scale bar: 2mm) (adapted from Laternser et al., 2018 [114]); B) Low (B1) and high magnification (B2-4) of 3D printed scaffold made of layers of PU and C2C12 for muscle (B2), interface region (B3) and PCL and NHI3T3 for tendon (B4). B5 shows how C2C12 (green) and NIH3T3 (red) are distributed on the scaffold and how they interact at the interface (yellow) (adapted from Merceron et al., 2015 [97]); C) Co-electrospun scaffolds made of PCL/collagen fibres for muscle (C1), interface region (C2) and PLLA/collagen fibres for tendon (C3) (adapted from Ladd et al., 2010 [92]).*

5.4. Bioreactors for the regeneration of tissue interfaces *in vitro*

Bioreactors are used in tissue engineering to provide physiological relevant mechanical stimulation for cells in culture to recreate an environment more similar to the *in vivo* state. Mechanical stimuli have been shown to enhance cellular activity and promote differentiation and the deposition of ECM. Parameters like pH, temperature, oxygen tension and medium perfusion can be controlled. Bioreactors can be used to improve the *in vitro* development of new tissues [133–135]. When bioreactors are designed, some principles need to be considered, including the ease of assembly, promotion of tissue formation, ease of sterilisation and maintenance of sterility and cell culture monitoring through embedded sensors [135]. A wide description of types of commercial bioreactors developed for tissue engineering regeneration, such as rotating wall bioreactors, flow perfusion, compression bioreactors, bioreactors and hydrostatic bioreactors, can be found in Reinwald et al., 2016 [136]. This article also gives an overview on the importance of the use of stem cell in tissue engineering and how bioreactors can influence their differentiation [136].

For bone, cartilage, tendon and muscle it is essential to recreate a dynamic and continuous flow within the bioreactor. A dynamic flow helps to obtain a homogeneous distribution and higher number of cells [137–140]. Besides a hydrodynamic stimulus, each tissue needs other specific mechanical stimuli. Various bioreactors have been developed for orthopaedic tissues. For example, in the body, bones are subjected to load. To mimic this condition, several chambers made of a piston that applies compressive loads directly on the scaffolds were created. Cells showed an improvement in cell size, extracellular matrix deposition, higher alkaline phosphatase activity and increased bone markers expression [141–143].

Because of the massive amount of water bound to proteoglycans, cartilage is subjected to osmotic pressure in the body [138]. Chondrocytes seeded in 3D and cultured in a bioreactor that provides hydrostatic pressure and perfusion, showed a better distribution, viability and phenotype maintenance compared to cells seeding in tissue culture plastic plates [139,144].

Tendons undergo cycling stretch and torsion. Bioreactors for tendons usually contain clamps that hold the scaffold while torsion is applied. In these conditions, cells increase their dimensions, alignment; and mesenchymal stem cells differentiate into tenocytes and express tendon markers [145–147].

In vivo, skeletal muscles receive electrical stimuli from nerves and consequently contract. Bioreactors can be connected to a stimulator circuit that provides controlled electrical pulses**.** Cell contraction is promoted, and cells increase in size and express tissue specific markers [148–150].

Few bioreactors for the regeneration of interfaces can be found in literature. For osteochondral interface regeneration, Chang et al., 2004 created a two-chamber bioreactor with magnetic-bar stirrer that mixes medium and provides shear stress to the construct. The bioreactor also has two independent circulation systems that facilitate the addition different medium in the chambers. The bioreactor was originally designed for the co-culture of bone and cartilage cells, and was utilised to test the ability of a biphasic scaffold to promote the formation of hyaline cartilage *in vitro*, demonstrating new cartilage formation after 4 weeks [151]. Doroski et al., 2010 developed a bioreactor that provides tensile stress to constructs promoting the differentiation of MSC into tenocytes to repair damaged tendon at the bone-tendon or tendon-muscle interface. However, this study

only evaluated the formation of single tissue, multiphase tissues and tissue interfaces. Results showed that culturing MSC under tensile stress promoted their differentiation in tenocytes.

In clinic, bioreactors can be used to culture elevate numbers of stem cells and other cells, like lymphocytes [152], to use for cell and/or gene therapy [153,154]. Bioreactors are also used to prepare the scaffold for the implantation, by promoting, for example, a higher cell matrix production [155]. Despite all the advantages of using bioreactors, their use in clinic and preclinic is still limited. This is primally due to the high costs required for their development, fabrication, sterilization and staff training [155–157]. Moreover, safety, scalability and ease of handling need to be taken into account when a bioreactor is developed [155].

6. Preclinical and clinical studies on the musculoskeletal interface regeneration

6.1 Preclinical studies

Preclinical studies ensure the clinical applicability and suitability of newly developed approaches for scaffold-based tissue regeneration. After successful completion of preliminary *in vitro studies*, scaffolds are tested *in vivo* using animal models to analyse the response in real time and to verify their safety and efficacy. The use of *in vitro* and *in v*ivo studies is crucial to avoid harm to people that the unknown graft could cause [158].
Several factors need to be considered before choosing a particular species as animal model. Most importantly the animal model should have physiological and pathophysiological features comparable to humans. It should also be manageable to operate and it should allow doing multiple studies post-surgery within a short period [159]. Also costs of acquisition and care, availability, ethics and tolerance to captivity need to be taken into account. International standards indicate that also the facilities that will host the animals have to be suitable in terms of size and capacity [160]. Various animal models are used for studying the regeneration of musculoskeletal interfaces. Among these, rabbits seem to be the most commonly used to study the enthesis/osteoligamentous junction [111,161–164] and osteochondral interface [103,165–167] regeneration. Rodents, are another commonly used as animal model because of their low cost, ease of handling and the ability to have a large numbers of animals [168]. Mice and rats are usually used in studies related to ageing, due to their restricted life span [169,170]. However, rodents have also been used to study the regeneration of bone-tendon/ligament [109,120,171] and bone-cartilage[118,172] interfaces. To replicate human joint diseases or injuries, it is important to use animals with size and anatomy as close as possible to humans [54,168]. For this reason, sheep [173–175] and mini-pigs [89,93,94] are used. Table 3 summarizes more examples of preclinical studies on musculoskeletal interface regeneration.

6.1.1. Recent preclinical studies on osteochondral interface

Nie et al. 2020 described an attempt to repair full-scale osteochondral defects. In this study, a sacrificial alginate porous scaffold was used to seed porcine chondrocytes. After new extracellular matrix was deposited by the cells, alginate was leached with citrate buffer, resulting in a sponge-like network of ECM proteins and chondrocytes. Afterwards, decellularization of the same scaffolds was performed. Cellular and decellularized scaffolds were implanted in New Zealand white

rabbits with massive patellar defects. Osteochondral regeneration occurred in animals that received normal and decellurized implants 50-100 days post-surgery. Osteochondral defects were fully filled with new ECM. The newly formed articular cartilage and subchondral tissues were physically differentiated from each other, but well integrated along the tidemark. Both tissues exhibited similar composition, structure, phenotype and mechanical properties of native tissues [103].
Hsieh et al. 2018 fabricated a scaffold made of poly(ethylene glycol)-poly(ε-caprolactone) (mPEG-PCL) and hydroxyapatite using fused deposition modelling to heal osteochondral defects in mini pigs. The scaffolds were coated with hyaluronic acid and modified with glycolmethacrylate to allow the adhesion of TGF-β1. Scaffolds were then implanted in mini pigs with defects to the knee. One year post surgery, histological analysis showed formation of new cartilage and subchondral bone in the lesions. Live magnetic resonance imaging showed bone defects were fully filled 6 months after surgery [54]. To improve the healing of large osteochondral defects, Wei et al., 2019 tested their novel scaffold in goats. The scaffold was composed of two parts, namely a platform of porous tantalum (pTa) seeded with bone marrow MSCs; and collagen membranes (CM) seeded with chondrocytes. Goats were divided in three groups; one of those was treated with pTa/CM only, one with pTa/CM and cells; while the third was not treated. After 16 weeks from surgery, complete repair of the defects in the treated groups was observed. In both cases, the new bone and cartilage tissues integrated with the host tissues, but the new chondrocytes formed in the acellular scaffolds were immature, small and flat. The interface between pTa and CM was uniform and smooth. In the non-treated group, degeneration of the cartilage and periosteum ossification occurred [178]. The scaffold developed by Radhakrishnan et al., 2018, discussed in section 5.1 was also tested *in vivo*, in New Zealand white rabbits with osteochondral defects. The alginate/PVA-based scaffold with hydroxyapatite and chondroitin sulphate gradients promoted the closure of the defects in 8 weeks, neo-matrix formed and tissue host integration [93].

6.1.2. Recent preclinical studies on enthesis interface

Qian et al. 2019 reported an approach for the repair of the bone-tendon interface. In this work, rabbit bone marrow-derived mesenchymal stem cells (BMSC) were seeded on scaffolds made of collagen/silk hydrogels fibres, which were either aligned or not aligned. Their efficiency was tested in New Zealand white rabbits that suffered damage at the tendon of the rotator cuff. Around 8 weeks post-surgery, animals that received randomly oriented scaffolds demonstrated newly formed tendon tissue and chondrogenesis had occurred at the fibrocartilage area of the interface. The new tissues grew further until the 12th week post-surgery and new collagen type I was produced in these rabbits [111]. Cao et al., 2020 fabricated a multiphase porous scaffold using 3D printing technique for bone-tendon interface engineering. The scaffold was composed of three sections. The bottom consisted of a two-layered PCL section for the tendon with fibroblasts embedded in gelatin methacrylate (GelMA). The top section was a three-layered PCL/ tricalcium phosphate (TCP) section for bone, with osteoclast embedded in GelMA; and the middle section for the fibrocartilage, which was made of four layers of PCL/TCP canals with bone marrow-MSCs still embedded in GelMA. This scaffold was tested in rats. The expression of tendon-bone interface related genes was assessed after 8 weeks from surgery. The genes involved in chondrogenesis increased their expression after 30 days post-surgery, while tendon markers had their peak of expression at week 8. Osteogenic marker Runx2 increased till week 4 then decreased. The scaffolds were well

integrated with the host tissues, new matrix deposition was shown and new blood vessels formed [109].

Another approach to improve the healing of damages at the enthesis interface was developed by Chen et al., 2019. They fabricated a book-shaped scaffold, by decellularizing fibrocartilage from rabbits. Grafts were formed by two book-shaped structures, one reversed and inserted into another; and layers of autologous adipose-derived stromal cells (ASCs) were seeded into the pages. Results compared the efficiency of acellular scaffold (AFS) and scaffold with cell layers inserted into the pages (ASCs/AFS). Rabbits underwent partial patellectomy, then the graft was implanted. After 8 weeks, for both groups, regenerated tissue bridging the residual patella and tendon was visible; while the ASCs/AFS group showed a larger region of cartilaginous metaplasia and more new bone formation than the AFS group. Moreover, in the ASCs/AFS group, the fibrocartilaginous junction was more robust than in the AFS group [179]. The decellularized scaffold developed by Su et al., 2019 and discussed in section 5.2. was also tested in New Zealand white rabbits with femur-tibia defects. Here, 8 weeks after surgery, new bone and tendon tissue were formed, there was no sign of loss of weight, infection and wound split in the animals [112].

6.1.3. Recent preclinical studies on myotendinous junction

Very few preclinical studies on the regeneration of the myotendinous junction (MTJ) have been reported to date. Zhao et al., 2018 obtained a scaffold from the decellularized Achilles tendon MTJ obtained from pigs and tested it New Zealand rabbits that suffered from muscle defect. After 30 days from surgery, abundant new myofibers were formed in the defect region and muscle-specific markers were expressed indicating the differentiation of endogenous muscle satellite cells. Furthermore, scaffolds were not rejected by the recipients and a low inflammatory response was observed [115].

6.2. Clinical trials

After successful completion of pre-clinical *in vitro* and *in vivo* studies, implants' safety and efficacy are investigated in clinical trials in humans. Their successful outcome will allow for the implants to be placed on the market [180]. Information on past, current and upcoming clinical trials can be accessed on clinical trial repositories such as Clinicaltrials.gov [181] or International Standard Randomised Controlled Trial Number (ISRCTN) [182]. These databases provide information on procedures, new prosthetic implants, drugs or scaffold developed to improve musculoskeletal disorders.

Table 4 presents a list of ongoing clinical trials on implants and procedures applied to the regeneration of the joint that can be found on clinicaltrials.gov. Current studies focus solely on disorders related to tendon and ligaments such as rotator cuff tear and anterior cruciate ligament, probably due to the high incidence in sport and work injuries [73,183]. These studies aim to improve patients' recovery after surgery, reduce failure in massive tears of the rotator cuff; and enhance the reconstruction of the anterior cruciate ligament. Moreover, studies on joint replacement due to damages caused by osteoarthritis are reported as well as repair of articular cartilage defects and meniscus fractures. [181]

On the ISRCTN repository, there are several studies on musculoskeletal diseases. These studies are aimed at reducing pain, testing new drugs and new surgery techniques or approaches; and improving the patients' conditions by implementing diet with supplements and/or performing exercises or physiotherapy. However, none of the studies mentioned the possibility to repair or regenerate interfaces or single organs with customised scaffolds [182].

The Australia and New Zealand Clinical Trials Registry reported a clinical trial using a scaffold made by the company Orthocell. CelGro ® is a collagen membrane, which was tested for the regeneration of dental bone and soft tissue (dental implant), the repair of rotator cuff tear and hip cartilage. While their other product, Ortho-ATI® (Autologous Chondrocyte Implantation) was tested for tennis elbow treatment and partial rotator cuff tear repair [184,185]

6.2.1. Concluded clinical trials on Rotator cuff tear and tendinopathies

Past clinical trials on the rotator cuff focused on tendon repair and the reinforcement of soft tissues. Two devices, both from Tornier and LifeCEll™, were tested. Biofiber™ (Tornier) is a collagen-coated absorbable scaffold, tested on patients of 18 years and older with full-thickness rotator cuff tears. Results showed that most patients experienced improved quality of life and almost total recovery of motion. Only 4% of patients had a reoccurring rotator cuff tear 12 months post-surgery, while 2% had infections. Conexa ™ (LifeCEll™) is a surgical acellular mesh derived from porcine derma. Adults, between the age of 40 to 70 years, with large irreparable rotator cuff tears, were enrolled in the study. Results showed an improved quality of life and recovery of motion over a 24 months period. Around 10% of patients experienced a reoccurrence of the tear and mild inflammation.
For treating epicondylitis and other tendinopathies, CollPlant developed an injectable gel made of bioengineered recombinant human type I collagen combined with autologous platelet-rich plasma. This matrix had the role of recruiting cells to the defect site promoting tissue repair. After 6 months post-implantation, significant clinical improvements were observed in treated patients, with only a single application. The mean Patient-Rated Tennis Elbow Evaluation had an improvement, i.e. a reduction of 59%. Grip strength increased by 28% from 28.8 kg at baseline to 36.8 kg. Improvements in sonographic tendon appearance were evident in 68% of patients. Moreover, there was no evidence of systemic or local severe adverse events [186].

6.2.2. Concluded clinical trials on regeneration of skeletal muscle

To reconstruct the knee joint after resection of soft tissue sarcoma, Müller D., et al. 2018, developed an allograft that includes the quadriceps tendon, the patella, the patellar tendon, and the tibia tuberosity. Results showed a local tumour recurrence in 33.3% of patients 5 years after surgery; 66.7% of patients showed presence of metastasis and 33.3% of patients needed a revision surgery. The flexion mean of the knee was 82.5° (range: 25-120°) and the mean extension lag was 10° (range 0-30°) [35], making this approach a reasonable approach for reconstructing the joint.
Sicari et al., 2014 performed a clinical trial on musculotendinous tissue repair and its reinforcement by implanting decellularized porcine urinary bladder scaffold in the injury site. Six months

post-surgery, a formation of dense tissue was evident, consistent with skeletal muscle at the implantation site. Biopsies performed after 8 weeks and 6 months from surgery, showed presence of perivascular stem cells (PVSC), blood vessels and the expression of desmin, a skeletal muscle cell-specific marker. These findings indicated that the regeneration of skeletal muscles and angiogenesis occurred. In 3 out of 5 patients, an increase in strength of 20% to 136% was observed. All the patients showed an improvement in the functional outcomes including dorsiflexion and knee extension [187].

6.2.3. Concluded preclinical trials on osteochondral regeneration

For the regeneration of cartilage after injuries, "TruFit Plug" was developed. It is a synthetic biphasic scaffold made of poly(lactide-co-glycolide) (PLG) and calcium sulphate for the bone region and only PLG for cartilage. Short-term results showed clinical improvement, decreased pain and 43% of patients had completely filled defects [188]. However, to date improvement of conventional microfractures and osteochondral allograft transplantation procedures have not been proved [189].

Table 3: Pre-clinical studies involving scaffolds used for musculoskeletal interface regeneration in vivo.

Application	Type of scaffold	Animal model	Study duration	Outcome	Reference
Osteochondral interface	Type I collagen bi-layered scaffold with bioactive magnesium doped hydroxyapatite in the bone layer	Nude mouse	4-8 weeks	• No sign of inflammatory reaction • The layers where intact • Cell colonization • Connective tissue penetration up to the inner part • Chondrogenic differentiation and bone formation Angiogenesis	Sartori, M. et al., 2017 [190]
	Decellularized iliac bone from pigs	Mini pigs	6-24 weeks	Defects were with new articular cartilage. New tissue well integrated with the normal cartilage. New tissue had similar physical and mechanical properties to normal cartilage.	Dai, L. et al. 2019 [191]
	Tissue engineered cartilage made of chondrocyte matrix and Calcibon®	Mini Pigs	26-52 weeks	Construct well integrated with native tissue. Only one animal presented infection. New bone formed from the sides of the construct. New cartilage formed with no gap between construct and native cartilage. New matrix was formed.	Petersen et al., 2007 [192]
	PEGDA triphasic scaffold with pore gradient	Immunodeficient mouse	4-8 weeks	Host cells were growing on the mineralized layer. Top of the gel presented chondrocyte characteristics and bone matrix and tissue formation.	Kang, H. et al., 2018 [193]
	3D printed porous scaffold of Alginate/Pluronic F-127 and lithium-calcium-silicate ceramic powder	New Zealand rabbit	8-12 weeks	No sign of inflammation was present. Host tissues integrated with the graft. There were presence of calcified tissue and neo-cartilage. The bone volume fraction increased.	Chen, L. et al., 2019 [194]
	3D printed scaffold of alginate/Pluronic F-127 and copper-incorporated bioactive glass-ceramic	New Zealand rabbit	8-12 weeks	No sign of inflammatory reaction was present. Host tissues integrated with the graft. There was presence of calcified tissue. New bone formation and the bone volume fraction increased. New cartilage formed. Interface integrated well between new bone and cartilage tissues.	Lin, R. et al., 2019 [195]
Enthesis	PLLA and calcium phosphate silicate material film	New Zealand rabbit	6-12 weeks	No sign of inflammatory reaction was present. No contractures limited the range of shoulder motion. Repaired supraspinatus tendon was connected to the bone. New bone formed and scaffold degraded. Bone mineral density increased.	Guo, J. et al., 2019 [196]
	Electrospun keratin fibres	Wistar-Han rat	16 weeks	No sign of inflammatory reaction was present. Formation of tendon-like tissue structures that bind inflicted defects. Tendon-bone was continuous. New fibrocartilage formed and mineralized.	Sevivas, N. et al., 2018 [197]
	Dual-layer aligned-random silk fibroin PLLA PCL nanofibrous scaffold	New Zealand rabbit	6-12 weeks	Metachromasia of area increased. Interface width decrease. Collagen maturation and organisation improved at enthesis. New bone formed.	Cai, J. et al., 2018 [198]
Myotendinous junction	3D skeletal muscle constructs co-cultured with self-assembled tendon constructs (Larkin et al. 2006)	Fisher 344 rat		Constructs maturated and integrated to native tissue. In force production increased.	Kastrominova TY. et al., 2009 [199]

Table 4: Ongoing clinical trials on scaffolds and devices used for the regeneration of the joints (information collected from clinicaltrials.gov [181]).

Condition	Title	Purpose of study	Device/ Treatment	Features	Duration	
Rotator cuff tear	Extracellular Matrix Scaffold Graft Augmentation in Rotator Cuff Repair: A Prospective, Randomized, Controlled Trial	Reduction of failure of large and massive tendon tears	ArthroFLEX® ECM	Decellularized human dermal scaffold	2018	2020
	A Prospective, Randomized Evaluation of Bioinductive Augmentation for High Risk Rotator Cuff Tears	Improve tendon healing after surgery	Regeneten	Collagen based bio-inductive implant	2020	2023
	A Prospective Randomized Multicenter Evaluation of Rotator Cuff Healing Using a Nanofiber Scaffold in Patients Greater Than 55 Years	Augment rotator cuff repair	Rotium ™ nanofiber graft	Biodegradable synthetic scaffold made of poly L-lactide-co-caprolactone and polyglycolide	2020	2024
Anterior Cruciate Ligament Injuries	Comparison of Anterior Cruciate Ligament Isolated Reconstruction or Combined With Lateral Extra-articular Tenodesis in Knee Laxity, Graft Failure and Patient-reported Outcome Measures	ACL reconstruction comparing isolated ACL reconstruction with bone-tendon patellar autograft and ACL reconstruction with LEAT (Lateral Extra-Articular Tenodesis)	Isolated ACL reconstruction/ ACL + LEAT reconstruction	Lateral Extra-articular Tenodesis	2020	2023
	A Randomized Clinical Trial Comparing Three Methods for Anterior Cruciate Ligament Reconstruction: Patellar Tendon, Quadruple Semitendinosus/Gracilis and Double-Bundle Semitendinosus/Gracilis Grafts.	ACL reconstruction comparing a patellar tendon, quadruple hamstring tendon or a double-bundle hamstring tendon autograft	Patellar-Tendon/ Hamstring-Tendon/Double-Bundle	Patellar-tendon; Hamstring-tendon; Double-bundle Autograft	2007	2020
	BEAR - MOON: A Two Arm Non-Inferiority Randomized Clinical Trial Comparing ACL Repair With BEAR Device vs. Autograft Patellar Tendon ACL Reconstruction	Determine if age is a risk factor for a worse outcome after a bridge-enhanced ACL repair (BEAR)	Bridge Enhanced ACL Repair (BEAR)	Implant is placed between the torn ends of the ACL and the patient's own blood is added to the implant to stimulate ligament healing	2020	2024
	Randomized Controlled Trial Comparing the Endobutton CL BTB Fixation System With Metal Interference Screws in the Femoral Fixation of Patellar Tendon Grafts in Anterior Cruciate Ligament Reconstruction	ACL reconstruction comparing two different methods of femoral fixation of a bone-patellar tendon-bone autograft	Endobutton CL BTB/Metal interference screw	Endobutton CL BTB is an oval shaped titanium bottom with polyester suture[200]	2016	2027
	The Effect of Bone Marrow Aspirate, Demineralized Bone Matrix, and InternalBrace™ on the Outcomes of Anterior Cruciate Ligament Reconstruction in Young Adults; Failure Rates and Return to Play	Improving ACL reconstruction by combining InternalBrace and autogenous bone marrow aspirate	InternalBrace™	Bridging concept based on braided suture tape and knotless bone anchors [201]	2019	2020
	Pilot Study: Augmentation of ACL Reconstruction With Bone Marrow Stem Cells and Amnion Collagen Matrix Wrap	Reestablish the natural synovial lining of the reconstructed ACL	Bio ACL	Collagen based membrane derived from amniotic tissue combined to hamstring or patellar tendon grafts and bone marrow aspirate	2017	2021
	Randomized Controlled Trial for the Use of an Osteoconductive Scaffold in ACL-Reconstruction	Evaluate efficacy of the surgical technique for ACL reconstruction using an osteoconductive scaffold, enlaced into the hamstring tendon	Osteoconductive scaffold-hamstring	Osteoconductive scaffold made of bovine derived composite bone substitute	2017	2024

		autograft, compared to the traditional technique	tendon composite repair			
Osteoarthritis	Evaluation of the Calypso Knee System for Symptom Relief in Subjects with Medial Knee Osteoarthritis, OUS	Implant a cushion to replace degraded cartilage	Calypso Knee System	Extra-capsular knee implant made of polycarbonate Urethane supported by a structure of titanium and cobalt	2019	2025
	Randomized Controlled Non-Inferiority Trial Assessing the Osseointegration of THA Grafted by PolyNASS (ACTISURF-CERAFIT®) Versus Non-grafted THA (CERAFIT®)	Prevent bacterial contamination and promote osteointegration	PolyNASS (ACTISURF-CERAFIT®)	Titanium graft covered with poly (stat-sodium styrene sulfonate) (PolyNaSS)	2017	2035
Defect of articular cartilage of the knee	A Prospective, Randomized, Active Treatment-controlled, Evaluator-blinded Multicenter Study to Establish the Superiority of Hyalofast® With BMAC in the Treatment of Articular Knee Cartilage Defect Lesions in Comparison to Control	Evaluate the safety and efficacy of Hyalofast® scaffold with bone marrow aspirate concentrate (BMAC) compared to microfracture in the treatment of symptomatic cartilage defects of the knee	Hyalofast®	Biodegradable non-woven pad composed of HYAFF-11®, a benzyl ester of hyaluronic acid	2015	2021
Meniscal injury	A Multicentre Study Evaluating the Treatment of Meniscal Defects with a Meniscal Repair Scaffold, FibroFix™ Meniscus	Assess the performance of FibroFix™ when implanted to replace removed or damaged meniscal tissue in humans	FibroFix™	Non-resorbable silk fibroin scaffold	2020	2021

7. Current challenges and outlook

A vast number of studies that investigated the regeneration of bone, cartilage and tendon however fewer studies on muscle repair were reported in literature [2]. Furthermore, studies of osteochondral and osteotendinous interfaces have been described, but still little is known about the myotendinous junction, probably because of the lack of information on the muscle ECM [2]. Improving the understanding of the muscle tissue and its interfaces could aid the development of more successful treatments for muscle regeneration. Most of the evidence in literature considers whole tissue regeneration. In addition, most studies are on single tissues. However, in their native environment, tissues are connected through heterogeneous interfaces, which work together to maintain the body's function. Yet little is known about their regeneration and genesis. In order to understand how to recover the functionality of the tissue/organ after damage, multiphase tissues and cellular cross-talk at their interfaces should be investigated [202]. Many studies involve the development of complex 3D model with gradients and different cell lines are co-cultured, as described in section 5. However, often the phenotype and/or genotype of cells at interfaces is not investigated [12,98,106,112]. Moreover, many studies on tissue interfaces lack the application of human cells [55,76,92–94,97,100,105,116,130,203–205]. The use of human cells is fundamental for translating novel approaches to the clinic. Another problem is the use of short time culture periods in vitro [95,97,100,205] and examination of implants is carried out too soon after implantation in vivo [52,76,110,115,206]. Some tissues require months and years for complete regeneration [206,207], so long-term experiments are needed to better understand the real potential of novel treatments. Finally, when using products of animal origin [92,105,115,130,208,209], there is a well-known risk of pathogen transfer from animal to human. For this reason, safety regulations should be applied during all the stages of research, from bench to clinic, to avoid contaminations.

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