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BILAYER SCAFFOLDS AND tSVF TO ENHANCE OSTEOCHONDRAL REPAIR

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INTRODUCTION

Osteochondal lesions (OCLs) is an injury involving the subchondral bone and the overlying cartilage. Different etiologies have been advocated, such as acute trauma, repetitive microtrauma, ischemia. Although the exact causes leading to OCLs are unknown, genetic predisposition is though the be involved. Such lesions can potentially affect any joint, however they are most common in knee and ankle. People playing sports, such as soccer, football, rugby and golf may be at risk of an OCLs, but also abnormal bone development can cause OCLs in children. The overall prevalence of OCLs in unknown, however, three quarter of the lesions involves the knee. Males are two to three times more frequently involved. OCLs do not heal properly either spontaneously, even when treated by the current variety of treatments (e.g. by Pridie's marrow stimulation or by mosaicplasty treatment). In fact, OCLs often lead to osteoarthritis (OA) of the involved joint. This inevitably has a high impact on the public health system, with the direct costs of the treatment, but it also has repercussions on the general economy (social costs and loss of economic production).

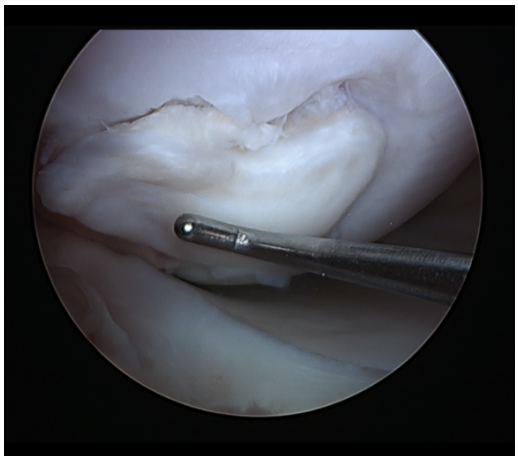


Fig. 1: arthroscopic view of a medial femoral condyle OCL, approximately 2 x 2.5 cm

OCLs encompass a variety of conditions that may manifest as a localized alterations of the subchondral bone, and the overlying articular cartilage. Specifically, it should be distinguished between *Osteochondritis dissecans,* in which the mechanism producing the lesion is an alteration of the blood flow, and *Osteochondral defect.* This latter will be the main object of this thesis.

OSTEOCHONDRAL DEFECTS

Osteochondral defect (OCD) is defines a localize lesion of the articular cartilage and the underlying subchondral bone. Acute trauma or chronic repetitive stress can lead to such condition, creating a localized defect of the articular cartilage and subchondral bone. (Fig 1a, 1b, 1c).

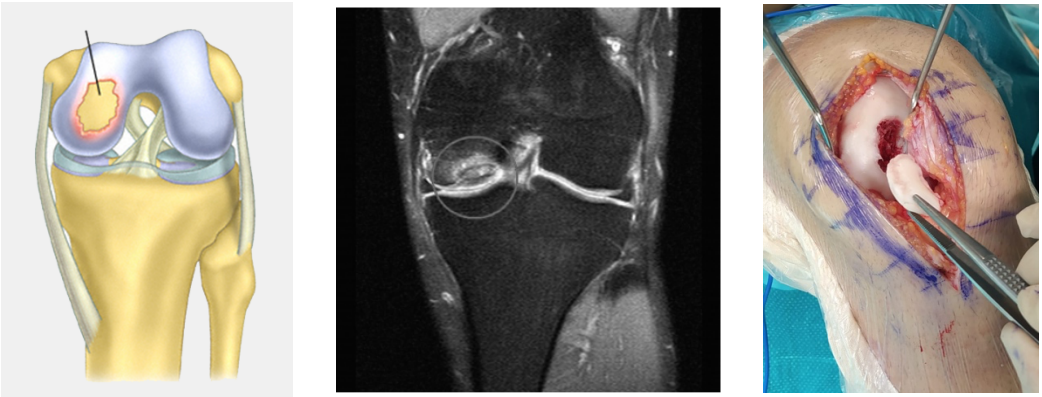


Fig 1: a) schematic drawing of OCD, b) MRI appearance, c) intraoperative findings of the same patients

An OCD can result from an acute injury or can result from a variety of chronic lesions such as (a) separation of the osteochondral fragment caused by an acute traumatic injury, (b) acute osteochondral impaction of the bone with resultant contour deformity, and (c) a collapse of the subchondral bone in a subchondral insufficiency fracture (SIF) or avascular necrosis (AVN) or a bone collapse uncovering a large subchondral cyst, (d) it can be the result of an end-stage *osteochondritis dissecans* (Figure 2)[1] .

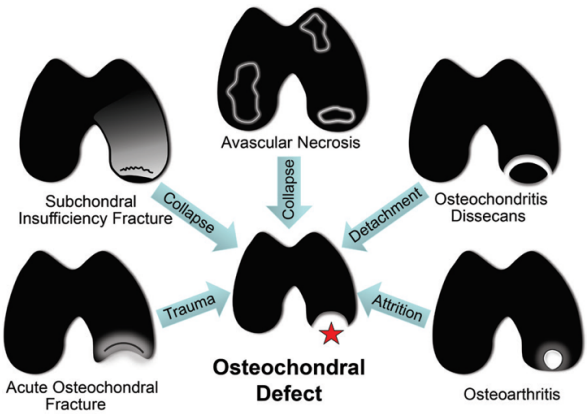


Fig 2: Osteochondral defect. Adapted from Gorbachova T et al, RadioGraphics 2018

The fragmentation of the articular cartilage and the underlying bone results in rapid progression of cartilage wear leading to degenerative changes and therefore function impairment of the affected compartment.

OCD occurs in the knee 75% of the time, the elbow 6% of the time, and the ankle 4% of the time. In the knee, OCD occurs in the medial femoral condyle 85% of the time, on the weight-bearing surface of the lateral condyle 10% of the time, and in the anterior intercondylar groove or patella 5% of the time[2,3]. In the ankle, OCD occurs in the posteromedial aspect of the talus 59% of the time and in the anterolateral aspect of the talus 41% of the time[4]. The prevalence of OCD is estimated at 15 to 30 cases per 100,000.

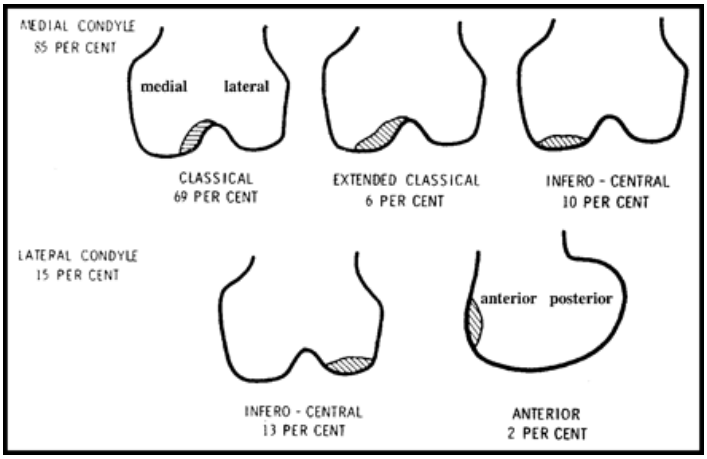


Fig. 3: frequency distribution of OCD in the knee

Cause, Natural History, and Prognosis

There still uncertainty on the exact mechanisms leading to OCDs; a number of factors have been claimed to be involved in variable degree: repetitive microtrauma, acute stress and injury, restricted blood supply, endocrine abnormalities, and genetic predisposition[5,6]. Acute traumatic event is considered as the leading factor contributing to the development of OCD. Chronic repetitive stress to the joint leads to redundant healing, altered blood supply to subchondral bone, ultimately leading to avascular necrosis [111]. High impact sports put at higher risk of developing OCD, as well as endocrine abnormalities affecting calcium and phosphorus homeostasis or anomalies of bone formation can compromise the blood supply to subchondral bone [111].

The natural history of OCD is poorly known. According to the literature, OCD occurring in the adulthood have an higher chance to progress to degenerative joint disease, compared to OCD in childhood. Prakash and Learmonth in a report on 15 knees affected by

OCD, reported at a mean follow-up of 109 months, that the 6 patients who had less than 18 years at the time of the lesion showed either no abnormality or a healed lesion, on the contrary, patient who had onset of the lesion in the adult age showed features osteoarthritis on the MRI scan[7]. Similarly, Linden et al in a very long-term retrospective study (average follow-up 33 years) on patients with OCD of the femoral condyles, reported that OCD occurring prior to closure of the physes (JOCD) does not lead to additional complications later in life, but patients who manifest OCD after closure of the physes (AOCD) develop osteoarthritis 10 years earlier than the normal population[8].

Nonoperative treatment for symptomatic adult onset OCDs is rarely an option, because of the poor regenerative potential of the articular cartilage. Several surgical techniques have been described through the year: loose body removal, drilling, internal fixation, marrow stimulation, autologous chondrocyte implantation (ACI), or osteochondral autograft/allograft transplantation, to replace the damaged cartilage [111].

Osteochondral defect of the knee

Presentation

The most common complaints from patients affected by OCD are pain and swelling of the affected joint, which can be worsened by physical activity [111]. If the OCD has become loose, mechanical symptoms can be also present, such as clicking, popping, and locking. At examination, patients present with tenderness at palpation of the area overlying the OCD region. Patients often present with an antalgic gait. The Wilson sign (ambulating with the affected leg in relative external rotation to decrease contact of the lesion with the medial tibial eminence) typically is present in those patients with a typical location of the OCD in the lateral aspect of the medial femoral condyle [111].

Joint effusion, decreased range of motion, and quadriceps atrophy are also variably present, depending on the severity and duration of the lesion [9,10].

Operative Treatment

Operative treatment of OCD encompasses a variety of options including include loose body removal, drilling of the subchondral bone, internal fixation of the fragment, microfracture, osteochondral autografting and allografting, and autologous chondrocyte implantation (ACI)[11,12]. The aim of those surgeries is promoting and favoring the healing of the subchondral bone, stabilize when possible the unstable osteochondral fragment or replace it

with implantable tissue when it is not suitable for fixation. The type and extent of surgery necessary for OCD depend on the patient's age and characteristics of the lesion [111] (Figure 4).

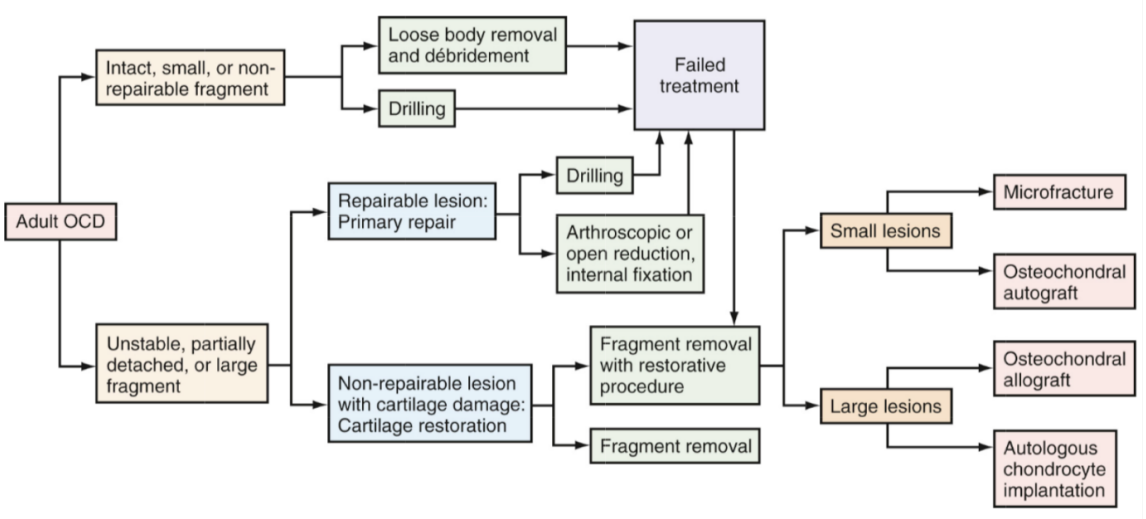


Fig. 4: treatment algorithm. From Insall and Scott, Surgery of the knee 6th edition.

Outcomes

Microfracture

In a study with a mean follow-up of 7 years, 80% of patients evaluated themselves as improved after microfrature (MF); in particular, the largest improvement was shown by patients younger than 35 years[13]. A study reporting histological outcomes after MF noted that approximately 10% had hyaline cartilage, with the majority having predominantly fibrocartilage [14]. Lesions smaller than 4 cm^2 are likely to respond best at MF in the first 2 years [112]. Systematic reviews have demonstrated a clear improvement in knee function at 24 months after MF but inconclusive durability and treatment failure beyond 5 years [15].

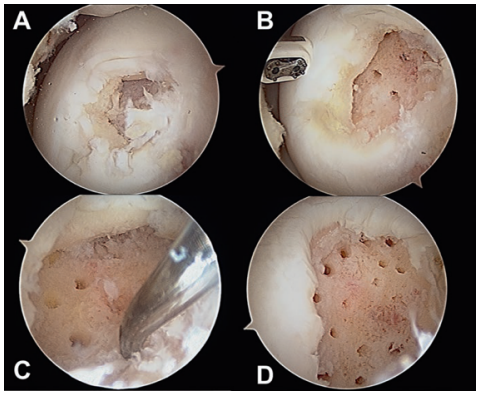


Fig. 5: Microfractures of the knee, arthroscopic view.

Autologous matrix-induced chondrogenesis

The Autologous matrix-induced chondrogenesis (AMIC) technique is a combination of MF with the application of bilayer collagen membrane, which is supposed to guide and enhance the repair process coming from the bone marrow. A multicenter trial comparing AMIC with MF alone showed at 12 months greater lesion filling and superior repair tissue quality compared with MF treatment alone; however, clinical symptoms were equivalent between groups[16].

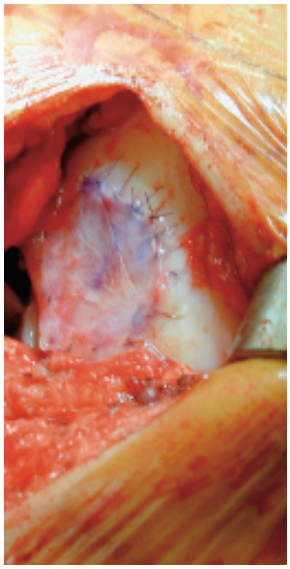


Fig.6 : AMIC of the medial femoral condyle. From Insall and Scott, Surgery of the knee 6th edition.

Autologous Chondrocytes implantation

Autologous Chondrocytes implantation (ACI) is a procedure in which between 200 and 300 milligrams cartilage is sampled arthroscopically from a non-weight bearing area of the knee. After enzymatic digestion of the extracellular matrix, chondrocytes are expanded in culture for about 6 weeks. Eventually, through a second surgery, chondrocytes are implanted on the area of the lesion. First-generation ACI used a periosteal patch, leading to the second generation, in which a type I–type III collagen membrane is used to cover the autologous chondrocytes, and ultimately the third generation, in which autologous chondrocytes are seeded onto the scaffold itself. In a prospective study by Ebert et al[17], a third generation ACI showed significant improvement at clinical and radiological (MRI) scores. At 5 years after surgery, 67% of cell carrier grafts demonstrated complete infill, whereas 89% demonstrated good to excellent filling of the chondral defect. Ninety-eight percent of patients satisfied with the ability of cell carrier surgery to relieve knee pain and 73% with their ability to participate in sport 5 years after matrix-induced ACI.

Osteochondral Autograft Transfer

The Osteochondral Autograft Transfer (OAT) is a procedure by which one or more osteochondral plugs are harvested from a non-weight-bearing area and implanted in the area of the lesion. Studies demonstrated good to excellent outcomes in up to 90% of patients at up to 10 year follow-up[18]. However, there are concerns about donor site morbidity, a variability of the outcomes based on age, sex, and size of the lesion. Poorer outcomes are reported for patients older than 40 years, women and lesions larger than 3 cm^2[19].

Osteochondral Allograft Transplantation

This technique is performed by implanting an osteochondral plug from cadavers. It is useful when the defect is too large for an autograft or a patient has failed a cartilage repair procedure [111]. Survivorship is reported to be about 85% at 10 years[20,21], decreasing over time up to 66% at 20 years[21].

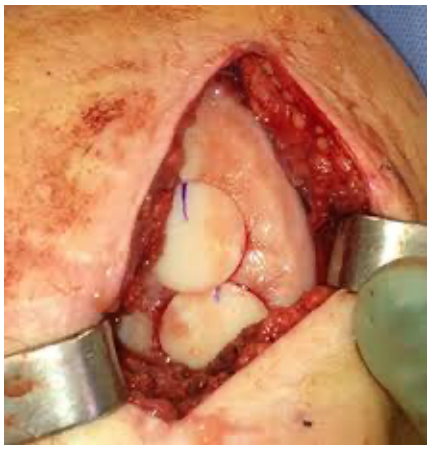


Fig. 7: Osteochondral Allograft Transplantation, two plugs

Osteochondral defect of the talus

Presentation

Osteochondral defect of the talus occurs in up to 70% of ankle sprain[22]. Although trauma is the most common mechanism of injury, similarly to the knee there is a number of non-traumatic conditions leading to OCD. There is non specific physical examination finding that can help in diagnosing an OCD of the talus, so clinician should have high level of suspicion for those patients complaining of pain after an ankle sprain despite having gone through an appropriate treatment. First line radiological exams are often unable to identify an OCD, therefore a second level imaging such as Magnetic Resonance (MRI) or Computed Tomography (CT) are necessary. Despite scoring systems have been developed to grade the OCD, studies have shown low correlation between MRI grading and clinical outcomes[23,24].

Treatment

Despite conservative treatment has been demonstrated to be successful in about 50% of patients in terms of symptoms relief[25], the long term outcomes have been not investigated, and the high level of inflammatory cytokines found in the OCD ankle can lead to a global degeneration of the joint[26].

Operative treatment encompasses basically two options: a reparative option, consisting in bone marrow stimulating technique, and replacement procedures.

Similarly to the knee, the decision whether to repair or to replace is based primarily on the size of the lesion. Traditionally, lesions of smaller sizes (< 15 mm in diameter or < 150 mm^2 in area) are treated with bone marrow stimulation, while larger lesions are treated with replacement procedure [113].

Outcomes

Microfracture

Data from literature suggests that lesions smaller than 15 mm[27] in diameter or < 150 mm^2[24], non-shoulder lesion[28] and without a subchondral cyst[29] are successfully treated by bone marrow stimulation. However, there are concern about mechanical properties of the repaired area, and long-term outcomes. It has been reported as the fibrocartilage deteriorates over time in up to 35% of patients within the first five years, and second look arthroscopies showed as in only 30% of cases there is integration of the repair tissue with the surrounding healthy cartilage at 12 months[23,30].

Osteochondral Autograft Transfer

Lesions greater than 15 mm in diameter or 150 mm^2, or in patients were bone marrow stimulation have failed, OAT is a good option. Several studies have reported good clinical outcomes following AOT at both short- and mid-term follow-up. Good to excellent outcomes have been also reported not only for the general population but also for athletes. A systematic review reported on 11 studies, including a total of 310 patients, and evaluating surgery outcome using the American Orthopaedic Foot and Ankle Society (AOFAS) both preoperatively and postoperatively. Score improvement was shown from 57.5 to 87.1 points. A total of 278 complications were reported including 173 ankle joint complications, 35 donor site-related complications and 70 general complications[31]. Concerns over donor site morbidity have gained increasing attention. Valderrabano et al[32] reported on the outcomes of 12 patients undergoing AOT, of whom 50% experienced donor site morbidity with all patients showing MRI signs of cartilage change, joint space narrowing, or cystic changes in untreated donor sites.

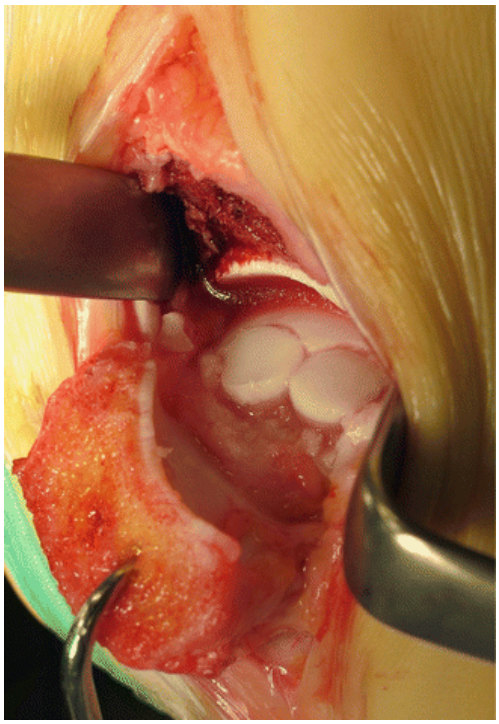


Fig. 8: Osteochondral Autograft Transfer

Autologous chondrocyte implantation

A meta-analysis by Niemeyer et al [33] reported a clinical success rate of 89.9% in 213 patients following ACI. Gobbi et al [34] reported no difference in AOFAS scores following chondroplasty, microfracture, and AOT. Disadvantages of ACI include the cost of culturing hyaline cells, the need for two surgical procedures, hypertrophy of the graft and the durability of the graft[35].

Second generation ACI (MACI) is a good option, with multiple case series[36–39] demonstrating increases in functional outcome scores after the use of MACI. However, most of the studies are small case-series with short-to-long term follow ups.

Osteochondral Allograft Transplantation

There is high variability in the success rate of this technique within the literature. El-Rashidy et al[40] performed one of the largest studies published on patients who received small cylindrical allografts and reported positive outcomes in 28 of 38 patients at a mean follow-up of 37.7 months. Raikin[41] evaluated patients who received bulk allografts and demonstrated improved AOFAS scores in 15 patients at a mean follow-up of 44 mo. Lastly, Haene et al[42] reported in a case series that only ten of 17 cases who underwent allograft transplantation had good or excellent results at an average follow-up of 4.1 years.

TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Tissue engineering and regenerative medicine (TERM) aims to replace through regeneration the damaged tissue rather than restoring its function. There is a number of TERM strategies that could be extremely helpful in treating OCDs. Since the poor potential of spontaneous healing, those lesions are prime candidates for stem cell therapies and tissue engineering approaches.

Stem cells

Chondrocytes are the most effective cells in producing cartilage tissue, however only 5% of the cartilage weight is composed by cells, therefore it is hard to regrowth new cartilage from the pool of chondrocytes after a chondral or osteochondral lesion occurred. Autologous sources of chondrocytes have been used, however not without drawbacks. Autologous chondrocytes should be harvested from an healthy area of the joint. This process can potentially initiate a secondary arthritis. Moreover, the restricted area of biopsies leads to harvesting few cells. Therefore, those cells have to be firstly expanded. Since the chondrocytes are characterized by a slow proliferation rate, the expansion process is often quite long, and the longer is the time of expansion, the higher is the risk of dedifferentiation[43–45].

Alternative sources of cells have been searched to overcome the limits of autologous chondrocytes. Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate in specific lineages of tissues such as the bone, cartilage, fat, muscle, etc. and present no immunogenicity after transplantation. Those cells are virtually present in every tissue of the adult human body, and it is thought they are responsible for tissue repair. The injured blood vessels or the extra cellular matrix can recruit, by the action of signaling molecules (BMPs, FGF, TGF-β, and SDF-1) together with the low oxygen tension, MSCs to the injury site. By mean of those stimuli, MSCs are not only recruited but also pushed to proliferate and differentiate, ultimately repairing or regenerating the injured tissue[46,47].

Synovium-derived stem cells (SMSCs) showed to have a great chondrogenic potential, better than MSCs from bone marrow, periosteum, muscle and adipose tissue[48]. In addition, they showed to deposit a great amount of extracellular matrix compared to bone-derived MSCs.

However, the yield of cells is relatively low, having only around 14 cells per mL of synovial fluid[49].

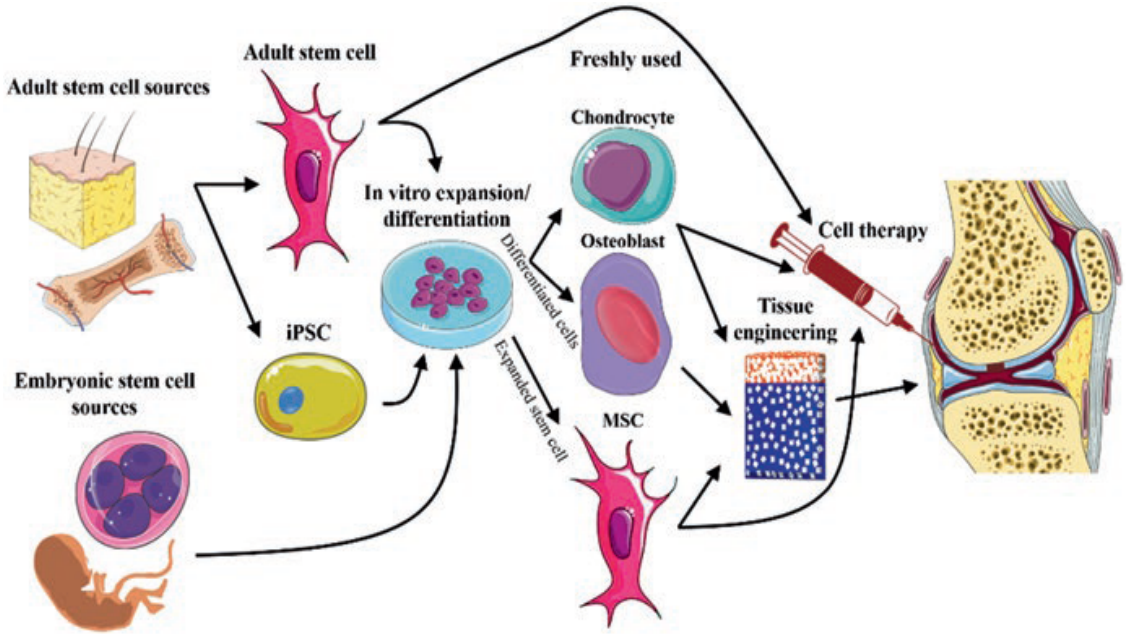


Fig. 9: Stem cell-based strategies for bone and cartilage engineering. From Osteochondral Tissue Engineering Challenges, Current Strategies, and Technological Advances. Oliveira et al. Springer, 2018.

MSCs derived from bone marrow or from adipose tissue showed the ability to differentiate into chondrocytes or osteoblasts, which would be really helpful in addressing OCDs. Although they have a less chondrogenic potential, those cells have the advantage of being largely available compared to autologous chondrocytes. Bone marrow mesenchymal stem cells (BMSCs) are the most studied source of MSCs in TERM, however the harvesting of those cells is often burdened with a significant degree of morbidity to the patient [50]. An alternative source of MSCs is the fat tissue. Adipose-derived mesenchymal stem cells (ADSCs) and BMSCS have similar surface receptor profile, but they may require different induction conditions [51]. For instance, to increase the expression of aggrecan (a marker of chondrogenic expression) by BMSCs, stimuli with transforming growth factor-β (TGF-β) should be provided; on the contrary, to have ADSCs expressing aggrecan, bone morphogenetic protein-6 (BMP-6) is necessary [52]. What really makes the difference between the two sources of MSCs, bone marrow versus adipose tissue, is the definitively larger amount of cell availability of the latter. Excised fat or lipoaspiration provide 400.000 cell per mL, while bone marrow aspirate yields between 100 and 1000 cells per mL [53].

Cell types	Benefits	Challenges
Autologous chondrocytes	Native phenotype Immunocompatibility	Insufficient cell number Prone to dedifferentiation
Allogeneic chondrocytes	Larger cell number Off-the-shelf solution	Risk of disease transmission Lack of donor availability Immune rejection
Adult stem cells (MSCs, ADSCs, SMSCs, BMSCs)	Reliable potential for differentiation Large availability Various tissue sources Easy extractability No ethical complications	Large variety of proliferative capacity, phenotype Heterogeneous cell population Differentiation problems

Fig. 10: Advantages and disadvantages of various cell. Adapted from Osteochondral Tissue Engineering Challenges, Current Strategies, and Technological Advances. Oliveira et al. Springer, 2018.

Scaffolds

Traditionally, scaffolds were made by homogeneous synthetic or natural polymers, with or without growth-factors and cells. However, those constructs do not provide an adequate environment for promoting the regeneration of both the subchondral and cartilage layers [54]. Thus, a second generation of scaffolds came out, characterized by a double or triple layer with different mechanical strengths and spatial structures of different parts. The upper layer has a chondrogenic profile, supporting cartilage restoration, while the bottom layer has an osteogenic profile to support the subchondral bone regeneration. Often those biphasic scaffolds are loaded with different growth factors simultaneously into different parts, which are specific for the chondrogenic (such as TGF-β) and osteogenic (such as bone morphogenetic protein-2, BMP-2 or Fibroblast growth factor-2, FGF-2) processes [55]. In addition, the bilayer scaffolds are commonly charged with cells, mostly chondrocytes or MSCs of variable origins. Many studies have demonstrated the superiority of scaffolds with transplanted cells versus cell-free ones [56–59]. The cartilage layer is generally made by natural biomaterials or synthetic polymers. Common natural biomaterials encompass materials include chitosan, collagen, hyaluronic acid, and biobased polymers. Those polymers have the advantages of providing a more comfortable environment for cell proliferation with less unfavorable reactions. Indeed, it has been showed that natural polymers may enhance cell proliferation and guide cellular differentiation to more desirable levels [60,61]. However, natural polymers aren't without drawbacks. They may lack for mechanical vigor, with lower mechanical strength compared to scaffolds from synthetic polymers. However, since weight-bearing could be controlled in the postoperative period after the surgical procedure, high mechanical properties could not be a true issue [62]. Synthetic polymers-based scaffolds can be built with different mechanical properties, porosity and

degradation rates. On the downside, they face a poorer cell adhesion capacity compared to natural biomaterials. However, that issue can be overcome by adding mixing natural material such as chondroitin sulfate, silicate or chitosan [54].

A variety of different cells have been seeded in bilayer scaffolds: chondrocytes, MSCs, pre-differentiated MSCs. While the chondrocytes are always implanted into the cartilage layer, MSCs can be seeded into one or both layers [63–65]. Several studies have investigated the outcomes of cells seeding on bilayer scaffolds for regeneration of osteochondral tissue. However, outcomes have been variable, some of those supporting the role of cell-seeded scaffolds [66,67] and some others of cell-free scaffolds [63,68].

THE REGENERATIVE POTENTIAL OF FAT TISSUE – THE STROMAL VASCULAR FRACTION (SVF)

The Stromal Vascular Fraction

ADSCs since their characterization in 2001 have been widely studied and used as a source of regenerative potential, with similar features of MSCs of different origin [69]. That source of MSCs benefit of being easily harvested and having large amount of cell availability per mL of excised fat (400.000 cell per mL) [53], however they are characterized by a lower chondrogenic potential compared to BMSCs and Synovium-derived MSCs [48]. ADSCs are isolated from the aqueous fraction of the lipoaspirate which is obtained after enzymatic digestion. The aqueous fraction is composed by combination of ADSCs, endothelial precursor cells (EPCs), endothelial cells (ECs), macrophages, smooth muscle cells, lymphocytes, pericytes, and pre-adipocytes among others. This is otherwise known as the stromal vascular fraction (SVF) [70]. The SVF includes ADSCs, which account for 30% of the total SVF cells [71]. Studies from a variety of different application fields showed as the tissue regeneration potential of SVF seems to be above the one of ADSCs [72–75]. The advantages of SVF have been explained by two main factors. Firstly, SVF is a compound of heterogeneous cells providing immunomodulation, antiflammatory and angiogenesis, so the whole compound can have a wider spectrum of action compared to ADSCs alone [72,74,75]. Secondly, SVF can be also obtained by non-enzymatic isolation (by mechanical of physical processes that do not require cell separation or culturing), therefore it is promptly read for use [76]. In 2013 the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) set recommendations to define cells isolated from adipose tissue. Cells that are not cultured are classified as "Cellular Stromal Vascular Fraction" (cSVF), which is a heterogeneous mixture including mature adipocytes, preadipocytes, fibroblasts, pericytes, macrophages and blood cells, endothelial progenitor cells, and mesenchymal stromal cells. Collagenase-free (enzymatic-free) methods for SVF isolation use mechanical or physical forces to break the structure of the harvested adipose tissue. Those methods are less effective in releasing the cells from the adipose extracellular matrix (ECM) and from their niche. What is achieved by non-

enzymatic processes is defined "Tissue Stromal Vascular Fraction" (tSVF) [77]. Both those methods have advantages and drawbacks, which are summarized in figure 10.

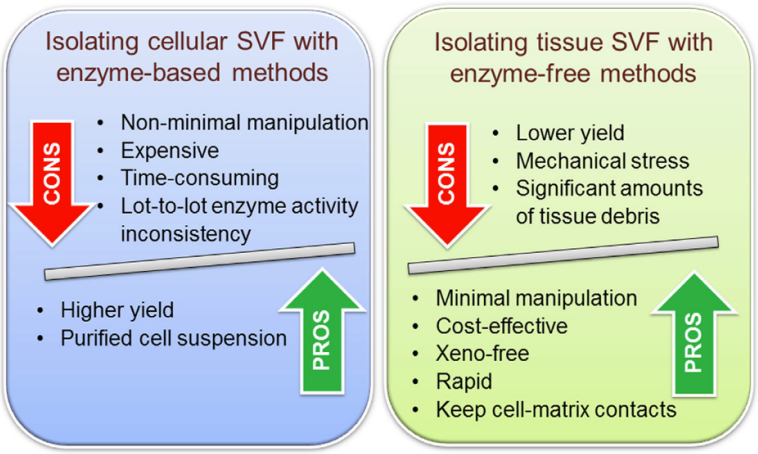


Fig. 10: Comparison between methods for cellular stromal vascular fraction and tissue stromal vascular fraction isolation from the adipose tissue complex [77].

Two of the main advantages of using tSVF are that mechanical disruption is generally provided by devices at point-of-care. Secondly, the native ECM and perivascular structures, comprising a three-dimensional "scaffolding," are maintained providing biophysical support, thus possibly reducing cell death and improving graft retention [78,79].

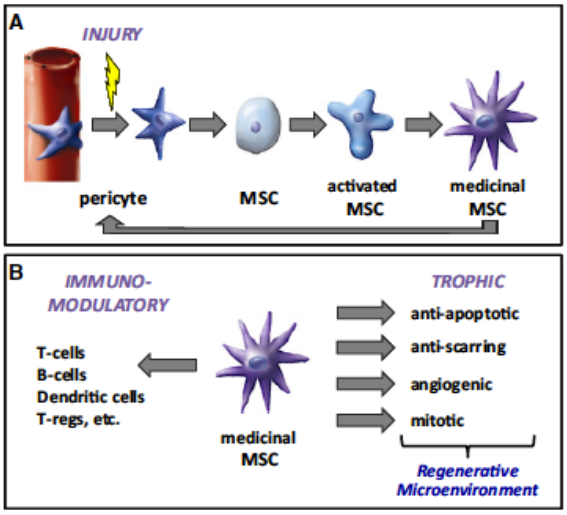


Figure 11. MSCs: Immunomodulatory and Trophic actions[80]

SVF acts by proliferation and differentiation of stem cells, in addition it has been proposed that the activated MSC, deriving from pericytes secrete bioactive molecules that are

immunomodulatory and serve to protect ischemic or injury sites from immunosurveillance and thus provide a barrier for protecting against the invitation of autoimmune reactions. Also, the MSCs secrete agents that are trophic which contribute to establishing a regenerative microenvironment[81–83] (figure 11). There is a variety of methods to achieve mechanically emulsified fat (figure 12). Since adipocytes are fragile cells, they are susceptible to rupture when facing a mechanical stress; these procedures therefore aim to reduce the number of mature adipocytes, which account for about 90% of adipose tissue volume[84]. SVF have been tested in a number of regenerative clinical trials attempting to restore damaged tissue by acute injuries or chronic conditions[85,86].

Method	Yield	References
Condensation	0.5×10^6/ml[a]	[24]
Emulsification		
Nanofat	1.2×10^6/ml[b]	[25–27], [28]
Millifat	3.6×10^7/g[b]	[29]
Millimicrofat	1.3×10^6/ml[b]	Trivisonno (u.w.)
Superficial enhanced fluid fat	n.a.	[30]
Lipogems	n.a.	[31, 32]
MyStem EVO	2.0×10^6/ml	[33]
Squeezed fat	1.1×10^6/ml[a]	[34]
Vortexing	1.5×10^5/ml	[35]
Liposuction aspirate fluid	$2.5–8.0 \times 10^5$/ml	[36–39]

[a]Number of ASCs after expansion in culture.
[b]Number of cSVF after collagenase-mediated isolation of processed samples.
Abbreviations: ASCs, adipose tissue-derived stromal cells; cSVF, cellular stromal vascular fraction; n.a., not available; u.w., unpublished work.

Fig. 12: Collagenase-free methods for adipose tissue processing. Adapted from [77]

A recent systematic review on eleven studies (9 prospective, 2 retrospective) reporting outcomes of SVF injection in the treatment of knee OA, showed that majority of patients reported improvement in pain, range of motion (ROM), functional rating, six metre walking distance, and functional outcome scores, without major donor-site morbidity and one case of knee joint infection [87].

.BILAYER SCAFFOLDS AND tSVF TO ENHANCE OSTEOCHONDRAL REPAIR

Osteochondral defects still challenge orthopaedic surgeons. It is currently possible to address chondral lesions with a variety of treatment options, though they do not always lead to highly satisfying outcomes. Even more so, there is limited choice in treating osteochondral lesions and either simple, low-cost techniques such as bone marrow stimulation, or more complex and expensive alternatives, have shown to be of limited effectiveness in long-term follow-ups[15] or linked with downsides, such as donor-site morbidity for autologous osteochondral transplantation[88]. Recently, thanks to the progresses in the biomaterial field, an increasing interest have been raised by the healing potential of combining scaffolds and stem cells[61]. Currently, a variety of different natural or synthetic materials have been used to develop scaffolds [89]. Scaffolds have been tested both empty or embedded with growth factors and cells, either autologous chondrocytes, or BMSCs or ADSCs. All of these cell populations need to be harvested from the patient and cultured and expanded in vitro before being seeded on the scaffolds [90]. These techniques therefore necessitate two-step surgeries. The aim of this study was to assess the feasibility of using the whole tissue-Stromal Vascular Fraction as a source of stem cell for bilayer scaffold-based osteochondral lesion repair. The potential main advantage of this technique would be a one-step surgery, in which the adipose tissue is firstly harvested, then processed to obtain tSVF and finally seeded on the osteochondral scaffold. In the first phase of the study, three different methods to achieve tSVF from lipoaspirate, available on the market, have been compared to assess whether one was superior to the others. In the second phase, the method that showed to be better than the others was compared to a newer mechanical processing method, the modified-Nanofat grafting [91]. Finally, ADSCs culture-expanded and tSVF, obtained by two different processing methods, were seeded on bilayer scaffolds to evaluate their cell viability.

Phase 1

Comparing biological and clinical features of tSVF obtained by different systems.

BACKGROUND

Different systems are available for processing lipoaspirate to obtain tSVF [92]. The function of these devices is to purify the lipoaspirate sample from oily and hemorrhagic fractions, minimizing the risk of complications and maximizing the biological yield for subsequent grafting [93,94]. However, few studies compared the efficacy of the different processing devices. This study aims to evaluate three different mechanical processing systems such as (1) micro-fragmentation, (2) filtration, or (3) slow centrifugation in terms of cell proliferation in vitro and clinical results of intraarticular injections for the treatment of knee OA.

MATERIALS AND METHODS

Study population

The study received approval by the local ethics committee (Prot: 21.19 TS ComEt CBM). Inclusion criteria were symptomatic knee OA (stage II and III according to Kellgren-Lawrence scale) with a duration of symptoms longer than three months, unresponsive to non-injective conservative therapies (rehabilitation, cryotherapy, rest, NSAIDs), and without other injective treatments or previous surgery on the affected knee in the last 12 months. Patients with documented knee instability, osteochondral lesion, ipsilateral hip and ankle OA, rheumatoid arthritis, immunodepression, and severe general comorbidities (ASA grade 4) were excluded.

Harvesting of adipose tissue

The adipose tissue was harvested from the abdominal area. The subcutaneous tissue of the donor site was injected with 250 ml of Klein's solution [95] under sterile conditions. Ten minutes after the injection, around 60 ml of fat tissue were suctioned using a 2 mm multi-port small-hole cannula.

Processing of adipose tissue

The adipose tissue was then processed with three different methods.

1) Micro-fragmentation: The adipose tissue is injected into the processing unit previously filled with saline solution (0.9% NaCl). The processing unit, containing metallic spheres, is then shaken, exerting a mechanical reduction of the lipid clusters, and inducing cell activation. At the same time, the saline solution clears the sample from residues and blood components. At the end of the process, the lipoaspirate is separated from the saline solution and is pushed through a filter into a 10ml syringe.

2) Filtration: The lipoaspirate is injected into the processing unit through a portal and is washed with the addition of Ringer's lactate solution and is then purified, passing through a membrane. At the end of the process, the final product is then collected by suction with a 10ml syringe from the device.

3) Slow centrifugation: The lipoaspirate is injected into the processing unit previously filled with saline solution (NaCl 0.9%). The sample is purified through the thrust induced by a mechanical rotor propeller. The final product is then collected by suction with a 10ml syringe from the device.

Intra-articular Injection

Knee injection was performed with a superolateral approach under sterile conditions in the operative room immediately after the harvesting procedure. An average of 7 ml of the product was injected with a 20-g needle.

Tissue fraction, separation, and cell isolation

For each procedure, 1 ml of SVF product was collected, and 3 ml of PBS added to isolate the cells and centrifuge at 300 g for 5 min at room temperature to separate the tissue into the following layered fraction: blood/saline fraction in the inferior layer, fat tissue fraction in the intermediate layer and oily superior layer. The purified fat fraction separated was washed with PBS and then digested with 2mg/mL collagenase (Worthington) for 1h at 37°C under gentle agitation. Enzymatic digestion was blocked by adding 4 mL of fetal bovine serum. The digested tissue was filtered through a 70-μm cell strainer (Corning) to remove residual tissue. The cell suspension obtained was centrifuged at 200 g for 5 min. The cell pellet was used to assess cell proliferation. All the cells were seeded into T25 tissue culture flasks with Dulbecco's modified eagle medium; supplemented with 10% fetal bovine serum, 15% penicillin, and 1% streptomycin; and incubated in a humidified atmosphere at 37°C, with 5% CO_2. After 24h, the culture medium was replaced to remove non-adherent cells.

Cell proliferation

The cell proliferation of the isolated cell populations was measured using the trypan blue exclusion assay. Trypan blue staining (Abcam) was performed according to the manufacturer's instructions. From each sample, cells were collected after trypsinization (Corning) and were subsequently counted using a Bürker's chamber. Cell count was executed weekly, for a total of 4 weeks of culture; the assay was performed in triplicate for each donor patient.

Clinical evaluation

Patients were clinically evaluated pre-operatively and at 1, 6, and 12 months after the procedure using a numeric Pain Rating Scale (NPRS) and the Western Ontario & McMaster Universities Osteoarthritis Index (WOMAC).

Statistical analysis

The entire statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc. CA, US). The data are presented as median (interquartile range) in a non-Gaussian distribution and compared with each other by the Friedman test for paired data or by the 2-Way ANOVA test for unpaired data.

RESULTS

From December 2017 to June 2018, 25 procedures were performed in 25 patients (8M, 17F) with a mean age of 60.5 years (SD=15) and a body mass index (BMI) of 25.6 (SD=5.5). The lipoaspirate was processed using a (1) micro-fragmentation system in 10 patients, (2) a filtration system in 8 patients, and (3) a slow-centrifugation system in 7 patients. None of the patients experienced local or systemic complications related to the operating procedure.

Cell proliferation

At the four weeks' time point, the micro-fragmentation system obtained a tSVF sample with a cell count of 8297.5±2350.8: it was a higher cell proliferation compared to the other two methods, although not statistically significant. At the same time point, the tSVF obtained by filtration showed a cell count of 5691.5±1694.8, while the cell count of the tSVF obtained by slow centrifugation was 5187.8±877.1 (Fig. 13). Interestingly, it is was noted that at the T0 time point (after processing the lipoaspirate) , the number of cells of tSVF obtained by the filtration and slow-centrifugation systems was higher than the micro-fragmentation one.

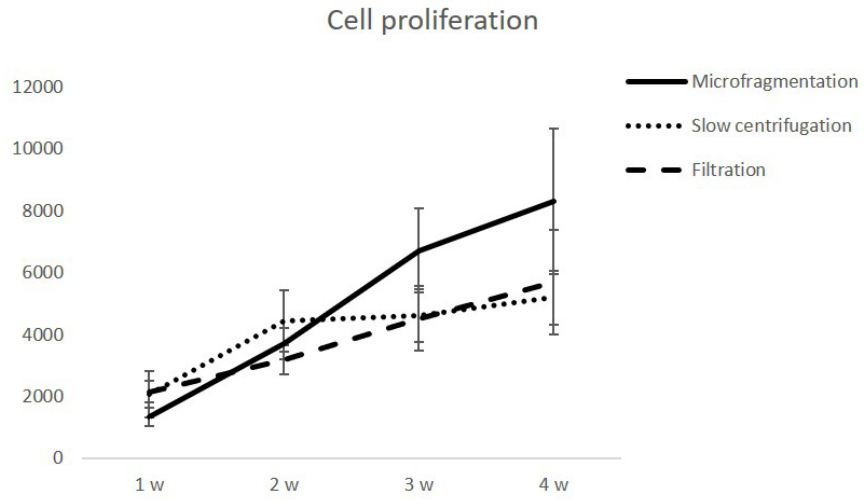


Fig. 13. *Graph showing cell proliferation of the three different systems analyzed.*

Clinical outcomes

The collected data indicate a considerable improvement of the clinical condition in almost all patients already at one month after treatment. Considering the whole population study, the mean preoperative NPRS was 7.96±1.19 while at the one-month follow-up, it was 5.07±1.99. The mean preoperative WOMAC index was 52%±12%, while at the one-month follow-up, was 32%±15%. The improvement was statistically significant both for NPRS and WOMAC ($p<0.0001$, Friedman test). The improvement achieved at the first post-treatment month was maintained stable at the further follow-ups (at the six and twelve months). The mean NPRS at six-month follow-up was 4.59±1.9 while at the twelve-month follow-up was 4.81±2.05. ($p<0.0001$ with Friedman test, when compared to the preoperative value). The mean WOMAC index at six-month follow-up was 31%±13% while at the twelve-month follow-up was 33%±16% ($p<0.0001$ with Friedman test when compared to the preoperative value) (Fig. 14).

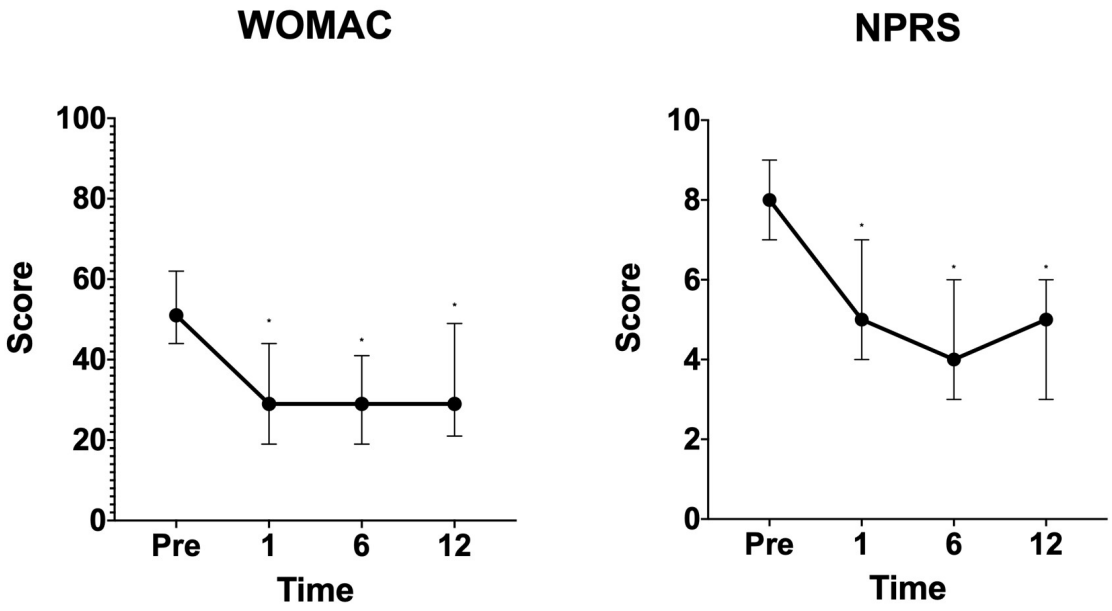


Fig. 14. Linear graph of WOMAC and NPRS index before (pre) and after operation. *=p<0.0001

The analysis of clinical outcomes clustered by the processing methods are showed in table 1 and 2.

NPRS					
	preop	**1 month FU**	**6 month FU**	**12 month FU**	**p (FUs vs preop)**
MF	7.75±1.75	4.5±2.27	4.25±2.34	4.33±2.27	0.0004
FT	8.25±1.03	5.37±2.06	4.75±1.75	5±2.07	0.001
SC	8±1.16	5.71±1.25	5±1.29	5.43±1.71	0.0062

Table 1: NPRS values for the three methods. MF= micro-fragmentation. FT= filtration. SC= slow centrifugation. Preop= preoperative value. FU: follow-up.

WOMAC					
	preop	**1 month FU**	**6 month FU**	**12 month FU**	**p (FUs vs preop)**
MF	52±145%	31±18%	30.5±17.53%	29.75±19.81%	0.0004
FT	56±12.6%	35±13.6%	32.38±10.6%	35.88±12.81%	0.0026
SC	49±11%	32±13%	31.86±12.05%	34.71±14.66%	0.0054

Table 2: NPRS values for the three methods. MF= micro-fragmentation. FT= filtration. SC= slow centrifugation. Preop= preoperative value. FU: follow-up.

tSVF obtained by the micro-fragmentation system resulted significantly more effective in reducing knee pain and improving joint function at one month, six months and twelve months after treatment, compared to those obtained by filtration or slow centrifugation systems ($p>0.05$ with 2 Way ANOVA test) (Fig. 15).

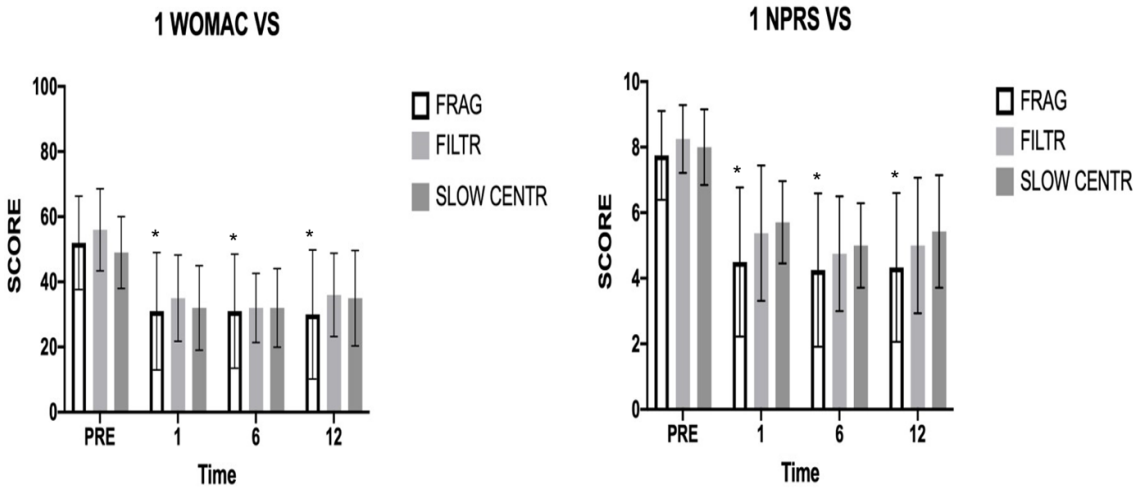


Fig. 15. Bar graph of WOMAC and NPRS index before (pre) and after operation: systems evaluation (Micro fragmentation, filtration and slow centrifugation). *=$p<0.05$

DISCUSSION AND CONCLUSION

The main finding of this phase of the study was that all the three mechanical methods to process the lipoaspirate have demonstrated to be effective in improving patients' knee pain and functioning at each follow-up point. This data is in accordance with other case-series reporting good clinical outcomes from tSVF articular injection [96]. Another finding of interest was that among the others, micro-fragmentation showed a significantly better performance in clinical scores. The cell count of samples coming from the micro-fragmentation process were the lowest at T0, but at the fourth week it turned to be the highest, although it didn't reach a significant difference with the others. Since cell count/cell proliferation data come from *in vitro* cell cultures and not from patients' joint fluid, it is not possible to assess if better clinical outcomes are related to an higher cell count. It has been demonstrated that the micro-

fragmentation method leads to a highly preserved vascular architecture and high number of Pericytes/MSCs, in fact the perivascular niche is preserved when lipoaspirate is processed by micro-fragmentation [93]. It has been assessed that the native ECM and perivascular structures provide for a biophysical support to stem cells, thus possibly reducing cell death and improving graft retention [78,79]. This could be a possible explanation for the better performances of the micro-fragmentation method. However, in this study only a quantitative analysis (cell count/proliferation rate) was performed, while a qualitative analyses of cell yield, cell viability and SVF composition would bring more light on the clinical outcomes.

Phase 2

Evaluation of the osteogenic and chondrogenic differentiation of ADSCs and cSVF obtained by modified Nanofat method, seeded on bilayer scaffolds

BACKGROUND

A number of scaffolds have been developed around the world and some of them are available on the market. However, clinical outcomes are still unsatisfactory. Clinical trials of commercialized scaffolds show poor cartilage fill and associated fibrocartilaginous repair rather than the hyaline cartilage, as well as poor osteochondral repair [97–100]. Preclinical studies of scaffolds loaded with stem cells, either bone marrow-derived [101] or adipose-derived [102] reported promising results, showing high degree of cell differentiation, tissue regeneration and defect filling. However, currently available clinical studies have investigated cell-free scaffolds. BMSCs- or ADSCs-loaded scaffolds would need a staged surgery to allow for harvesting of the stem cell source tissue in the first step, culturing and expansion of the cells in the cell factory and, eventually, implantation of the cell-loaded scaffold by a second surgical step. Basic science studies showed that tSVF intraoperative isolation procedures had comparable cell yield, cell viability and SVF composition compared to enzymatic-based processes [103]. This study aimed to study the feasibility of using tSVF as a source of stem cells to load bilayer scaffolds, and to compare tSVF-loaded to ADSCs-loaded scaffolds. Firstly, two non-enzymatic methods of processing the tSVF were compared to enzymatic fat tissue digestion in terms of genes expression and cell phenotype. Secondly, the differentiation potential of stem cells obtained by the three methods were assessed. Finally, ADSCs culture-expanded and tSVF, obtained by the two different processing methods, were seeded on bilayer scaffolds to evaluate their cell viability.

MATERIALS AND METHODS

Preparation of the bilayer scaffolds [104]

Scaffolds were kindly prepared and gifted by the 3B's Research Group I3Bs - Research Institute on Biomaterials, Biodegradables and Biomimetics of University of Minho, Portugal.

Purified aqueous SF solution were extracted from Bombyx mori cocoons and obtained at high concentration (16 wt.%). Powders of β-TCP, pure and doped with 10 mol.% of Zn and Sr, were synthesized by aqueous precipitation. The cartilage-like layer of the scaffolds was produced with the HRP-crosslinked SF (HRP-SF) solution at 1/0.26‰/1.45‰ (SF/HRP/H_2O_2), while the subchondral bone-like layer was prepared with 80/20 (w/w) ratio HRP-SF/ZnSr-β-TCP, and HRP-SF/β-TCP with no ion incorporation, for comparison purposes. Firstly, the subchondral bone-like layers were produced by mixing ZnSr-β-TCP or β-TCP powders with the HRP-SF solution and transferred into cylindrical shaped silicone molds (9 mm inner diameter), followed by adding 2 g of granular sodium chloride (particle size 500-1,000 μm). After complete gelation at 37ºC in the oven, the molds were soaked in distilled water and the salt was subsequently extracted (porogen) for 72 hours. Finally, a biopsy punch (8 mm inner diameter; Smith & Nephew, Portugal) was used to remove the subchondral bone-like layers' scaffolds from the molds and cut into pieces. Secondly, the subchondral bone-like layers were placed in the bottom of new silicon molds (8 mm inner diameter) and the HRP-SF solution was added on the top of these scaffolds in order to produce hierarchical bilayered scaffolds, followed by adding granular sodium chloride particles. After complete gelation and salt-leaching process, the bilayered scaffolds were frozen at -80ºC overnight, and freeze-dried (Telstar Cryodos-80, Barcelona, Spain) for 7 days. The bilayered scaffolds are abbreviated as BdTCP for bilayered HRP-SF|HRP-SF/dTCP scaffolds. Moreover, scaffolds were embedded with TGF- β in cartilage layer and FGF-2 in bone layer, both at 3 ug/mL.

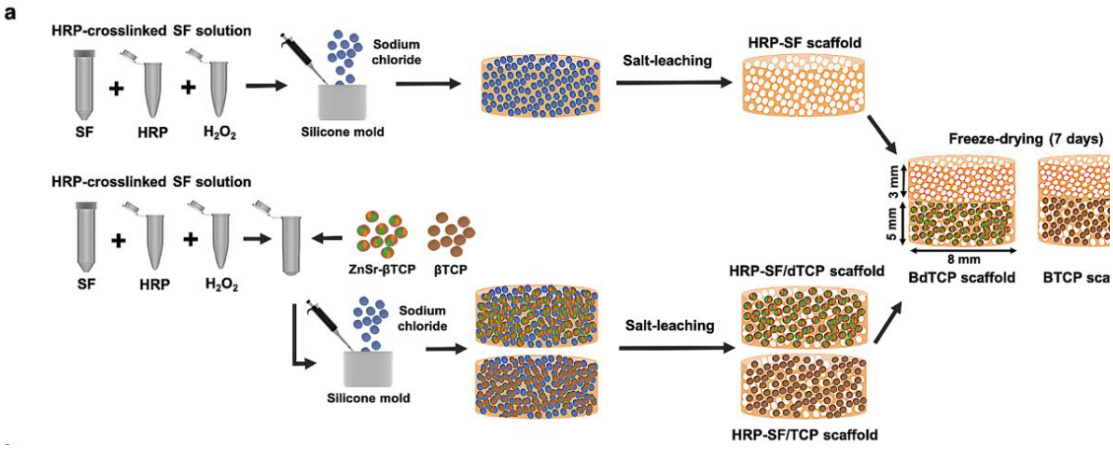


Fig. 16: Methodology used for preparing the BdTCP scaffolds. Adapted from [104]

Harvesting the adipose tissue

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Campus Bio-Medico University of Rome. All patients signed an informed consent. The study received approval by the local ethics committee (Prot: 21.19 TS ComEt CBM).

Lipoaspirate tissue was obtained from 11 patients undergoing intraarticular injection of tSVF (mechanically processed) for treating knee osteoarthritis. The adipose tissue was harvested from the abdominal area. The subcutaneous tissue of the donor site was injected with 250 ml of Klein's solution under sterile conditions. Ten minutes after the injection, around 60 to 80 ml of fat tissue were suctioned using a 2 mm multi-port small-hole cannula. Either the micro-fragmentation method (Lipogems, Lipogems International S.p.A., Milan, Italy) or the modified Nanofat method, as previously described by Segreto et al. [91] were used to obtain tSVF from lipoaspirate. Aliquots from tSVF obtained by migrofragmentation (5 patients) or by modified Nanofat method (6 patients) and aliquots of not processed lipoaspirate (from all the 11 patients) were used for this experimental study.

Tissue fractionation, separation and cell isolation

ADSCs were isolated from lipoaspirate and tSVF (both micro-fragmentation and modified Nanofat methods). The tissue from each specimen was washed with PBS, and then digested with d 0.2% collagenase type II (Worthington, Lakewood, NJ, USA) for 40 min at 37°C under gentle agitation. Enzymatic digestion was blocked by adding fetal bovine serum (FBS). The digested tissue was filtered through a 100-µm cell strainer (Franklin Lakes) to remove residual tissue. The cell suspension obtained was centrifuged at 200 g for 5 min. The cells washed, were resuspended in alpha-MEM (all from GIBCOLife Technologies) with 10% FBS and 1% P/S. The culture medium was changed twice weekly and cultures were allowed to grow until reaching 80–90% confluence. Cell pellet at passage 4 - 5 were used for the experiments. Distinct aliquots of the resulting cell pellet were used to perform, alternatively, cell seeding and viability assessment, gene expression analysis and culture expansion for multilineage potential assay.

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from lipoaspirate and from tSVF (obtained both from micro-fragmentation and modified Nanofat), not expanded in cultures, using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was

produced using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The mRNA levels were measured through quantitative real-time polymerase chain reaction (qRT-PCR) using TaqMan Gene Expression Assays and TaqMan Universal Master Mix II with UNG-Real Time PCR System Instrument 7900HT FAST according to manufacturer's instructions. Gene expression assays SOX2 (Hs01053049) and NANOG (Hs02387400) were performed and compared to the ones of cultured expanded ADSCs.

Phenotypic characterization using flow cytometry

Cultured expanded ADSCs, from lipoaspirate and from tSVF (obtained both from micro-fragmentation and modified Nanofat), were washed and analyzed by flow cytometry. After culture expansion for a total of 4–5 passages, the cells were trypsinized, washed and analyzed by flow cytometry. Briefly, the human adipose-derived cells were harvested by trypsinization, washed and centrifuged at 300g for 5 min. The cells were counted prior to flow cytometry and a total of 2 X 10^5 were transferred to fluorescence-activated cell sorting (FACS) polypropylene tubes. The antibodies listed in the table below were used for these experiments. Optimal amounts of monoclonal antibodies (mAbs) were determined and added to each tube for 60 min at 4 °C in the darkness. Most antibodies were conjugated with fluorescein isothiocyanate (FITC) or phycoerithrin (PE) and were specific for markers associated with mesenchymal and hematopoietic lineages.

Antibody	**Specificity**	**Source**
FITC-CD45	Leukocyte common antigen (LCA)	BD Pharmingen
PE-CD73	Ecto-50 -nucleotidase	BD Pharmingen
FITC-CD90	Thy-1	BD Pharmingen
FITC-CD105	Endoglin, SH2	BD Pharmingen

In Vitro Differentiation Assays

Cultured expanded ADSCs from enzymatically digested lipoaspirate and from tSVF (obtained both from micro-fragmentation and modified Nanofat) were evaluated *in vitro* for differentiation potential.

Adipogenic differentiation

ADSCs at the 4th–5th passages were detached using trypsin-EDTA, seeded at 1.5 X 10^5 cells/cm^2 in a chamber slide and cultured in growth medium until confluent. Adipogenesis was induced by culturing for three weeks in Bullekit Adipogenic Differentiation Medium (Lonza, Barcelona, Spain), following the manufacturer's instructions.

Osteogenic differentiation

ADSCs at the 4th–5th passages were detached using trypsin-EDTA, seeded at 1.5 X 10^5 cells/cm^2 in a chamber slide and cultured in growth medium until confluent. Osteogenesis was induced by culture for three weeks in hMSC Bullekit Osteogenic Differentiation Medium (Lonza). This culture medium was changed every 2–3 days.

Chondrogenic differentiation

Chondrogenesis was assessed using the micropellet formation (2.5 X 10^5 cells) technique [105], with some modifications. ADSCs from the 4th to 5th passages were detached using trypsin-EDTA and centrifuged at 300g for 10 min. The resulting pellet was cultured in chondrogenic differentiation medium (Lonza), following the manufacturer's instructions. The culture medium was changed every 2–3 days.

Histological analyses

After 21 days of culture in differentiation medium, each cell culture was compared to a control corresponding to cells cultured for the same period of time in αMEM with 10% FBS.

For adipogenesis evaluation, differentiation was confirmed by detection of cytoplasmic lipid droplets by oil red O staining after cell fixation in 4% paraformaldehyde.

For osteogenesis evaluation, differentiation was analyzed by Alizarin red staining after cell fixation in 4% paraformaldehyde, to assess the presence of calcium deposits.

For chondrogenesis evaluation, 4 μm-thick frozen sections of aggregates were stained with hematoxylin and eosin (HE), alcian blue (AB), and safranin O (SaO) for proteoglycans and collagens.

Cell Seeding and Cellular viability

Seeding of ADSCs cultured expanded on the bilayer scaffolds

For the culture system, BdTCP-GF (SF/TCP scaffolds with TGF-B1 in cartilage layer and FGF-2 in bone layer, both at 3 ug/mL) and BdTCP-C (SF/TCP without growth factors) were used and sterilized by β-radiation (IONMED sterilization SA, Tarancón, Cuenca, Spain). Before the cell seeding, all scaffolds were hydrated in alpha-MEM, in the CO2 incubator overnight. The hydrated scaffolds were transferred to 24-well culture plates (Corning Incorporated, Life Sciences, Durham, NC, USA). ADSCs of passage 4-5 were detached and the live cells count was determined by trypan blue dye exclusion method. A 10 µL cell suspension was used for seeding onto the surface of the HRPSF/dTCP and HRP-SF/TCP layers, at a density of 3 x 10^5 cells/scaffold.

Cells Viability in the Scaffold

Viability and toxicity of cell pellet from ADSCs and from tSVF in the scaffold was assessed using the Live/Dead assay (Life Technologies) after 7, 14 and 21 days of culture in growing medium in a 5% CO2 and 95% air incubator. Briefly, scaffolds were incubated in serum free medium supplemented with 10 mM calcein AM green and 1 mM ethidium homodimer-1 for 30 min. The scaffolds were visualized on fluorescent Microscope (Nikon Eclipse Tí light microscope, Tokyo, Japan). Red and green cells were counted**.**

Statistical Analysis

The experimental data from all the studies are expressed as mean ± standard deviation (SD) Single-factor analysis of variance (ANOVA) was used to assess the statistical significance of the results. A p-value of less than 0.05 was considered statistically significant. Results are expressed as the mean ± standard deviation (mean ± SD). The entire statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc. CA, US).

RESULTS

RNA Extraction and Gene Expression Analysis

Gene expression analysis showed that cells from lipoaspirate and from tSVF (obtained both from micro-fragmentation and modified Nanofat), not expanded in cultures, expressed a set of genes (NANOG and SOX2) that are embryonic stem cell markers, confirming the presence of ADSCs in all the tissues. From real-time PCR, a quantitative analysis of the gene expression was obtained. ADSCs from modified-Nanofat and cultured expanded ADSCs showed an higher expression of the SOX2 gene compared to lipoaspirate and tSVF obtained by micro-fragmentation (post-test for linear trend p=0.0127). Similarly, the NANOG expression showed a linear trend towards a higher expression by modified-Nanofat and cultured expanded ADSCs, although it was significant only for ADSCs (post-test for linear trend p=0.0114) (figure 17, table 3).

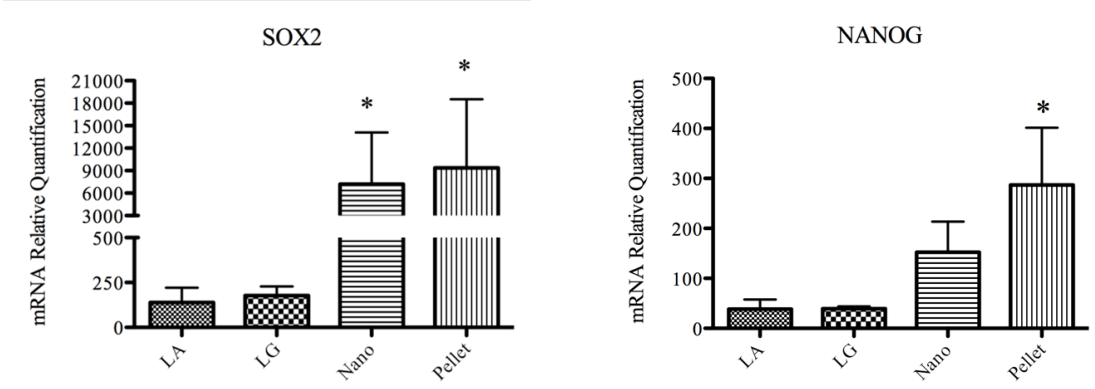


Figure 17: qRT-PCR analyses of SOX2 and NANOG expression. LA=lipoaspirate. LG= tSVF obtained by micro-fragmentation (Lipogems). Nano= tSVF obtained by modified Nanofat. Pellet= cultured expanded ADSCs. *p<0.05.

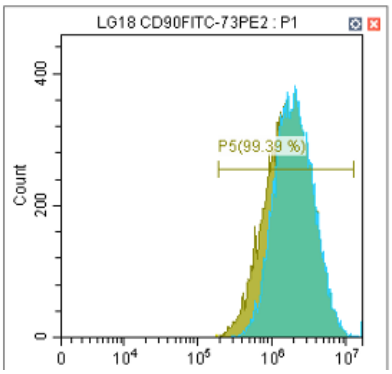
	NANOG	SOX2
LA	38.4±12.5	138.8±72.2
LG	39.35±9.4	177.3±95.4
Nano	152.5±82.1	7195±3872
Pellet	287.1±129.1	9363±4792

Table 3: mean value ± standard deviation for qRT-PCR analyses of SOX2 and NANOG expression. LA=lipoaspirate. LG= tSVF obtained by micro-fragmentation (Lipogems). Nano= tSVF obtained by modified Nanofat. Pellet= freshly isolated ADSCs obtained after collagenase digestion.

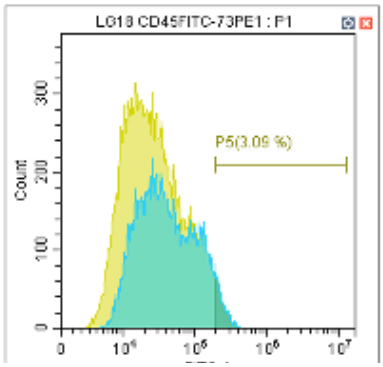
Flow Cytometry Stem Cell Phenotype Characterization

The cell-surface antigenic characteristics of cultured expanded ADSCs from all tissues' aliquots at passage 4-5 were analyzed by flow cytometry. All of them showed >99% positivity to CD90 and CD73 and >75% CD105 positivity, while > 90% of cells where CD45 negative.

a)



b)



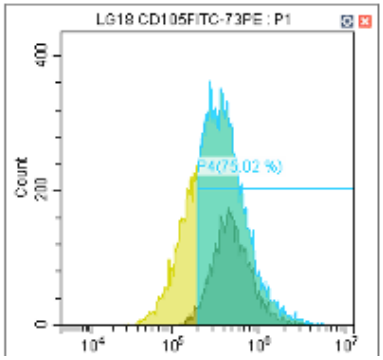
c) 

Figure 18a ,b, c: cytofluorimetric analysis for CD90, CD45 and CD105

In Vitro Differentiation Assays: Histological analyses

Adipogenic Differentiation. After 21 days of culture with the appropriate differentiation medium or with αMEM with 10% FBS for the control, the cells were stained with Oil Red O for lipid droplets to evaluate adipogenic differentiation. All the controls resulted negative for staining, showing the absence of lipid droplets. The cells grown in differentiation medium in conditions were positively stained, indicating differentiation (Figure 19 a and b).

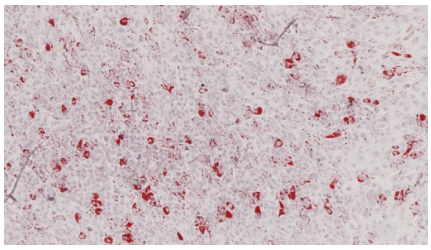
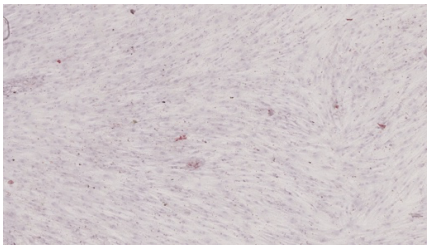
a)  b) 

Figure 19 a and b: Adipogenic Differentiation, Oil red O staining of the cells differentiated (a) compared to their control point (b)

Osteogenic Differentiation. After 21 days of culture with the appropriate differentiation medium or with αMEM with 10% FBS for the control, the cells were stained with Alizarin Red for calcium deposits. The controls were negative for staining. The cells grown in differentiation medium were positively stained, showing the presence of calcium deposits and the presence of differentiation (Figure 20 a and b).

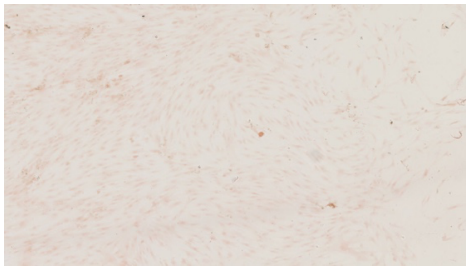
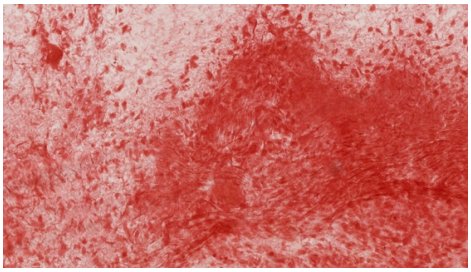
a)  b) 

Figure 20 a and b: Staining techniques of osteogenic differentiated cells. Alizarin red staining of the cells differentiated (a) compared to their control point (b)

Chondrogenic Differentiation. After 14 days of culture with the differentiation medium or with αMEM with 10% FBS for the control, the aggregates were analyzed by histostological stainings using hematoxylin eosin (HE), alcian blue (AB), and Safranin O (SaO) staining for proteoglycans and collagens. These techniques confirmed chondrogenic differentiation for cells grown in chondrogenic media. As shown in Figure 21, the presence of collagens and proteoglycans could be seen only for aggregates of cells grown in chondrogenic media.

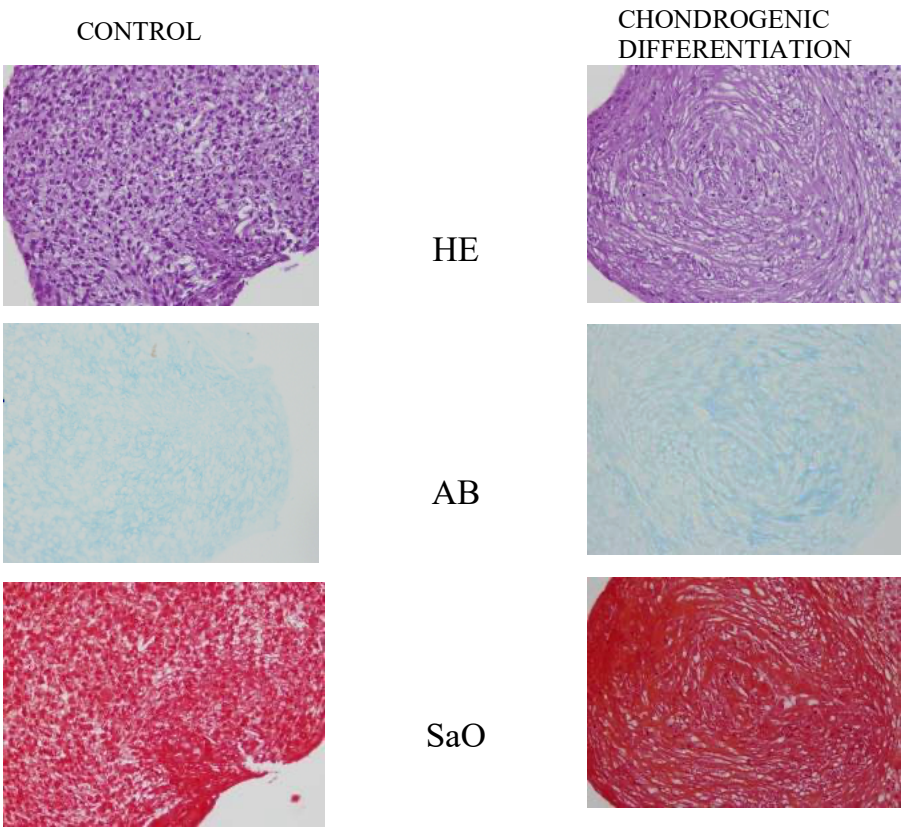


Figure 21: histological stainings using hematoxylin eosin (HE), alcian blue (AB), and Safranin O (SaO).

Cell Seeding and Cellular viability

ADSCs cultured expanded and tSVF were seeded on bilayer scaffolds. The Viability and toxicity of cell pellet from ADSCs and from tSVF in the scaffold was assessed using the Live/Dead assay at 7, 14 and 21 days (figure 22). Unfortunately, only ADSCs cultured expanded were available for cellular viability assay. Cultures of tSVF on the scaffolds, either from micro-fragmentation or from modified Nanofat showed extensive tissue necrosis (figure23), therefore it was not possible to perform a cell count comparison among the three cell sources.

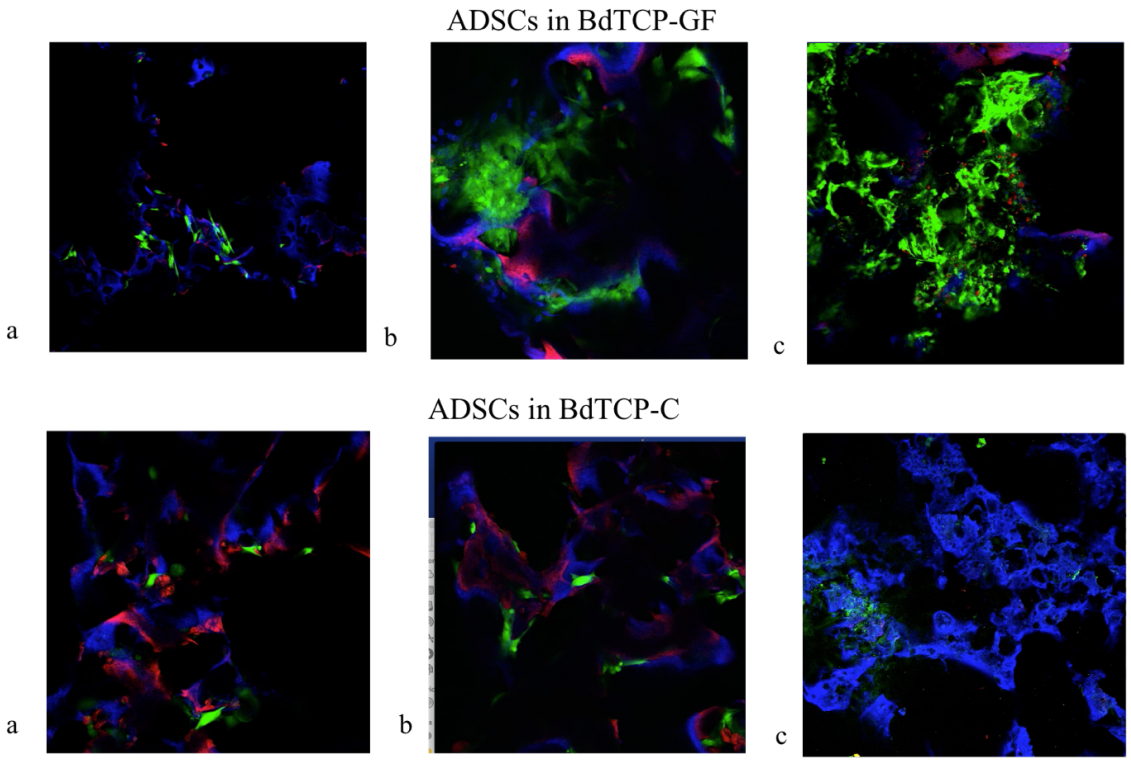


Figure 22: live/dead assay of ADSCs seeded scaffolds, at 7 (a), 14 (b) and 21 (c) days using both BdTCP-GF (SF/TCP scaffolds with TGF-B1 in cartilage layer and FGF-2 in bone layer) and BdTCP-C (SF/TCP without growth factors).

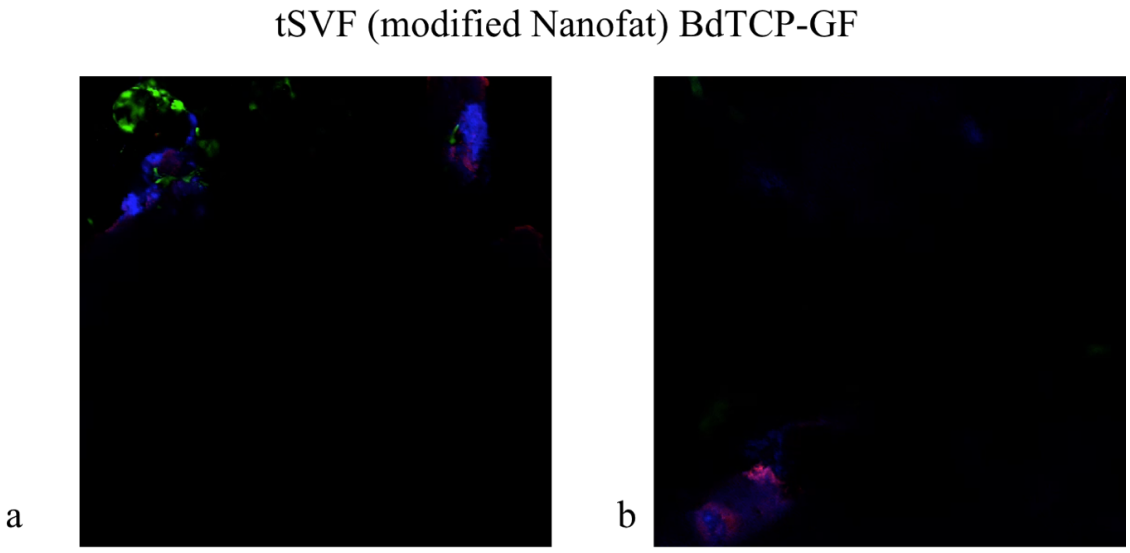


Figure 23: live/dead assay of tSVF seeded scaffolds, at 7 (a), 14 (b)) days of BdTCP-GF (SF/TCP scaffolds with TGF-B1 in cartilage layer and FGF-2 in bone layer). No live cells were counted, while extensive necrosis was noticed.

DISCUSSION AND CONCLUSION

The main finding of this study was that the modified Nanofat and the micro-fragmentation methods to obtain tSVF both lead to a population of adult Adipose-Derived Stem Cells which showed gene expression, stem cells CD markers and differentiation potential similar to ADSCs cultured expanded obtained by the traditional and well-established enzymatic collagenase-based digestion. These outcomes are in line with the current available literature. NANOG and SOX2 are known embryonic transcription factors which are expressed by Mesenchymal Stem Cells [106]. It has been shown that bone marrow, adipose tissue, dermis and heart derived MSCs express OCT4 and NANOG genes, while SOX2 is expressed in adipose tissue derived, dermal and heart MSC samples [107]. Analyzed cells from this study were positive for NANOG and SOX2 expression, confirming that all the processing methods yielded to ADSCs. It was noted a trend towards a higher expression of NANOG by enzymatic-digestion derived ADSCs compared to mechanically methods. This is obviously explained by the different cell yield by enzymatic and non-enzymatic methods. The higher expression of SOX2 by modified-Nanofat can be explained by the fact that this method may lead to significantly higher cell yield compared to the other mechanical methods. However, this is a point that should be investigated, since the current available studies on Nanofat do not allow for relative yield calculation [103] and the modifications provided by Segreto et al. [91] to the original Nanofat technique may have changed what is already known in terms of cell yield, viability of cells and composition of SVF. According to the international criteria which defines multipotent MSCs, Dominici et al. [108] stated that ADSCs were positive for MSC markers CD90, CD105, and CD73, and negative for CD45, CD11b, HLA-DR, CD34, and CD19 according to the international criteria which defines multipotent MSCs[109]. Findings of cytofluorometry analysis from this study showed as cultured expanded cells from either enzymatic digestion or mechanical processing expressed the correct CD markers to be defined ADSCs (CD90, CD105 and CD73 positive, CD45 negative). Cell seeding of cultured expanded ADSCs on bilayer demonstrated good cell viability by the live/dead assay. Recently, an *in vitro* study demonstrated chondrogenesis and osteogenesis by ADSCs cultured on a poly(l-glutamic acid) and chitosan bilayer scaffold[102]. The current study was the first one to attempt culturing the whole tSVF on a bilayer scaffold, however the live/dead assay at 7 and 14 days showed extensive tissue necrosis. A previous *in vivo* (animal) study that investigated the fate of SVF after transplantation showed necrosis of most mature adipocytes and endothelial cells during the ischemia at the early stage of transplantation, while only the CD34+ ASCs survived; these CD34+ migrated to the periphery

of the graft, moving from a hypoxic region to an oxygenated region, which raises the possibility that ASCs in the graft respond to a signal from an existing blood vessel lying in peripheral region of the transplant [110]. Although this is an *in vivo* study on animal, it showed that the graft suffers the hypoxic condition. Even more so this should be true for *in vitro* cultures. This study used a standard *in vitro* model of static culture, that showed to be effective for culturing isolated cells, which showed to be clearly inadequate for culturing a whole tissue. However, since this was the first study to attempt the whole tSVF culturing on a scaffold, this effect couldn't be expected. It was hypothesized that using a dynamic culture, i.g. using a bioreactor, would avoid tissue necrosis. However, further scaffolds would be needed for continuing the study. The COVID-19 pandemic started at the beginning of 2020 forced the staff of the 3B's Research Group to smart-working from home, therefore preventing them from providing new scaffolds for the study purpose. Thus, the dynamic culturing system could not be tested yet. This study is not without furhter limitations. The main one is the limited number of samples. In addition, the outcomes could have been affected by the variability in the donors. Simple lipoaspirate was harvested from all the patients, but some of them underwent a tSFV injection obtained by micro-fragmentation (Lipogems, Lipogems International S.p.A., Milan, Italy) while others by modified Nanofat method [91]. The gold standard would have been to get from the same patient all the three tissues, but this was made difficult from economic issues (Lipogems is a quite expensive method) and from the longer operative time that would have been required to obtain tSVF by both methods from the same patient. In conclusion, it can be said that micro-fragmentation and modified-Nanofat methods yield to a population of cells which is reach in ADSCs (CD90+, CD73+, CD105+ and CD45-), which express genes characteristic of stemness and which showed differentiation potential towards osteogenic and chondrogenic tissues. A clear superiority of one mechanical method over the other was not found, although cells from modified-Nanofat showed a tendency towards a higher expression of SOX2, however this result has to be further investigated. It was not possible to establish the feasibility of culturing the whole tSVF of a bilayer osteo-chondrogenic scaffolds, therefore further research is needed.

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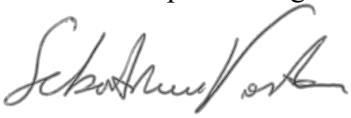

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