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EFFECTS OF ADIPOSE-DERIVED STEM CELLS TREATMENT IN RECALCITRANT CHRONIC LEG ULCERS: A PHASE II RANDOMIZED CONTROL TRIAL

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1. INTRODUCTION

Wound healing constitutes an intricate process, which requires coordinated interaction between diverse immunological and biological systems and includes different cell types and molecules. When the healing process fails to result in structural integrity or to follow an orderly and timely sequence, a chronic wound develops [1].

Chronic wounds are a major problem in medicine today as their incidence is continuously increasing due to an ageing population and a growth in the incidence of underlying diseases [2], causing a reduction in patient quality of life and rising health care costs. The incidence of severe burns in the United States is estimated at 70,000 per year by studies [2,3]; venous leg ulcers occur at a level of between 600,000 and 1,500,000 [3]; and the prevalence of chronic foot wounds in diabetics is 15 to 20 percent [4]. The cost of dressings alone to care for the above-mentioned cases has been estimated at $5 billion per year from healthcare budgets [5].

These wounds fail to heal due to several factors, which can include: prolonged or excessive inflammation, persistent infections, the formation of drug-resistant microbial biofilms, and the inability of dermal and/or epidermal cells to respond to reparative stimuli [6-9]. In such situations conventional therapies which incorporate surgery, dressings and topical negative pressure among other treatments are increasingly reaching their limits, motivating the use of skin grafts, advanced therapies and the search for substitute treatment options, including stem cell-based therapies [2].

Over the last few years, stem cell application has been put forward as a promising novel therapy for regenerative medicine. This is due both to the stem cells' infinite capacity for self-renewal and the ability to differentiate into multiple cell types under appropriate stimuli [10]. In addition, stem cells are pluripotent and secrete a variety of growth factors. Following the initial attention paid to embryonic pluripotent cells however, different types of adult stem cells have since been studied as a valid and continuous source of stem cells that are readily obtained. The most common source is bone marrow, probably because it can be easily and swiftly accessed and also because various devices are applicable for marrow harvesting (from bone marrow transplantation). The marrow contains hematopoietic cells, mesenchymal cells, and other cell types which may contribute to the

promotion of tissue regeneration. Yet the limited amount of source material harvested and the low yield of cell isolation protocols are a major limitation to intraoperative stem cell therapy approaches. To surmount these challenges and obviate the requirement for highly invasive bone marrow harvesting, a procedure causing pain at the aspiration site, alternative sources should be sought from which to isolate autologous stem cells [11]. In this field, adipose derived stem cells (ADSCs) are the stem cell of choice, being abundant, harvestable via a minimally-invasive procedure, providing an elevated yield when isolated and proving suitable for clinical application without prior manipulation [12]. The mechanism for healing chronic wounds using ADSCs is built around direct differentiation towards lineage-committed cells, or on the production of angiogenic growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF): ADSCs migrate to the wound site through paracrine effects and catalyse wound healing, as well as fusion and differentiation [13].

Although stem cell therapy is a comparatively new tool, there have been several cases where their capacity to heal wounds has been established as well as several studies which have shown that these types of cells can be used safely.

1.1 Physiological wound healing

Cutaneous wound healing represents a complex biological process involving regenerating dermal and epidermal tissues consisting of four different but temporally and spatially overlapping steps: haemostasis, inflammation, proliferation (with formation of granulation tissue) and remodeling [14]. Hemostasis occurs immediately after injury and is characterized by vasoconstriction and blood-clotting cascade, preventing excessive bleeding and providing the provisional matrix for cell migration to ensure temporary protection of the wound area. PDGF and transforming growth factors A1 and 2 (TGF-A1 and TGF-2) are released during this process, attracting inflammatory cells such as leukocytes and macrophages. Cytokines initiate the healing process by attracting fibroblasts, endothelial cells, and immune cells. The subsequent inflammation phase lasts up to 7 days, involving apoptosis of inflammatory cells such as neutrophils and macrophages. Proteases and reactive oxygen species (ROS) are released by neutrophils, preventing contamination of bacteria and cleansing the wound of cellular debris. Blood

monocytes reach the wound site and differentiate into macrophages. These macrophages remove bacteria and nonviable tissue through phagocytosis and also release various growth factors and cytokines. Endothelial cells, keratinocytes and fibroblasts, are recruited to repair the damaged vessels [15].

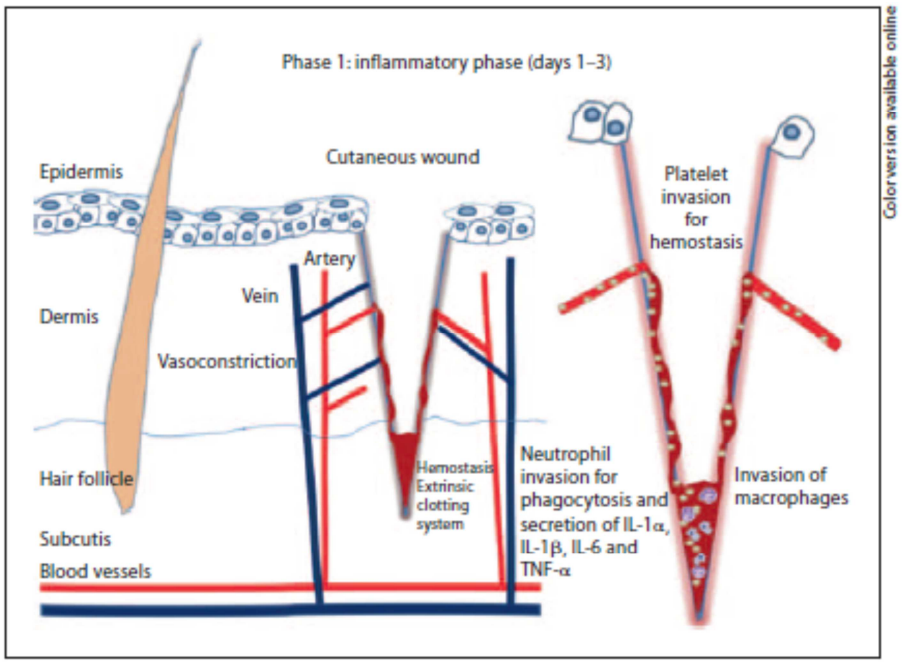

Figure 1. *Inflammatory phase after skin lesion: hemostasis and invasion of inflammatory cells.*

The proliferation phase begins as the inflammatory phase subsides accompanied by apoptosis of immune cells. The production of growth factors and activation of dermal and epidermal cells in this phase leads to tissue granulation, angiogenesis and epithelialization. Endothelial progenitor cells, which are important for physiological wound healing, are mobilised by VEGF, matrix metalloproteinase (MMP9) and nitric oxide. Formation of extra-cellular matrix (ECM) rich tissue occurs in response to insulin-like growth factor (IGF) and stromal cell-derived factor (SDF) [2,15].

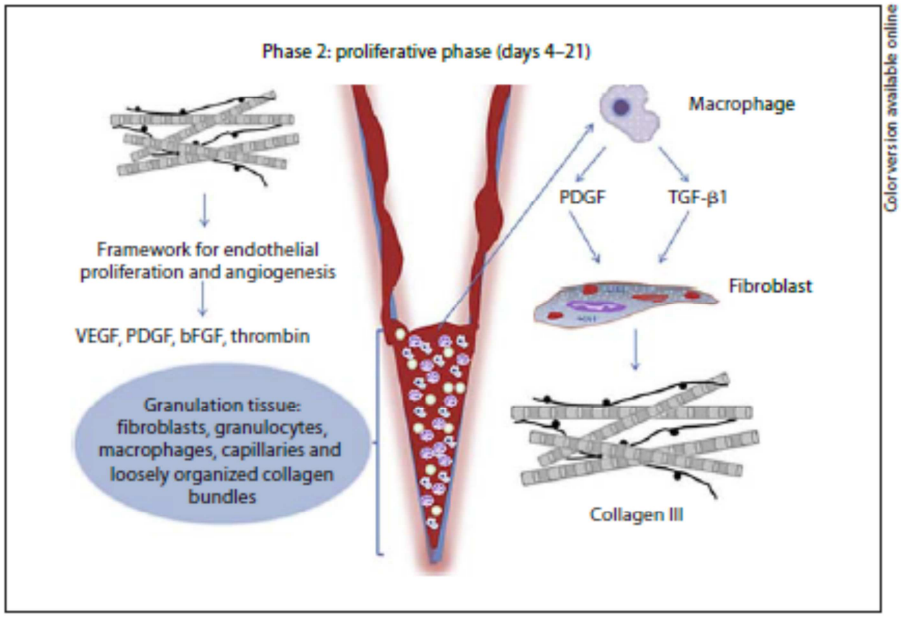

Figure 2. *Proliferative phase: organisation of the clot, secretion of growth factors, synthesis of collagen III and initiation of angiogenesis.*

The last phase takes place once the wound has closed and may last 1–2 years. During this phase, matrix remodelling into organized collagen bundles [16,17] and/or scar formation through cellular migration, proliferation and angiogenic induction is initiated by TGF-A, matrix metalloproteinases (MMPs) and tumor necrosis factor (TNF) [18].

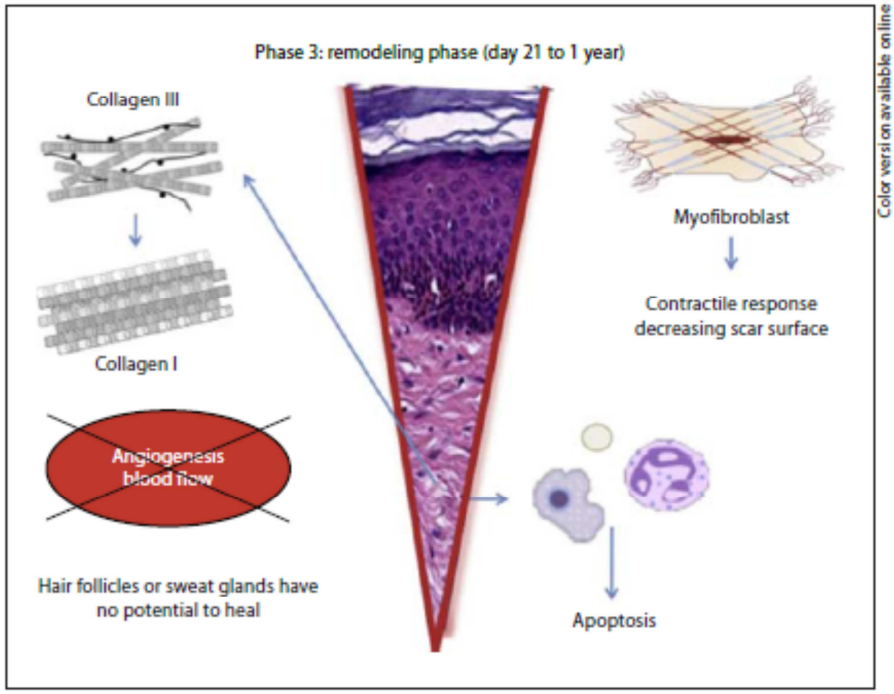

Figure 3. *Remodelling phase: the regenerative process concludes and is followed by matrix remodelling and scar formation.*

1.2. Pathophysiology of chronic wounds

Chronic wounds are the result of an interruption in the progression of one or more of the four phases of the normal cellular and biochemical processes towards re-establishing skin's integrity. Thus, they represent a failure to reach complete re-epithelization in the proper temporal sequence of tissue repair [19].

Ninety per cent of all chronic wounds are comprised of venous ulcers, pressure ulcers and diabetic ulcers, which are the most common types of chronic wounds [20]. Both local and systemic factors can be involved in chronic wound etiopathogenesis. Ischaemia, arterial/venous insufficiency, local toxins, trauma and radiation are very important local infection factors. Ageing, chronic diseases, alcoholism, smoking, drugs, nutritional deficiencies, chronic kidney disease and neuropathies seem to be the most important systemic factors [21].

Local Factors	Systemic Factors
Oxygenation	Age and gender
Infection	Sex hormones
Foreign body	Stress
Venous sufficiency	Ischemia
	Diseases: diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia
	Obesity
	Medications: glucocorticoid steroids, non-steroidal anti-inflammatory drugs, chemotherapy
	Alcoholism and smoking
	Immunocompromised conditions: cancer, radiation therapy, AIDS
	Nutrition

Table 1. *Local and systemic factors involved in chronic wound etiopathogenesis.*

Although there are differences in etiology at molecular level, chronic wounds share certain common features, which include: the prolonged inflammatory phase, the lack of appropriate metabolism and clearance of toxic substances from the wound resulting in very high levels of pro-inflammatory cytokines, proteases, ROS, and senescent cells, the existence of persistent infection, the formation of drug resistant microbial bio-films, the inability of dermal and/or epidermal cells to respond to regenerative stimuli [2], and a deficiency in stem cells, which are often also dysfunctional.

The incessant and chronic inflammatory state which characterises chronic wounds is the basis for ECM degradation due to the loss of important wound healing products, such as PDGF and hepatocyte growth factor (HGF), which are respectively broken down by ROS or MMPs and elastases secreted by neutrophils [22]. In particular, platelet-derived factors such as transforming growth factor-beta (TGF-β) or ECM fragment molecules and microorganisms stimulate the constant influx of immune cells, due to repeated tissue injury; the pro-inflammatory cytokine cascade is therefore amplified and continues for an extended period, leading to high levels of proteases. Proteases are tightly regulated by their inhibitors in acute wounds, while in chronic wounds, protease levels exceed those of their respective inhibitors. Therefore, in chronic wound fluid, pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) as well as MMPs and neutrophil elastase are enhanced, leading to the destruction of ECM and a degradation of growth factors and their receptors. Although the production of growth factors is often increased in chronic wound fluid by comparison with acute wound fluid, their quantity and bioavailability are decreased [18]. Moreover, proteolytic destruction of ECM both precludes the wound from progressing into the proliferative phase and attracts more inflammatory cells, amplifying the inflammation cycle [23]. Immune cells produce ROS, providing defense against microorganisms when in low concentrations. In chronic wounds, however, the predominant hypoxic and inflammatory environment boosts ROS production, damaging ECM proteins and causing damage to cells. This sequence of events brings about an enhanced stimulation of proteases and inflammatory cytokines [24]. Furthermore, chronic wounds are characterized by senescent cell populations with impaired proliferative and secretory capacities, which means that they do not respond to normal wound healing signals [17]. Accumulated data also show that chronic wounds comprise senescent keratinocytes, endothelial cells, fibroblasts, and macrophages [25-28]. Oxidative stress is thought to be the cause of the senescent phenotype of cells in chronic wounds. This leads to DNA damage-related cell cycle arrest, or to abnormal metabolic changes in diabetic patients, which in turn bring about defects in intracellular biochemical pathways such as the GSK-3β/Fyn/Nrf2 pathway [27-29].

1.2.1. Venous and mixed leg ulcers. A prominent problem of social medicine

Chronic venous disease (CVD) is a common condition that afflicts the general population, and it certainly represents the most widespread vascular pathology. In the majority of

cases, it is a minimally disabling disease, but a significant proportion of cases may progress towards chronic venous leg ulceration (CVU) [30].

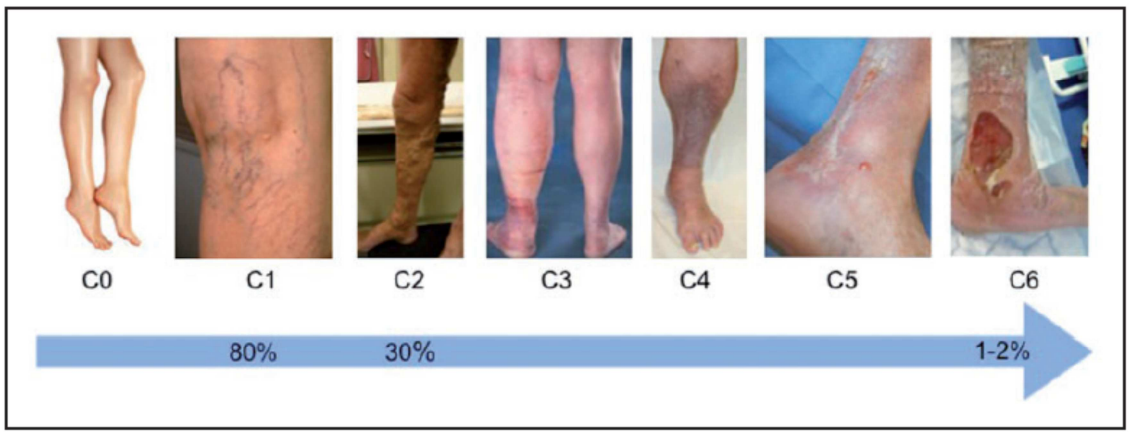

Figure 4. *Clinical representation of CVD severity, following the CEAP classification (Clinical, Etiology, Anatomic, Pathophysiology).*

In the "Edinburgh vein study" Robertson et al. [31] stated that the CVD ratio in a peer-investigated population is higher and higher, with a 1 % increase year-on-year. The deep venous system was affected in 1/3 and the superficial venous system was affected in 2/3 of this population.

Despite superficial venous insufficiency being the pattern most frequently associated with venous leg ulcers, the prognosis is worsened when co-morbidities (rheumatoid arthritis, malnutrition, diabetes, chronic anaemia, etc), second stage peripheral arterial disease or any other vascular pathology are involved. For this reason we usually speak of mixed venous leg ulcers [30,32-34].

Impairment of venous hemodynamic is an essential but insufficient factor in explaining the progression of the disease to the point of skin lesion, or even to explain the frequent presence of non healing CVU [35,36].

Risk factors for venous ulceration

Direct risk factors

- Varicose veins
- Deep vein thrombosis
- Chronic venous insufficiency
- Poor calf muscle function
- Arterio-venous fistulae
- Obesity
- History of leg fracture

Indirect risk factors

- All risk factors leading to deep vein thrombosis including protein-C, protein-S, and anti-thrombin III deficiency
- Family history of varicose veins
- A history of minor trauma prior to the development of ulceration may also be identified

Table 2. *Risk factors for venous ulceration.*

Venous surgery can be helpful in achieving healing and more than all the other options in preventing recurrences [37-42].

However, not all CVU patients are candidates for surgery due to comorbidity conditions; compression, wound-care and debridement still remain the first-line treatments for such disabling diseases. Finally, it has been reported that the CVU healing rate is highly variable in the literature (35%-85%), and various controlled trials report a significant proportion of recalcitrant ulceration, which responds to treatment but does not heal [41, 43, 44]. In such situations skin grafts and advanced dressings are currently used to achieve healing, and new regenerative approaches have been proposed. One of the more interesting among the latter is ADSCs.

2. REGENERATIVE MEDICINE IN WOUND HEALING

The goal of the novel field of regenerative medicine is to reestablish function and structure in damaged tissue through the use of three tools: cell-based therapy, biomaterials (or scaffolds), or/and scaffolds seeded with cells [45].

2.1 Cell-based therapy

Cell-based therapy represents a set of strategies which use live cells with therapeutic aims. It includes stem cells, which are undifferentiated cells with the capability to autorenew and differentiate into progenitor or precursor cells of one or several specific cell types [46,47]. The most commonly used stem cells in regenerative medicine are adult stem cells. In particular, mesenchymal stem cells are a cluster of stem cells originating at the mesodermal germinal layer [48] [49] and can be isolated from various tissue sources in adults [50]. The use of these cells does not raise any ethical concerns, in contrast with using embryonic cells, and they are relatively easy to obtain. Bone marrow derived stem cells have been the focus of regenerative medicine research strategies for many years. However, current research interest is focusing on the development of ADSCs, which can be isolated directly from liposuction during plastic surgery procedures.

There have already been several clinical reports of the successful application of ADSCs in wound healing [51]. Wound healing benefits because ADSCs are multipotent, with the ability to differentiate into other specialized cells, secrete or suppress the growth hormones and cytokines necessary in the environment. They are also capable of increasing in number while displaying a stable phenotype.

Both acute and chronic wounds can be treated using cell therapy. ADSCs can improve wound healing, reduce scar contracture, and minimize donor-site morbidity in the treatment of acute wounds. Conversely, in chronic wound treatment, the wound bed is the environment where maximum healing can be achieved through the transplant of cells with excellent wound healing capacities [52].

We emphasize that wound bed preparation needs to be meticulous before implantation, in accordance with the TIME protocol (tissue, infection, moisture, environment (protocol))

[53]. The TIME principles include the basic principles of wound care that are critical for managing chronic wounds.

2.2 Scaffolds

The use of scaffolds and cellular matrices is essential to differentiate mesenchymal stem cells into the cells required and use them to produce three-dimensional tissue for use in reconstructive medicine.

An essential strategy for tissue engineering is the choice and the construction of a good quality scaffold. Preferably, the scaffold should be a structural and functional platform able to imitate the native extracellular matrix and support the morphogenesis of multiple tissues. Building on the literature, tissue-engineering scaffolds should 1) be biodegradable 2) not trigger inflammatory responses 3) have surface properties which enhance the attachment, proliferation and differentiation of cells 4) mimic skin *in vitro* 5) have the relevant mechanical properties and 6) be well suited for manufacture into different forms [45,54].

2.3 Scaffolds seeded with cells

Engineered tissue regeneration uses a biocompatible scaffold which replaces, repairs or regenerates damaged tissue in combination with living cells and/or bioactive molecules. This kind of scaffold should be biocompatible, porous and permeable to support cell adhesion and proliferation [45].

Suitable scaffolds available for adipose tissue engineering include type I collagen sponge, non-woven polyglycolic acid (PGA) and hyaluronic acid gel. Hyaluronic acid (HA) is a naturally occurring non-sulphated glycosaminoglycan which is present in connective tissue, the synovial fluid of articular joints and the vitreous humor of the eye. It is one component of the natural extracellular matrix and is important in tissue hydration, cell differentiation and tissue reparation.

HA is defined as a “suitable scaffold for adipose tissue engineering”. It is highly biocompatible, does not cause adverse reactions and is reabsorbed by the host tissues [55]. In particular, *in vivo* experiments have confirmed the ideal tissue repair and restoration of

full-thickness wounds for the treatment of ulcers in a placebo-controlled study [56]. Moreover, HA and its derivatives are actively angiogenic: preliminary results in *in vivo* models demonstrate whole vena cava regeneration inside hyaluronic acid-based prosthesis, introducing innovative perspectives in microvascular surgery applications [55,57].

In a recent study, Altman at al. [58] showed that a new scaffold consisting of silk fibroin-chitosan when combined with ADSCs supports the engraftment of stem cells and their differentiation into epithelial and fibrovascular components, increasing the repair and healing capacity of damaged tissues.

The microenvironment for wound regeneration depends mainly on interaction between stem cell progenitors and their niche [59]; consequently, any tissue-engineered reconstruction should provide an appropriate microenvironment for the cells to proliferate and differentiate.

2.4 Scaffolds and nanotechnology

Stem cell differentiation can also be induced by physical factors and modulation of extracellular matrix nanostructures. Indeed, since the majority of signaling molecules interact with stem cells at the nanoscale level, scaffolds with surface nanostructures have potential applications for stem cells in the field of tissue engineering and regenerative medicine. The literature offers several different methods to induce such differentiation through the use of high quality nanoparticles of varying chemical composition. However, before introducing nanoparticles into clinical practice, the real biological effects of their use should be carefully assessed: releasing active peptides may conceivably cause interference with some biological functions and cellular processes [45,60].

3. ADIPOSE-DERIVED STEM CELLS (ADSCs) PROPERTIES

Adipose tissue is one of the largest tissues in the body, representing an important energy and endocrine reservoir [61]. It is mainly composed of adipocytes arranged in lobules, accounting for more than ninety per cent of the tissue volume, and of pericytes, fibroblasts, macrophages, vascular endothelial cells and an extracellular matrix [62].

Zuck et al. [63] first identified ADSCs in 2001 as a population of fibroblast cells with the ability to differentiate into myogenic, adipogenic, osteogenic and chondrogenic cells through specific induction factors. Since this time, easier isolation without major surgical procedures and donor-site morbidity, along with better availability, has sparked great clinical and research interest in ADSCs. In particular, ADSCs have attracted a lot of attention as an alternative to bone marrow stem cells. ADSCs are multi-potent stem cells, with characteristics similar to bone marrow-mesenchymal stem cells. These are demonstrated by their expression of identical cell surface markers, their comparable gene expression profiles and their similar differentiation potentialities [64]. For this reason, ADSCs have the capacity to form fat, muscle, bone and cartilage, under appropriate stimuli. They are multi-potent and produce a range of growth factors [65] such as bFGF, Keratinocyte growth factor (KGF), TGF-β, HGF and VEGF [66].

Human adipose tissue offers several advantages as a stem cell source. These include the ease with which ADSCs can be isolated and expanded, their abundance within the adipose tissue and frequency ranges from 1:100 to 1:1500 cells, far exceeding the prevalence of mesenchymal stem cells in bone marrow [67]. There is only 1 per 100,000 nucleated cells of mesenchymal stem cells in bone marrow and their quantity decreases with age [68].

Many groups have tried to optimise the isolation and expansion of ADSCs [65,69-79]. ADSCs are commonly extracted from adipose tissue in a stepwise procedure. However, the most commonly used ADSCs isolation technique is that described by Coleman.

3.1 Coleman's Technique

This method was introduced by Coleman in 1995 [80] and it was first used for facial remodeling, in breast reconstruction and breast augmentation [81,82]. It provides a

multiple stepwise procedure including aspiration, centrifugation and the subsequent re-injection of autologous fat. The fat donor sites are the abdomen, waist adipose deposit, flanks and inner sides of the thigh and knees [45].

First, Kleine's solution made up of 250 mL normal saline, 20 mL of 1% carbocaine, 1 mL adrenaline and 2 ml bicarbonate is injected into the fat donor area [83] (Figure 5).

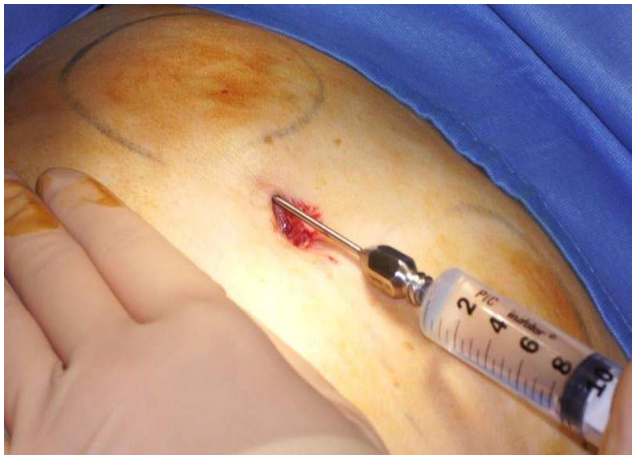

Figure 5. *Tumescent local anaesthesia infiltration.*

After diffusion of the solution the harvesting procedure is undertaken, using a two-hole blunt cannula (Byron Medical, Tucson, AZ, USA) fitted directly to a 10 mL Luer Lock syringe (BD Syringe Luer-Lok tip; Becton Dickinson, Franklin Lakes, NJ, USA), which helps to lessen the pressure caused during the harvesting procedure and preserves the fat [84] (Figure 6).

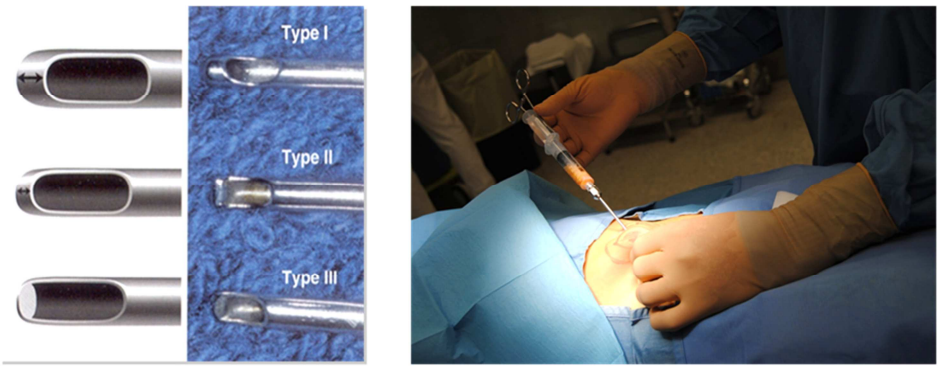

Figure 6. *On the left are examples of various blunt Coleman cannulas used during the harvesting phase; on the right, the harvesting phase according to Coleman's procedure.*

Following the fat harvesting, the lipoaspirate is processed via centrifugation of the lipoaspirate at 3000 rpm (rotor size: 16 cm; g force: 580) for 3 min in 10 ml syringes. This separates the fat into 3 layers (Figure 7).

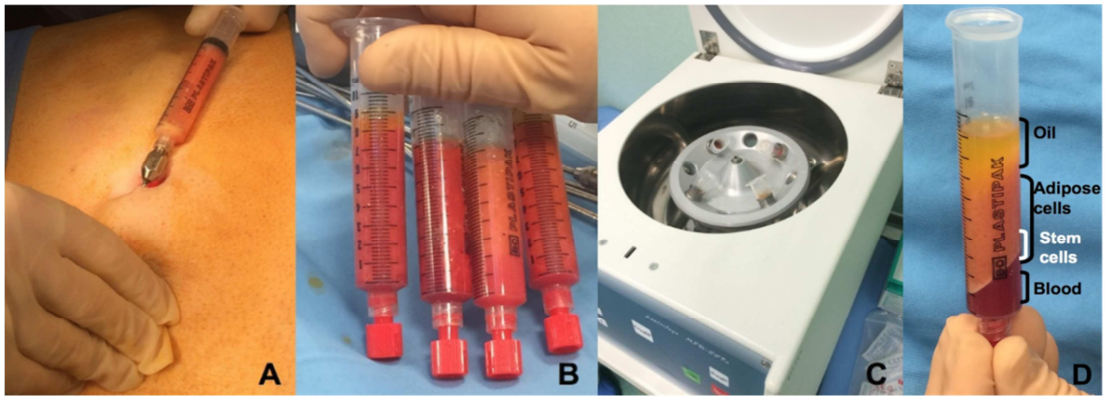

Figure 7. *(A) Coleman's technique for harvesting adipose tissue-derived stem cells. In this particular case, the harvesting is performed in the periumbilical area with tumescent local anaesthesia; (B) Adipose tissue aspirated. The areas with more fat are not always the best to harvest tissue for regenerative purposes; (C) The adipose tissue is then centrifuged in order to separate the different components; (D) The lower part of the adipose layers is particularly rich in stem cells, containing more than 90% ADSCs.*

The upper layer (supranatant) and lower layer are discarded, as they are composed of oil from destroyed fat and blood respectively (Figure 8 E, F), leaving the middle layer, which contains a high concentration of stem cells [85,86], stromal cells, vascular endothelial and mural cells, termed the "stromal vascular fraction".

Once the middle layer has been extracted, the micro-fat graft is transferred into 1ml syringes prior to injection into the tissue to be grafted (Figure 8 G, H).

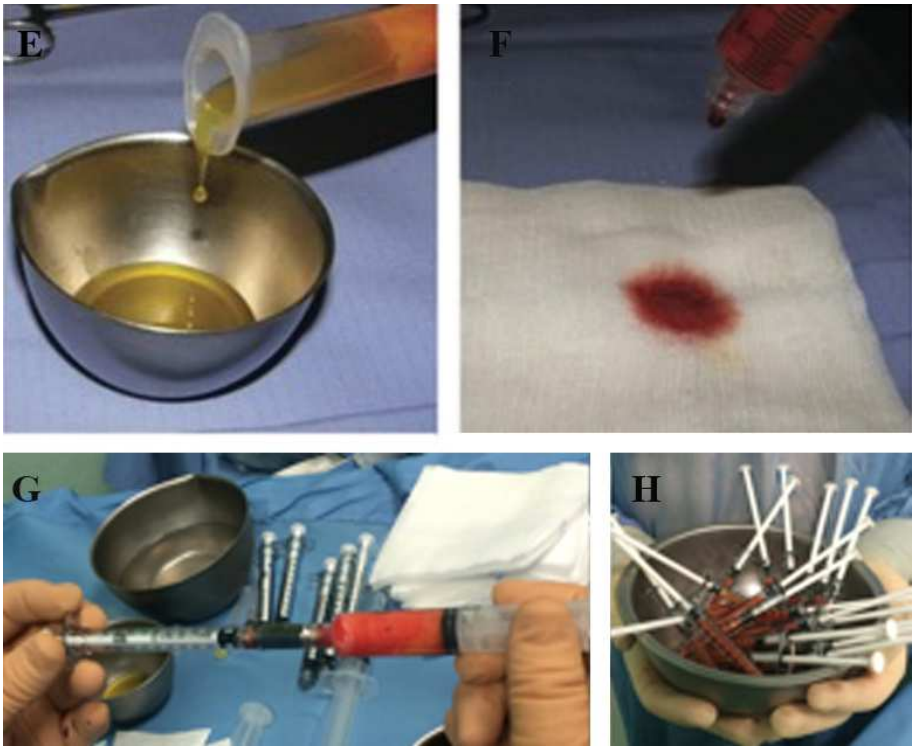

Figure 8. *After oil (E) and blood (F) elimination the ADSCs are aspirated by Luer-Lock syringe technique (G); Syringes loaded with stem cells (H).*

A multilayer technique is used to implant the aliquots of fat, with very small amounts of fatty tissue released into the recipient area in order to optimize successful implantation of the graft. A blunt Coleman microcannula is used to deposit the micro-fat graft via a number of sub-dermal and hypodermal tunnels through numerous tissue planes (Figure 9).

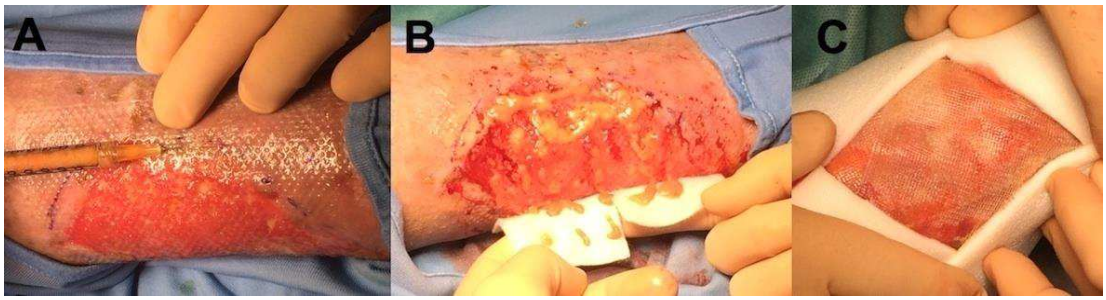

Figure 9. *(A) Margin injection with a suspension of ADSCs. The wound bed preparation is apparent; (B) Islets of adipose cells in the wound bed together with a scaffold of hyaluronic acid; (C) Foam advanced dressing protects the healing area before application of compression.*

This technique of minimising the amount of micro-fat graft released each time the cannula is inserted increases the area between the grafted fat and the tissue receiving it. In this way there is a reduction in fat damage and adipocyte necrosis, with an improvement in graft vascularization and three-dimensional fat distribution. The recently grafted fat has a readily available blood supply, which facilitates its survival and reduces the possibility of fat necrosis or calcification [45,87].

3.2. Safety of Coleman's Technique for ADSCs harvesting

The focus of current stem cell harvesting techniques is on bone marrow, umbilical cord blood and peripheral blood. The method for harvesting and fat grafting initially introduced by Coleman [80] yields a higher number of stem cells when compared with these techniques. The stem cells can be used intra-operatively with minor organizational and legal limitations that might otherwise prove expensive for clinical use [45].

ADSCs are also competitive with regard to complications. Zollino et al [45]. undertook a review of the scientific literature to evaluate complications reported in studies on the fat harvesting and grafting process employing Coleman's Procedure for reconstructive surgery purposes. A total of 5089 patients who underwent plastic surgery and maxillo-facial surgical procedures including facial restoration and post mastectomy reconstruction were

selected [88-101]. There were complications reported in 169 cases (3.3% of the overall number). 141 (2.77%) patients were classed as having minor complications, while 28 patients (0.55%) were classed as having major complications. A detailed breakdown of the minor complications recorded revealed: nodularity and/or induration 93 (1.83%), dysaesthesia 14 (0.26 %), hematoma 12 (0.23 %), superficial infection 11 (0.21 %), pain 7 (0.13 %), poor cosmesis 3 (0.06 %) and abnormal breast secretion 1 (0.02 %). The list of major complications recorded revealed: deep infection 22 (0.43 %), sepsis 3 (0.06 %), abdominal hematoma requiring percutaneous surgical drainage 2 (0.04 %) and pneumothorax 1 (0.02 %) (Table 3).

Author	Patient population	Complications (%)
Burnouf (Reference 88)	33	0
Hardy (Reference 89)	12	0
Dollfus (Reference 90)	6	0
Clauser (Reference 91)	47	0
Guijarro-Martínez (Reference 92)	4	0
Claro Jr. (Reference 93)	4601	• nodularity and/or induration (2.02) • deep infection (0.41) • dysaesthesia (0.30) • hematoma (0.26) • superficial infection (0.19) • pain (0.06) • sepsis (0.06) • abnormal breast secretion (0.02) • • pneumothorax (0.02)
Guisantes (Reference 94)	8	0
Seth (Reference 95)	90	• pain (4.4) • poor cosmesis (3.3)
Arcuri (Reference 96)	19	• abdominal hematoma requiring percutaneous surgical drainage at 2 days postoperatively (5.2)
Weichman (Reference 97)	100	deep infections (1.0)
Biglioli (Reference 98)	8	0
Endara (Reference 99)	21	• deep infections (9.5) • superficial infection (4.7) • hematoma requiring drainage and closure on the day of surgery (4.7)
Kaoutzanis (Reference 100)	108	superficial infection (1.0)
Piombino (Reference 101)	32	0

Table 3. *Complications for harvesting and fat grafting procedures.*

In comparison, data recorded on the recovery and safety profiles following bone marrow collection in 9245 donors identified 345 cases (3.7%) with potential complications. In 125 cases (1.35% of the 9245 total) post-harvesting, these complications were classed as serious. Of the 125 serious cases, 116 were classed as being directly related to the collection, namely: mechanical injury to tissue, bone or nerve 69 (0.7%), anesthesia 45 (0.5%), infection 1 (0.01%) and grand mal seizure 1 (0.01%). Among the 116 patients with serious complications, 67 (0.7% of the 9245 total) experienced prolonged recovery times due to mechanical injury to tissue from needle aspirations, and required interventions ranging from limited physician involvement and/or physical therapy to surgical intervention and ongoing disability (1 to 10 years). Among the remaining 49 patients (0.5% of the 9245 total) with severe reactions, most issues were due to severe acute reactions related to anesthesia (complicated post-spinal headaches, cardiac arrhythmia, and pulmonary edema) [102-104].

The use of Coleman's Procedure for ADSCs harvesting therefore seems advantageous when compared with other techniques for stem cell harvesting. It seems a safer option, involving less discomfort and a reduced risk of complications for patients (Figure 10).

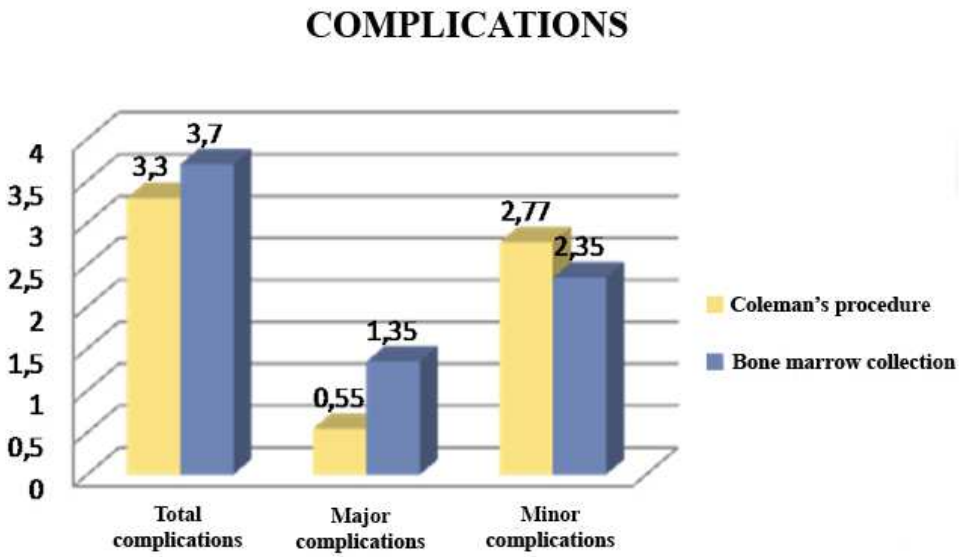

Figure 10. *Complications post harvesting (percentages).*

3.3. ADSCs in wound healing

As conventional treatment strategies for chronic wounds increasingly reach their limits and often fail, advanced healing therapies are being used to rectify the irregular and

dysfunctional cellular pathways which are present in chronic wounds. These treatment strategies include skin substitutes, growth factor-based therapies, biological dressings, and synthetic acellular matrices [105].

Increasing vasculogenesis and angiogenesis is vital to the investigation of pioneering wound healing strategies [106]. Both fibroblast growth factor (FGF) and VEGF are potent angiogenic factors. Increased granulation, decreased contraction, and increased angiogenesis have been demonstrated through the application of FGF to wounds [107]. In addition, KGF and FGF have been clarified in order to activate fibroblast and keratinocyte proliferation and migration, collagen synthesis, and induce angiogenesis [108,109].

Vascularization plays a crucial part in wound healing and is thus a significant parameter for new therapies [110]. For this reason, innovative and alternative treatment options including stem cell-based therapies have been investigated over the last decade [20]. ADSCs offer prodigious potential through their capacity to release angiogenic factors, displaying increased angiogenesis in wound healing whenever injected or delivered via a scaffold [111]. Increased vascular tissue has been observed in models using endothelial cells and fibroblasts have yielded increased vascular tissue. Moreover, ADSCs have displayed improved healing in both burn models and full-thickness wound models [20].

ADSCs have also shown a positive impact on wound healing in clinical and pre-clinical studies, in addition to their angiogenic potential. Recent ADSCs applications *in vitro* and *in vivo* have demonstrated that they are attracted to the wound site and affect regeneration processes by means of paracrine mechanisms in addition to fusion and differentiation, for instance, into keratinocytes or dermal fibroblasts [112-114].

Ongoing clinical trials are using ADSCs for regenerative medical and tissue engineering applications [115,116]. Rigotti et al [117]. used ADSCs to treat patients with severe symptoms or irreversible function damage due to side effects from radiation treatment (LENT-SOMA scale grades 3 and 4). Irradiated areas were treated through the application of purified autologous lipoaspirates. In the majority of patients treated, both ultrastructural tissue regeneration with neovessel formation, and significant clinical improvement were observed.

Promising applications in wound and ulcer healing have been reported, although thus far these are small studies with a total of only 98 patients [12,118-122]. Bartsich and Morrison

[123] have discussed the long-term treatment of chronic sickle cell ulcers and the possible use of a skin graft and fat grafting to achieve healing. Current treatment using skin grafting and local wound care is often unsuccessful over the long-term, as wounds that have healed break down again. Treatment involving enduring modification of the wound bed with enrollment of a new cell population and subsequent fat grafting was satisfactorily applied in Bartsich and Morrison's study. Raposio et al. [12] presented their experience in regenerative surgery of chronic skin ulcers, evaluating the effects related to the use of adipose-derived stem cells (ASCs) added to platelet-rich plasma (PRP), to obtain an enhanced PRP (e-PRP). e-PRP significantly enhanced wound closure rates when compared to standard wound care through a faster recovery and without causing any serious complications. In 2010 Cervelli et al. [118] showed the capability of combining autologous adipose grafts and PRP injected intralesionally or perilesionally to regenerate tissue and induce epithelialization with wound closure in patients with a loss of substance on the lower limb. They reported a significant decrease in healing time (57% of patients achieved complete healing within 3 months) and a noticeable improvement in quality of life, along with a diminution in cost due to the decreased amount of medication required. Their subsequent research [119] suggests a new therapeutic plan: the use of Enhanced Stromal Vascular Fraction (e-SVF). As reported, e-SVF and PRP mixed with fat grafting applied to the bed of ulcers are two treatments that showed improvement in the healing process in post-traumatic extremity ulcers. Results showed that wounds treated with e-SVF healed better than wounds treated with hyaluronic acid alone. After 9.7 weeks, the patients treated with e-SVF experienced a 97.9%±1.5% re-epithelization rate compared to 87.8% ± 4.4% of the first control group (hyaluronic acid); while patients treated with PRP and Lipostructure after 9.7 weeks experienced a 97.8% ±1.5% re-epithelization rate compared to 89.1%±3.8% of the second control group (PRP). Marino et al. [120] have used purified adipose-derived stem and regenerative cells (ADRCs) obtained from autologous fat for the treatment of chronic lower limb ulcers of arteriopathic patients. In all cases, injections into the edges of the ulcer produced a decrease in both the diameter and depth of the ulcer. There was healing with complete re-epithelization in over half of the cases. In the first phase I trial, Bura et al. [121] have proved that adipose-derived stroma cell (ASC) transplantation greatly increases tissue revascularization, with ulcer evolution and wound healing improvement in non-revascularizable critical limb ischemia patients. Lee et al. [122] have demonstrated that a safe alternative method to achieve therapeutic angiogenesis in patients with critical limb ischemia who are refractory to other treatment modalities

could be the use of multiple intramuscular adipose tissue-derived mesenchymal stem cell (ADMSC) injections. The Authors have observed clinical improvement in 66.7% of cases. Their patients have displayed a significant decrease on the pain rating scale and improved claudication walking distance.

WOUND TYPE	CELL TYPE	DELIVERY SYSTEM	OUTCOME	REFERENCE
Chronic venous, diabetic, and ischemic ulcers (n=16 patients)	e-PRP	Injection into skin edge and at the bottom of the lesion	Complete healing in 71% of patients	Raposio et al. (2016) [12]
Non-revascularizable critical limb ischemia with/without ulcers (n=7 patients)	ASCs	Intramuscular injection	Increased trans-cutaneous oxygen pressure, improvement of ulcer evolution and wound healing	Bura et al. (2014) [121]
Chronic ulcers of the lower limbs of artheriopatic patients (n=10 patients)	ADRCs	Injection at the edges of the ulcers	Complete healing in 60% of patients	Marino et al. (2013) [120]
Critical limb ischemia with/without non healing ulcers (n=15 patients)	ADMSCs	Intramuscular injection	Clinical improvement occurred in 66.7% of patients (minor amputation in 5 patients)	Lee et al. (2012) [122]
Post-traumatic lower extremity ulcers (n=20 patients)	e-SVF in 10 patients, AT+PRP in 10 patients	Injection in small tunnels in the perilesional area	Complete healing in 97.9%-97.8% of patients	Cervelli et al. (2011) [119]
Vascular, diabetes-correlated, or post-traumatic diseases with ulcers or loss of substance of the lower limbs (n=30 patients)	AT+PRP	Injection of AT + PRP covered with 3-dimensional polymerized hyaluronic acid - medicated biological dressing	Complete healing in 57% of patients	Cervelli et al. (2010) [118]

Table 4. *Summary of the use of cell therapy in ulcer healing.*
Key: ADSCs (or ASCs) = Adipose derived stem cells, PRP = Platelet-rich-plasma, e-PRP = ADSCs (or ASCs) + PRP, ASCs = Adipose-derived stroma cells, ADRCs = Purified adipose-derived stem and regenerative cells, ADMSCs = adipose tissue-derived mesenchymal stem cells, e-SVF = Enhanced Stromal Vascular Fraction, AT = Adipose tissue, HA = hyaluronic acid.

Moreover, various studies on mouse, rat and rabbit models have provided encouraging evidence of ulcer evolution and wound healing improvement [112,113,124-140]. Likewise, the positive results of ADSCs on wound healing have also been demonstrated using animal models with chronic diseases or artificially induced impaired wound healing. An increase in collagen intensity, capillary density, VEGF and TGF-β3 expression was displayed as a consequence of ADSCs transfer. Animals with autologous ADSCs transplantation on wounds demonstrated appreciably increased survival, angiogenesis and epithelialisation rates [131]. The ADSCs accelerated wound healing of radiation ulcers in a modified rat model, where they were co-localised with endothelial cell markers in ulcerated tissues. Treatment using ADSCs achieved smaller wound sizes and was linked to development of new blood vessels [141]. Finally, Hong et al [129]. showed in a rabbit ear *in vivo* model that topically delivered rabbit ADSCs are engrafted and proliferate in wounds, where they exhibited an activated fibroblast phenotype. Furthermore, ADSCs led to augmented endothelial cell and macrophage enrollment. In contrast to bone marrow-derived stem cells and fibroblasts they also increased granulation tissue formation.

3.4 Histology of the wound healing process and potential role of ADSCs

The normal wound healing process in vascular ulcerations is usually aborted for a variety of reasons depending on each clinical case. Usually, the first phase of wound healing is the inflammatory phase. Immediately after injury, a hemostatic reaction begins. Blood platelets release growth factors, cytokines, and other survival or apoptosis-inducing agents. Leukocytes release reactive oxygen species (ROS) with antimicrobial and proteases roles. Resolution of the inflammatory phase is accompanied by gradual inflammatory cell apoptosis a few days after the event.

As the inflammatory phase ends, the proliferative phase of repair begins. Wound healing angiogenesis begins immediately after injury and this event stimulates the production of pro-angiogenic factors. In response, endothelial cells degrade basal membrane, migrate into the wound site, proliferate, and form new blood vessels (Figure 11 A, B).

More recently, it has been revealed that endothelial progenitor cells (EPCs) are also required for wound revascularization [142,143]. Endothelial progenitor cell mobilization is mediated by nitric oxide, VEGF, and matrix metalloproteinases (MMP9 particularly)

[143,144]; in this phase ADSCs could play a main role secreting bFGF, KGF, TGF-β, HGF and VEGF. Although more research needs to be done to further elucidate the mechanisms of EPC and ADSC recruitment and homing, it is clear that these progenitor cells are necessary for normal wound healing associated neovasculogenesis and injury repair [2,111,145].

Reestablishment of a normal blood supply provides a favourable microenvironment for epidermal and dermal cell migration and proliferation. Fibroblasts proliferate within the wound and synthesize extra-cellular matrix (ECM) forming granulation tissue perfused with newly formed blood vessels. It seems that ADSCs, when applied after wound bed preparation according to the TIME protocol, particularly enhance blood vessel proliferation as well as synthesis of matrix components. In figure 11 C, one week after ADSCs treatment, blood vessel proliferation together with a provisional matrix can easily be seen.

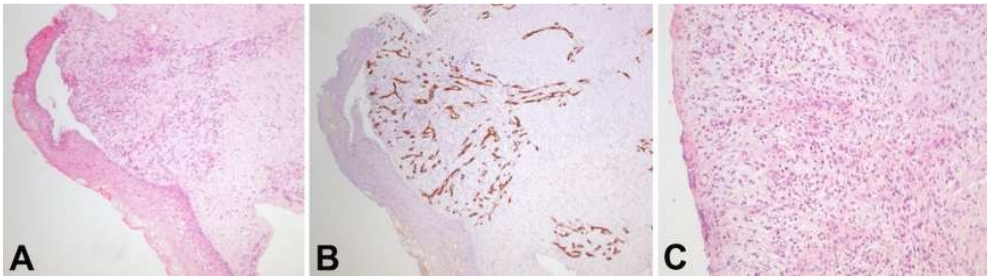

Figure 11. *Exemplification of the healing process 2 weeks after implantation. (A) Wound site at 4X, Hematoxylin - Eosin. At this magnification, the acanthosis of epidermidis detached by papillary dermis cohabited by granulation tissue rich in vascular structures and remodelling collagen can be seen. In the lower portion of the dermis, the incipient remodelling reaction is evident; (B) Ulcerated site at 10X, Hematoxylin - Eosin. No epidermidis covers the superficial dermis that is repopulated by granulation tissue rich in neovascular structures and collagen; (C) CD34 immunohistochemistry marks neovascular endothelial cells and underlines the typical wound site morphology during repair time characterized by the presence of granulation tissue and scar formation with fibrosis and loss of adnexae structures in the lower dermis.*

We know that such a matrix mainly consisting of collagen III, fibrin, fibronectin, and hyaluronic acid is progressively substituted with ECM mainly containing collagen I. Next, wound contraction and matrix remodelling take place. Finally, apoptosis of fibroblastic cells occurs, leading to the formation of a relatively acellular scar tissue whose tensile strength is comparable with unwounded skin.

3.5. Limitations of using ADSCs

Donor specificity is a recognised phenomenon. Recent studies have investigated ADSCs function within the context of donor age and gender. Donor specificity of human ADSCs has been reported by Shu et al. [146], who observed links between donor age and cell differentiation as well as anti apoptosis ability. Another study with human ADSCs demonstrated that equal amounts of ADSCs could be isolated, regardless of donor age. However, infant-derived cells have shown different morphology and enhanced angiogenic and osteogenic capabilities [147]. Donor age specificity has also been investigated by Guercio et al [148]. and has revealed a higher proliferation capacity of ADSCs in younger dogs compared with older animals.

There is scant evidence thus far regarding the effect of gender on ADSCs potential. Fossett and Khan [149] surmised that females have a notably higher yield of mesenchymal stem cells than males and that oestrogens play an excitatory role in controlling levels of cytokines and growth factor production. Furthermore, the gender of ADSCs donors influenced the proliferation, differentiation, paracrine and anti-apoptosis capacities of human ADSCs [146]. As well as age and gender, body mass index, chronic disease, a western lifestyle and many other features cause donor-specific variations. With progenitor cells, it has been seen that cells harvested from patients with chronic diseases have reduced regenerative potential [150,151].

Another influencing factor in the applicability of ADSCs in chronic wound treatment is the composition of wound fluid, which differs markedly between acute and chronic wounds. In an *in vitro* wound model, wound fluid has been shown to influence ADSCs function inversely. While acute wound fluid has a strong chemotactic impact and stimulates ADSCs proliferation, chronic wound fluid has an inhibiting effect on ADSCs migration and proliferation. Chronic wound fluid strongly induces expression of bFGF, VEGF and MMP9 [152]. Furthermore, the risk of inducing cancer by transplantation of ADSCs has not yet been fully excluded [66].

4. DIAGNOSIS OF ULCER PATHOGENESIS AND SYNCHRONOUS VASCULAR TREATMENTS AND WOUND CARE

Impairment of venous and arterial hemodynamics represents an essential factor in the pathogenesis of CVU. However, other associated medical conditions are reported: obesity, smoking, diabetes, hemolytic anemia and/or iron-deficiency anemia and/or malnutrition, inability to walk, severe cardiac and/or hepatic and/or renal and/or pulmonary insufficiency or chronic administration of cortisones for chronic inflammatory disease and/or auto-immune disease [153,154].

ADSCs grafts in successful CVU outcomes need a significant improvement of the venous function by means of associated and customized case-by-case treatments. All of these treatments aim to improve tissue drainage and perfusion and include: compression [37,40,155-158], intermittent pneumatic compression and other novel pneumatic devices [159,160], superficial venous surgery complemented by drugs [161-163].

Before starting, it is important to obtain a healthy, clean wound bed without devitalized tissue, with increased drainage and around-wound tissue that is not macerated or indurated [53].

Moreover, in addition to wound care and infection, the level of proteinases also needs to be corrected before implantation of ADSCs. There is now substantial evidence in animals and humans that proteases in general, human neutrophil elastase (HNE) and matrix metalloproteinases (MMP-9) in particular, are highly elevated in wounds with delayed healing compared to normally healing wounds [164,165]. In healing wounds, there is a rapid initial increase in protease levels. If healing progresses normally, levels peak at about day three and start to reduce by about day five. In non-healing wounds, not only do proteases reach higher levels than in healing wounds, but they persist, resulting in a highly destructive environment.

The neutrophil-derived protease elastase is also important. This is because it has been found to be the biggest contributor to fibronectin degradation in non-healing wounds and fibronectin degradation products stimulate the release of MMPs [166,167]. Intact fibronectin (which is necessary for cell adhesion and growth factor signalling) is absent in

non-healing wounds, but has been shown to reappear in the wound bed as a wound begins to heal [168].

It appears clear from the above how important it is to include treatments which improve drainage and perfusion, wound care, and proteases management to prepare the tissue for subsequent ADSCs to achieve successful implantation.

5. MOTIVATION AND NOVELTY OF THE RESEARCH

Different technical approaches for ADSCs implantation have been proposed, but a standardized technique verified through parallel clinical-lab investigation is currently lacking. The quality and the therapeutic potency of the stromal vascular fraction (SVF), from which ADSCs are derived, is heavily dependent on how the adipose tissue (AT) is processed, so different methods may produce dramatic differences in the cell composition of SVF and gene expression profile of ADSCs [169]. Moreover, an analytical approach to the study of the immunophenotypic and functional features of SVF is needed. Further studies are therefore required to standardize the technique and diversify the various processes (from collection to implantation) in accordance with the different diseases where cell treatment has been proposed. From this point of view, few studies [12] are currently available on venous leg ulcers (VLU) addressing the questions above.

5.1 Aim of the study

The aim of our study is to preliminarily and randomly evaluate the efficacy and safety of the use of ADSCs in the healing process of VLU, which do not respond to the gold standard treatment, using a reproducible methodological approach verified in parallel with a lab cell characterization.

5.2. Material and Methods

5.2.1. Patient recruitment

From an initial cohort of 38 patients referred to the Vascular Diseases Center of the University of Ferrara, 8 patients (5 men, 3 women) affected by VLU entered the study. Clinical assessment consisted of physical examination, echo-color-Doppler to assess venous etiology, the presence of peripheral artery disease with the measurement of the ankle brachial index (ABI) and evaluation of bottom line wound area. Patients were selected in accordance with the following inclusion criteria: age range 18-90 years old; chronic pure venous and/or mixed VLU refractory to standard treatments in stall for at least 9 months; surface area > 8 cm^2; chronic pain refractory to home-based therapy. The

exclusion criteria were: clinical evidence of bacterial infection; peripheral arterial disease with ABI < 0.8; anemia with Hb < 10.0 gr/dl; malnutrition (BMI < 17,5); inability to walk; multi organ failure; malignancy; immunosuppressive or cytotoxic agents. After applying the above criteria, 16 patients were selected and randomized according to a computer generated 1:1 list into two arms, respectively ADSCs treated and Controls. The study protocol was approved by the Ethical Committee of the Azienda Ospedaliero-Universitaria Sant'Anna, Ferrara, Italy.

5.2.2. Procedures

In the two arms the debridement of ulcers was performed by means of Versajet device (VERSAJET™, Versajet Hydrosurgery System, Smith and Nephew, Hull, UK) (Figure 12), a debridement tool able to cut and remove necrotic tissue and fibrin based on a high pressure jet of water which produces the Venturi effect [170].

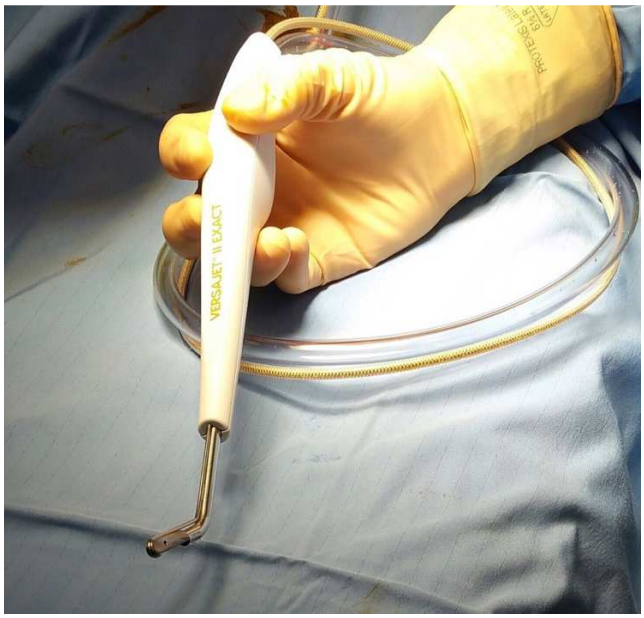

Figure 12. *Versajet.*

In the experimental arm, the study design contemplated ADSCs harvesting using Coleman's technique [80] as previously described [45,171]. Approximately 20 ml of AT was removed by suction. The samples were sealed and centrifuged at 1300 r/min (rotor size: 16 cm; g force: 580) for 3 minutes. Before transfer into the tissue, the oil and the blood were eliminated and the high-density layer (HDL) [85] micro-fat (approximately 15 ml for each patient) with a high concentration of stem cells was placed into 1 ml syringes

with an 18-gauge needle. Multiple sub-dermal and hypodermal tunnels were designed on the withdrawal phase of the needle; in this way we reduced to small pearls the amount of micro-fat graft released each time with a retrograde motion, minimizing damage to the cells and adipocyte necrosis, improving graft vascularization and three-dimensional graft distribution. The samples were injected at a depth of 1 cm, with care taken to spread them in all directions both into the bed and to the edges of ulcers. After the injection, a scaffold of hyaluronic acid was applied to the wound and an advanced foam dressing was applied to protect the healing area. A sample of 1 cc of lipoaspirate was sent to the Laboratory of Molecular Biology and a punch biopsy (5 mm in diameter) was performed for the histological evaluation.

Both arms, after the procedures, were treated with a multilayer bandage (zinc oxide non-elastic bandage- Lohmann, Rengsdorf, Germany- and hypoallergenic adhesive bandage) applied from the foot to just below the knee. In cases of clinical suspicion of infection, swab cultural examination and antibiogram based antibiotics were prescribed. Patients were advised to walk. Wound dressings and bandaging were applied weekly until healing.

5.2.3. Outcome measures

Ulcers were treated weekly following the TIME criteria [17,53]. The visit included evaluation of adverse events, and of the changes in area size of the lesion. The contour of the ulcer was traced on a transparent dressing and then assessed by means of software that was able to calculate any irregular area (Visitrak Capture, Smith & Nephew, London, UK). If multiple ulcers were present on the same limb their areas were summated. The patients were asked to indicate the intensity of chronic pain using the Numeric Pain Rating Scale (NRS) [172].

Furthermore, photographic images, swab cultures and three punch biopsies (5 mm in diameter) at baseline, week 1 and week 2 after treatment, were performed. Specimens were analyzed by the Laboratory of Molecular Biology and by the Department of Experimental and Diagnostic Medicine, Sant'Anna University Hospital of Ferrara-Italy.

The primary outcomes were: the Safety and the Healing Time expressed in weeks from randomization.

The secondary outcomes were: NRS post treatment to evaluate whether ADSCs treatment could modulate pain [173]; rate of patients reaching complete wound healing at 24 weeks

identified through the Margolis Index (MI = the percentage change in area of an ulcer over the first 4 weeks of treatment represents a practical prognostic indicator and a predictive measure of healing or non-healing at 24 weeks) [174]; the healing process evaluated as a reduction in the initial area per week (cm^2/week); Histopathology was also performed through punch biopsy at week 1 and week 2.

5.2.4. Isolation and expansion of autologous ADSCs

Adipose samples were obtained with informed consent and cells were taken as described above [45]. The SVF fraction was separated using a procedure modified from Zuk et al [63,175]. Briefly, various aliquots of 2 ml of AT were digested with 0.075% collagenase in DOM completed medium: Dulbecco's modified Eagle Medium (GIBCO Italy) supplemented with 12,5% bovine serum, 12,5% horse serum, 1% penicillin/streptomycin, 2% glutamine (GIBCO) and vortexed for 2 minutes then at 37°C for 15 minutes. After incubation at 37°C, the cells were centrifuged for 10 minutes at 1200 rpm in order to separate mature adipocytes from the pellet. After centrifugation, the pellet was divided and resuspended in PBS, for FACS analysis of the fresh samples before culture as well as for CFU-F assay. For the *in vitro* expansion, the SVF was plated in 5100 medium (StemCell Technologies Inc., Vancouver, B.C., Canada) at a density of 5.000 cells/cm^2 in T25 tissue culture flasks (BD BioCoat Collagen I Cellware Flasks; Becton-Dickinson, San Jose', CA, US). The cells were maintained in media until they achieved 75%–90% confluence and than trypsinized for further analysis after expansion.

5.2.5. Multicolour flow cytometric analysis of freshly isolated SVF

Freshly isolated SVF fraction was characterized through a four-color flow cytometric approach. Multiparametric flow cytometric analysis was performed on a FACS Calibur equipped with the four-color option (Becton Dickinson, San Jose, CA, US). In order to optimize the FACS analysis, non-viable cells that had lost membrane integrity were identified by uptake of 7-amino-actinomycin D (7-AAD). 7-AAD was purchased from Molecular Probes (Leiden, The Netherlands) and we used 4ug/ml for each sample. Ten thousand gated events were acquired. Freshly isolated cells from SVF were maintained on ice and incubated simultaneously with different specific monoclonal antibodies in order to quantify and characterize the amount and the type of stem cell cellular content. Different monoclonal antibodies such as: anti-CD34-PercP (cl.8G12), anti-CD45-APC (clone 2D1,

IgG1), anti-CD271, anti- CD271, anti- CD146-PE (cl. P1H12), anti CD31-FITC (clone WM-59, IgG1), anti-CD90 (clone 5E10, IgG1 k, FITC), and MSCA-1 (all from Becton-Dickinson) were used in different combinations. The non-hematopoietic stem cells were analysed in the gate of CD45 med low events. In particular, the percentage of CD45 med low CD34-positive cells was considered as expression of the non-hematopoietic stem cell content in the SVF.

The percentage of the content of SVF-nonhematopoietic stem cell and the type of other cells, including ADSCs, was correlated to the patient follow up. The nonhematopoietic stem cell immunophenotype was analysed by excluding CD45-positive hematopoietic cells and 7-AAD positive non viable cells.

5.2.6. CFU-F assay of SVF

Primary cultures-expanded from SVF were established by plating all the SVF cells derived from 2ml of ADSCs onto BD BioCoat Collagen I Cellware Petri dishes (35mm) (Becton-Dickinson, San Jose', CA, US) in Dexter's type long term culture medium 5100 (StemCell Technologies Inc., Vancouver, B.C., Canada) (M5100). The *in vitro* clonogenic output was evaluated at the 15th day, as the number of fibroblast aggregates that were scored as colony forming unit fibroblasts (CFU-F detection) as described by Castro & Malaspina group [176,177]. Clones of more than 50 cells were considered to be colonies. At the first confluence, SVF derived stromal layers were trypsinized for FACS analysis. The presence of possible endothelial progenitor cells EPC colonies was also scored.

5.2.7. Multilineage potential of SVF

The plasticity of SVF derived cells was assessed after 2/3 culture passages. To promote adipogenic and osteogenic differentiation, the cells were trypsinized and plated in BD BioCoat Collagen I 12 multiwell plates (Becton-Dickinson, San Jose', CA, US) for three weeks respectively in adipogenic and osteogenic commercially available specific media (respectively NHOsteoDiff and NHAdipoDiff media from Miltenyi Biotec). BM-derived MSCs (bone marrow-derived mesenchymal stem cells) were used as a positive control. The lipid droplets in the generated adipocytes were visualized by staining with Sudan Black IV (Sigma). The percentage of adipocytes was estimated by counting 100 cells on 4 random fields (400 total cells counted). The assessment of mineralization areas was

visualized through Von Kossa staining. Osteogenic differentiation was evaluated as a percentage of the mineralized area against the total culture area.

5.2.8. Immunophenotypic analysis of cultured ADSCs-derived SVF fraction

Primary culture-expanded SVF derived adherent cells from patients were studied using flow cytometry. Adherent cells were detached with trypsin /EDTA (GIBCO), suspended in 0.5 ml PBS supplemented with 0,2 % bovine serum albumin (BSA, Sigma). For the panel of MoAbs used and the cytometric analysis see our previous studies [178-180].

5.2.9. Histology

Microscopic evaluation of routinely hematoxylin-eosin stained paraffin derived sections was performed to evaluate the healing process in both arms.

5.2.10. Statistical analysis

Clinical data were expressed as mean ± standard deviation and tested for significance by the Mann-Whitney U-test depending on variable distribution. The comparison of the outcome measure achievement in the two arms was performed by Fisher's exact test. Cellular expression for the various immune-phenotypic markers in patients was assessed using Mann-Whitney non parametric statistics. The percentage of the different non-hematopoietic stem cell subsets (such as CD34+CD45-, CD34+CD146+/-, CD271+/-CD146+/-, etc..) present in the ADSCs was correlated to patient follow up (see previous paragraph about outcome assessment in particular evaluated as healing time, NRS and MI). p values lower than 0.05 were considered to be significant.

5.3. Results

5.3.1. Patients

In Table 5, patient population demographics are given, with no significant differences between the 2 arms after randomization. The etiology of patients' ulcers was chronic pure venous (75%, meaning reflux and or venous obstruction with ABI > 0.8 and no significant comorbidities) and mixed venous (25%, meaning reflux/obstruction coupled with significant comorbidities or ABI < 0.8) with homogeneous distribution between the two arms. The mean ulcer size in the ADSCs group was slightly increased with respect to

controls, although not significantly, as described in Table 5. The chronic pain assessed by NRS before treatment had a mean rating of 7 ± 3 in the study group *vs* 6 ± 3 in the control group.

	ADSCs (N°;%)	**CONTROL (N°;%)**	**P**
AGE (Y)	74 ± 6.7	68 ± 12.8	0.79
GENDER (M/F)	5/3	5/3	1.00
ETIOLOGY	VENOUS 6;75% MIXED 2;25 %	VENOUS 5;62,5% MIXED 3;37,5%	0.50
WOUND SIZE (cm^2)	31.3 ± 59.0	29.0 ± 42.0	0.95
COMORBIDITY AFFECTING HEALING	Hypertension 8;100% Diabetes 2;25 % DVT and PE 1;12,5% Heart failure 2;25%	Hypertension 8;100% Diabetes 3;38 % DVT and PE 1;12,5% Chronic Kidney failure 2;25%	
NRS BEFORE TREATMENT	7.0 ± 3.0	6.6 ± 2.9	0.63

Table 5. *Patient population demographics with no significant differences between the 2 arms.*

5.3.2. Primary endpoint

We did not record any major adverse events related to the procedure. In one case we registered perilesional dermatitis shortly after the cells were implanted, which spontaneously resolved in the second week.

The primary outcome, mean healing time, was significantly different between the 2 arms: 17.5± 7.0 weeks in the experimental group as compared to 24.5 ± 4.9 weeks recorded in the control group ($p<0.036$) (Table 6).

	ADSCs	CONTROL	P	95% C.I.
Healing Time (weeks)	17.5 ± 7.6	24.5 ± 4.9	**0.036**	**-**
NRS post treatment	2.7 ± 2.3	6.6 ± 2.9	**0.01**	**-**
MI +(N°)	4	2	0.60	0.3-24
Healing (N°)	6	4	0.60	
Healing process (cm^2/week)	3.2 ± 8.0	0.2 ± 0.4	0.37	-

Table 6. *Primary and secondary endpoints.*

5.3.3. Secondary endpoints

Shortly after the application of ADSCs, NRS dropped after the first week to 2.7 ± 2.3 whereas in the control group it was 6.6 ± 2.9, ($p < 0.01$). Conversely, the MI was not significantly different between arms: in the experimental group, 50% of patients obtained the MI + *vs* 25% of the control group (O.R. 3.0; 95% CI 0.3-24; $p<0.608$).

Finally, complete healing was reached in a higher proportion in both groups, being 6/8 in the ADSCs patients (Figures 13-16) *vs* 4/8 in the controls with no significant difference between arms ($p<0.608$). The wound healing process proceeded in the ADSCs group at 3.2 ± 8.0 cm^2/week; not significantly faster with respect to 0.2 ± 0.4 cm^2/week in the control group, yet ($p < 0.371$) (Table 6).

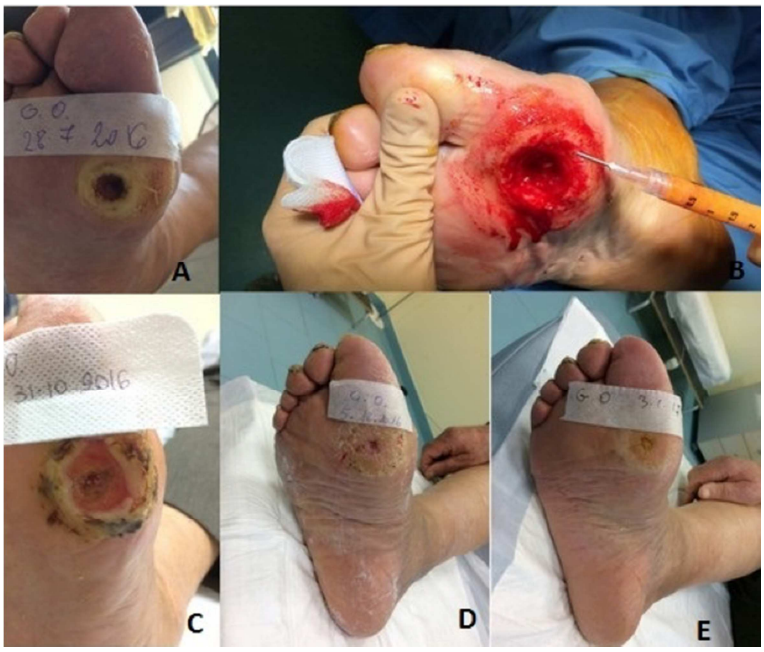

Figure 13. *Pre-operative mixed venous leg ulcer (A); intraoperative injection of ADSCs by 18 Gauge needle (B); post-operative ulcer after 1 week (C) and after 6 weeks (D); ulcer healed after 8 weeks of treatment (E).*

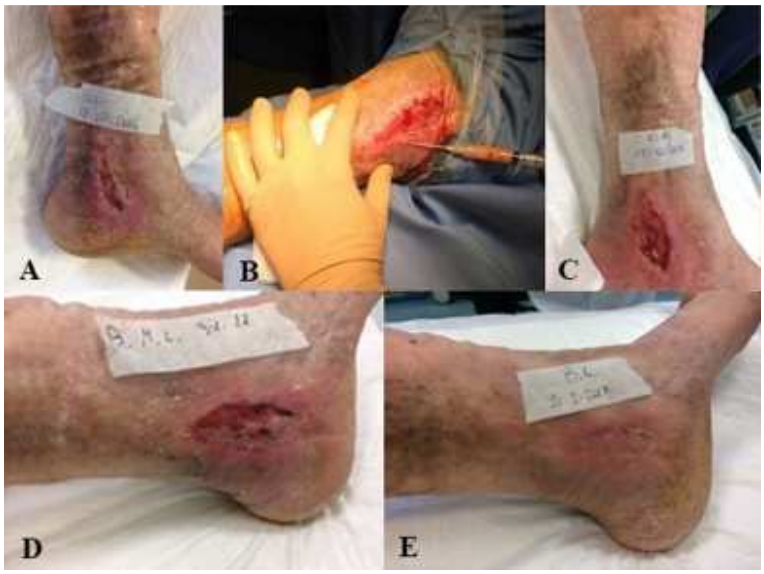

Figure 14. *Pre-operative pure venous leg ulcer (A); intraoperative injection of ADSCs by 18 Gauge needle (B); post-operative ulcer after 6 weeks (C) and after 10 weeks (D); ulcer healed after 16 weeks of ADSCs treatment (E).*

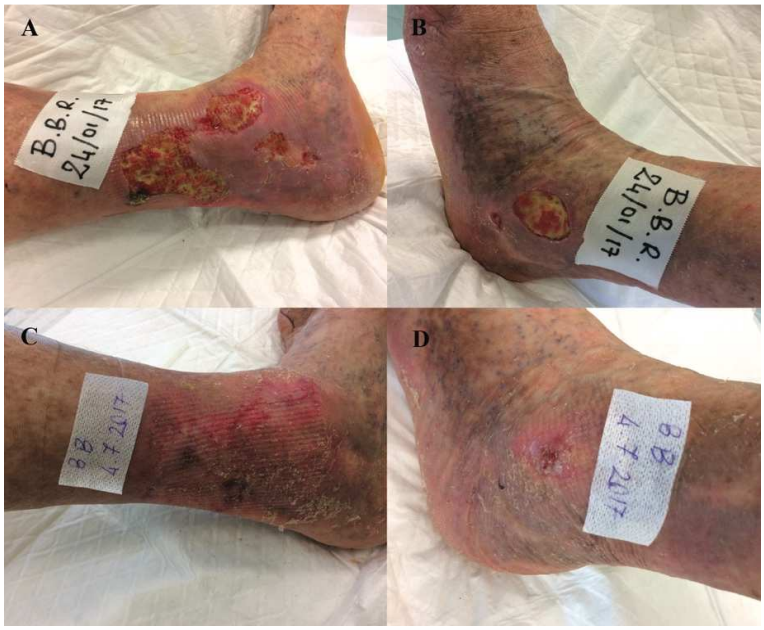

Figure 15. *Pre-operative venous leg ulcer over the medial malleolus (A) and over the lateral malleolus (B); leg ulcers healed after 23 weeks of ADSCs treatment (C-D).*

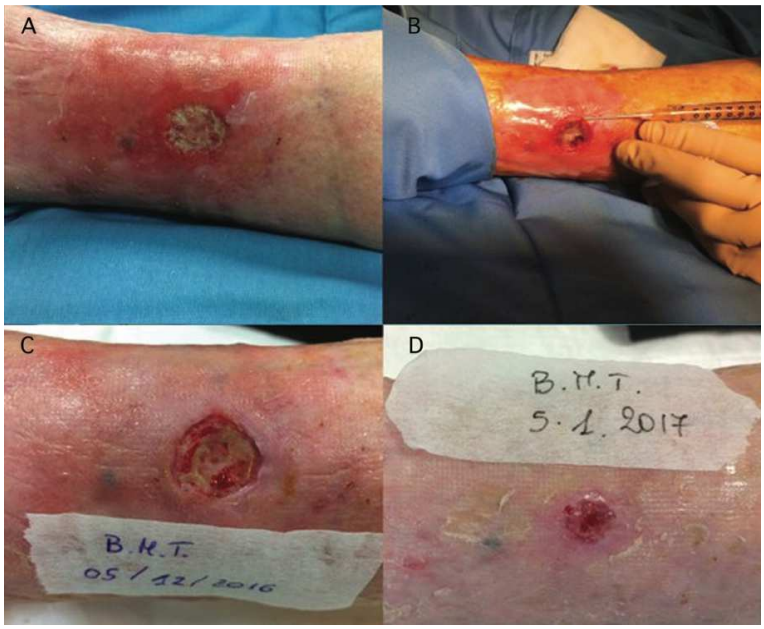

Figure 16. *Pre-operative Martorell's ulcer (A); intraoperative injection of ADSCs (B); post-operative leg ulcer after 4 weeks of treatment (C) and after 8 weeks of treatment (D).*

5.3.4. Histopathological results

The healing process induced by ADSCs involves a preliminary stage where the granulation tissue remodels and repairs plastically the ulcer damage, which is in turn re-structured and replaced due to fibroblast intervention, so as to develop the various types of collagen. Type III collagen is subsequently replaced by stronger type I collagen that will form the main component of the end point scar.

Neoangiogenesis appeared earlier in the first week in the cells treated arm, where new blood vessels encircled by endothelial cells were widely apparent in the wound bed at the second week (Figure 17).

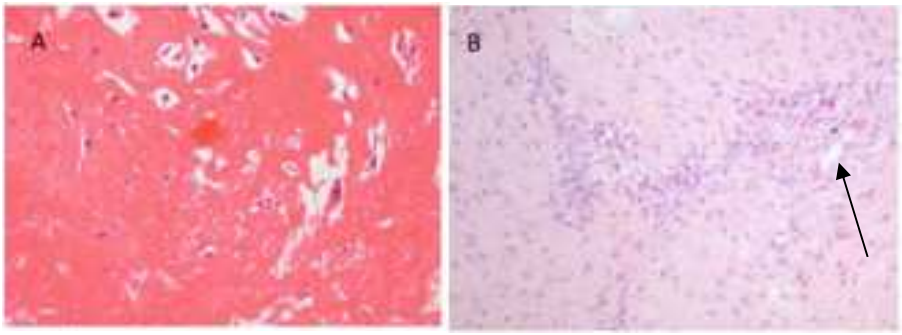

Figure 17. *(A) (Hematoxylin - Eosin 20x) The wound bed with fibroblasts producing type III collagen at week 1. The matrix consists of a collagen type III network, stained in red, rapidly synthetized following ADSCs implantation. (B) (Hematoxylin - Eosin 10x) Neoangiogenesis becomes well and widely apparent in the wound bed in the second week. Vascular endothelial cells indicated by the arrow form new blood vessels.*

5.3.5. Multicolour flow cytometric analysis of freshly isolated SVF

Since AT is a heterogeneous source of multipotent stem cells which are associated with small vessels and are easily recovered by mechanical and enzymatic digestion in an SVF, it becomes necessary to distinguish the possible different stem cell populations by their immunophenotype. Hematopoietic cells and dead cells were excluded from analysis by gating on CD45negative/med low and 7AAD negative events. As reported in Table 7, our first results showed a variable percentage of the CD45 med low fraction (mean percentage: 14,3+/-16 with a range of 1.9-42). By gating on this fraction, a very high number of CD34+/CD45- stem cells was observed (mean of 82.6+/-16) even though a variable percentage of these CD34+ stem cells out of the total cells were observed among patients (range: 52-92). Moreover, the CD34+/CD45- subpopulation in the SVF fraction was differently enriched in CD146 and/or CD271 positive cells (see Table 7 for the percentage of antigen expression and Figure 18 A-E). In particular, the most represented immunophenotypic subsets were the CD34+CD146- cells that could represent supra-adventitial adipose stromal cells (SA-ASC that are prevalent in 40% of patients) and the CD271+CD146- cells described as more mesenchymal stromal cells (MSC prevalent in 60% of patients). Pericytes considered as CD34-CD146+CD31-CD90-CD45- were also present with a variable percentage among patients. A very low percentage of CD34+/-CD146+/CD31+ endothelial progenitor cells was found.

ANTIGENS	CD45medlow population	CD34+7AAD-	CD146+7AAD-	271+7AAD-	CD271+146- MSC prog	CD271+CD146+ MSC prog	CD271-CD146+ MSC perivasc	CD34+CD146- SA-ASC	CD34-CD146+ pericytes
% +/-SD on P1 GATE (morphol.) (range)	14,3+/-16 (1.9-42)	18.28+/-17.4 (0.9-45)	11,36+/-7 (1.8-15.4)	13.4+/-23.1 (0.3-54)	4.9+/-10.3 (0.2-25.9)	0.28+/-0.5 (0-14)	0.75+/-1.4 (0-3.6)	13.8+/-13 (0.5-27)	2.9+/-4.3 (0-7.9)
% +/- SD on P2 GATE (CD45-medlow) (range)	100%	82.6+/-16 (52-92)	18+/-12 (9-39)	49.5+/-48 (0-95)	45.1+/-46 (0-92)	4.8+/-5.4 (0-14)	7.2+/-13.7 (0-31)	39+/-28 (8-64)	11.1+/-9.6 (0.2-18)

Table 7. *The table shows the immunophenotype of the freshly analyzed cells constituing the SVF fraction as percentage of expression and co-expression of the different surface antigens. The immunophenotype allows us to distinguish the different non-hematopoietic progenitor cell subsets present in the ADSCs. P1= % of cells analysed in the morphological gate SSC vs FSC. P2= % of cells analysed in the CD45 negative/med low. MSC prog = mesenchymal progenitor stromal cells. MSC perivasc = mesenchymal cells with more perivascular immunophenotypical features. SA-ASC = supra-adventitial adipose stromal cells.*

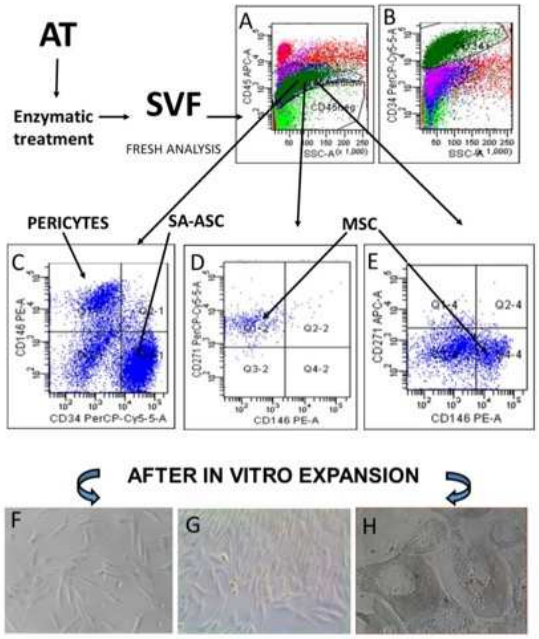

Figure 18. *Adipose Tissue was treated by enzymatic process and the SVF was analysed by flow cytometry. The non-hemopoietic stem cells were analysed in the gate of CD45 med low events (plot A: green population plot). Most of the CD45 med low green cells expressed the CD34 antigen (plot B: green cells). By gating only on the CD45 med low green cells we analysed the expression of other non-hematopoietic stem cell markers such as CD146 and CD271. As represented in plots C-D-E we identified a variable percentage of some phenotypic subsets such as pericytes. In plots F-G-H the cells that rapidly generated a monolayer with mesenchymal like morphological features are shown.*

5.3.6. In vitro expansion of SVF and CFU-F assay

The SVF fraction was successfully expanded *in vitro*. Nevertheless, after dissociation and elimination of mature adipocytes by centrifugation, the stromal vascular fraction remained heterogeneous. Adherent cells generated after SVF expansion were observed after 10-15 days in culture. The cells rapidly generated a monolayer with mesenchymal like morphological features as represented in Figure 18 (F-H). In some samples pericytes, some differentiating adipocytes and endothelial cells could also be observed. After 15 days of culture, the clonogenic potential of the relative SVF derived non-hematopoietic stem cell adherent fraction resulted in a variable number of CFU-F/2ml AT cell colonies with a mean of 16,7+/-7,2 (range 5-25). These colonies grew in culture forming adherent monolayers that were tested for their osteogenic and adipogenic differentiating potential as described for the non-hematopoietic progenitor cells. Since we observed a variable number of CFU-F derived from 2ml of starting AT, we calculated for each patient the total amount of inoculated progenitor cells that ranged from 50 to 375 CFU-F/15ml of inoculated AT.

5.3.7. Immunophenotypic analysis of cultured ADSCs-derived SVF fraction

Adherent cells generated after SVF expansion were observed after 4-7 days in culture from all patients. The cells rapidly generated an adherent monolayer of cells with fibroblastic-like morphology. At the second passage, cells were analyzed by flow cytometry. These cells were negative for hematopoietic markers and gave a positive result for the different mesenchymal markers such as CD105, CD90, CD73, CD44, CD29, and negative for HLA-DR, CD80 as expected. Variable expression was found for the CD146 marker.

5.3.8. Multilineage potential of SVF

We evaluated both the osteogenic and adipogenic potential of the *in vitro* expanded adherent SVF fraction at the second passage of the culture. Morphological changes of osteogenic differentiation appeared after 3 weeks of culture, since some calcium crystals were clearly visible in culture. Nevertheless, two patients showed a reduced *in vitro* capacity to form a mineralization area. Adipogenic differentiation was also investigated. All patients showed abilities, albeit at differing levels, to give rise to differentiated progeny with accumulated lipid vesicles as revealed by microscopical observations and Sudan black staining. Nevertheless, the differentiation capacity towards osteogenic and adipogenic lineages of SVF derived fraction was reduced compared to the bone marrow-derived mesenchymal stem cells (BM-derived MSCs) used as a control.

5.3.9. Correlation between the clinical outcomes and cell characterization

In this study we correlated the percentage and the amount of the CD34+/CD45- stem cells, CFU-F and pericytes with the main clinical outcomes, in particular with the healing time, the NRS and the MI.

The plot (Figure 19 A) shows a reverse correlation between the % of CD34+/CD45- and the healing time ($r= -0.894$, $p<0.041$): a very significant correlation between % of CD34+/CD45- and NRS ($r=-0.934$, $p<0.020$) (Figure 19 B). There was also a positive correlation between the MI and the % of the CD34+ cells ($r = 0.94$ $p < 0.005$). Moreover, the *in vitro* expanded non hematopoietic progenitor cells expressed as a N° of CFU-F present an inverted correlation with the healing time ($r=-0.868$, $p<0.057$) (Figure 19 C) and a significant correlation with NRS ($r=-0.883$, $p<0.047$) (Figure 19 D). The relationship between the MI and % of CFU-F is significantly associated ($r=0.97$ $p < 0.002$), as is also the correlation between MI with the total number of calculated CFU-F reinfused ($r=0.98$ p

< 0.002). Finally, the number of pericytes described as the % of CD146+/CD271-CD45low cells did not correlate significantly with the healing time (r=-0.743, p<0.150) or with the NRS (r=-0.692, p<0.195). On the contrary the relationship with the MI (r = 0.98 p < 0.002) was significantly associated. The MI also correlated with the % of the SA-ASC (r = 0.99 p < 0.002) defined as CD34+CD146-CD45 low cells, presenting a positive correlation.

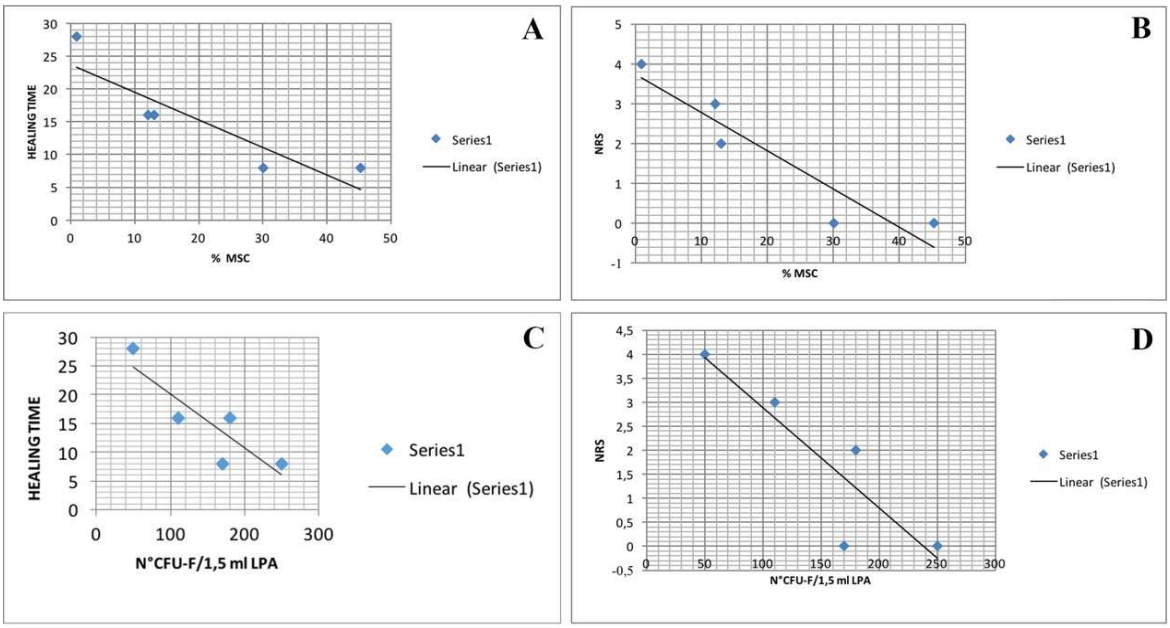

Figure 19. *The number of CD34+/CD45- (%MSC) correlates significantly with healing time (A) and NRS (B). The number of CFU-F demonstrates an inverse correlation with healing time (C) and NRS (D).*

5.4. Discussion

For many years, subcutaneous AT has been used for plastic and aesthetic surgery indications such as lipofilling and breast augmentation [181,182]. After the identification of a conspicuous proportion of ADSCs in AT, the potential use of fat in regenerative medicine was proposed [183]. However, the mechanism of action, the technique of application, the safety and the clinical effects had never been tested in a clinical trial regarding VLU.

The primary outcome of our trial is a significant reduction in healing time in the treated arm, as well as a confirmation of the safety of ADSCs in the clinical setting. The study of Cervelli et al. [119], albeit in the less challenging field of post traumatic wounds, confirmed our primary outcome, and demonstrated the ability of stem cell therapy to

regenerate tissue up to epithelialization. Furthermore, no major adverse events were recorded in our trial, confirming the safety of the ADSCs approach.

The main finding of our study is the significant reduction in healing time observed in the treated arm. The added value of the study was the meaningful and blind cell characterization in the lab setting performed on the same lipo-aspirates used in the surgical procedure. The synchronous lab cell characterization enabled us to understand what the biological determinants of successful cell treatment are. Endothelial progenitor cells expressed by the % of CD34+/CD45- showed a strong and significant correlation with healing time, confirming how the vascular component of the mesenchymal stem cells play a pivotal role in modulating the healing process [184,185]. Although MI was not significantly different in the reduced sample size of our trial, the correlation found between CD34+/CD45-, even with this clinical parameter, tells us how endothelial progenitor cells work from the beginning in order to favor the healing process, as was also clearly shown by histology (Figure 2).

A very important secondary endpoint demonstrated by our explorative study was the significant reduction of pain, with improvement of NRS shortly after the application of the cell treatment. We know that VLU is a condition leading to low quality of life, mainly due to chronic pain [186-188]. This finding is extremely relevant because VLU patients are outpatients, and it is very difficult to control pain at home. Again, the translational nature of our research allow us to understand how the prediction of such a desired outcome depends respectively on CD34+/CD45- and CFU-F. Speculatively we hypothesize that the stem cells treatment completely changes the cytokine cascade in the wound bed, thereby acting as an anti-inflammatory local treatment [189,190].

The establishment of ADSCs strategies required meticulous preparation of all steps of the tissue engineering process: the collection of a cell source, scaffold biomaterial and native microenvironment to contribute to cell growth [45,191]. However, successful ADSCs therapy requires compliance with the defined principles of VLU management including debridement, infection control and compression [40,45,155-158,171,191]. The latter were perfectly respected in both arms in order to evaluate the effects of cell treatment.

Few studies have analyzed all the different cellular components of the SVF. In the present study by the means of a multicolor flow cytometric strategy, we demonstrated the presence of a variable number of CD34+ cells in the uncultured fresh SVF component, and

according to Zimmerlin et al. [192] we also observed that the SA-ASC component as well as the pericytes are the most represented stem cell populations in the SVF of our patients. In particular, the percentage of CD34 + in the non-hematopoietic fraction gave higher than expected results, being an average of 82% in the samples (range 52-92%).

Our observations are in agreement with some other studies on whole AT revealing that stem cell components organized around small vessels are dominated by a prevalent supra-adventitial (SA) layer of CD34+ MSC-like multi-potentiality [63]. However, the ISCT considers both CD34+ and CD34- as ADSCs [193] These SA-ASC components surround arterioles and venules, which are colonized on their surfaces by CD146+ perivascular cells or pericytes [194]. A CD34+/CD31+ endothelial component associated with the luminal layer could be also be present. In particular, as expected for AT, our results showed a variable percentage of CD34+/CD45- non-hematopoietic stem cells among patients; we also showed that the CD34 positive population was differently enriched in CD146 and/or CD271 positive cells. In particular, 40% of patients showed a prevalent SA-ASC subpopulation since the cells resulted in being CD34+CD146-CD31-CD90+/- while the CD34+ subset of CD146+ pericytes was also present. A very low percentage of endothelial progenitor cells was observed, while it was possible to score a different level of CD45+ "contaminating" hemopoietic cells in the starting SVF that was excluded from analysis. After *in vitro* expansion, the ADSCs lose the CD34 expression and display a typical mesenchymal cell immunophenotype as described by other Authors [195].

Concerning the nature and the quality of the the ADSCs isolated from patient's and *in vitro* expanded in this work, we observed that the cellular SVF content is enriched in clonogenic progenitors since we observed the *in vitro* presence of a variable number of CFU-F. Related to the plastic potential of the CFU-F, we observed a normal adipogenic capacity with a reduced differentiation potential (it results not so significant, statistically speaking) compared to bone marrow-derived mesenchymal stem cells (BM-MSCs), especially towards osteogenic lineage.

Based on our preliminary positive results we have also provided evidence that Coleman's procedure seems safe, minimally invasive and well tolerated by patients because there were no related serious complications or side effects. Moreover, the technique is easy to perform, highly reproducible and inexpensive [45,171]. Since SVF from AT by enzymatic or through mechanical dissociation is considered to be "more than minimal manipulation"

[196] and since the quality and the therapeutic potency of SVF is heavily dependent on how the AT is processed, according to our results, Ibatici et al. [197] observed that centrifugation does not impair cell viability, can augment the content of ADSCs and the frequency of CFU-F and reduce the number of pro-inflammatory blood cells.

Finally, we recognize that the major limitation of our study is the sample size. An underpowered may expose to type I error. A second limitation of our study is the lack of a double-blind, sham controlled study design.

5.5. Conclusion

The aim of our study was to explore the effects of cell treatment in non-healing VLU, possibly correlating the clinical findings with lab cell characterization. The correlations found with the lab analysis allow us to hypothesize that healing time or pain reduction could both be candidate primary outcomes to be explored in a further multi-center, double-blind, sham controlled clinical trial.

List of abbreviations

ADSCs Adipose derived stem cells

PDGF Platelet-derived growth factor

bFGF Basic fibroblast growth factor

VEGF Vascular endothelial growth factor

TGF-A1 and TGF-2 Transforming growth factors-A1 and 2

ROS Reactive oxygen species

MMP9 Matrix metalloproteinase

ECM Extra-cellular matrix

IGF Insulin-like growth factor

SDF Stromal cell-derived factor

MMPs Matrix metalloproteinases

TNF Tumour necrosis factor

HGF Hepatocyte growth factor

TGF-β Transforming growth factor-beta

TNF-α Tumor necrosis factor-alpha

GSK-3β/Fyn/Nrf2 Glycogen synthase kinase-3β/Fyn kinase/Nuclear factor erythroid 2-related factor 2

CVD Chronic venous disease

CVU Chronic venous leg ulceration

TIME protocol tissue, infection, moisture, environment protocol

PGA Polyglycolic acid

HA Hyaluronic acid

KGF Keratinocyte growth factor

FGF Fibroblast growth factor

ASCs adipose-derived stem cells

PRP platelet-rich-plasma

e-PRP enhanced PRP

e-SVF Enhanced Stromal Vascular Fraction

ADRCs Purified adipose derived stem and regenerative cells

ASC adipose-derived stroma cell

ADMSC adipose tissue-derived mesenchymal stem cell

EPCs endothelial progenitor cells

HNE human neutrophil elastase

SVF Stromal vascular fraction

AT Adipose tissue

VLU Venous leg ulcers

ABI Ankle brachial index

HDL High-density layer

NRS Numeric Pain Rating Scale

MI Margolis Index

7-AAD 7-amino-actinomycin D

CFU-F colony forming unit fibroblasts

BM-derived MSCs bone marrow-derived mesenchymal stem cells

SA-ASC supra-adventitial adipose stromal cells

SA supra-adventitial

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APPENDIX

In this section, our results regarding the new generation bioscaffold and ADSCs are reported.

Keratin scaffold

Several natural polymers were studied for biomedical applications in the search for biocompatibility, biodegradability and promotion of cell adhesion. In particular, protein-based materials were proposed for various biomedical and biotechnological applications due to their ability to function as extracellular matrices which facilitate cell-cell and cell-matrix interactions. From these, keratin-based materials have emerged as promising due to their biocompatibility, biodegradability and stimulation of cell adhesion.

Keratin is one of the most plentiful non-food proteins in nature, found in hair, nails, hooves, horn and wool. It has the highest sulphur content of any protein due to its high cysteine content - an amino acid which gives rise to intra- and intermolecular bridges, making keratin a protein with chemical stability and high tenacity [1]. Scientific studies have also shown how keratin plays a positive role in stimulating an anti-inflammatory response mediated by cytokine [2], has a hemostatic action [3] and adapts to the cultivation of several types of cell, for example fibroblasts [4] and osteoblasts [5]. This innate capacity is also due to the presence of specific amino acid sequences in the protein structure which promote the adhesion of cells to the culture medium (arginine-glycine - aspartic acid: RGD and leucine - aspartic acid - valine: LDV).

Wool fibers are formed of keratin and present a complex histological structure [6]. The external shell is made up of overlapping cuticular cells which surround the cortex and intercellular cement. The cortex makes up the bulk of the fiber and consists of cuticular cells with a fusiform structure oriented parallel to the axis, while the intercellular cement glues together the cortical cells (fibril).

The keratin is generally extracted from natural substances through oxidising or reducing agents, to obtain a protein solution from which gel, sponge or fibres are produced. For this study, keratin biomaterials were produced from wool fiber using ultrasonic fibrillation,

exploiting the histological structure of the fiber itself (fibril) to produce porous material suitable for use as a scaffold. The fibrillation of the wool fiber structure produces an aqueous suspension of fibril which, after a salt-leaching process consisting of the addition of salt of controlled size in quantities above the saturation point and then its removal by washing, becomes a spongy scaffold (Figure 1).

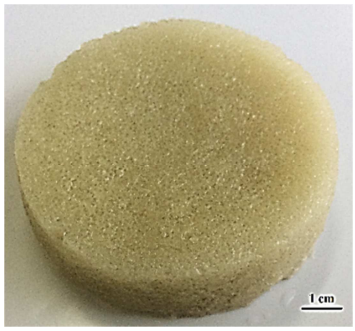

Figure 1. *Visual appeareance of the wool fibril sponge (wet state).*
(Courtesy of Dr. Alessia Patrucco)

The scaffolds obtained have both macro- and micro-porosity. The macro-porosity is useful for cell settlement, derived from the salt added and removed during the salt-leaching process, while the microporosity, which aids the passage of nutrients and facilitates cell-cell interactions, comes from the fibril network. The total interconnected porosity is high (93%) and the microscopic structure mimics the ECM [7].

The procedure that leads to the fibril sponge requires a mild alkaline treatment before the application of ultrasound. The keratin reaction to the alkaline converts the disulphide bonds present within the cysteine into shorter monosulphide bonds of lanthionine, which leads to an increase in thermal stability and in aqueous solutions [7].

The sponges demonstrate an enhanced swelling in water of 38% due to the combination of porosity and the raised hydrophilicity of the wool keratin. Despite the high swelling, the sponges are stable in aqueous solutions without structural changes and show excellent resilience to repeated cycles of compression (Figure 2).

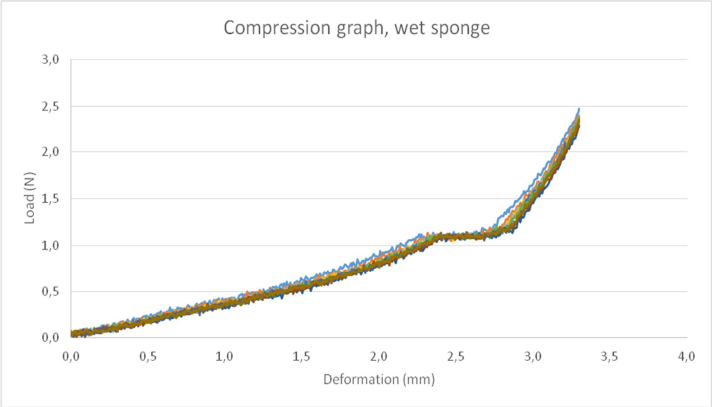

Figure 2. *Behavior under compression of sponges respectively dried and soaked, exposed to 10 compression cycles. (Courtesy of Dr. Alessia Patrucco)*

To verify the functionality of these scaffolds, both stem cells derived from the lipoaspirate of patients suffering from chronic venous leg ulcers and cells derived from the ulcers of the patients themselves were cultivated using keratin fibril sponges. The good adhesion and the proliferation of cells reported are due to the excellent biocompatibility of wool keratin, as well as to the micro and macroporous structure with high interconnection due to the cortical cell network and the salt-leaching process.

Within tissue repair processes, this is an innovative scaffold, as the most widely used media are mainly based on hyaluronic acid [8-10].

Scaffold preparation and co-culture

Once lipoaspirate cells and cells from the ulcers were isolated, we trypsinized these cells and kept them in suspension in culture medium. Multiple well plates were then set up in which thin scaffold slices were deposited. In the well we added suitable culture medium 5100 (DOM) type Dexter (StemCell Technologies Inc., Vancouver, B.C., Canada), supplemented with 12,5% fetal serum, 12.5% horse serum, (StemCell Technologies Inc., Vancouver, B.C., Canada), 1% penicillin/ streptomycin, 2% L-glutamine (both products supplied by Invitrogen Ltd, Paisley, UK) and subsequently co-cultured with scaffolds two cell wells with stem cells derived from the lipoaspirate and two other wells with cells derived from the ulcers. As a control, in two further wells, scaffold fragments were left in culture without cells. The cultures were observed daily for at least 15 days.

Growth of cells on the scaffold

After 15 days, we picked up scaffold fragments left in co-culture with the cells for 15 days and proceeded immediately with glutaraldehyde fixation for the preparation of the pieces at scanning microscopy observation in order to verify any growth of the two types of cells on the scaffolds. After fixation, the pellets were dehydrated in a descending alcohol scale and prepared according to standard scanning microscopy procedures.

The first results were good, since, as can be seen in Figures 3 and 4, the adherent cells derived from ulcer and lipoaspirate cells are shown on the narrow scattered fiber texture of the scaffold.

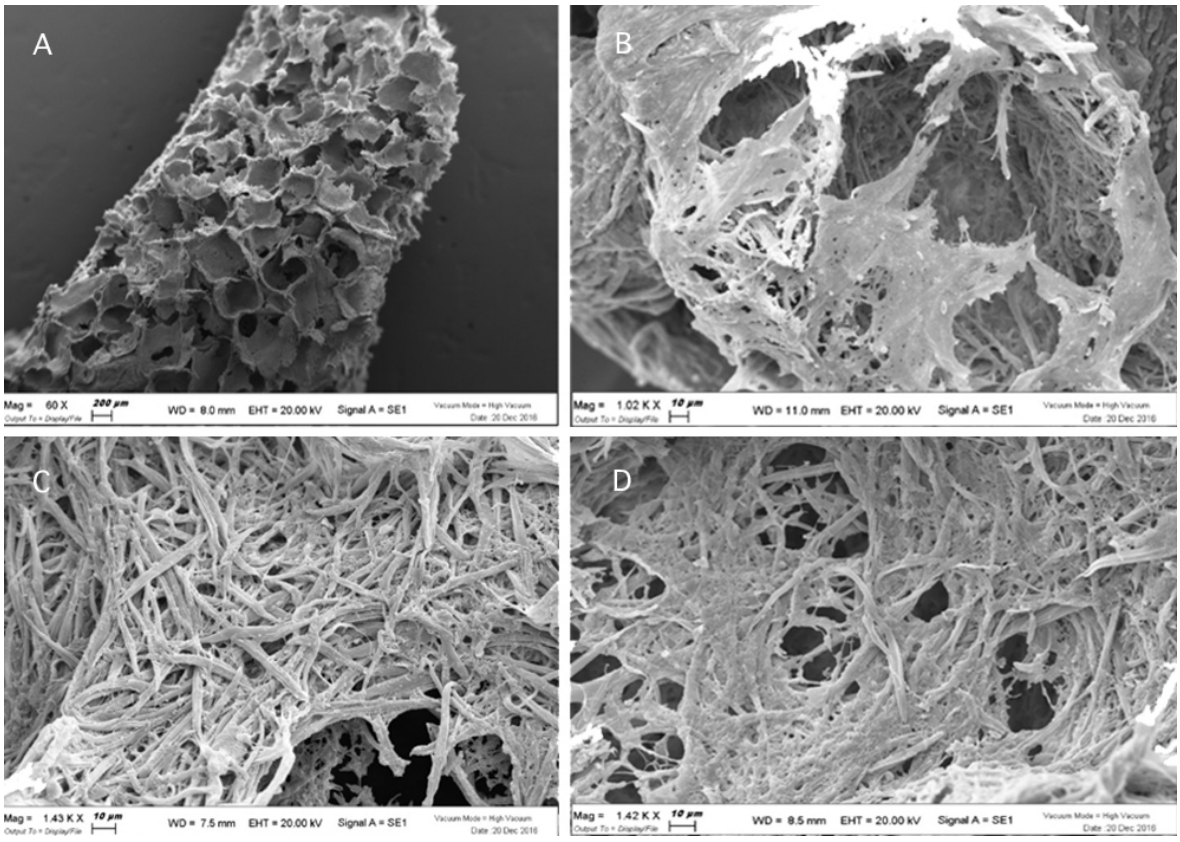

Figure 3. *Micrographs with scanning electron microscope: (A) and (C) Network structure of the scaffold remaining in cell culture at different magnifications; (B) and (D) the scaffold plot shows visible cells derived from the ulcers of the patients. The cells appear as wide and continuous areas resting on the scaffolds.*

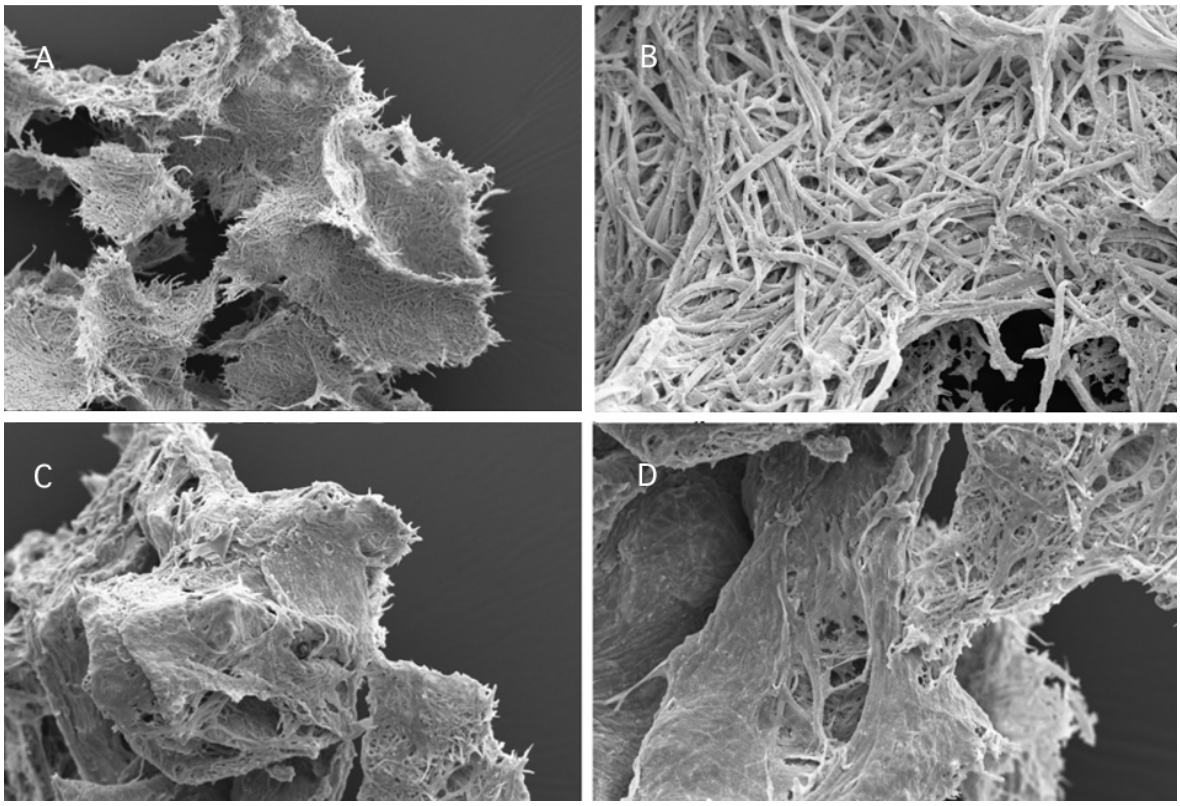

Figure 4. *Micrographs with scanning electron microscope: (A) and (B) structure of the scaffold remaining in culture without cells at different magnifications; (C) and (D) co-culture of the scaffold with lipoaspirated stem cells: it is visible that the cells are attached to the base of the scaffold and that they retain adhesion after culture.*

Conclusion

These early results are encouraging, as they demonstrate good adhesion compatibility between the new generation bio-scaffold and the ulcer microenvironment cells, as well as with the stem cells to be transported inside it.

List of abbreviations

RGD Arginine-glycine - aspartic acid

LDV Leucine - aspartic acid - valine

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