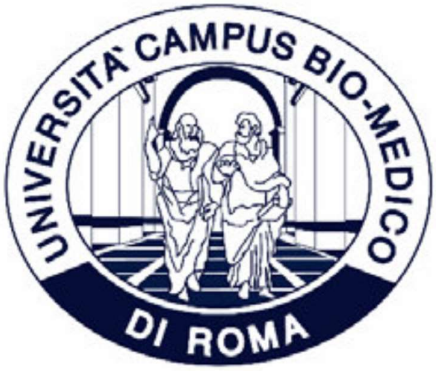

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The use of the "stem graft" in chronic wounds: in vitro analysis, clinical and comparative study

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INTRODUCTION

Chronic wounds affect 0.2% to 1% of the population in developed countries. In the United States, annual health care costs are in the billions of dollars. They are more prevalent in the elderly population and, as the population ages, the number of chronic wounds is expected to rise. Such wounds are a major issue for patients and a challenge for clinicians and surgeons. Wound healing is a complex and highly regulated process consisting of inflammation, proliferation, matrix formation, and remodeling. When the process is disrupted, chronic non-healing wounds will develop. Although the pathophysiology of chronic wounds is still not entirely understood, impaired vascularization and consequent hypoxia, persistent and increasing inflammation, and the ineffectiveness of the immune system in controlling bacterial infections are all crucial factors that negatively affect the physiologic wound closure. Due to the imbalance between pro- and anti-inflammatory signals able to alter the microenvironment, chronic wounds fail to progress beyond the inflammatory phase, which precludes proliferation, matrix deposition, and, ultimately, wound resolution. Hyper-inflammation also leads to an over-expression of metalloproteinases (MMPs), with consequent degradation of growth factors, their receptors, and the provisional extracellular matrix (ECM) essential for cell migration. Also, the lack of growth factors and the accumulation of senescent cells in the injured area slow or block the wound repair process. Figure 1 outlines the pathophysiology of healing and non-healing wounds.

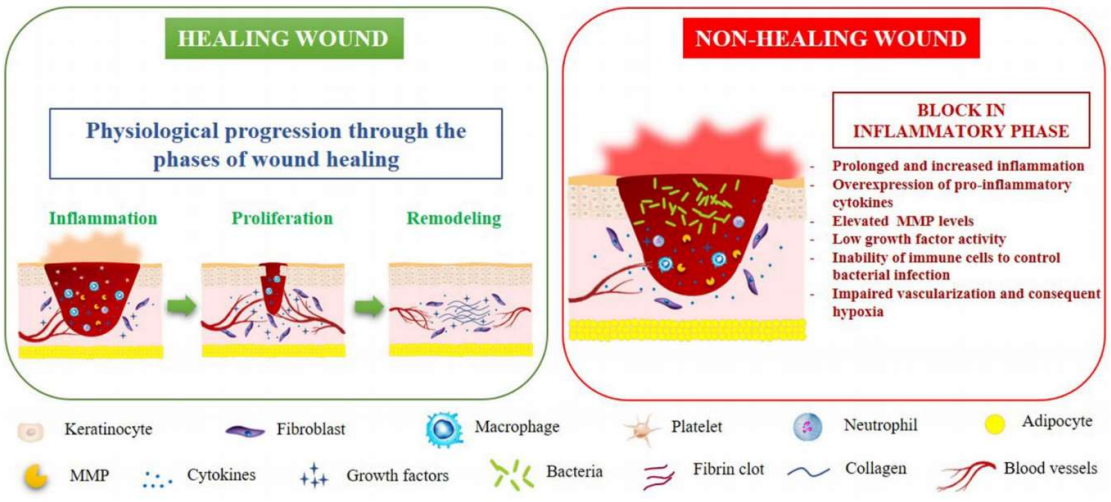


Figure 1: the pathophysiology of healing vs non-healing wound (from Lombardi F. et al)

Chronic wounds include venous ulcers, pressure ulcers and diabetic ulcers. Venous ulcers are the most common and the main object of this thesis.

VENOUS ULCERS

Chronic venous disease and insufficiency often complicate with venous leg ulcers (VLUs). VLUS re defined as an open skin lesion of the leg or foot that occurs in an area affected by venous hypertension. They represent up to 80% of all leg ulcers and have a prevalence of approximately 1% in the general population, increasing with age. Moreover, they are chronic, slow to heal and have a high rate of recurrence within 6 months (50–70%). As a consequence, VLUs impose a heavy burden on patients and substantially reduce their quality of life (QoL). The socioeconomic impact is conspicuous due to the cost and duration of care (10,000 to 12,000 USD/year per patient) and can account for up to 1% of national healthcare budgets. Lost productivity of patients and family members who provide home care, premature disability and other factors are further indirect burdens and costs.

Chronic venous disease (CVD) progression leads to poor venous return, venous hypertension, damage to venous valves and chronic inflammation. The chronic oedema that develops as a result increases capillary permeability and lymphatic damage to the superficial veins and skin. Pathologic skin changes along with reduced capillary blood flow and capillary leakage contribute to the breakdown of the epidermis and lead to ulceration. Progression is common, with the disease expected to worsen in 50% of those affected. In 30% of patients with varicose veins, skin changes develop over time along with progression to chronic venous insufficiency (CVI) and a high risk of ulceration. CVD tends to progress faster in patients with a history of deep venous thrombosis. This is likely due to venous hypertension and reflux, which are more severe in these patients, stemming from persistent obstruction, damaged veins and/or valvular incompetence.

MANAGEMENT

Dressings and compression therapy are generally the primary therapeutic option, followed by surgery, if necessary, to remove incompetent veins, to debride or reconstruct the wound.

COMPRESSION AND SYSTEMIC THERAPY

Standard compression to reduce venous insufficiency stands as a mainstay (Figure 1).

Compression stockings		
Class	Pressure at ankle (mm Hg)	Indication
I	14-17	Mild varicose veins
II	18-24	Prevention of recurrence of venous ulcers on narrow legs and in slim patients and for mild oedema
III	25-35	Chronic venous insufficiency and oedema, and large heavy legs

Figure 1: compression stockings

Intermittent pneumatic compression (IPC) has improved healing rates in some studies when used with standard compression, but it is not yet clear whether it is superior to standard compression and for which patients it is most beneficial. To date, IPC is recommended for patients with VLUs that have failed to heal with standard compression therapy or for patients who cannot tolerate compression stockings or bandages. Patients with VLU may sometimes have nutritional deficiencies impacting on VLU healing and recurrence. Zinc intake was found to be below recommendations in a minority of VLU patients. Other nutritional characteristics of VLU patients may be low levels of serum vitamin D, vitamin C and zinc as well as fatty acid imbalances. In a meta-analysis of 20 studies, vitamin D, folic acid and flavonoids were associated with some beneficial effects on ulcer healing. However, dietary supplements have not been shown to be efficient therapies for VLU, and further investigation into the role of micronutrient deficiencies in wound healing is needed. Systemic treatment with veno-active drugs (VADs) in combination with compression can be highly effective in healing VLUs. Adjunct treatment with VAD can decrease the inflammation associated with venous hypertension, promote VLU healing and improve QoL. Micronized purified flavonoid fraction is a widely prescribed VAD to treat CVD. The pharmacologic actions of MPFF include reductions in endothelial cell activation, serum concentrations of endothelial cell adhesion molecules and growth factors, leukocyte adhesion and activation, venous valve deterioration and reflux, proinflammatory mediator production and release, and capillary leakage. These properties result in clinical benefits that improve venous tone and the clinical signs and symptoms of CVD, oedema, skin changes, VLU healing and QoL. Two other drugs, pentoxifylline and sulodexide, both of which are not VADs, have also been shown to improve VLU healing and are recommended in addition to compression therapy. Pentoxifylline, a methylated xanthine derivative, is a competitive non-selective phosphodiesterase inhibitor that has been shown to have antioxidant properties and to reduce inflammation. In addition, pentoxifylline reduces blood viscosity and decreases the potential for platelet aggregation and blood clot formation. Sulodexide, a combination of fastmoving heparin and dermatan sulfate, also has antithrombotic and profibrinolytic properties as well as antiinflammatory effects. Local antibiotic

therapy is generally unadvised due to the high risk of resistance development. Systemic antibiotic therapy must be based, when possible, on microbiological culture and resistance.

DRESSING

Dressing selection can be challenging due to the vast market of available devices combined with a general lack of high-quality evidence. The goals of wound dressing should include the following:

1. maintaining a moist wound environment;
2. preventing infection
3. minimizing skin irritation or friction between the wound and clothing or devices such as wheelchairs.

Standard medications only act on the external factors that may hamper tissue healing. On the other hand, advanced medications act on both external and internal factors, creating a local micro-environment promoting tissue healing.

STANDARD GAUZE

Sterile gauze dressings are the standard to which other wound care products are compared. Wet-to-dry packing consists of moistened gauze placed into the wound with changes at least once daily, which provides debridement. This technique is widely used but can result in a dehydrated wound bed, preventing granulation and matrix regeneration. Dry wounds are painful, increasing patient discomfort. If wet-to-dry packing is used, it should not be in contact with the adjacent intact skin around the wound because it causes maceration of healthy tissue, enlarging the wound.

ADVANCED DRESSINGS

Many advanced wound dressings are available (Figure 2), but little high quality evidence supports their use.

Class	Composition	Characteristics and function	Commercial examples
Gauze	Woven cotton fibers	Permeable with desiccation; debridement; painful removal	Curity, Mepilex, Mepitel
Tulles	Open-weave cloth soaked in soft paraffin or chlorhexidine, textiles, or multilayered or perforated plastic films	Low adherent, suitable for flat, shallow wounds with low exudates	Adaptic, Grassolind, Jelonet, Tullegras, Urgotul
Film	Plastic (polyurethane); semipermeable	Allows water vapor permeation; adhesive; impermeable for liquids and bacteria	OpSite, Tegaderm
Foam	Hydrophilic (wound side) and hydrophobic (outer side) polyurethane or silicone foams; semipermeable	Highly absorbent; for necrotic and exudative wounds	Lyofoam, Allevyn, Tielle
Hydrogel	Water (96%) and polymer (polyethylene oxide)	Aqueous environment; requires secondary dressing; no adherence; not recommended if infection is present; semipermeable	Aquaform, Intrasite, Purilon, Vigilon, Aquasorb
Hydrocolloid	Hydrophilic colloidal particles and adhesive	Absorbs fluid; necrotic tissue autolysis; little adherence; occlusive	Comfeel, DuoDERM, IntraSite, Tegasorb
Absorptive powder, paste and fiber	Starch copolymers, hydrocolloid particles	Absorbs exudate; used as a filler; good for deep wounds	Aquacel, Geliperm, GranuGel paste, DuoDERM granules
Alginate	Calcium and sodium salts of alginic acid found in brown seaweed	Absorbs exudate and forms a hydrophilic gel after ion exchange with wound fluid; not suited for dry wounds	Algisite, Algosteril, Kaltostat SeaSorb, Sorbsan, Suprasorb
Antimicrobial dressings	1. Silver (in ionic or nanocrystalline form) 2. Iodine (a) as povidone-iodine (b) as cadexomer iodine 3. Metronidazole gel 4. Octinidine gel	Suited for colonized or infected wounds: 1. Absorptive: cadexomer iodine (caution: thyroid diseases) 2. Control of odor caused by anaerobic bacteria, used for fungating malignant wounds 3. Used for burns	1. Acticoat, Actisorb, Silver 200, Aquacel Ag 2. Iodosorb 3. Metrotop Gel 4. Octinidine/Lavanid Gel
Silicone	Silicone sheets	Sheet induces a localized electromagnetic field and increased skin temperature; decreases scar formation?	Sil-K, Mepitel, Mepilex
Subatmospheric pressure	Vacuum pump, sponge, plastic film	Sponge conforms to wound and vacuum removes edema fluid and bacteria; stimulation of granulation, vascularization, and wound cell proliferation	VAC device

*Multiple brands within each class are available, and a partial list is given. No particular brands are recommended.
(Modified from Lorenz et al.[7] and Enoch et al.[96])

Figure 2: Some of the commercially available medications

Hydrocolloids

Sodium carboxymethylcellulose, gelatin, pectin, elastomers, and adhesives are bonded to a carrier of semipermeable film or a foam sheet to produce a flat, occlusive, adhesive dressing that forms a gel on the wound surface, promoting moist wound healing. Cross linkage of the materials used influences the viscosity of the gel under the dressing. This gel, which may be yellow and malodorous, may be mistaken for infection by the unwary. Hydrocolloids are virtually impermeable to water vapour and air and can be used to rehydrate dry necrotic eschar and promote autolytic debridement. They are reported to reduce wound pain, and their barrier properties allow the patient to bathe or shower and continue with normal daily activities without disturbing or risking contamination of the wound. Caution should be exercised when using hydrocolloids for wounds that require frequent inspection—for example, for diabetic foot ulcers.

Hydrogels

Hydrogels consist of a matrix of insoluble polymers with up to 96% water content enabling them to donate water molecules to the wound surface and to maintain a moist environment at the wound bed. As the polymers are only partially hydrated, hydrogels have the ability to absorb a degree of wound exudate, the amount varying between different brands. They transmit moisture vapour and oxygen, but their bacterial and fluid permeability is dependent on the type of secondary dressing used. Hydrogels promote wound debridement by rehydration of non-viable tissue, thus facilitating the process of natural autolysis. Amorphous hydrogels are the most commonly used and are thick, viscous gels. Hydrogels are considered to be a standard form of management for sloughy or necrotic wounds. They are not indicated for wounds producing high levels of exudate or where there is evidence of gangrenous tissue, which should be kept dry to reduce the risk of infection.

Alginates

Alginates are produced from the naturally occurring calcium and sodium salts of alginic acid found in a family of brown seaweed (Phaeophyceae). They generally fall into one of two kinds: those containing 100% calcium alginate or those that contain a combination of calcium with sodium alginate, usually in a ratio of 80:20. Alginates are rich in either mannuronic acid or guluronic acid, the relative amount of each influencing the amount of exudate absorbed and the shape the dressing will retain. Alginates partly dissolve on contact with wound fluid to form a hydrophilic gel as a result of the exchange of sodium ions in wound fluid for calcium ions in the dressing. Those high in mannuronic acid can be washed off the wound easily with saline, but those high in guluronic acid tend to retain their basic structure and should be removed from the wound bed in one piece. Alginates can absorb 15 to 20 times their weight of fluid, making them suitable for highly exuding wounds. They should not be used, however, on wounds with little or no exudate as they will adhere to the healing wound surface, causing pain and damaging healthy tissue on removal.

Foam dressings

Foam dressings are manufactured as either a polyurethane or silicone foam. They transmit moisture vapour and oxygen and provide thermal insulation to the wound bed. Polyurethane foams consist of two or three layers, including a hydrophilic wound contact surface and a hydrophobic backing, making them highly absorbent. They facilitate uniform dispersion of exudate throughout the absorbent layer and prevent exterior leakage (strike-through) due to the presence of a semipermeable backing. Polyurethane foam dressings are also available as a cavity dressing—small chips of hydrophilic polyurethane foam enclosed in a membrane of perforated polymeric film, giving a loosely

filled bag. Silicone foams consist of a polymer of silicone elastomer derived from two liquids, which, when mixed together, form a foam while expanding to fit the wound shape forming a soft open-cell foam dressing. The major advantage of foam is the ability to contain exudate. In addition, silicone foam dressings protect the area around the wound from further damage.

Dressing selection according to exudate and wound bed is summarized in figure 3

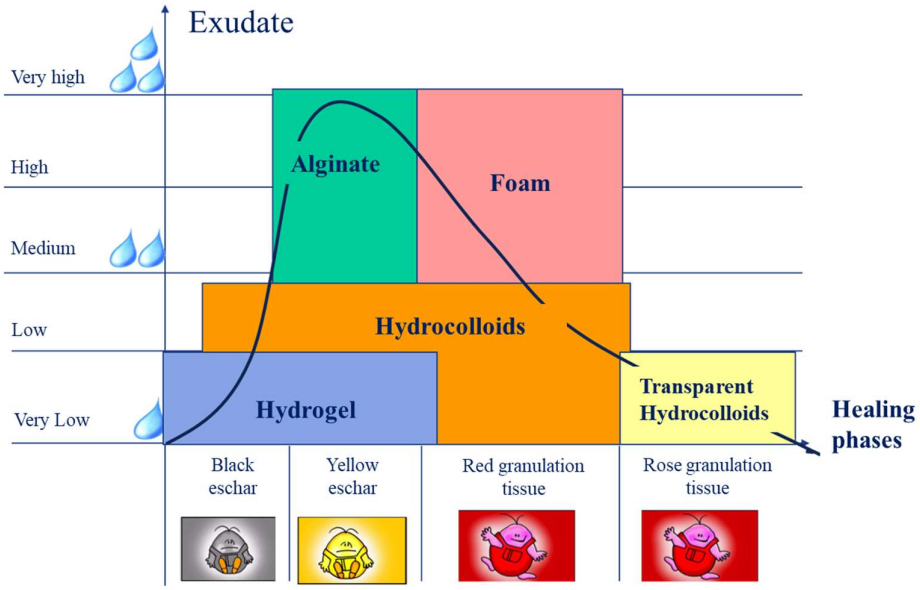


Figure 3: Dressing selection

Negative-Pressure Wound Therapy

Wound vacuum devices deliver negative-pressure wound therapy (NPWT) (Figure 4). These devices are composed of a sterile foam dressing that covers the wound, which is then enclosed by an occlusive film that adheres to the adjacent, normal skin. Suction is applied to the dressing and a drainage tube connects toa portable vacuum canister. High quality evidence has shown that NPWT reduces wound exudate, debris, and bacterial contamination while increasing vascular perfusion and granulation of the wound base.3 A meta-analysis of randomized trials showed that NPWT, compared with standard wound care, was associated with decreased wound size and shorter time to healing

Figure 4: NPWT device

DEBRIDEMENT

Surgical debridement is paramount to promote re-epithelization in non-healing wounds. Since necrotic tissue can also harbour pathogenic organisms, removal of such tissue helps to prevent wound infection. Necrotic tissue and slough should be debrided with a scalpel so that the wound bed can be accurately assessed and facilitate healing. Eschar may be adherent to the wound bed, making debridement with a scalpel difficult. Further debridement, as part of wound management, may be required using other techniques (Figure 5).

Types of debridement

Sharp—At the bedside (using scalpel or curette)
Surgical—In the operating theatre
Autolytic—Facilitation of the body's own mechanism of debridement with appropriate dressings
Biological—Larval (maggot) therapy
Enzymatic—Not widely used; pawpaw (papaya) or banana skin used in developing countries
Mechanical—Wet-to-dry dressings (not widely used in the UK)

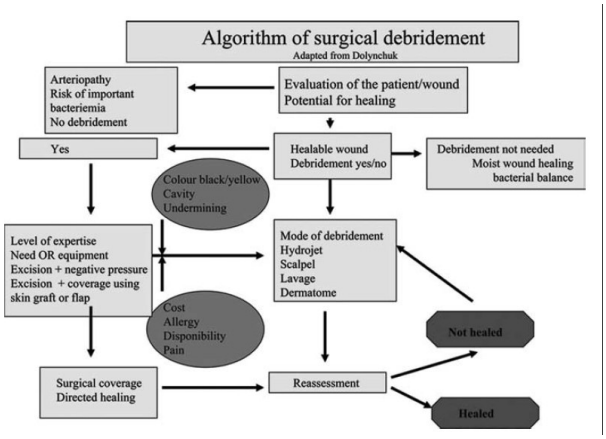
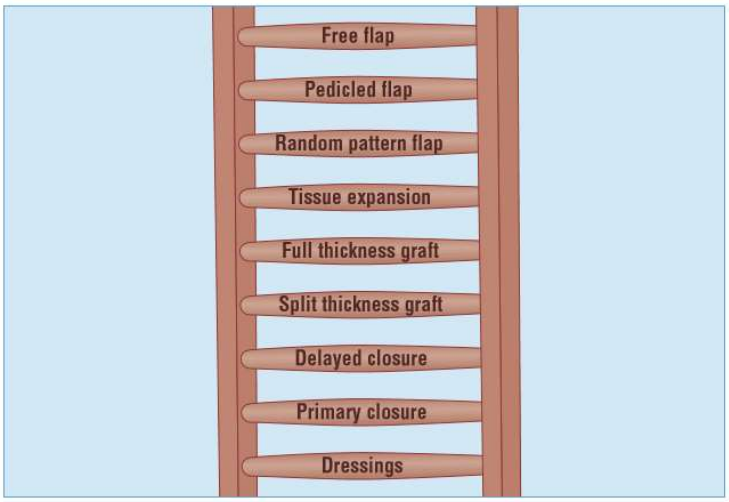


Figure 5: Above, types of debridement; below: Dolynchuk algorithm of surgical debridement

SURGICAL RECONSTRUCTION

Reconstructive surgeons use the concept of a "reconstructive ladder"—the more problematic the wound, the higher up the ladder the surgeon has to climb.



Chronic wounds are very seldomly closed by primary suturing and they often require complex reconstruction, including free tissue transfer, in hospital. All wounds should undergo debridement and thorough irrigation before primary closure. The aim of debridement is to remove all potentially contaminated and devitalised tissue along with foreign material. Primary suture is never indicated in heavily contaminated wounds, where the risk of infection is high. In such cases the wound should be debrided, with "delayed closure" carried out later. Occasionally, wounds may be allowed to heal by secondary intention, where areas of skin loss are initially replaced by granulation tissue. The skin defect continues to heal as a result of proliferation or migration of epidermal cells within and around the wound and by contraction of the wound by specialised cells (myofibroblasts) within the granulation tissue. Healing by secondary intention is slow and may lead to contractures, scarring, and restriction of movements. Where skin defects are too large for skin apposition, and healing by secondary intention is inappropriate, skin grafts may be used. Free skin grafts are taken from another part of the body and rely on revascularisation from a healthy, well vascularised wound bed. Grafts will not be successful on non-vascularised beds, such as exposed bone or tendon. Split skin grafts consist of the epidermis and a variable amount of dermis. They are usually harvested from the thigh using a specially designed knife or powered dermatome. The donor area will heal within 10-14 days from remaining dermal adnexal structures. Such grafts are the mainstay of treatment of large wounds such as burns. Full thickness grafts consist of the epidermis and dermis and show several advantages, but are size limited as the donor area must be directly closed. Many wounds are not suitable for grafting, and techniques further up the reconstructive ladder, such as a flap reconstruction, must be used. A flap is a unit of tissue that can be moved to cover a wound while surviving on its own vascular supply. Random pattern flaps rely on random cutaneous vessels for their blood supply. Greater lengths of flap can be used by including the underlying deep fascia and also by including a perforating blood vessel in the base of the flap. In some circumstances better cosmesis may be obtained by raising the flap as fascia only, leaving the overlying skin behind. Islanding a flap on its vascular pedicle allows even greater pedicle length and thus greater mobility and versatility. Occasionally no options are available for local wound cover, and tissue has to be harvested from elsewhere around the body

by using microvascular techniques. This transfer of tissue, known as a free flap, represents the top rung of the reconstructive ladder. Any tissue that can be isolated on a suitable vascular pedicle can be used, and it may include muscle, skin, fascia, fat, nerve, and bone. However, most patients affected by chronic wounds cannot undergo such complex procedures due to comorbidities.

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TISSUE ENGINEERING AND REGENERATIVE DEVICES

The term "tissue engineering" was thought to have been first introduced in 1987 during a meeting of the National Science Foundation. Although the definition has matured and expanded, the author generally regards The National Institute of Health's definition of tissue engineering and regenerative medicine as representative, defining it as a multidisciplinary field involving biology, medicine, and engineering likely to revolutionize the ways we improve health and quality of life by restoring, maintaining, or enhancing tissue and organ function.

SKIN SUBSTITUTES

Tissue engineering of skin substitutes has generally focused on the development of an ideal, universal skin substitute, which possessed identical structure, composition, and vital potential, or one which when applied to a wound site encourages and directs autologous mechanisms of regenerative healing. The universality aim, however noble, have to date failed to varying degrees. Currently, it is felt that for a functional construct to truly succeed in clinical practice, a more comprehensive and directed composition, which addresses the underlying pathophysiology of the specific and differing wound types is required. Specifically, a dressing to improve wound re-epithelialization in burns must have different attributes from those required for chronic wounds or scar revision as the individual patho-physiology differ. Ideally these constructs would:

1. biointegrate and vascularize in a timely fashion;
2. re-establish commensal flora while resisting infection;
3. optimize moisture vapor transmission;
4. withstand expected shear forces;
5. be cost effective;
6. lack inhibitory antigenicity while hosting normal regulatory function;
7. have a long shelf-life;
8. be easily stored and applied;
9. easily conformable, visualized, and secured;
10. durable;
11. painless.

Moreover, even the most ideal tissue-engineered construct will likely fail without attention to meticulous wound bed preparation and haemostasis, minimizing biologic and bacterial burden, shear,

and further trauma, as well as control of oedema, removing contaminants and debris, optimizing vascular status, nutrition, patient comorbidities, and homeostasis. The catalogue of commercially available tissue-engineered constructs is huge.

Non-cultured Products

Temporary skin substitutes are generally employed to protect the wound bed and to facilitate timely definitive coverage by either promoting primary re-epithelialization or optimizing the status of the wound bed for engraftment. Both xenogeneic and allogeneic skin have been employed (Figure 1).

Natural biological materials						
Alloderm® [44]	Lifecell Corporation, Branchburg, NJ, USA	Acellular human dermis	0.79–2.03 2.05–3.30 mm	n.a.	No	Partial and full-thickness (burn) wounds, soft tissue replacement, interpositional grafts
Strattice™ª	Lifecell Corporation, Branchburg, NJ, USA	Acellular porcine dermis (α-Gal removed)	1.5–2.0 mm	n.a.	No	Soft tissue
Dermamatrix®	Synthes, Westchester, PA, USA	Acellular human dermis	0.2–0.4, 0.4–0.8, 0.8–1.7, 1.7> mm	n.a.	No	Soft tissue replacement
Graftjacket® [136]	Wright Medical Technology, Inc, Arlington, TN, USA	Acellular human dermis	1, 1.4 or 2 mm	n.a.	No	Chronic wounds, ligament repair, soft tissue replacement
Oasis®ª	Healthpoint Ltd., Fort Worth, TX, USA	Porcine small intestine submucosa	~0.15 mm	~20–30 µm -	No	Chronic wounds
-			–	~20–30 µm	-	-
Oasis burn matrix®ª			~0.30 mm)			Partial and full-thickness burn wounds
Permacol™ª	Covedien, Mansfield MA, USA	Acellular porcine dermis	0.4 or 1.5 mm	n.a.	Yes	Full-thickness wounds
Tiscover®ª	A-SKIN B.V., Amsterdam, Netherlands	Acellular human dermis with autologous fibroblasts	1–2 mm	n.a.	No	Chronic wounds
Glyaderm®ª	Euro Skin Bank, Beverwijk, Netherlands	Acellular human dermis	0.2–0.6 mm	n.a.	No	Full-thickness wounds
Matriderm®ª	Skin and Health Care AG, Billerbeck, Germany	Bovine collagen 1 with elastin	1 or 2 mm	~75 µm	No	Burn wounds, chronic wounds
Orcel®ª	Forticell Bioscience New York, USA	Collagen 1 sponge + gel with allogenic fibroblasts and keratinocytes	~1mm	50–250 µm	Yes	Chronic wounds skin graft donorsites,
Renoskin®ª□	Groupe Perouse, Bornel, France	Bovine collagen 1 with GAG	1.5–2.5 mm	~100 µm	Yes	Burn wounds, tissue defects

Figure 1: Some of the available non-cultured skin substitutes

Thin allograft is generally preferred to promote re-epithelialization of partial-thickness wounds. Thicker allograft skin helps to develop fibrovascular ingrowth of tissue at the wound bed when allowed to biointegrate. On removal of the allograft, this newly well-vascularized bed usually remains and is then suitable for autografting. Interestingly when allograft is maintained for protracted periods of time, the more immunogenic epidermis separates leaving behind the biointegrated and vascularized dermal substrate suitable for cell transplantation.

Porcine-derived biologics have long been favored for human use not only because of availability but also as a result of biocompatibility. Porcine skin demonstrates comparable epidermal thickness, dermal elastin structure, hair distribution, epithelial turnover and migration, and dermal collagen structure. **E-Z Derm** (Figure 2, Molnlycke Health Care, US, LLC, Norcross, Ga.), a silver-impregnated aldehyde cross-linked porcine dermis formulation is thought to provide antimicrobial protection. Zhang et al reported its efficacy in promoting healing, reduce hospitalization and complications in perineal and lower limb wounds. Zajicek and colleagues demonstrated that such dermal substitute promoted the proliferation and differentiation of human keratinocytes. Moreover, it was found to increase collagen synthesis, stem cells proliferation as well as PDGF, EGF and FGF expression.

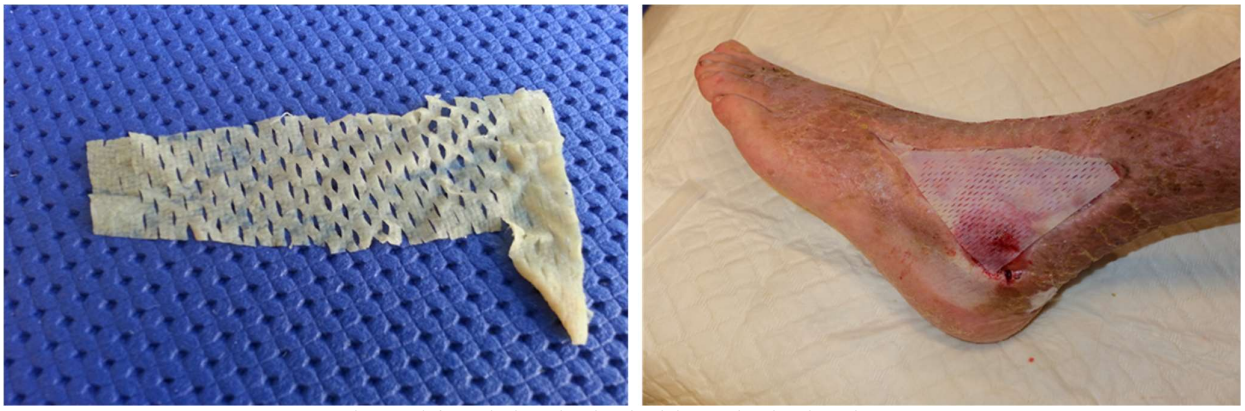

Figure 2: left, meshed porcine dermis; right, porcine dermis onsite

Potential problems associated with allogeneic and xenograft skin include the transfer of viral infections. The quality and uniformity of the individual grafts especially allogeneic can often be quite variable reflecting not only availability but also technical limitations. Biologic properties and efficacy of these products can similarly vary depending on the type of preservation employed. Fresh, irradiated products have generally maintained the highest biologic properties as defined by adherence. Glycerol-preserved allogeneic skin is nonviable and poses the lowest risk for viral transmission. The viability of remaining cells in current xenogeneic products depends on processing methodology and may be low or absent. It is interesting to note that the immunologically privileged bio-integrated allograft may leave behind a well-integrated dermal layer, often successfully used to bond and accept cultured epidermal autograft preparations. Biobrane (Mylan and Smith & Nephew), perhaps the first widely utilized biosynthetic, is composed of a nylon mesh bonded to a silicone rubber membrane and impregnated with porcine collagen type I peptides facilitating adherence and promoting re-epithelialization on partia lthickness wounds and burns. Challenges with the product, as with so many biosynthetics, are infection and moisture vapor transmission rates. Some clinicians found requisite

fibrovascular ingrowth and adherence on excised wounds to be inadequate, and as such Biobrane has not been universally accepted as a temporary skin substitute for excised full-thickness wounds. Human keratinocytes have similarly successfully grown and transferred on Biobrane. Integra Dermal Regeneration Template is a commercially available bilayer in common use since the mid-1990s. It consists of an outer layer made of a thin silicone film that protects the wound from both heat and moisture loss. The inner layer is the active matrix constructed of cross-linked bovine collagen fibers and shark glycosaminoglycans. A defined porosity encourages vascular and cellular ingrowth. The product matures and biointegrates acting as a vascularized biologic scaffold forming repair characteristics somewhat more consistent with a regenerative pattern of healing than pure scar and contracture. Once dermal skin has regenerated, the silicone outer layer is removed and replaced with a thin autologous skin graft. Integra is indicated for the post-excisional treatment of life-threatening full-thickness or deep partial-thickness thermal injuries where sufficient autograft is not available at the time of excision or not desirable because of the physiologic condition of the patient. It is also utilized for the repair of scar contractures when other therapies have failed. This methodology allows one to take particularly thin donor grafts decreasing donor-site morbidity while improving availability and promoting improvements in recipient pliability. Challenges include time to biointegration and infection. Of particular interest is the capacity of the product to carry, grow, and transfer adipose-derived regenerative cells, suggesting a novel therapeutic option for healing wounds. Another material (Matriderm) produced from bovine collagen and elastin is advocated for simultaneous transplantation with a split-thickness skin transplant. Good take rates and improved appearance of the wounds have been reported. Matriderm has also been used for the successful growth of keratinocytes and fibroblasts with subsequent delivery to full-thickness wounds or delivery of mesenchymal stem cells. Amnion, part of the fetal placental membrane, consists of a single layer of epithelium covering a stromal layer and has been used since at least the beginning of the last century for the treatment of wounds, ophthalmic injuries, and burns. This was particularly true in the era before HIV and AIDS when freshly donated amnion was washed and separated from the chorion and then applied to the wound bed. The tissue is generally considered minimally immunogenic, is easily visualized through and promotes re-epithelialization while modulating inflammation. Several recent studies have shown promising results in treating diabetic neuropathic ulcers and venous stasis ulcers with these products. As with all biologics, donor screening and preservation methods ultimately determine immunogenicity, viability, efficacy, and infectious risk.

Cultured Keratinocytes

Commercially available cultured epithelial sheets such as Epibase (Laboratoires Genévrier, Antibes, France), Epicel SM, Genzyme (Cambridge, Mass.), Tissue Repair, Keratinozyten Sheets (DIZG, Berlin, Germany), and Epibase PIBASE (Laboratories Genéevrier) are utilized in the treatment of extensive skin loss in the absence of sufficient autologous donor skin. Unfortunately, development, culturing, and processing time requirements remain high, and cultured epithelial sheet grafts are usually not available for at least 3 weeks after skin biopsy and initiation of cultures. Sequelae like early and late graft losses, infections, and friability of healed skin have been reported. Laserskin or Vivoderm (Fidia Advanced Polymers, Italy) is based on a biodegradable carrier composed of esterified hyaluronic acid with autologous keratinocytes seeded on this matrix. Hyaluronic acid being a major constituent of extracellular matrix (ECM) is thought to be a hospitable host for cellular integration and promotion. The ideal methodology to successfully deliver viable and uninjured cells with minimal loss or unintentional exposure to staff continues to be explored. Currently cell suspension sprays are often transferred in fibrin glue or membrane delivery systems like collagen, polyurethane films, and polymeric films. Epidermal cells suspended in liquid media (ReCell; Avita Medical, Melbourn, United Kingdom), and keratinocytes cultured on a membrane (MySkin, Celltran, Great Britain) are currently commercially available.

Cultured Fibroblasts

Cultured fibroblasts synthesize a variety of matrix molecules and growth factors known to promote wound healing. A variety of products capitalize on these properties with the consideration that cultured and actively dividing fibroblasts might either incorporate into the wound site or at least stimulate wound healing in chronic wounds, which are typically characterized by senescent fibroblasts. Dermagraft (Organogenesis) a living dermal replacement tissue consisting of human neonatal fibroblasts that are cultured on a biodegradable polyglactin mesh (Vicryl). Because fibroblasts cultured in a 3D mesh appear to be nonantigenic, Dermagraft can be considered a permanent replacement. Dermagraft has been tested in clinical trials and has demonstrated efficacy in the treatment of chronic wounds (diabetic foot ulcers). Hyalograft (Fidia Advanced Biopolymers, Abano Terme, Italy) is composed of autologous fibroblasts cultured on esterified hyaluronic acid. Various observational studies and clinical trials have been performed with this material in chronic wounds and acute full-thickness wounds. The promise of single-cell suspensions for the definitive treatment of deeper wound states, while beneficial, has not to date proven to be a panacea. Augmenting their potential with the use of extracellular matrices and mesenchymal stem cells might offer improved therapeutic potential.

Cultured Composite Skin Constructs

The concept of engineering a more anatomic appearing skin construct using cultured dermal–epidermal constructs was perhaps most comprehensively promoted by Dr. Steve Boyce from the University of Cincinnati (Cincinnati, Ohio), in which a dermal lattice composed of collagen and glycosaminoglycans (collagen-GAG) was inoculated with autologous fibroblasts and seeded with autologous keratinocytes in vitro. Follow-up clinical studies have demonstrated promising results. A newer formulation is reportedly in progress, currently named NovaDerm. Graftskin (Apligraf; Organogenesis, and Novartis Pharmaceuticals) is an allogeneic bilayered cultured skin equivalent containing keratinocyte and fibroblasts. Clinical trials in chronic wounds have shown efficacy of this allogeneic tissue-engineered material in chronic wounds. Similar to Graftskin, OrCel (Forticell Bioscience, New York, N.Y.; Ortec International) is also a bilayered construct composed of human neonatal allogeneic keratinocytes and fibroblasts cultured on a type I collagen sponge with atelocollagen. Although the aforementioned products have demonstrated measured improvements, definitive differentiating studies are lacking. More recent approaches have focused on the composite skin constructs with the addition of adipocyte, preadipocytes, or endothelial cells. In vitro studies have shown that such constructs can be grown in culture.

3D Printing

Recently, several teams started applying 3D printing technologies to repair wounds. Among the earliest promoters of this technology, the Wake Forest team along with military research funding support utilizes skin biopsy as autologous donor substrate. The wound site is mapped by a laser scanner, and a modified inkjet printer is employed to fill the wound in a layered cell fashion. A team in Canada (PrintAlive Bioprinter) has developed a process that creates a hydrogel bilayer of fibroblasts and keratinocytes in an effort to create a more structured matrix for transplantation. A Dutch company (SkinPrint, Goshen, N.Y.) reportedly produces a universal transplantable skin graft derived from induced pluripotent stem cells from autologous hair follicles. Advocates of these strategies are particularly encouraged by increased availability and decreasing costs of commercially available 3D inkjet printer technologies.

Whole-organ Decellularization

Whole-organ decellularization and regeneration might well offer potential functional advantages. This emerging technology maintains native ECM scaffolds constituting the established cellular microenvironment and provides overall geometry and structure that is known to support tissue and organ function. The process as currently described utilizes a detergent perfusion through native

vasculature, to solubilize and remove cellular components (ie, intracellular proteins and nucleic acid material), which generates an acellular whole-organ scaffold with perfusable vasculature. Ongoing preliminary efforts have demonstrated successful transplantation of rudimentary structures in animal models, resulting in rudimentary organ functions such as urine production in the kidneys, electromechanical contraction in hearts, and even gas exchange in the lungs. Most recently, this methodology has been translated to humans, using human cells. Although whole-organ decellularization and regeneration is promising, very significant challenges remain with regard to revascularization patency and homogenous cellular distribution. It is hoped that by improving ex vivo maturation, many of these obstacles might be overcome.

Polynucleotides-rich hyaluronic acid

Hyaluronic acid (HA) is a polysaccharide common in all species. Several studies found it to improve the healing of wounds resulting from burns, venous insufficiency, diabetes and surgery. The combination of HA and polynucleotides (PAHA) was demonstrated to be more effective than HA alone in promoting re-epithelialization of venous ulcers. Specifically, such polynucleotides are poly-deossi-ribonucleotides (PDRN) and they are made of linear polymers of purine and pirimidine monomeric units. The product is obtained by trout gonads, then purified and sterilized to obtain 50-2000bp-long chains. It stimulates A2a and A2b adenosine receptors, promoting VEGF production and increasing angiogenesis. Moreover, it stimulates fibroblast differentiation as well as collagen and fibronectin production. The combination with HA increases tissue hydration, fibroblasts survival, cell migration increasing PDRN effects of 20%.

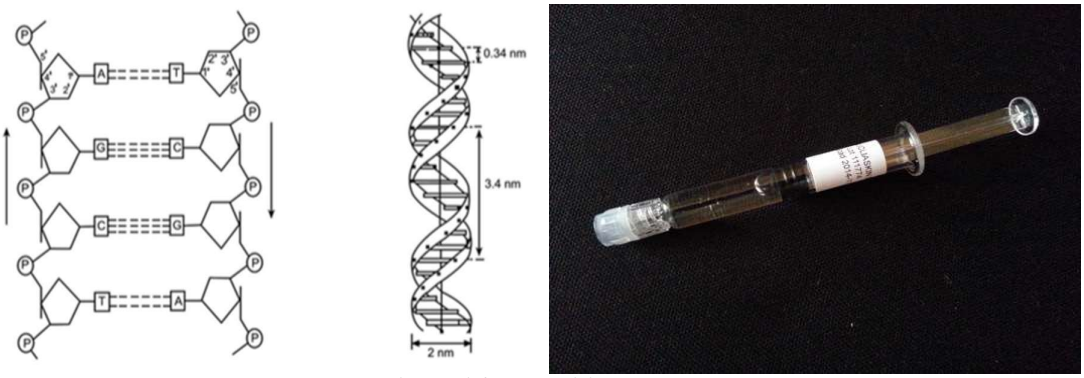

Figure 3: left, PDRN; right, PAHA

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THE REGENERATIVE POTENTIAL OF FAT TISSUE

Fat tissue was widely used to fill volume defects resulting from congenital, degenerative, traumatic diseases or aging. Adipose derived stem cells (ADSCs) are mesenchymal cells contained into fat tissue. They were identified in 2005 and, in the following years, the regenerative potential of fat grafting was solidly demonstrated, extending its clinical indications well beyond volumizing procedures, such as in neurodegenerative diseases, inflammatory disorders and complex wounds. ADSCs express similar surface markers to bone marrow mesenchymal stem cells such as CD10, CD13, CD29, CD44, CD54, CD71, CD90, CD105, CD106, CD117, and STRO-1. They are negative for the hematopoietic lineage markers CD45, CD14, CD16, CD56, CD61, CD62E, CD104, and CD106 and for the endothelial cell (EC) markers CD31, CD144, and von Willebrand factor. ADSCs show high resistance to ischemia and differentiation potential in mesodermal, ectodermal and endodermal lines. They can promote angiogenesis, secretion of growth factors and differentiate into multiple lineages upon appropriate stimulation, thus supporting human dermal fibroblast proliferation by directly contacting cells and paracrine activation. However, adipose tissue is composed of a heterogeneous population of different cells along with adipocytes and ADSCs. Cells isolated from fat tissue are referred to as stromal vascular fraction (SVF). Generally, SVF consists of various components such as pericytes, ECs, and macrophages, and it is thought that this composition may offer unique benefits in wound healing applications. The components of SVF may work synergistically to enhance the ASCs regenerative potential.

ADSCs IN WOUND HEALING

ADSCs can promote wound healing by several mechanisms, summarized in figure 1.

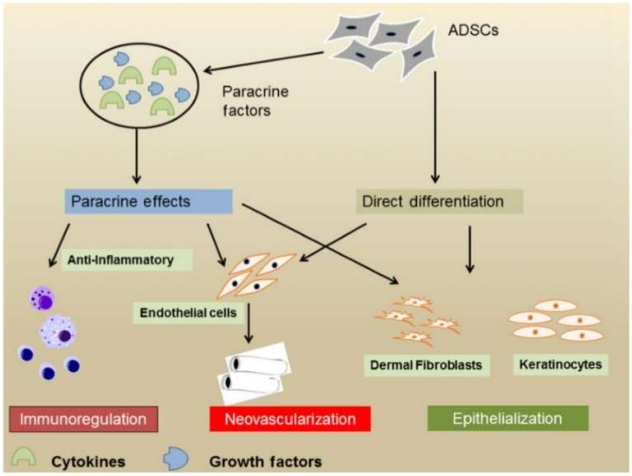

Figure 1: ADSCs effects on wound healing (from Hassan et al.)

DIFFERENTIATION

ADSCs can differentiate into fibroblasts, keratinocytes, and endothelial cells. Ebrahimian found that GFP-positive cells appeared in the epidermis and dermis after injection of GFP-transfected ADSCs on the dorsal surface of the rat, and it was able to express two epidermal keratinocyte marker proteins, cytokeratins CK5 and CK14, confirming that ADSCs differentiate into keratinocyte to enhance wound regeneration. However, Sivan also found that ADSCs can be differentiated into keratinocytes by culturing autologous ADSCs in an imitation eco-environment containing fibrin complexes. One of the most important reasons for prolonged wound repair is the reduction of blood flow to the wound. The regeneration of endothelial cells is especially important in the wound repair process, especially in refractory wounds such as ischemic diseases and diabetes. Planat-Benard first discovered that adipocytes and endothelial cells share a common origin. ADSCs not only participate in the formation of vascular-like structures, but also enhance the neovascularization of ischemic tissue, suggesting that adipocytes can be a source of cells for angiogenesis in ischemic diseases. This study indicated the direction of adipose-derived stem cells in the study of ischemic diseases. Similarly, Cao had also found in in vivo and in vitro studies that ADSCs can differentiate into endothelial cells and can improve blood perfusion and angiogenesis in ischemic lower limbs. Nie found that ADSCs transfected with GFP not only can differentiate into vascular endothelial cells and epithelial cells to enhance the vascular structure and epithelial formation of wounds, but also can secrete angiogenic factors such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) to promote wound angiogenesis. Huang transplanted ADSCs in a rat model with acute radiation skin ulcer, it was found that ADSCs can differentiate into endothelial cells and ultimately promote wound regeneration and angiogenesis. In the full-thickness injury model of rabbit ears, Hong found that ADSC transplantation can activate the fibroblast phenotype, increase the recruitment of endothelial cells and macrophages, and promote the formation of granulation tissue, while BM-MSC reach less than this effect. In a vocal cord wound repair experiment, Hu found that ADSCs can differentiate into fibroblast-like cells under the regulation of connective tissue growth factors, and injected ADSC-differentiated fibroblast-like cells in the vocal fold wounds of dog can significantly enhance wound healing. Additional studies have shown that ADSCs seeded onto a biomaterial such as a silk fibroin-chitosan scaffold transformed into fibrovascular, endothelial, and epithelial elements of repaired tissue and improved wound healing. The magnitude of microvessels was considerably higher at the site of the wound, and ADSCs that homed to the site of injury and adhered were positive for fibroblastic markers, heat shock protein, smooth muscle actin and von Willebrand factor.

PARACRINE EFFECT

Many evidences indicate that ADSCs secrete a variety of cytokines, growth factors, and chemokines to promote the proliferation and migration of endogenous cells, thereby stimulating angiogenesis, epithelial regeneration, and wound remodeling. They secrete a variety of angiogenesis-related cytokines such as GM-CSF, PDGF, SDF-1, VEGF, b-FGF, HGF, TGF-α, MMP, IL-6, and IL-8. VEGF is the most important growth factor in the process of angiogenesis. It can promote the mobilization, recruitment, and migration of endothelial progenitor. HGF also plays an important role in new vessels formation. Some cytokines such as IL-6 and IL-8 play an important role in epithelial regeneration of the wound during the proliferative phase. Heo found that TNF-α-activated ADSCs might produce pro-inflammatory cytokine IL-6 and IL-8 to promote angiogenesis, regeneration of epithelium and ultimately accelerating the healing of skin wounds. As a kind of paracrine product of stem cells, exosomes have the same functions as their stem cells and are rich in proteins, mRNA, miRNA, and other substances. Adipose stem cell-derived exosomes have naturally become a hot topic of research. Wang stated that intravenous injection of adipose stem cell-derived exosomes can increase the ratio of collagen fiber III to collagen fiber I and the ratio of TGFβ3 to TGF β1 and improve MMP3 expression of skin fibroblasts by activating the EPK/MARK pathway to promote ECM remodeling, ultimately promotes wound healing and reduces scar formation. Co-culture of adipose stem cell-derived exosomes with fibroblasts revealed that concentration of exosomes was found to be proportional to the proliferation of fibroblasts and the expression of N-cadherin (Cyclin-1, PCNA). Furthermore, the exosomal concentration of 50 μg/ml promoted the expression of collagen III and collagen I, suggesting that exosomes may be able to promote wound repair by optimizing fibroblasts.

CLINICAL TRIALS

The first clinical application of ADSCs used SVF in a case report to treat defect of the calvaria after injury. In this case study, fibrin glue was used along with SVF. Three months post treatment, new bone formation was detected and resulted in near complete healing of calvaria defect. The efficacy of SVF in the cardiovascular field for acute myocardial infarction was also tested in another clinical trial. In some clinical trials, ADSCs were utilized after being purified from SVF to obtain a pure population of ADSCs. In another report that was successfully completed and documented with 36 months follow-up, it was shown that ADSCs along with BMP-2 and tricalcium phosphate scaffold lead to successful healing of osteogenic defect. Although encouraging, this is only a single report and conclusions for long-term safety of these cells cannot be drawn until successful phase I/II clinical trials are completed. Other ADSC-related clinical trials focus on auto-immune inflammatory diseases

such as Crohn's disease and fistula complications that result from tissue degeneration following an uncontrolled inflammatory process and graft-vs-host disease (GvHD). Garcia-Olmo et al. have shown successful healing with expanded ADSCs (rather than the freshly prepared cells) in treating Crohn's disease. In trials that focused on treating fistula, ADSCs were shown to be very efficient in controlling inflammation and improving the healing process. Therefore, the results of currently ongoing clinical trials, if encouraging, are opening up the field for ADSC related regenerative medicine.

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THE USE OF THE "STEM GRAFT" IN CHRONIC WOUNDS: IN VITRO ANALYSIS, CLINICAL AND COMPARATIVE STUDY

BACKGROUND

A wound that has failed to re-epithelialize after 3 months is defined as chronic. Over 4 million patients in the United States are affected by chronic wounds, and around 50 billion US dollars are spent every year on their treatments.[1] Such wounds are a major issue for patients and a challenge for clinicians and surgeons. They can complicate with infection, sepsis or cancerization, sometimes leading to amputation. The patient's quality of life is reduced by pain, need for frequent medications, as well as by functional and social limitations. Surgical reconstruction is often unfeasible due to poor local tissues, comorbidities and local infection. As a consequence, in most cases, patients are treated with simple debridement and then allowed to heal by secondary intention. It would be paramount for these patients to speed up the re-epithelization process, possibly improving their symptoms.

Advanced medications, such as polyurethane foam (PF), can promote healing. They became the standard of care (SOC), although they are expensive and require to be changed every other day.[1] On our current era of increasing health care costs, aging population, and increasing prevalence of obesity and diabetes, it is important to identify novel therapeutic options to better address the growing burden of chronic wounds.

Porcine dermis (PD) and polynucleotides-added hyaluronic acid (PAHA) were previously reported to reduce hospitalization and to promote healing, as well as cell proliferation and matrix production in skin ulcers.[2-6]The use of porcine dermis in cutaneous ulcers was previously investigated. Zhang et al reported its efficacy in promoting healing, reduce hospitalization and complications in perineal and lower limb wounds.[2,3] Zajicek and colleagues demonstrated that such dermal substitute promoted the proliferation and differentiation of human keratinocytes.[4] Moreover, it was found to increase collagen synthesis, stem cells proliferation as well as PDGF, EGF and FGF expression.[7] Hyaluronic acid is a polysaccharide common in all species. Several studies found it to improve the healing of wounds resulting from burns, venous insufficiency, diabetes and surgery.[5,6] The combination of HA and polynucleotides was demonstrated to be more effective than HA alone in promoting re-epithelialization of venous ulcers.[7]

Fat grafting is an emerging treatment for chronic wounds.[9] Since 1995,[10] it was widely used to fill volume defects resulting from congenital, degenerative, traumatic diseases or aging. Adipose derived stem cells (ADSCs) are mesenchymal cells contained into fat tissue. They were identified in 2001[11] and, in the following years, the regenerative potential of fat grafting was solidly demonstrated,

extending its clinical indications well beyond volumizing procedures, such as in neurodegenerative diseases, inflammatory disorders and complex wounds.[12] Specifically, ADSCs showed high resistance to ischemia and differentiation potential in mesodermal, ectodermal and endodermal lines.[13] The different techniques of harvesting and processing the fat tissue may affect the number, viability and differentiation capability of ADSCs. As an example, the aspirated fat tissue may be decanted or centrifuged (3000 rpm or 1200 g for 3 minutes) to allow stratification and subsequent removal of blood and anesthetic solution: in the former technique ADSCs are mostly present in the adipose layer, while with the latter approach they stratify in the caudal pellet of the lowest layer.[14] The "Cell-assisted lipotransfer" (CAL)[15] is a technique that allows to isolate ADSCs from fat tissue and then enrich the fat graft with the concentrated cells. However, such approach requires dedicated facility and enzymatic treatment of the harvested fat before re-implantation, thus being expensive and even forbidden in some countries. In 2013, Tonnard et al. described the "nanofat graft", a technique that allowed, by repeated shifting of fat tissue between 2 connected syringes, fragmentation of mature adipocytes with preservation of the ADSCs.[16] The filling capability of the "nanofat" graft was limited due to adipocyte fragmentation. As a consequence, it was mainly indicated to achieve tissue regeneration instead of filling. Opposite to the CAL, it was cheap, straightforward and did not require enzymatic treatment. Moreover, when compared to both CAL and conventional fat graft, it showed several advantages:

1. it could be injected by needles instead of cannulas, allowing easier injection in chronically inflamed or fibrotic tissues;
2. it could be injected in thin layers, such as intra-dermally;
3. by using thin needles, the cells could be injected into more tissue "channels", thus maximizing their contact with the surrounding vascularized tissues and ultimately promoting their survival;
4. local metabolites were only used by stem cells and not "stole" by mature adipocyte for their survival;
5. cell fragments acted as a biological matrix and contribute to the inflammatory activation sustaining the differentiation of local and injected stem cells.[17]

Nevertheless, "nanofat" graft also showed some limitations:

1. adipocytes fragmentation resulted in the release of oil in the solution to be injected, which is histo-toxic and can result in granuloma formation;
2. it had a lower number of ADSCs compared to CAL and conventional fat graft.[16]

AIMS OF THE STUDY

Aim 1 is to obtain, by minimal manipulation of fat tissue,[18,19] a product with high concentration of viable ADSCs and low oil content

Aim 2 is to assess the efficacy of such product (hereafter called Stem Graft (SG)) in the treatment of chronic wounds and to compare its outcomes and biological effects with the standard of care in advanced medications (polyurethane foam).

Aim 3 is to compare the efficacy and biological effects of SG with those of currently used devices in regenerative medicine, namely porcine dermis and polynucleotides-added hyaluronic acid

MATERIALS AND METHODS

Aim 1

Firstly, the literature was analysed focusing on every single step of fat harvesting and processing to increase ADSCs number, viability and proliferation. The following technique according to the literature findings. The harvesting was performed in the lower abdomen, as it was reported to be the region with the highest ADSCs concentration,[20,21] although no statistical difference in their viability was found when compared to other donor sites.[22] Lidocaine was previously shown to reduce ADSC proliferation and viability, while Ropivacaine had a negligible effect.[23-26] Epinephrine did not exhibit any cytotoxic effect[27] and was used in the infiltration solution. Aspiration was performed according to the micro-fat technique, by mean of a 3 mm cannula with 1 mm lateral holes, because such an approach was shown to allow for higher ADSCs concentration compared to macro-fat. [28] The harvested micro-fat was centrifuged at 3000 rpm for 3 minutes, to avoid any possible loss or viability impairment in ADSCs.[14] The oil in the upper layer and the infiltration in the lower layer were aspirated and discarded by mean of a 18-20 G spinal needle. The cellular pellet stratified in the caudal most layer of the syringe[3] was cautiously preserved. The product was then mechanically emulsified by shifting the fat for 60 times between two syringes connected to a 90 degrees Luer-Lock connector. Subsequently, the product was further centrifuged at 3000 rpm for 3 minutes and, again, the oil in the upper layer and the infiltration in the lower layer were discarded. No filtration was performed to avoid any loss in ADSCs and to keep the cell fragments resulting from the emulsification process, which may act as pro-inflammatory signals triggering the regeneration cascade.[16,29] The resulting product

was injectable with a 27 Gauge needle, allowing for easy tunneling in cases of advanced fibrosis. The "stem graft" technique is summarized in table 1 and Figure 1.

Infiltration	Solution of: • 1000 ml NaCl, • 225 mg Ropivacaine • 1 mg Epinephrine
Harvesting	• 3 mm cannula with multiple 1 mm lateral holes connected to a 10 cc luer-lock syringe
Processing	1. 1st centrifugation at 3000 rpm for 3 minutes; 2. 1st purification: removal of the oil in the upper layer and of the infiltration in the lower layer by mean of a 18-20 G spinal needle. The cellular pellet stratified in the caudal most layer of the syringe[3] must be preserved; 3. Emulsification: fat is shifted for 60 times between two syringes connected by a 90° Luer-Lock connector; 4. 2nd centrifugation at 3000 rpm for 3 minutes; 5. 2nd purification: removal of the oil and the residual infiltration as previously described, preserving the caudal most cellular pellet and 1 cc of product
Injection	• 27 G needle

Table 1: the stem graft technique

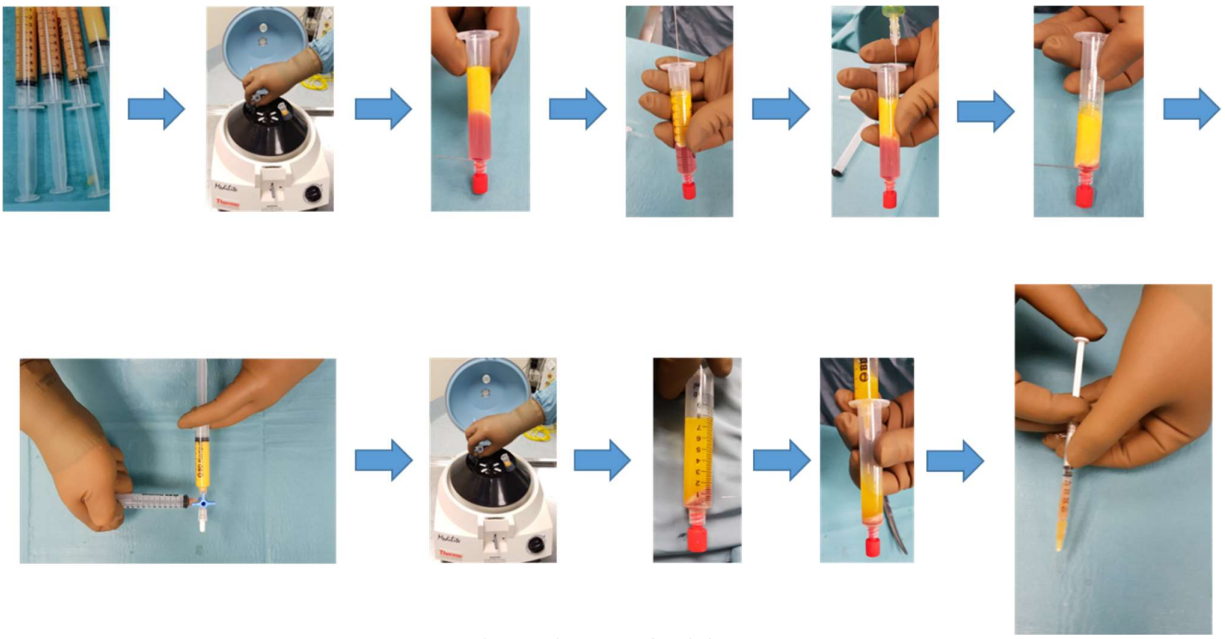

Figure 1: the stem graft technique

Nine patients undergoing abdominal liposuction were randomly enrolled in the study. For each patient, 10 cc of fat were decanted, while 10 cc were processed according to the stem graft technique. ADSCs from decanted fat and stem graft were isolated after centrifugation at 600 g for 10 minutes. Cell pellet was seeded at a density of 60-70% for adipogenic differentiation and 90% for osteoblast differentiation. ADSCs were then differentiated into adipocytes adding 3-Isobutyl-1-methylxanthine (IBMX) (500µM), Dexamethasone (1µM), Indomethacin (1µM) , Rosiglitazone (1mM) and Insulin (10µM) to medium culture (D-MEM High glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 250ng/mL amphotericin B). Adipocyte differentiation was evaluated at the end of differentiation protocol (13 days) using Oil Red-O staining. Osteoblastic differentiation was achieved as previously described by Iuliani et al. Specifically, ADSCs were seeded at an initial density of 5×104 in 24 well plate or at 3×106 in 75 cm2 flask and differentiated into osteoblasts adding 10 mM beta-glycerophosphate (Sigma-Aldrich), 50 µM ascorbic acid (Sigma-Aldrich) and 100 nM dexamethasone to culture medium. After 28 days of differentiation, mature osteoblasts were fixed with 4% formaldehyde for 20 minutes and stained with Alizarin red for 1 hour at room temperature (RT) to detect bone matrix deposition. Flow cytometry analysis was performed as follow. Lipoaspirate specimens were enzymatically digested by collagenase type II (1 mg/ml Sigma) in a shaking water bath at 37° C for 40 minutes, in order to obtain a heterogeneous cell suspension. After wash, cells were incubated with the monoclonal-antibodies: CD45-FITC, CD34-PE, 7AAD (tube n. 1) and CD45APC-H7, CD146-PE, 7-AAD,

CD34PE-Cy7 (tube n. 2), for 30 minutes at 4° C, protected from light. Then, tubes were washed and immediately measured. For each tube, 100.000 events were acquired in a FACS Canto II (BD Biosciences). The flow cytometry analyses were performed using FACS Diva Software (BD Biosciences). Paired t-test was used for statistical analysis. P values lower than 0.05 were considered as statistically significant. We performed a preliminary study on 6 consecutive patients (3 patients per group). Group sample sizes of 4 and 4 achieve at least 80% power with a significance level (alpha) of 0.05 using a paired t-test.

Aims 2 and 3

Study design. The study was conducted in compliance with the Declaration of Helsinki and the Guidelines for Good Clinical Practice. It was designed as a single blind randomized controlled trial. 40 consecutive patients were enrolled according to the following criteria:

- inclusion criteria: venous ulcer on the inferior limb not improving after 3 months of standard of care (including compression, where indicated); age over 18 years old; wound area larger than 5 cm^2.
- exclusion criteria: active systemic infection; connective tissue or rheumatic diseases; cancer; ongoing chemotherapy or therapy with biologic drugs (e.g. anti-TNF), corticoids or immunosuppressants; diabetes; neoplastic or Marjolin ulcer.

Patients were randomized by mean of block randomization and assigned to one of the 4 groups of treatment: advanced medications, stem graft, porcine dermis, polynucleotides-added hyaluronic acid. Mean age of the patients(±SD) was 69 (±13) years.

Endpoints. The primary endpoint was the percentage reduction of the wound area at 30 days post-operatively in the stem graft group vs the advanced medication group. The secondary endpoints were the percentage reduction of the wound area at 30 days post-operatively in the stem graft group compared to the advanced medication, the porcine dermis and polynucleotides added hyaluronic acid, as well as the changes in neo-angiogenesis, cellular proliferation, collagen deposition and myofibroblasts number among groups

Study Protocol

Two plastic surgeons were involved: one (operator 1) performed the pre-operative assessment and the procedure, the other (operator 2) was blinded to the treatment, conducted patient follow-up and analyzed the data. Before the surgical procedure, the operator 1:

- took a digital photo of the wound, with the patient in supine or prone position, from a distance of 50 cm and including a meter in the picture.

Intra-operatively, all the patients underwent skin biopsy of one margin of the wound, surgical debridement and treatment according to the assigned group: advanced medication (group 1), stem graft (group 2), porcine dermis (group 3), polynucleotides-added hyaluronic acid (group 4).

Advanced medication: polyurethane foam with silver complex (Biatain Ag non-adhesive, Coloplast) was applied immediately after debridement and changed every other day.

Stem Graft: the lower abdomen was infiltrated with 250 ml of a solution containing saline solution, ropivacaine and adrenaline. One hundred ml of fat was aspirated by mean of 3 mm blunt cannulas with multiple 1 mm holes (Sorensen cannulas, Tulip) and then processed by sequential series of centrifugation and emulsification according to the above described stem graft technique. Following debridement, 10 ml of stem graft were infiltrated in the bed and margins of the wound. The wound was then covered with a paraffin-embedded gauze, changed after 5 days and then every other day.

Porcine Dermis: porcine dermis (EzDERM, Mölnlycke Health Care AB, Gothenburg, Sweden) was applied once immediately after debridement

Polynucleotides-added hyaluronic acid: polynucleotides-added hyaluronic acid (NukliaSkin S, Mastelli SRL, Sanremo (IM), Italy) was applied once immediately after debridement.

30 days post-operatively, the operator 2 took a digital photo as previously described and performed a skin biopsy of one margin of the wound. Pictures were manually processed through the AutoCAD software (Figure 2).

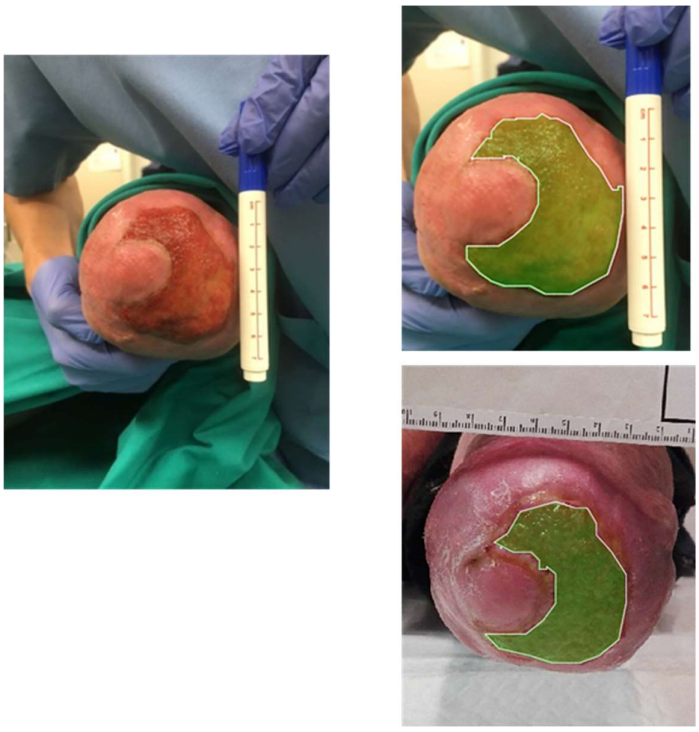

Figure 2: Image processing with AutoCAD software

The ulcerated area was calculated in square centimeters. The wound area reduction was calculated in square centimeters and as a percentage.

Biopsies and histological analysis: biopsies were performed with a conventional scalpel under local anesthesia and fixed in formalin. Tissue specimens were fixed in 4% neutral buffered formaldehyde, embedded in paraffin and cut into 3 µm slices to achieve a transversal view of the tissue layers. They were subsequently stained by hematoxylin/eosin and immunohistochemistry antibodies for alpha smooth muscle actin (α-SMA, as a marker of myofibroblasts, Clone 1A4 Dako), CD34 (as a marker of neo-angiogenesis, Clone QBEnd/10 Biocare Medical), Ki67 (in keratinocytes and dermal fibroblasts, as a marker of cell proliferation, Clone MIB-1 Dako), collagen type 1 (COL I Polyclonal IgG, AbD Serotec) and collagen type 3 (COL III, Polyclonal IgG, AbD Serotec). Secondary antibodies and processing were performed as previously described.[31] The positivity for α-SMA was evaluated at a 400x magnification in three randomly chosen fields, then averaged and expressed a percentage of positive cells. Neo-angiogenesis was evaluated by CD34 positivity, with the Weidner method.[32] Collagen positivity was assessed as previously described,[33] Ki67 positivity of keratinocytes and dermal cells was evaluated as the average percentage of positive cells in 3 randomly chosen 400x fields. Light microscopy images were captured by a videocam (SPOT Insight; Diagnostic Instrument, Inc., Sterling Heights, MI, USA) connected to an Olympus BX-51 light microscope (Olympus, Tokyo, Japan) and processed with an image analysis system (Delta Sistemi, Rome, Italy). The operator evaluating clinical and histological outcomes was blinded to treatment group and patient's data. Data were expressed as mean (± Standard Deviation) or median with 95% confidence interval and analyzed as both absolute value and percentage. Independent samples T test were performed to assess any statistically significant difference between groups. Paired T test was used to assess the eventual difference between pre-operative and 30 days post-operative values. Wilcoxon and Mann-Whitney U tests were performed where necessary. Correlations were evaluated by mean of Spearman correlation coefficient. All tests were two-tailed and considered statistically significant for p values lower than 0.05.

Preliminary Power Calculation. We performed a preliminary study on 6 consecutive patients (3 patients per group) who met the inclusion criteria, considering the reduction of the wound area at 30 post-operative days as endpoint. Mean wound area reductions (±SD) were 1.6 (±0.58)% in the advanced medication group, 74.14 (±32.94)% in the "stem graft" group. For *the primary aim*, group sample sizes of 4 and 4 achieve at least 80% power to detect a difference of 72.54 between the null hypothesis that both group means are 1.6 and the alternative hypothesis that the mean of "stem graft"

group is 74.14, with estimated group standard deviations of 0.58 and 32.94 and with a significance level (alpha) of 0.05 using a two-sided two-sample t-test.

RESULTS

Aim 1

One cc of stem graft was obtained by 10 cc of harvested fat. The mean content of CD34+CD45- cells was 9.2% (±2.1) in the stem graft, 2.5% (±3.8) in the decanted fat (Figure 3).

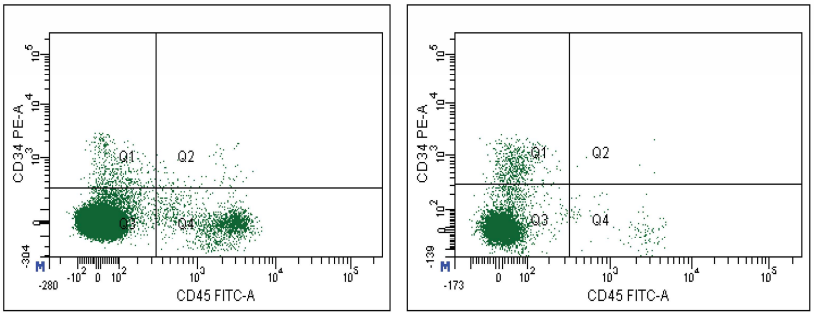

Figure 3: Dot plots showing the detection of different population of cells from stem graft and decanted fat. CD45+ cells were regarded as blood-derived cells, whereas CD45- cells were regarded as adipose derived stromal cells. In this specimen CD34+/CD45- cells were 6.8 % in the stem graft and 0.3 % in the decanted fat.

The concentration of ADSCs in the stem graft group was significantly higher than in decanted fat ($p<0.05$). Cells isolated from the stem graft were viable and able to differentiate in both adipocytes and osteoblasts (Figure 4).

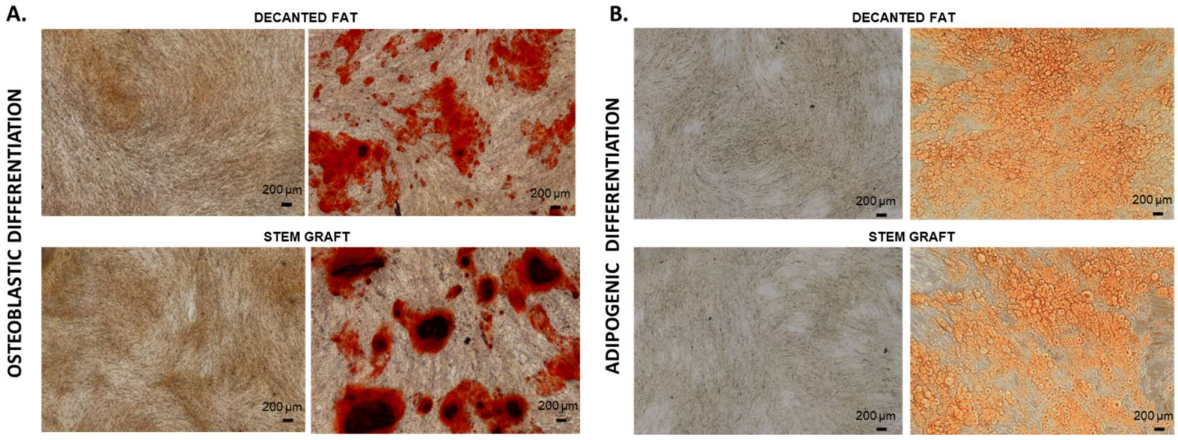

Figure 4: ADSCs differentiation into osteoblasts or adipocytes. (A) Osteoblastic differentiation of ADSCs was assessed after 28 days through Alizared Red assay that stains bone matrix deposition (in red). Undifferentiated ADSCs are shown on the left. (B) Adipogenic differentiation was evaluated after 13 days with Oil Red-O assay that stains neutral triglycerides and lipids in mature adypocytes Undifferentiated ADSCs are shown on the left. Scale bar= 200 µm

Aims 2 and 3

Clinical cases from different groups are shown in the following figures (figures 5 to 9)

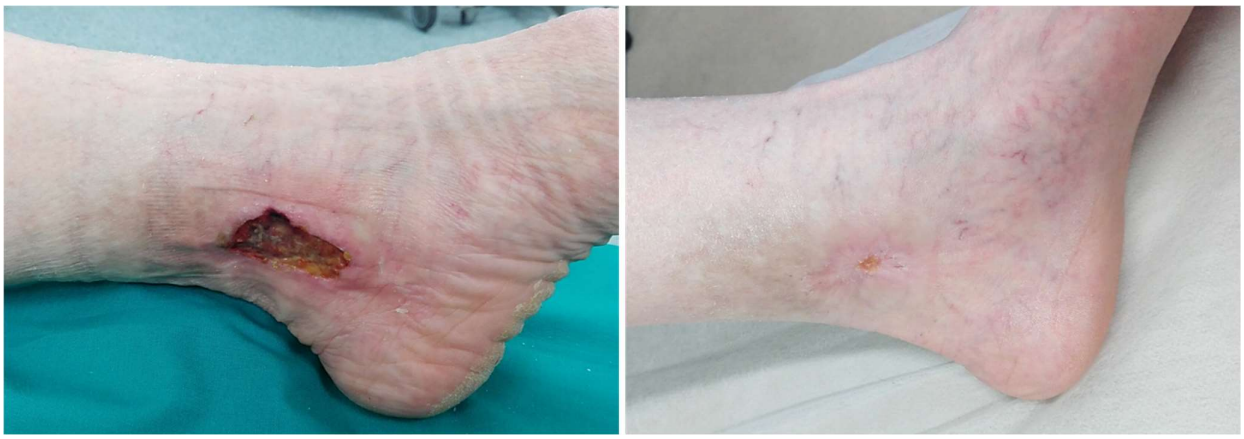

Figure 5: patient treated with the stem graft; left, pre-operative view; right, 30-days post-operative view.

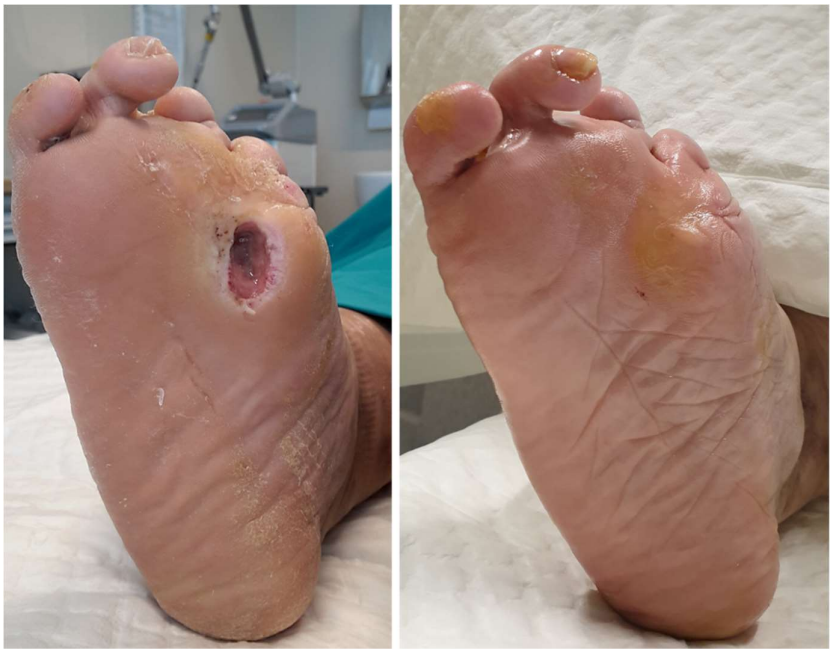

Figure 6: patient treated with the stem graft; left, pre-operative view; right, 45-days post-operative view.

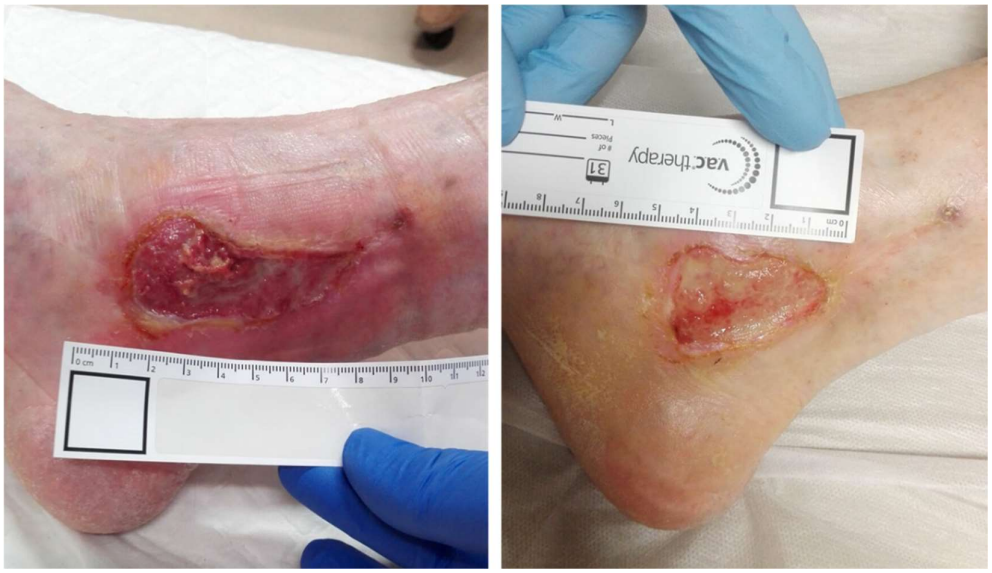

Figure 7: patient treated with the stem graft; left, pre-operative view; right, 30-days post-operative view.

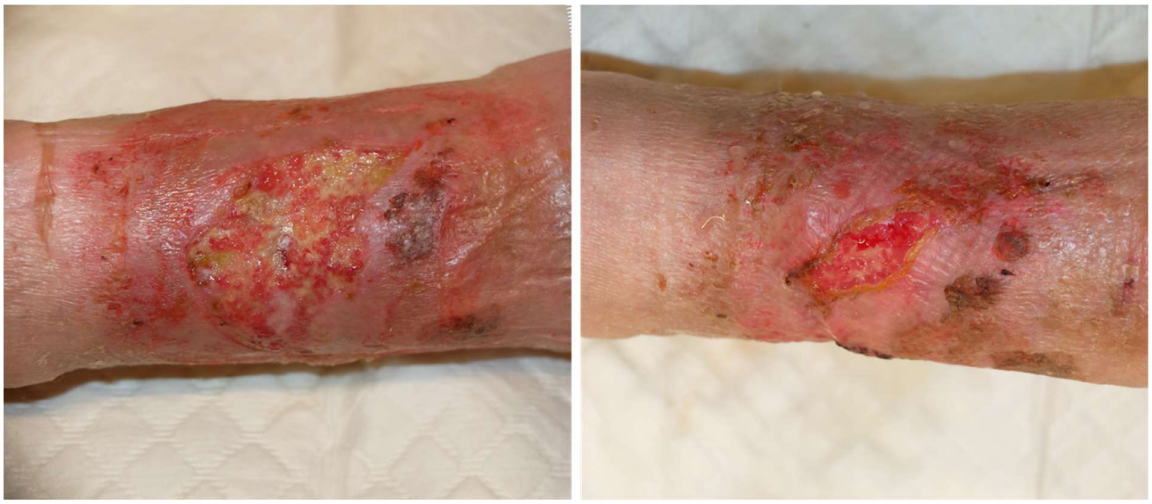

Figure 8: patient treated with porcine dermis; left, pre-operative view; right, 30-days post-operative view.

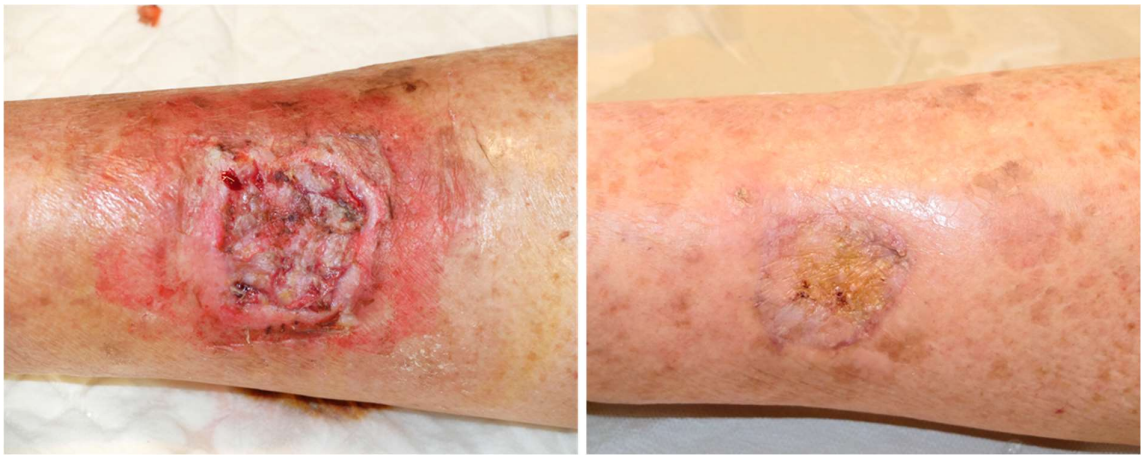

Figure 9: patient treated with polyurethane-added hyaluronic acid; left, pre-operative view; right, 30-days post-operative view.

Wound area and wound area reduction are summarized in table 2 and 3 and figures 10 and 11, respectively.

	PRE-OPERATIVE WOUND AREA (cm2)		30-DAYS POST-OPERATIVE WOUND AREA (cm2)	
	Mean	*±SD*	*Mean*	*±SD*
CONTROL	28.28	19.02	27.88	18.9
STEM GRAFT	20.2	17.82	14.3	17.06
PORCINE DERMIS	34.81	19.8	27.81	18.61
POLYNUCLEOTIDES-ADDED HYALURONIC ACID	20.76	19.25	15	18.01

Table 2: mean wound areas.

	30-DAYS WOUND AREA REDUCTION (cm2)		30-DAYS WOUND AREA REDUCTION (%)	
	Mean	*±SD*	*Mean*	*±SD*
CONTROL	0.4	0.17	1.59	0.58
STEM GRAFT	5.94	2.83	40.78	21.08
PORCINE DERMIS	7	2.96	23.53	11.69
POLYNUCLEOTIDES-ADDED HYALURONIC ACID	5.76	1.3	43.82	27.99

Table 3: mean wound area reduction at 30-days follow u

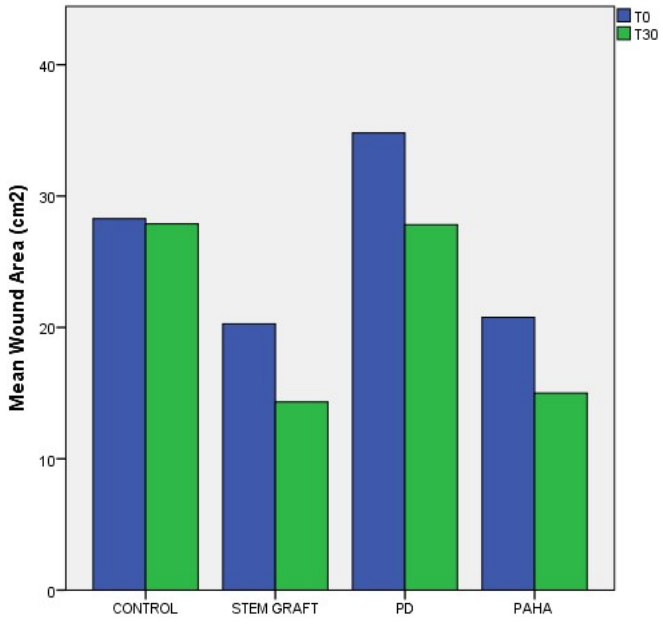

Figure 10: mean pre- and post-operative wound areas

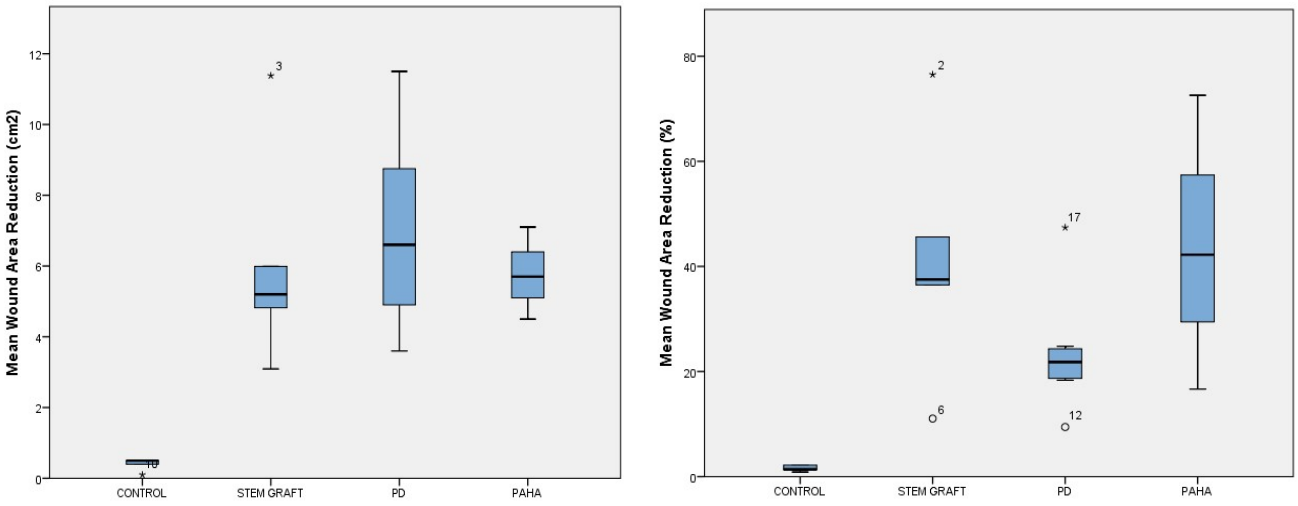

Figure 11: mean wound area reduction in cm2 (left) and percentage (right)

Compared to pre-operative values, the SG, PD and PAHA groups showed a statistically significant reduction in wound area at 30 days post-operative follow-up ($p<0.01$ in each group).

Mean wound area reduction, both as absolute value and percentage, showed a statistically significant difference in SG, PD, PAHA groups compared to control group ($p<0.01$, $p<0.01$, $p<0.01$, respectively)

At 30-days follow-up, the SG showed a statistically significant higher percentage of wound area reduction compared to PD ($p<0.05$)

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CD34 positivity is summarized in table 4.

	T0		**T30**		**Delta T30-T0**	
	Mean	*±SD*	*Mean*	*±SD*	*Mean*	*±SD*
Control	11.41	3.03	16.16	6.99	4.75	7.82
SD	5.57	2.32	8.68	2.21	3.11	1.88
PD	13.45	1.90	26.04	3.77	12.58	2.7
PAHA	12.86	1.78	22.66	5.62	8.06	5.67

Table 4: Mean CD34 positivity

Compared to pre-operative values, the increase in CD34 expression was statistically significant in SG, PD and PAHA groups ($p<0.01$, $p<0.01$, $p<0.05$, respectively). The increase of CD34 expression in PD group was statistically significant compared to control group ($p<0.05$) and to SG group ($p<0.01$) No difference was found

in the increase of CD34 expression between Control, SG and PAHA, as well as between PD and PAHA (figure 12 and 13).

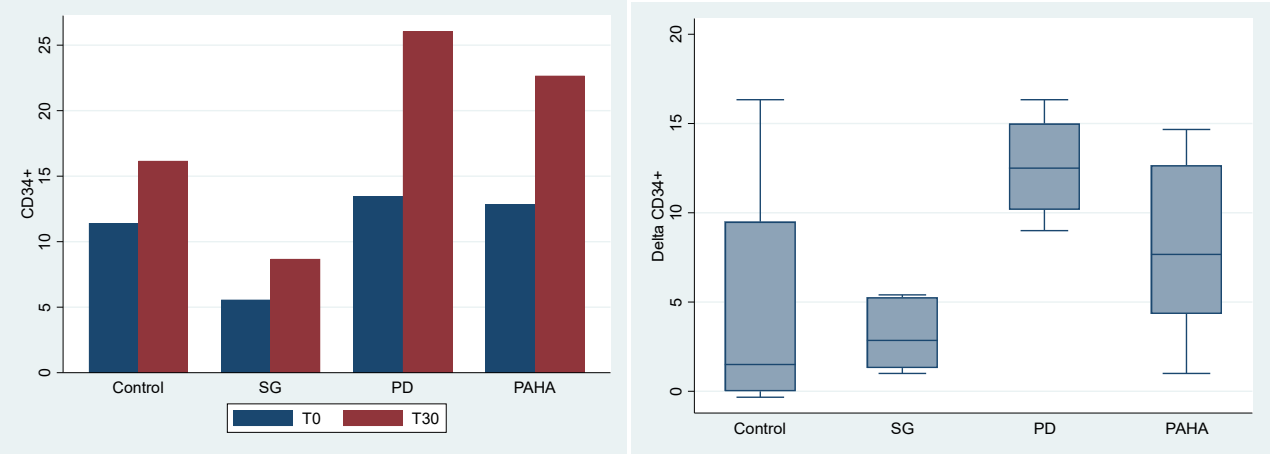

Figure 12: left, CD34 expression pre-operatively (T0) and at 30-days follow-up (T30); right, the change in CD34 expression between T0 and T30

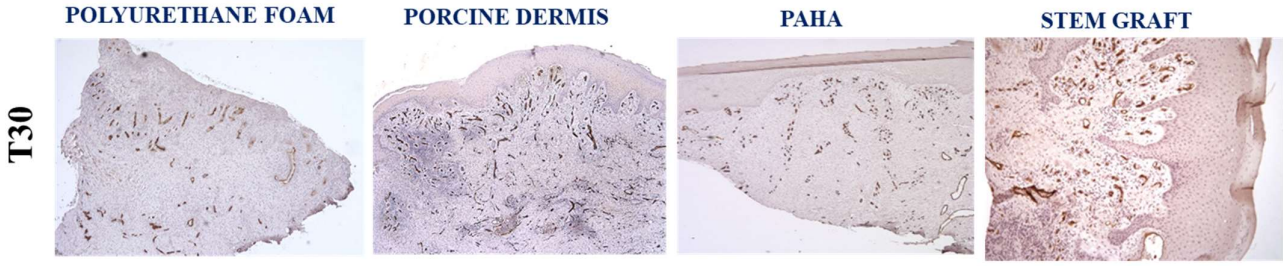

Figure 13: CD34 expression at 30-days follow-up in the different groups

Ki67 positivity is summarized in table 5.

	T0		T30		Delta T30-T0	
	Mean	*±SD*	*Mean*	*±SD*	*Mean*	*±SD*
Control	0.24	0.05	0.23	0.04	-0.01	0.43
SD	0.28	0.26	0.46	0.19	0.18	0.1
PD	0.24	0.14	0.49	0.18	0.23	0.24
PAHA	0.24	0.11	0.42	0.11	0.16	0.11

Table 5: Mean Ki67 positivity

Compared to pre-operative values, the increase in Ki67 expression was statistically significant in SG, PD and PAHA groups ($p<0.01$, $p<0.05$, $p<0.05$, respectively). Compared to the control group the increase of Ki67 expression was statistically significant in the SG and PAHA groups ($p<0.01$ and $p<0.05$). There was no statistically significant difference among the SG, PD and PAHA groups (figure 14 and 15).

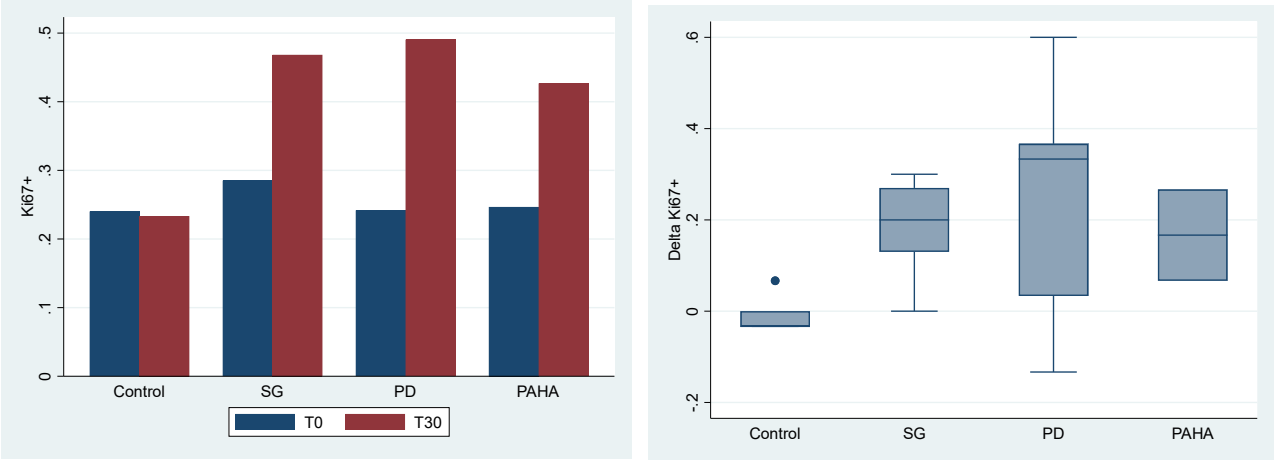

Figure 14: left, Ki67 expression pre-operatively (T0) and at 30-days follow-up (T30); right, the change in Ki67 expression between T0 and T30

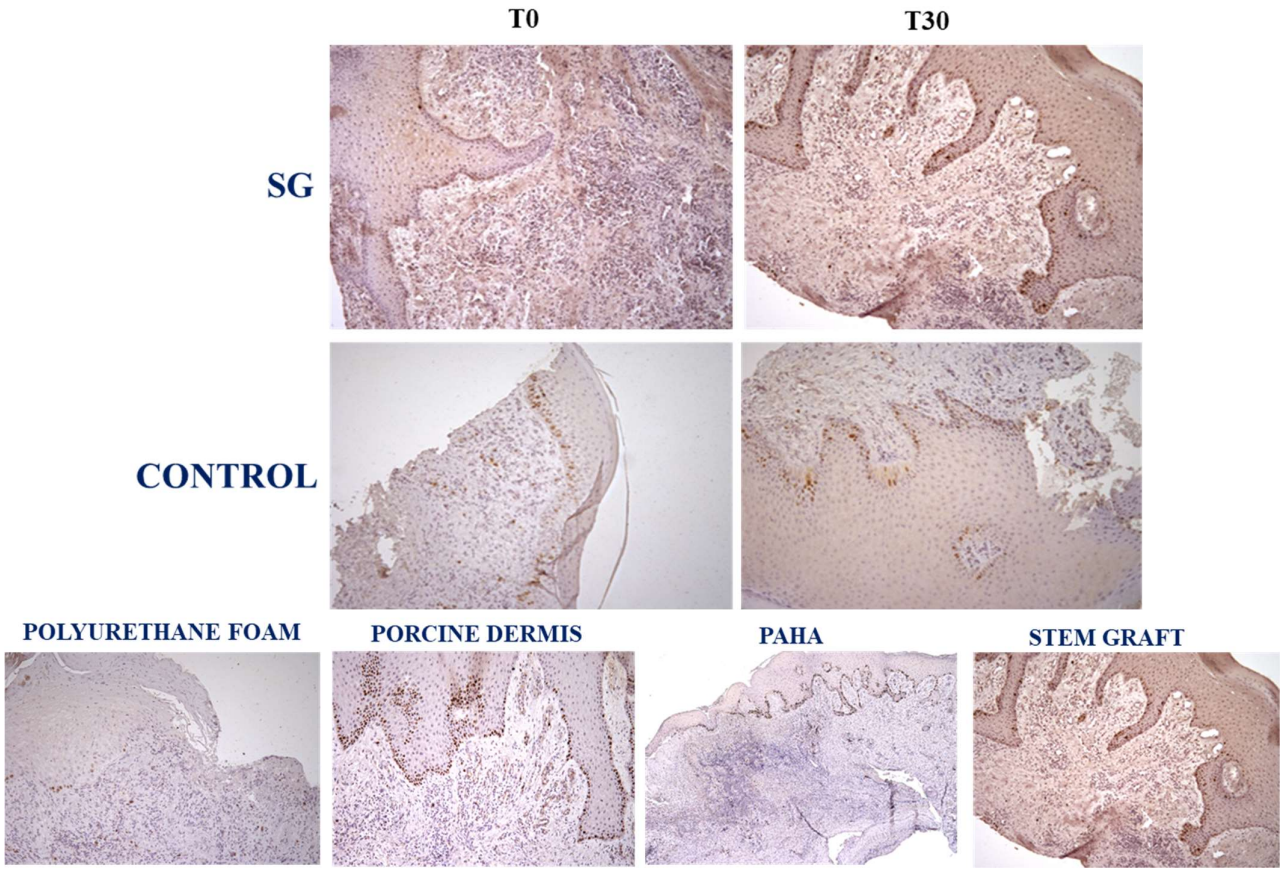

Figure 15: above, Ki67 expression in the SG and control group at T0 and T30; below, Ki67 expression at 30-days follow-up in the different groups

α-SMA positivity is summarized in table 6

	T0		T30		Delta T30-T0	
	Mean	*±SD*	*Mean*	*±SD*	*Mean*	*±SD*
Control	0.2	0.27	0.4	0.16	0.15	0.14
SD	0.13	0.53	0.10	0.05	-0.02	0.1
PD	0.46	0.16	0.64	0.23	0.17	0.06
PAHA	0.38	0.1	0.61	0.12	0.22	0.13

Table 6: Mean α-SMA positivity

Compared to pre-operative values, the increase in α-SMA expression was statistically significant in PD and PAHA groups ($p<0.01$, $p<0.01$). The increase of α-SMA expression in PD and PAHA group was statistically significant compared to SG group ($p<0.05$, $p<0.05$, respectively). There was no statistically significant difference between control, PD and PAHA groups (figure 16)

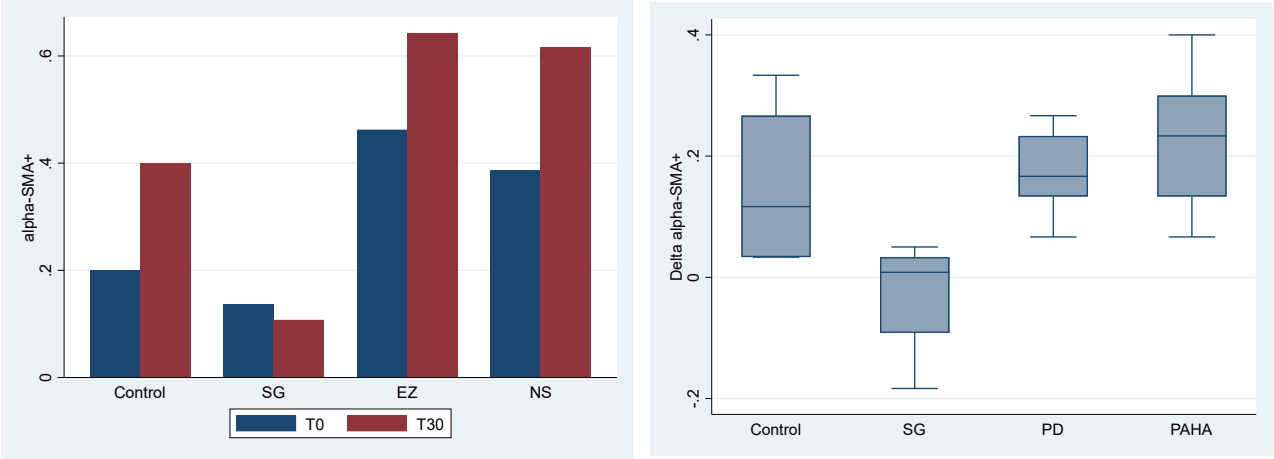

Figure 16: left, α-SMA expression pre-operatively (T0) and at 30-days follow-up (T30); right, the change in α-SMA expression between T0 and T30

Compared to pre-operative values, the increase in collagen expression was statistically significant in SG group only ($p<0.05$), Compared to the control group the increase in collagen expression was statistically significant in the SG, PD groups ($p<0.01$, $p<0.05$). The increase of collagen expression in the SG group was statistically significant compared to PAHA group ($p<0.01$) while no difference was found between PAHA and PD groups (figure 17).

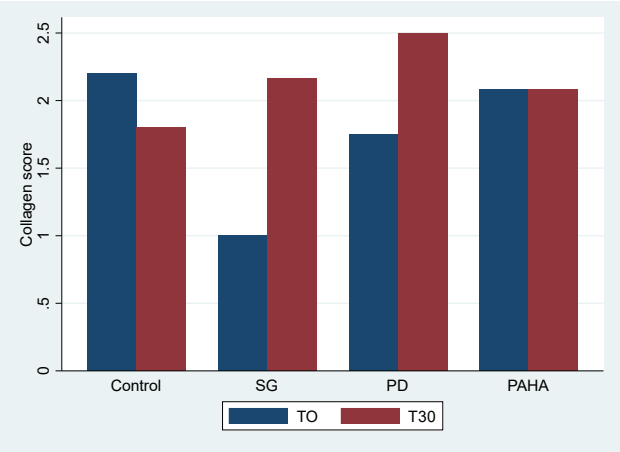

Figure 17: left, collagen expression pre-operatively (T0) and at 30-days follow-up (T30)

DISCUSSION

The reported technique achieved a significant increase in ADSCs concentration compared to decanted fat, while also preserving viability and differentiation capability. Having high concentration in small volumes is paramount when injecting superficial or scar tissues. In such cases, an exceeding increase of the interstitial pressure could impair the local blood supply, thus hampering stem cell take and retention within the recipient area. The lack of mature adipocytes in the graft is a further major advantage: it accounts for the reduced overall volume and the local metabolites are mainly used by stem cells and not for survival of mature adipocyte. Injection by small needles allows for the creation of multiple narrow tunnels, thus increasing the surface of the graft surrounded by vascularized tissue, as well as easy injection intra-dermally or in severely fibrotic areas. The stem graft was not filtered to avoid any possible loss in ADSCs and stromal vascular fraction (SVF). Moreover, the retained extracellular matrix and the cellular debris resulting from the processing may act as a biological matrix contributing to the inflammatory activation underlying the differentiation of local and injected stem cells.[17] The "stem graft" technique did not require any adjunctive cost or device[34] compared to conventional liposuction. It proved to be a cheap and effective method to achieve an oil-poor matrix containing high concentration of ADSCs. The injection was performed with a 27 G needle, thus making the procedure feasible for intra-dermal or intra-scar use.

Healthy skin wound healing is a complex and highly regulated process consisting of inflammation, proliferation, matrix formation, and remodeling. When the process is disrupted, chronic non-healing

wound will develop. In our cohort of patients, treatment with the SG resulted in a statistically significant reduction in wound area at 30 days. Moreover, the percentage area reduction was significantly higher in the SG group than in the control one (polyurethane foam). Such findings proved the clinical efficacy of the here-described technique. The underlying biological processes were investigated by mean of immunohistochemistry. Compared to pre-operative values, no statistically significant increase in angiogenesis, number of myofibroblast, dermal cell proliferation and collagen deposition was found in the control group. The SG, instead, was found to increase neo-angiogenesis (CD34), dermal proliferation (Ki67), and collagen deposition. On the other hand, there was no significant increase in the number of α-SMA positive cells (myofibroblasts) at 30-days follow up. Tissue healing goes through 3 phases: inflammatory, proliferative and remodelling. Myofibroblasts are mainly expressed in the first two phases, contracting the healing tissue and producing collagen. They gradually lose the contractile force by progressively de-differentiating into fibroblasts, which are mainly responsible for collagen production. The stem cells contained in the stromal vascular fraction of the SG may be responsible for an accelerated process, where the tissue healing rapidly progress to the last phase, with a low number of myofibroblast and a high number of Ki67+ proliferating fibroblasts massively producing collagen. The increases in Ki67+ and collagen deposition were also statistically significant compared to those of the control group at 30 days follow-up.

SG and PAHA resulted in higher average wound healing reduction (40.78% and 43.82%, respectively) than PD (23.53%), although the difference was not statistically significant. SG showed a significantly lower increase in neo-angiogenesis and α-SMA positive cells (myofibroblasts), compared to PD, with higher collagen deposition compared to PAHA. Taken together, such results support the above cited theory of an accelerated regenerative process by SG. Specifically, the inflammatory and early proliferative phases, typically characterised by neo-angiogenesis and myofibroblast proliferation, are reduced in duration and/or intensity, rapidly yielding the way to the remodelling phase. In future studies, it would be interesting to set an intermediate time-point, closer to the treatment time, in order to assess such a biological kinetics.

The Stem Graft proved to be a simple, cheap, reliable and effective technique to address chronic non-healing wounds. The clinical efficacy was comparable to those of dermal substitute (porcine dermis) and synthetic product (PAHA). However, SG is autologous, thus preventing possible allogenic or allergic reaction. Moreover, it's cheaper, as only routinely used surgical equipment is needed for its production. The possible use of SG in the clinical setting is also supported by a potential antibiotic role. Adipocytes extracts were found to inhibit Staphylococcus Aureus and Pseudomonas Aeruginosa

growths by mean of the antimicrobial peptide cathelicidin.[35,36] Interestingly, cathelicidin production initially increased in response to infection, then declined as the adipocytes matured, leading to the deduction that ADSCs or pre-adipocytes were responsible for its production. As a consequence, the SG could be extremely useful in infected non-healing wounds.

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