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EFFETTI BIOLOGICI DELLE FORZE MECCANICHE ESTERNE SUI TESSUTI MOLLI: OTTIMIZZAZIONE PRECLINICA PER L'APPLICAZIONE TRANSLAZIONALE IN CHIRURGIA RIGENERATIVA

(BIOLOGICAL EFFECTS OF EXTERNAL MECHANICAL FORCES ON SOFT TISSUES: PRECLINICAL OPTIMIZATION FOR TRANSLATIONAL APPLICATION IN REGENERATIVE SURGERY)

Coordinatore: Ch.mo Prof. Stefano Piccolo
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ABSTRACT

In reconstructive surgery, tissues are routinely transferred to repair a defect caused by trauma, cancer, chronic diseases, or congenital malformations. Surgical transfer intrinsically impairs metabolic supply to tissues placing a risk for ischemic complications such as necrosis, impaired healing, or infection. Pre-surgical induction of angiogenesis in tissues (preconditioning) limits ischemic complications and improves outcomes but very few preconditioning strategies have successfully been translated to clinical practice.

The first goal of our research was to improve current standard of care in reconstructive surgery by developing a translational technique that can effectively and safely increase the vascularization of soft tissues. To achieve this goal, we optimized, using preclinical animal models resembling clinical needs and scenarios in a controlled setting, a method that adopts non-invasive external suction (External Volume Expansion, EVE) to precondition tissues through the induction of hypoxia-mediated angiogenesis. Using a sequential approach in a rodent model we determined the parameters of application (frequency, suction levels, duration, and interfaces) that fine-tune the balance of enhanced angiogenesis, attenuation of hypoxic tissue damage, and length of treatment. The optimized parameters of application (short, cyclical stimulations at moderate suction) almost doubled tissue vascular density after only 5 days of treatment. Our outcomes also showed that the use of micro-deformational interfaces of treatment retain the biological effectiveness of EVE while further reducing the cutaneous damage by distributing forces across the stimulated tissue. Our model confirmed that the optimized technique significantly improves the survival of transferred soft tissues (+20-30%), such as adipose tissue grafts, and can achieve the same beneficial outcomes in animal models of pathologic cutaneous vascularization, such as the one occurring in the skin of patients affected type-2 diabetes. We assessed that EVE retains a beneficial effect on the vascularization and proliferation (adipogenesis) of soft tissues when used both as a pre-conditioning method (before surgeries) and as a post-conditioning method (after surgeries) As a second goal of our research we integrated the knowledge on the application of EVE on soft tissues, to the use of a shelf-ready, bio-mimetic, decellularized allograft adipose matrix (AAM) with the aim of developing an innovative and minimally-invasive strategy for in vivo regeneration of soft tissues. In an animal model we tested the potential of a human-derived, injectable AAM to regenerate soft tissues when used in combination with EVE. This strategy significantly improved long-term volume retention (50-80% higher) and histological quality of reconstructed tissues compared to current standard of care (adipose grafts). The AAM induced both adipogenesis and angiogenesis. Combined use of the AAM and adipose grafts mitigated efficacy.

Our studies suggest that EVE can improve the outcomes of reconstructive surgeries by safely and promptly enhance vascularity of soft tissues, in addition to its edema-/mechanically-induced adipogenic effect (confirmed by our study). EVE's use with an AAM, instead, can synergistically and effectively induce in vivo soft tissue regeneration. These translational principles are ready to be translated to clinical trials and, if outcomes will be confirmed, they could establish the basis for a novel therapeutic paradigm in reconstructive and regenerative surgery for the benefit of a large number of patients.

SOMMARIO

La chirurgia ricostruttiva si basa sul trasferimento di tessuti da un distretto corporeo ad un altro al fine di riparare un difetto tissutale causato da un trauma, un tumore, una malattia cronica, o una malformaizoen congenita. Questo trasferimento chirurgico compromette la vascolarizzazione (e quindi il support metabolico) dei tessuti trasferiti, mettendoli a rischio per complicanze ischemiche quali la necrosi, laguarigione inefficace delle ferite, o la sovrainfezione batterica. L'induzione di fenomeni angiogenici nei tessuti prima della chirurgia (pre-condizionamento) limita le complicanze ischemiche e migliora I risultati chirurgici; tuttavia, pochissime strategie di pre-condizionamento sono oggi disponibili nella pratica clinica.
Il primo obiettivo di questa ricerca era di migliorare gli attuali standard in chirurgia ricostruttiva attraverso lo sviluppo di tecniche traslazionali in grado di aumentare la vascolarizzazione dei tessuti in maniera efficace e sicura. Al fine di raggiungere tale obiettivo abbiamo ottimizzato, usando modelli preclinici animali rappresentativi di condizioni cliniche controllate, un metodo che adopera una stimolazione meccanica esterna non invasiva tramite pressione negativa (Espansione Volumetrica Esterna, EVE) per precondizionare I tessuti attraverso l'induzione di fenomeni angiogenici causati da una ischemia transitoria. Tramite questa strategia di ottimizzazione sequenziale in un modello murino abbiamo definite i parametri di trattamento ottimali di EVE (frequenza, livelli di pressione, durata, interfaccia di trattamento) in grado di bilanciare l'induzione di angiogenesis con l'attenuazione del danno ischemico causato ai tessuti, e con la durata di trattamento. L'ottimizzazione di EVE (brevi, cicliche stimulazioni a suzione moderata) ha dimostrato la capacita' di raddoppiare la densita' vascolare dei tessuti stimulati dopo solo 5 giorni di trattamento. I nostri risultati hanno anche dimostrato che l'uso di interfacce di trattamento a micro-deformazione garantisce il mantenitmento degli stessi effetti biologici di EVE ma allo stesso tempo reduce il danno cutaneo causato ai tessuti tramite la distribuzione delle forze meccaniche su tutto il tessuto stimulato. I nostri modelli sperimentali hanno confermato che l'ottimizzazione di EVE permette di aumentare significativamente (+20-30%) la sopravvivenza dei tessuti trasferiti (ad esempio il tessuto adiposo), e che gli stessi effetti possono essere osservati in modelli di vascolarizzazione cutanea patologica (ad esempio la cute di soggetti affetti da diabete di tipo 2). Inoltre, abbiamo confermato che EVE induce la vascolarizzazione e la proliferazione (adipogenesi) dei tessuti molli sia quando utilizzara come metodo di pre-condizionamento (prima della chirurgia) dei tessuti sia quando utilizzata come metodo di post-condizionamento (dopo la chirurgia).
Come secondo obiettivo di questa ricerca abbiamo integrato le conoscenze acquisite sull'applicazione di EVE ai tessuto molli all'uso di una matrice adiposa allogenica (AAM) -ottenuta tramite decellularizzazione di tessuto adipose umano, caratterizzata da proprieta' bio-mimetiche, e realizzata in una formulazione iniettiabile "pronta all'uso"- con lo scopo di sviluppare una strategia innovativa e mini-invasiva per la rigenerazione in vivo di tessuto molli. In un modello animale abbiamo testato il potenziale della AAM di rigenerare i tessuti molli quando utilizzata in combinazione con EVE. Questa strategia ha portato ad un significativo aumento volumetrico (+50-80% a 12 settimane) ed un miglioramento della struttura istologica

dei tessuti molli ricostruiti in comparazione ai risultati ottenuti con le terapie standard attuali (innesti di tessuto adiposo). Abbiamo evidenziato come la AAM sia in grado di indurre sia fenomeni adipogenici che fenomeni angiogenici: l'applicazione combinate di AAM e innesti di tessuto adiposo, invece, mitigano I risultati ottenibili con l'uso esclusivo della AAM.

In conclusion, i nostril studi suggeriscono che EVE e' in grado di migliorare i risultati ottenibili in chirurgia ricostruttiva attraverso un incremento, sicuro e rapido, della vascolarizzazione dei tessuto molli, in aggiunta all'efftto adipogenico (mediato da stimolazione meccanica diretta ed edema dei tessuti) gia descritto nella precedente letteratura e qui confermato dai nostril risultati. L'utilizzo di EVE con l'AAM, invece, puo', efficacemente e sinergisticamente, indurre fenomeni rigenerativi dei tessuto molli in vivo. Questi principi traslazionali sono pronti per essere validati in trial clinici e, qualora I loro risultati venissero confermati, potrebbero porre le basi per lo sviluppo di nuovi paradigm terapeutici in chirurgia ricostruttiva e in chirurgia rigenerativa, per il beneficio di un grande numero di pazienti

INTRODUCTION

The importance of tissue vascularization in reconstructive-regenerative surgery

Delivery of adequate metabolic supply to tissues is a cornerstone of surgery. Increased knowledge of anatomy and physiology has led to the development of more complex surgical procedures [1–4]: however, innovation of these strategies is limited by the need to retain sufficient vascular perfusion to tissues to guarantee their survival [5,6].

Reconstructive surgeons especially rely on vascularization of tissues to avoid complications after their surgical transfer. Tissue transfer (flaps or grafts) is routinely used in reconstructive procedures after oncologic surgery (e.g. breast cancer, melanomas, or head and neck cancer removal), traumas to soft tissues (e.g. burns, road traffic or work-related injuries), consequences of chronic diseases (e.g. chronic wounds caused by diabetes, pressure sores in patients with spinal cord injuries) or congenital malformations. Patients undergoing these procedures are extremely large in number and broad in variety. Inadequate tissue vascularization is the main cause of ischemic surgical complications which represent a significant burden for patients, surgeons, and healthcare systems. Ischemic complications lead to suboptimal outcomes, delayed healing of surgical incisions, multiple revision surgeries, infections, prolonged hospitalization, and increased treatment-associated costs [7–9].

Therapeutic innovations to counteract this problem have not fully addressed it. The distance between transferred tissue and capillaries at the recipient site determines the capacity of cells to receive adequate metabolic supply, such as oxygen, through diffusion and to survive. Optimization of this mechanism can be achieved by increasing the vascular density of the recipient site (for grafts) or by increasing the vascular density of transferred tissues (for flaps): this procedure is called “tissue preconditioning”. Very few preconditioning strategies have been successfully used in a clinical setting so far [10–14]. Today, tissue preconditioning is obtained mostly through a “delay” surgery (first described by Tagliacozzi over 500 years ago), an additional surgery performed few weeks before the actual procedure. Delay surgeries partially isolate the tissue to be transferred by interrupting a portion of its vascular supply: this causes an hypoxia-driven angiogenesis and an increase in the tissue vascular density later resulting in improved tissue survival and surgical outcomes after its transfer [15]. Extensive research has explored the potential of less invasive and more effective preconditioning strategies using stem cell, drugs, growth factors, gene delivery, hyperbaric oxygen, electrical stimulation, and other methods. Almost none of these approaches however has been successfully translated to clinical practice due to their invasiveness, complexity and unease of application, suboptimal effects, costs and challenging regulatory requirements.

The therapeutic potential of mechanical forces

The angiogenic potential of mechanical forces offers a unique opportunity to challenge this unmet need [16–31]. Mechanical stimulation has shown a capacity to induce angiogenesis in soft tissues leading to the development of novel therapies, especially for wound healing [32–34]. Non-invasive external suction (External Volume Expansion, EVE) has also shown a potential to precondition tissues before grafting procedures and improve post-operative graft survival: it has been proposed that these outcomes might be mediated by a hypoxia-driven and mechanical stretch-driven angiogenic effect. [20,22,24,25,29–31,35–37]. EVE holds substantial promise to be an easy-to-use, effective, and non-invasive preconditioning method in surgery. In addition, EVE can be delivered using FDA-approved devices in an out-patient setting. Preliminary clinical experience with EVE however has been limited by a suboptimal efficacy, a not negligible rate of complications (skin damage including sustained erythema, blistering and localized ulceration along the interface between the EVE device and skin), and a lack of compliance of patients to prolonged treatments. These limitations originate from the empirical use of EVE that lacks an optimization study aimed to design a clinically effective treatment [20,22,35,36,38,39]: notably, involved biological outcomes (angiogenesis, tissue damage) and non-biological factors (length of treatment) are related to the parameters of application of mechanical forces to tissues. The wide range of parameters and the complexity of theoretical combinations to be considered make the definition of an optimal regimen of treatment a challenging task, particularly in patients. Instead, the adoption of a sequential optimization approach in a controlled pre-clinical model allows for the careful investigation of these phenomena and a more accurate description of the biological basis behind their mechanisms.

We have previously developed a murine model of EVE and confirmed its angiogenic effect [23,25,27]. Several other authors have confirmed these outcomes in both small (rodents, rabbits) and large (swine) animal models [40]. Despite this initial progress, a comprehensive comparative analysis of all clinically-relevant parameters of applications of EVE (frequency, suction, duration, and treatment interface) has not been yet performed. Our group and others have previously demonstrated that mechanical forces can stimulate tissue growth and angiogenesis in preclinical models of wound healing and skin expansion.[19,32,41–43] In both wound healing and skin expansion models, a cyclical-intermittent application of forces achieved a higher induction of angiogenesis compared to a static application. This is related to the induction of a temporary sub-critical ischemia in tissues, triggering a hypoxia-driven angiogenic and vasculogenic response. Longer stimulations may limit effectiveness by partially causing a critical ischemic insult on tissues, leading to cell necrosis, blistering and fibrosis. Overall, waveform modification of dynamic mechanical forces can maximize the proliferative response of tissues and cells compared to continuous static forces.[32,41,44] Clinically, soft tissues sustain viability with ischemic times up to two hours (tourniquet in hand surgery) and when blood flow is restored, a reactive hyperemia develops. We hypothesize that the same phenomenon could apply to EVE.

A particular need for effective pro-angiogenic and pre-conditioning techniques: diabetic patients

In plastic surgery, an already vast, and yet still increasing, number of patients are affected by type-2 diabetes. The Centers for Disease Control and Prevention (CDC) report that 9.4 % of the U.S. population, over 30 million individuals, suffered from diabetes in 2015: in the elderly (>65 years) prevalence of the diseases exceeded 25 %.[45–47] Unfortunately, these numbers are expected to significantly increase in the near future and similar alarming trends have been described worldwide, including in Europe and in south-east Asia such as in China and in India.[48] Statistically, this data suggests that one in ten of all plastic surgeons' patients (or one in four of our elderly patients) might be diabetic; if considering specific sub-categories of patients that are routinely referred to plastic surgeons (e.g. those affected by impaired post-surgical/post-traumatic wound healing or by chronic wounds) this percentage is likely to result even higher.[49–51]

In fact, one of the most common consequences of diabetes is an impairment of the vascularity of soft tissues.[52–54] Several diabetes-induced pathological phenomena (e.g. vascular accumulation of advanced glycation end-products, direct glucose-mediated endothelial damage, increased oxidative stress, impaired vasodilation, endothelial dysfunction, chronic inflammation, and dysregulated coagulation) damage the micro-vascular network of soft tissue leading to a lower vascular density in tissues, a higher sensitivity to ischemia, and a reduced capacity to generate new blood vessels in response to trauma.[52–55] These conditions frequently result in the development of chronic non-healing wounds, surgical complications (e.g. wound dehiscence, higher risk for infections, or complete tissue necrosis/loss), or sub-optimal surgical outcomes (e.g. partial tissue necrosis/loss after surgical transfer or repair).

These evidences highlight the need for plastic surgeons to integrate in existing treatments with novel therapeutic strategies that specifically address the risks and challenges posed by diabetes. Regrettably, such solutions are mostly lacking. The use of non-invasive suction (External Volume Expansion, EVE) has been proposed as a method to increase vascularization of soft tissues and has been empirically adopted as an ancillary technique in adipose tissue grafting with unreliable outcomes.[56,57] Mounting experimental evidence suggests that the adoption of an optimized EVE in clinical care could improve surgical outcomes in reconstructive and aesthetic procedures. EVE's effects on categories of patients at higher-risk for sub-optimal outcomes or surgical complications, such as those affected by diabetes, could be even more beneficial. Yet, the intrinsic differences in ischemia-resistance of diabetic skin might require adjustments to the treatment to reduce the risk of tissue damage while retaining an angiogenic effect.

Soft tissue reconstruction by adipose tissue (fat) grafting

Adipose tissue ("fat") grafting has become an increasingly popular technique among plastic surgeons. The American Society of Plastic Surgeons reports that in 2016 in the United States over 100,000 aesthetic surgical procedures and over 30,000 breast reconstructions have been performed using fat grafting.[58] Given the widespread use of fat grafting as an ancillary procedure to several other reconstructive surgeries it is likely that these numbers underestimate its actual impact on plastic surgery. The popularity of fat grafting for both surgeons and patients derives from the fact that it provides an autologous, biologic, minimally invasive, relatively safe, and cost-effective alternative to other alloplastic-based or complex autologous reconstructive procedures.[59,60]

However, fat grafting has downsides; as a non-vascularized graft, transferred adipose tissue relies on the vascular density (or, more precisely, on the grafted tissue volume-to-vascular density ratio and the maximal diffusion distance for nutrients within the graft volume) of the recipient site to obtain sufficient metabolic support and survive.[61] High-volume tissue transfers limit the diffusion of nutrients from blood vessels to grafted cells, resulting in ischemic necrosis and formation of oil cysts or vacuoles.[62] Revascularization of the graft by angiogenesis requires several days. By then three zones become apparent within the graft: an external "surviving zone" of living cells that had received nutrients through diffusion from nearby capillaries, an intermediate "regenerating zone" of partially ischemic cells that initiate regenerative proliferation, and an inner "necrotizing zone" that becomes fibrotic tissue with oil cysts.[62] As a consequence, the survival (volume retention) of fat grafts at follow-up is usually limited (30-60% of initially grafted volume) and inversely proportional to the amount of tissue grafted. To achieve complete reconstruction of larger soft tissue defects surgeons (and patients) are compelled to multiple procedures with smaller grafts that are spaced-out several months (to allow for re-vascularization of a graft before transferring additional volumes).[63,64]

Several experimental approaches have been proposed to try to overcome the intrinsic limitations of fat grafting.[65,66] Among these, non-invasive external suction (External Volume Expansion, EVE) has been suggested as a method to pre-operatively (pre-conditioning) increase the vascular density (angiogenesis) and the proliferation of adipocytes (adipogenesis) at the recipient site of a subsequent graft. Clinical adoption (BRAVA Breast Enhancement and Shaping System, Brava LLC, Miami, FL, USA) and evidence-based preclinical in small and large animals research has shown that EVE increases the vascular density of tissues by inducing a sub-critical hypoxia that mediates a pro-angiogenic stimulus.[20] This increased vascularity allows a higher metabolic support to fat grafts and improves their survival at follow-up.[26,67–74] Other studies have also confirmed the adipogenic effects of EVE on tissues and the comparable effectiveness of different interface material. These results have provided an encouraging outlook on the use of EVE to improve the surgical outcomes of fat grafting.

Both preclinical and clinical evidence have extensively investigated the effects of EVE on adipogenesis and tissue edema. Importantly, EVE was originally proposed by Khouri et al. in 2000 as a method for breast augmentation without adipose tissue grafting uniquely based on its adipogenic and pro-edema effect.[75] In his beautiful letter, Denkler later traced the actual historical origin of the method up to 1895 and 1897 (Sears Roebuck Catalogues listing a breast vacuum pump system).[76] In the study by Khouri et al. on a case series of 17 female patients treated by means of EVE for 10.2 hours/day over a 10-week period (range 10-18 weeks), breast enlargement was monitored clinically (modified bead displacement technique and Grossman-Roudner device) and by means of by magnetic resonance.[75] According to authors a statistically significant immediate breast size increase (67-98% increase, on average 240ml) was observed with satisfaction by all patients after 10 weeks then gradually stabilizing after a partial decrease to a 15-115% enlargement (average 55%, 103ml +/-35ml) at a 30 weeks follow-up. Magnetic resonance confirmed results and showed proportionate enlargement of both adipose and fibro-glandular tissue components in absence of any pathologic signs, consistently with histological analysis. Authors provide their hypothesis on mechanism of action discussing bio-engineering principles and the role of stretch-induced tissue growth (Shear Forces, Pressure Distribution). Interestingly, the article highlights the role of “water tissue content” and of “reversible elastic deformation and extracellular fluid accumulation” as well as of the need for stimulation “continuous and sustained over a prolonged period of time”: these remarks are consistent with current preclinical evidences although authors exclude any role for “significant tissue inflammation”. Schlenz et al. treated 50 women with EVE for on average 11 hours a day for a median period of 18.5weeks (range: 14-52 weeks). Authors reported a median volume increase measured of 155ml (range: 95-300ml) and a chest circumference increase by a median of 4.4cm (range: 1-11 cm): volume expansion was measured 4 weeks after the end of the treatment remained constant until the latest follow-up.[77] In 2012 Khouri et al. published another work on EVE: in this prospective multicenter not-randomized cases series breast augmentation was achieved by fat grafting in breasts pre-treated/ post-treated by EVE.[78] According to authors their protocol provided a statistically significant two-fold mean augmentation volume at 12 months (233ml per breast, range: 60-619 ml) compared to fat grafting alone, supporting also a higher rate of fat survival (30% higher, $p<0.00001$) with reduced fat necrosis. Authors speculated that efficacy of the procedure would rely in the ability of EVE to increase fibro-adipose interstitial space, to promote angiogenesis and cell proliferation. Interestingly, Khouri et al. also state that stimulation through EVE alone would have limited breast enlargement to “a modest augmentation in the 100- to 150-ml range”. These clinical evidences are consistent with those observed in preclinical studies on animal models. In the study by Heit et al. on a murine model of EVE, authors observed local swelling after 21 of stimulation.[23] In addition, microscopic analysis of stimulated tissue revealed a statistically significant increase of the subcutaneous adipose layer and of the rate of proliferating cells, all up to two folds compared to controls. According to the authors, described biologic effects were induced by mechanical forces (tension, compression, shear, interstitial pressure) directly and indirectly by an edema-driven adipogenic stimuli mediated by hypoxia and inflammation. In a second

study by the same group, the same animal model was used to more closely investigate stimulation of adipogenesis by EVE.[79] These data confirmed the role of inflammation and edema in stimulating adipose tissue growth and remodeling by means. Other investigators have later provided similar evidence of the effects of mechanical forces on adipose tissue, showing how mechanical forces affect adipose tissue physiology and different stimuli provide varying effects.[67,80–84] In particular, it has been reported that compressive forces cause adipose atrophy whereas an expanding force (e.g. suction) stimulates adipogenesis, also by formation of tissue edema. These evidences are consistent with the results observed in previous clinical studies and in more preclinical recent research on EVE, including the one here described. In addition, static forces more effectively impact the physiology of adipose tissue as compared to cyclical stimulations.[80,81]

Acting through similar mechanisms, the post-operative use of EVE (post-conditioning) has also been postulated to improve outcomes in fat grafting. Yet, the sub-critical ischemic stimuli that allow EVE to trigger angiogenesis in well-vascularized tissues (pre-conditioning) might have deleterious results when applied to non-vascularized grafted tissues (post-conditioning), paradoxically causing further damage to tissues. This uncertainty highlights the need for evidence-based guidelines. Despite post-conditioning of adipose tissue grafts with EVE has been empirically adopted in clinical practice, no controlled research has examined its actual effects on transferred tissues and whether it might improve graft survival.[22,85] Extensive research on flaps and several other experimental models (e.g. myocardium) has shown that post-conditioning can moderately improve tissue survival; [86,87] yet, no post-conditioning method (other than EVE) has been used to improve outcomes in fat grafting in a clinical or preclinical setting. A better understanding of the effects that post-conditioning with EVE has on fat grafts could highlight whether these effects can improve graft survival, and determine if they could replace those achieved by pre-conditioning with EVE (in all those cases in which it is not possible to perform EVE before the surgery) or act synergistically to further enhance graft survival at follow-up.

Soft tissues restoration: from *Reconstructive Surgery* to *Regenerative Surgery*

Loss or impairment of soft tissues represents an extremely common consequence of traumatic injuries, surgery, chronic disease, or congenital malformations.[51,88–93] This broad spectrum of conditions includes very diverse scenarios ranging from the damage caused by trauma (e.g. workplace-related injuries, motor-vehicle accidents, battlefield injuries, or burns), the therapeutic (or prophylactic) removal of pathologic tissues in oncologic surgery (e.g. mastectomies or lumpectomies in oncologic breast surgery, tumor excisions in soft tissue sarcomas, or resection of tumor margins for epithelial cancers of the skin or the head and neck region), the effects of radiation therapy or radiation injury, the onset of infections and acute conditions (e.g. necrotizing fasciitis, septic shock, ischemia-reperfusion injury, or compartment syndrome), the presence of chronic disorders (e.g. severe diabetic wounds, deep pressure ulcers, or scleroderma and other auto-immune diseases), congenital malformations (e.g. Parry-Romberg syndrome, Poland syndrome, or other lipodystrophies), and many others.[51,88–93] In wider terms, "*soft tissues*" refers to different connective structures, mostly adipose tissue and skeletal muscles but also more specialized tissues including tendons, fascia, nerves, and blood vessels. We here refer to and focus our attention on adipose tissue and skeletal muscles since they are most frequently affected by disease or trauma and since clinical strategies to reconstruct defects of these tissues are either absent (skeletal muscles) or with limited effectiveness (adipose tissue).[64,94–96] Other than the morbidity related to morphological (volume and shape) alternations and disfigurement of body areas which affect patients' self or social psychological well-being, loss of these soft tissue is also frequently associated with severe functional disability. Soft tissues hold a relevant role in human physiology: they protect other sensitive structures (e.g. blood vessels, nerves) from direct damage, contribute to body thermal regulation, help provide structural support and musculoskeletal balance, and participate in the regulation of systemic metabolism.

The vast reach and substantial clinical impact that soft tissue defects have clearly highlights the significance of developing effective therapeutic solutions for patients. Despite intense research efforts to day no therapy has shown the capacity to reconstruct or functionally regenerate lost skeletal muscles in a preclinical or clinical setting:[94,95] as a consequence, skeletal muscle injuries with loss of volume are commonly left unrepaired or treated as an adipose tissue loss with the goal of restoring at least the original tissue shape/volume and its non-contractile functions. Instead, although adipose tissue defects can be reconstructed with several strategies, none of these is free from drawbacks. Smaller volumetric defects can be restored by grafting (injection) of autologous adipose tissue obtained through a liposuction: advantages of this technique include its minimal invasiveness, its relative simplicity, and the superior outcomes associated with the use of a biological, immune-compatible tissue.[60,97] In presence of larger defects, however, adipose tissue grafting often provides inadequate outcomes. As described above, immediately after surgery grafted tissue mostly survives on the diffusion of metabolites from the capillaries of the recipient site: when the volume of grafted tissue exceeds the capacity of metabolites to promptly diffuse to its core, cells contained in the inner portion of the graft become ischemic and undergo

necrosis.[62,64,96] This phenomenon results in the loss of tissue (and volume) at follow-up and the formation of oil cysts from the cell remnants. In order to achieve the final reconstructive goal, surgeons are compelled to either perform multiple grafting procedures or to adopt surgical alternatives that are substantially more invasive (flap transfers: e.g. the microsurgical transfer of an abdominal flap to reconstruct a breast after a mastectomy) or significantly less biologically effective (synthetic implants: e.g. the use of silicone implants).[9,91,98–100]

Substantial research efforts have been undertaken to develop novel strategies that adopted stem cells, growth factors or tissue-engineered scaffolds to improve outcomes in (adipose) soft tissue reconstruction: yet, translational of these approaches to clinical care has been limited by their low efficacy, complex regulatory path (e.g. stem cells and growth factors), and/or lack of scalability (e.g. tissue-engineered scaffolds).[90,101–104] Leveraging previous experience in skin tissue-engineering and regeneration, numerous studies have demonstrated that a promising area of research is the development of bio-mimetic acellular scaffolds that can promote in situ soft tissue regeneration while avoiding the ischemia-related necrosis observed in grafts of living tissues.[90,105–115] Among these, adipose tissue scaffold derived from living sources through decellularization have shown to retain the native structural and bio-chemical characteristics that make them robust inductors of adipogenesis.[90,105–116] Despite demonstrating effectiveness in preclinical models the described decellularized scaffolds have lacked some of the key characteristics needed to transform them in a scalable shelf-ready clinical therapy (e.g. scaffolds derived from surgical discards and treated with complex decellularization procedures). Development of genetically-modified swine might in the future represent a source of donor tissue, yet decellularized scaffolds derived from animal sources today pose the risk for rejection of a xenogeneic extra-cellular matrix (ECM) leading to a foreign-body reaction.

We have previously investigated the optimization of a novel, shelf-ready Allograft Adipose Matrix (AAM) derived from human cadaveric donor tissue through a minimal manipulation decellularization method in compliance with current FDA requirements. We observed that the AAM retains its volume after in vivo grafting, induces angiogenesis in recipient tissues, and improves reconstructive outcomes when combined to adipose tissue grafts. Here, we integrate this evidence to evaluate in an established preclinical model the reconstructive potential of the AAM in comparison to current standard of care (adipose tissue grafts). We postulate that a combine treatment integrating recipient site preconditioning with non-invasive mechanical forces (EVE) and the AAM might synergistically improve the outcomes of the AAM by providing an inductive micro-environment for soft tissue regeneration (migration and proliferation) Through its bio-physical properties and by lacking of ischemia-sensitive living components (cells) the AAM has the potential to retain a higher volume at long-term follow-up and avoid the formation of necrosis-associated cystic areas.

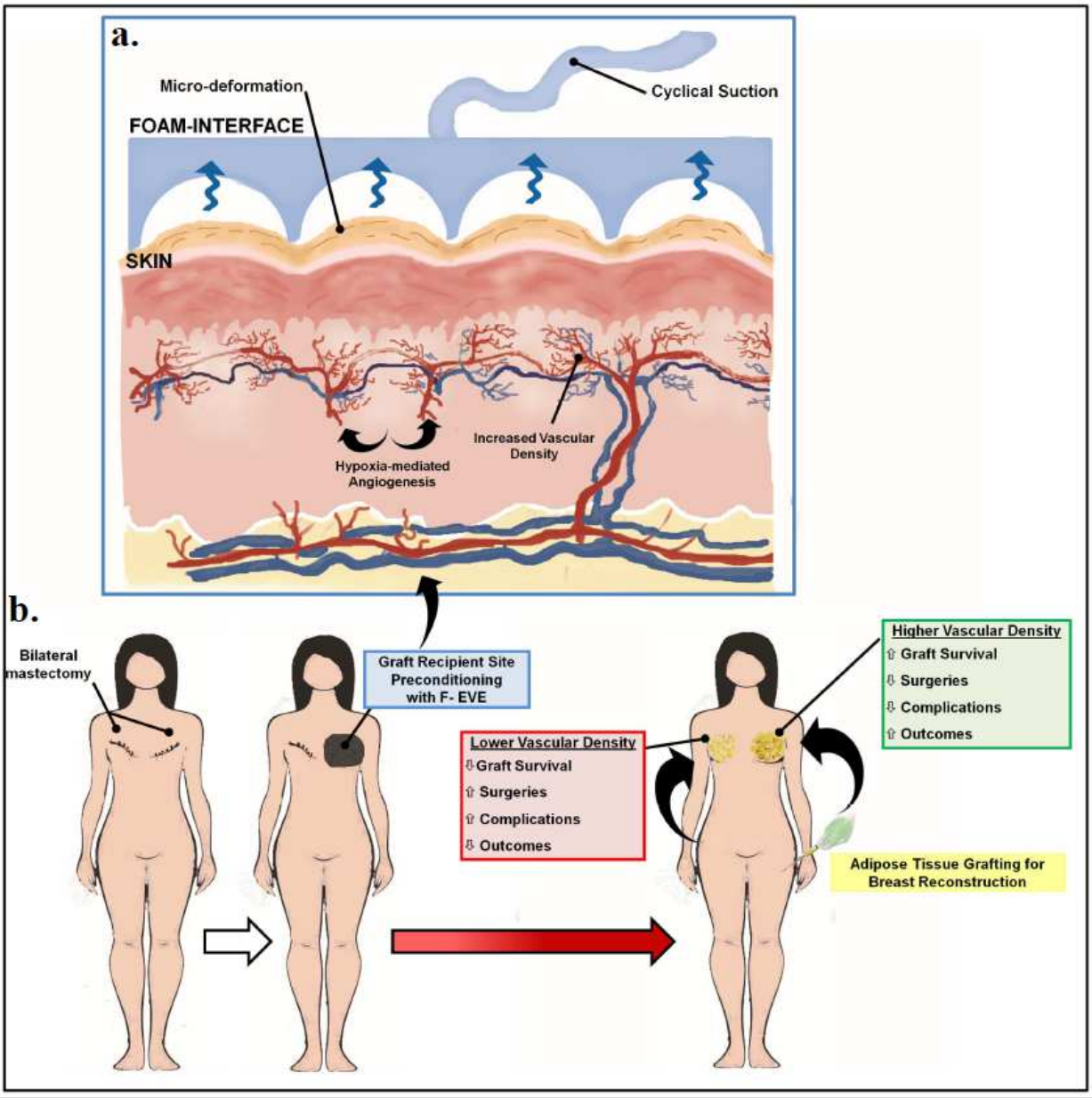
AIM OF THE RESEARCH

The overarching aim of this project is to establish -through preclinical research- evidence-based foundations that can guide the development of novel regenerative therapies in reconstructive surgery that use external, non-invasive, mechanical stimulation of soft tissues. By confirming the translational feasibility of the technique and by providing evidence of its biological effects we aim to support the design and clinical application of effective and safe therapies.

Here, we adopt an established murine model of EVE and design several studies with the goal of better assessing the biologic effects of EVE, optimizing the parameters of treatment that maximize the response elicited in physiological and pathological soft tissue, and integrating its use with biomaterials and tissue-engineered scaffolds.

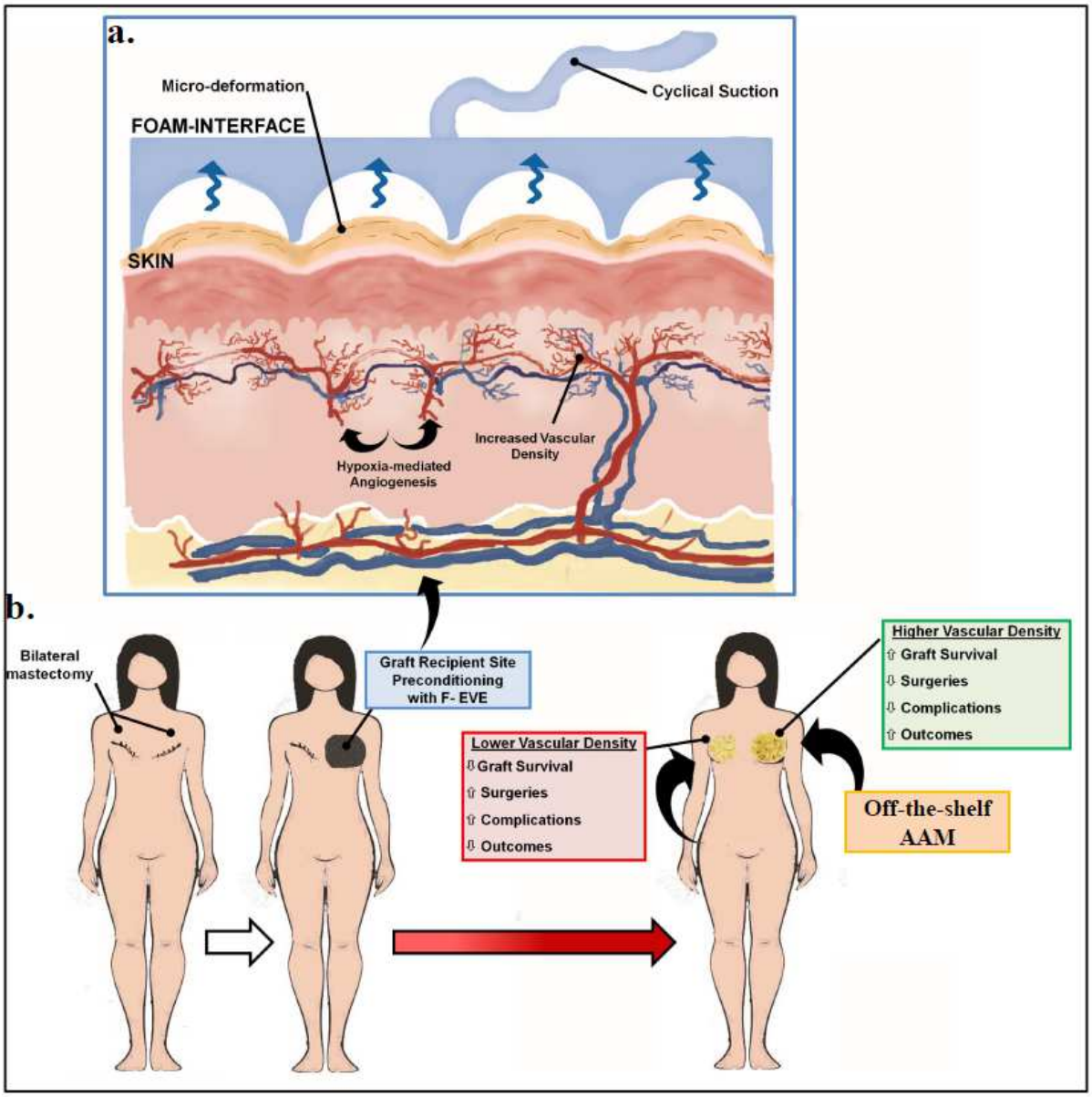
To achieve these goals, we determined:

1) The effects of cyclical-intermittent kinetics of application of EVE on soft tissues;
2) An optimized therapeutic regimen of EVE, defining the biological effect of each parameter of treatment (kinetic/frequency, suction force, duration, and interface) and an ideal therapeutic ratio ($Tr = \frac{Angiogenesis}{Tissue\ damage + Treatment\ length}$);
3) The capacity of the optimized pre-conditioning regimen of EVE to improve in vivo survival of tissue grafts;
4) The effects of an optimized post-conditioning regimen of EVE on the in vivo survival of tissue grafts;
5) The biological effects on pathological (diabetic) soft tissues of an EVE therapy using novel micro-deformational treatment interfaces (a polyurethane Foam-shaped interface – F-EVE - or a silicone Micro-array chamber interface – M-EVE);
6) The potential of a combined therapy integrating EVE and an Allograft Adipose Matrix (AAM) to induce effective and safe in vivo soft tissue regeneration.



Effective soft tissue *reconstruction* using EVE and adipose tissue (fat) grafts

Conceptual representation of non-invasive induction of angiogenesis in tissues (preconditioning) using external suction. **A:** External Volume Expansion (EVE) in a micro-deformational Foam-mediated interface (F-EVE). **B:** Mechanism of increased graft survival in reconstructive surgeries.



Effective soft tissue *regeneration* using EVE and an AAM

Conceptual representation of non-invasive induction of angiogenesis in tissues (preconditioning) using external suction. **A:** External Volume Expansion (EVE) in a micro-deformational Foam-mediated interface (F-EVE). **B:** Mechanism of increased graft (Allograft Adipose Matrix, AAM) recellularization in regenerative surgeries.

STUDY 1:

MODERATE-INTENSITY INTERMITTENT EXTERNAL VOLUME EXPANSION OPTIMIZES THE SOFT TISSUE RESPONSE IN A MURINE MODEL

Summary of the study

Aim: Intermittent External Volume Expansion (EVE) using suction enhances the vascular network of soft tissues possibly increasing fat graft survival. Yet, the optimal kinetics of application have not been determined. Based on our previous experience, we hypothesized that moderate-intensity intermittent EVE application may further enhance both the angiogenic and adipogenic potential.

Materials and Methods: Fifty, twelve-week old, wild-type mice were assigned to five experimental groups (n = 10) and underwent five different intermittent applications of EVE (Single-application Control, Low-intensity, Moderate-intensity and two groups of High-intensity). Five days following the final stimulation, skin biopsies were obtained from stimulated and contra-lateral non-stimulated areas. Microscopic sections were analyzed for angiogenesis, skin remodeling and adipogenesis.

Results: Moderate-intensity intermittent stimulation (0.5 hours, 6 times/day for 5 days at -25 mmHg suction) almost doubled cutaneous vascular density (1.9-fold increase), induced skin thickening (1.9 - fold increase) and expanded the subcutaneous tissue (2.3-fold increase) compared to control. EVE kinetics did not affect tissue inflammation at 5 days after treatment. High-intensity intermittent stimulations also increased density of blood vessels (1.6-fold increase compared to controls) but caused tissue damage, whereas low-intensity EVE did not induce significant changes.

Conclusions: Application of moderate-intensity intermittent EVE optimizes induction of angiogenesis and adipogenesis in soft tissues without tissue damage, holding potential for time-effective recipient site pre-conditioning before fat grafting.

Materials and Methods

Animal Model

We attached to the dorsum, lateral to the spine of animals, a custom-made dome-shaped rubber device with a diameter of 1 cm and an internal volume of 1 ml, connected it to a suction pump (ActiVAC; Kinetic Concepts, Inc., San Antonio, TX) at a suction level of -25 mmHg according to a previously published method (Figure 1a).[23,68]

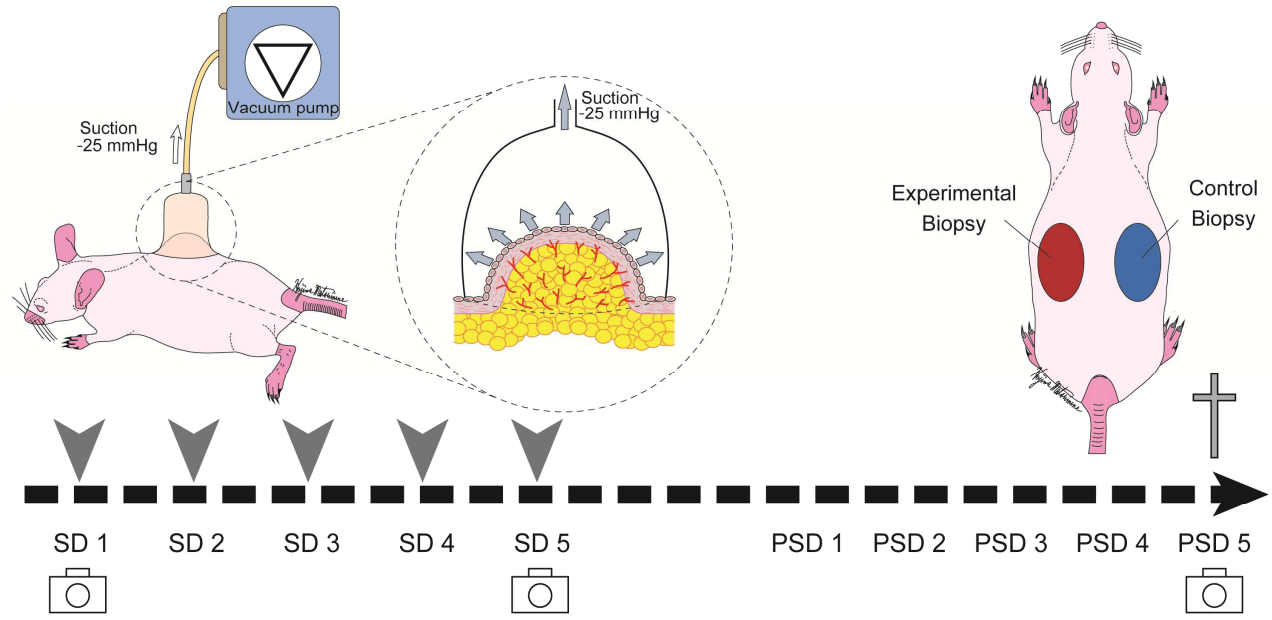


Figure 1 a: Study design. The red-labeled circle indicates the area pre-conditioned with External Volume Expansion (EVE), the blue-labeled circle indicates the internal control with no previous EVE. SD = Stimulation Day; PSD = Post-Stimulation Day; ▼= EVE (Single application control, 1-day: only SD1); †= Procurement of Samples; = Digital Photography

Study Design

Our study involved 50 female, 12-week-old, C57 BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) housed in an Association for Assessment and Accreditation of Laboratory Animal Care–certified facility and in accordance with our Institutional Animal Care and Use Committee guidelines under an approved protocol. Animals were divided into 5 experimental groups (n = 10 per group), pooled in 3 conceptual categories, each undergoing a different protocol of stimulation (Single application control: 1.5 hours, 1 time/day x 1 day; low-intensity: 1.5 hours, 1 time/day x 5 days; moderate-intensity: 0.5 hours, 6 times/day x 5 days; high-intensity: 1.5 hours, 3 times/day or 1.5 hours, 5 times/day both for 5 days) (Figure 1b-c). Mice received stimulation on one side of the dorsum, and contra-lateral not-stimulated areas were used as internal controls (Figure 1a-b-c). After the last day of treatment, we followed-up animals until post-stimulation day (PSD) 5. A single investigator procured full-thickness skin biopsies from stimulated areas

and contra-lateral control areas with a 10-mm biopsy punch. We fixed samples in 10% neutral-buffered formaldehyde for 24 hours and stored it in 70% ethanol at 4°C.

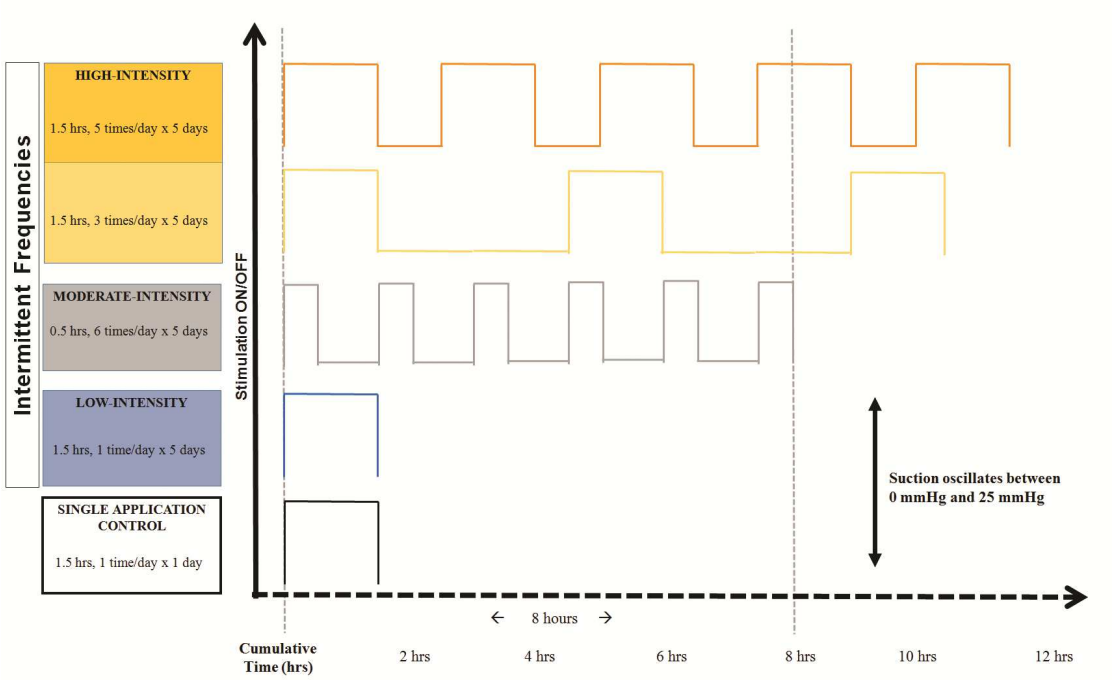


Figure 1 b: Daily kinetic of stimulation for each experimental group. Orange Line: High-intensity EVE. Grey Line: Moderate-intensity EVE. Blue Line: Low-intensity EVE.

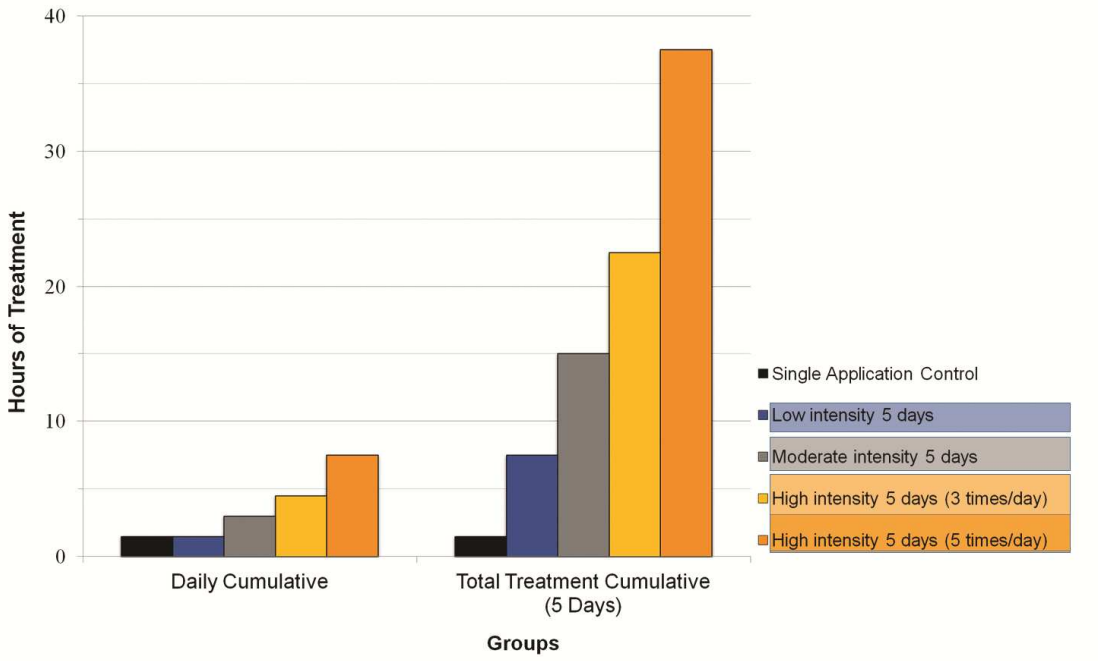


Figure 1 c: Total cumulative hours of stimulation for each experimental group.

Macroscopic Analysis

Three independent observers blinded to the treatment mode compared digital photographs (Nikon Coolpix S4; Nikon Co., Tokyo, Japan) captured on 5 days after the last stimulation with the initial photographs taken at day 0 before the first stimulation. Observers evaluated pictures for cutaneous complications.

Microscopic Analysis

We performed histology (Hematoxylin and Eosin) and immunohistochemistry (endothelial cell marker platelet endothelial cell adhesion molecule-1 CD 31, pan-leukocyte marker CD 45, Perilipin PLIN) of samples according to previously described standard protocols.[23,68] Images were acquired using a Nikon E200 microscope (Nikon Corp., Tokyo, Japan). We quantified the thickness of the entire skin (panniculus carnosus to the outer epidermal surface) and the subcutaneous tissues (panniculus carnosus muscle to the dermis), density of CD 45+ inflammatory cells, density of blood vessels and of adipocytes according to previously established methods. [23,68] For each sample, three fields per staining were evaluated by three independent observers with experience in skin histology and trained in the specific methods used in this study.

Statistical Analysis

We express results as the mean ± SD in text and figures. For inter-group comparison, samples of treated areas were standardized by the matched contra-lateral controls according to our previously published methods, and are expressed as a fold increase over controls. One-way analysis of variance (SPSS Statistics 20, IBM, NY, USA) with Tukey post-hoc correction was used to determine the differences in significance of quantitative immunohistochemistry. A value of $p < 0.05$ was considered statistically significant.

Results

Moderate -intensity EVE Does Not Cause Skin Damage

The low-intensity and moderate-intensity treatments did not cause any major (ulceration) or minor (inflammation, blistering) long-term complication at PSD 5. For high-intensity treatments (> 6 hours a day) we noted minor temporary cutaneous complications immediately after treatment (erythema and blistering), which resolved by PSD 5. (Data not shown)

Moderate-intensity EVE Optimizes Vascular Induction

We quantified the density of CD 31+ blood vessels in skin after EVE as an end-point of angiogenesis. All kinetics of EVE induced an angiogenic response in stimulated tissue but effectiveness significantly varied among groups. Moderate-intensity EVE best enhanced density of the vascular network compared to single application controls (average fold increase over internal control: 1.9 ± 0.3; $p < 0.01$) and high-intensity EVE (average fold increase over internal control for 3 times/day and 5 times/day: 1.5 ± 0.3 and 1.6 ± 0.2 respectively; $p < 0.01$) (Figure 2 a-b, Supplemental Table 1). Low-intensity EVE did not demonstrate a statistically significant increase over single application and internal controls. Histology showed that endothelial vessels have similar morphology in all groups (Figure 2b).

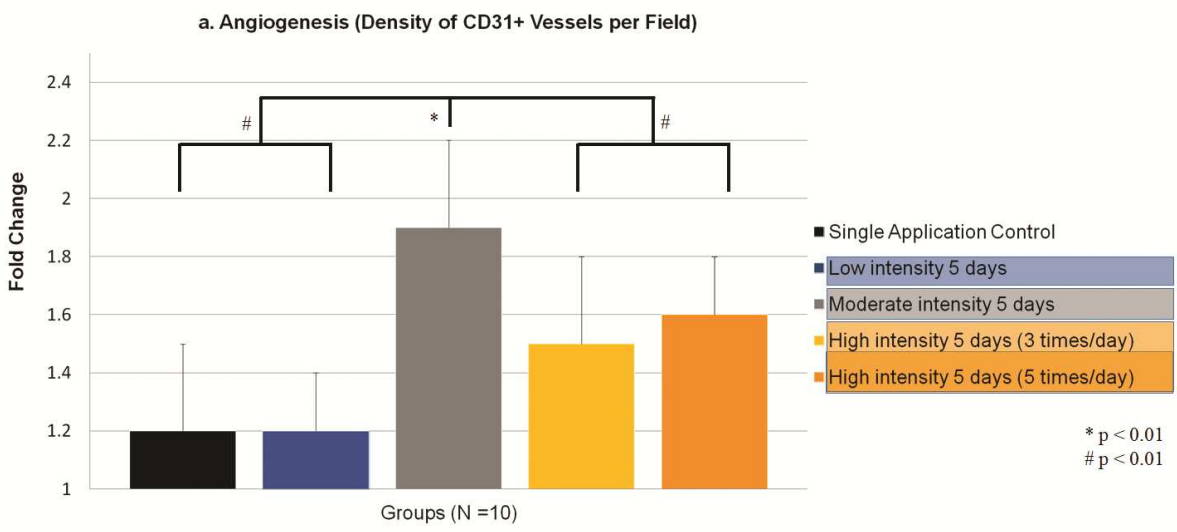


Figure 2: Angiogenic effect of External Volume Expansion (EVE). **a:** Outcomes of measurement on histological images. Single application control, Low-intensity EVE (5 days), Moderate-intensity EVE, High-intensity EVE.

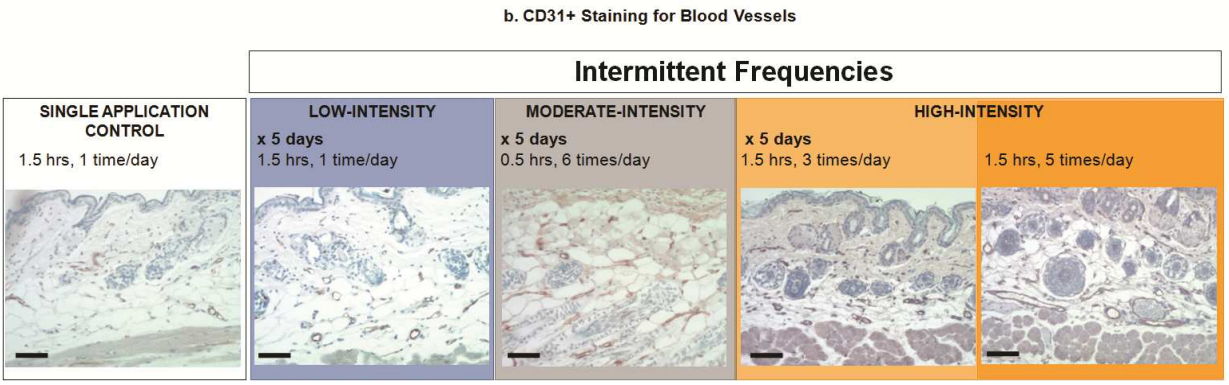


Figure 2: Angiogenic effect of External Volume Expansion (EVE). **b:** CD31 staining for blood vessels. (Magnification: 10X; Reference bar = 250 µm)

Measurement (fold change)	Internal Control	Single Application Control	Low-intensity 5-days	Moderate-intensity 5-days	High-intensity 5-days (3 times/day)	High-intensity 5-days (5 times/day)	P-value Moderate vs. Low/High-intensities [vs. Internal Control]
Daily Hours of Stimulation	0	1.5	1.5	3	4.5	7.5	n/a
Cumulative Hours of Stimulation (5 days)	0	1.5	7.5	15	22.5	37.5	n/a
Angiogenesis (CD31+ blood vessels)	n/a	1.2 +/- 0.3	1.2 +/- 0.2	1.9 +/- 0.3	1.5 +/- 0.3	[1.6 +/- 0.2]	p < 0.01 [p < 0.01]
Skin Thickness	n/a	1.2 +/- 0.3	1.3 +/- 0.2	1.9 +/- 0.3	1.3 +/- 0.3	1.4 +/- 0.2	p < 0.01 [p < 0.01]
Subcutaneous Tissue Thickness	n/a	1.0 +/- 0.3	1.5 +/- 0.2	2.3 +/- 0.3	[1.7 +/- 0.3]	1.5 +/- 0.2	p < 0.01 [p < 0.01]
Adipogenesis (PLIN+ cells)	n/a	1.6 +/- 0.3	1.6 +/- 0.2	1.8 +/- 0.3	[1.7 +/- 0.3]	1.9 +/- 0.2	p > 0.05 [p < 0.01]
Inflammation (CD45+ cells)	n/a	1.3 +/- 0.3	1.2 +/- 0.2	1.5 +/- 0.3	[1.2 +/- 0.3]	1.1 +/- 0.2	p > 0.05 [p > 0.05]

Supplemental Table 1: Summary of experimental data for angiogenesis, adipogenesis and skin remodeling, inflammation in all groups.

Moderate-intensity EVE Optimizes Structural Remodeling of Skin

EVE affected the histological structure of stimulated skin as seen at PSD 5. Moderate-intensity EVE increased skin thickness compared to single application controls (average fold increase over internal control: 1.9 ± 0.4; $p < 0.01$). High-intensity or low-intensity EVE collectively showed a less pronounced effect in comparison to single application control (Figure 3-4a, Supplemental Table 1). In particular, suction affected the thickness of the subcutaneous layer (Figure 3-4b, Supplemental Table 1). Moderate-intensity EVE induced a two-fold thickening of the subcutaneous tissue over single application controls (average fold increase over internal control: 2.2 ± 0.4; $p < 0.01$). This result was 1.5 times higher than both high-intensity and low-intensity stimulations (Figure 3-4b, Supplemental Table 1).

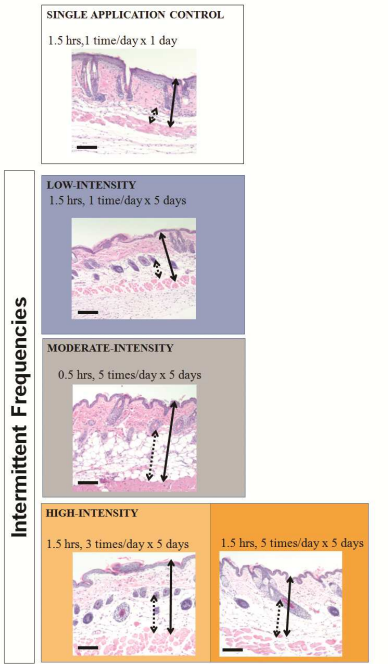


Figure 3: Histological hematoxylin and eosin staining of skin showing differences in overall thickness and differences in thickness of the subcutaneous tissue. (Magnification: 10X; Reference bar = 250 µm).

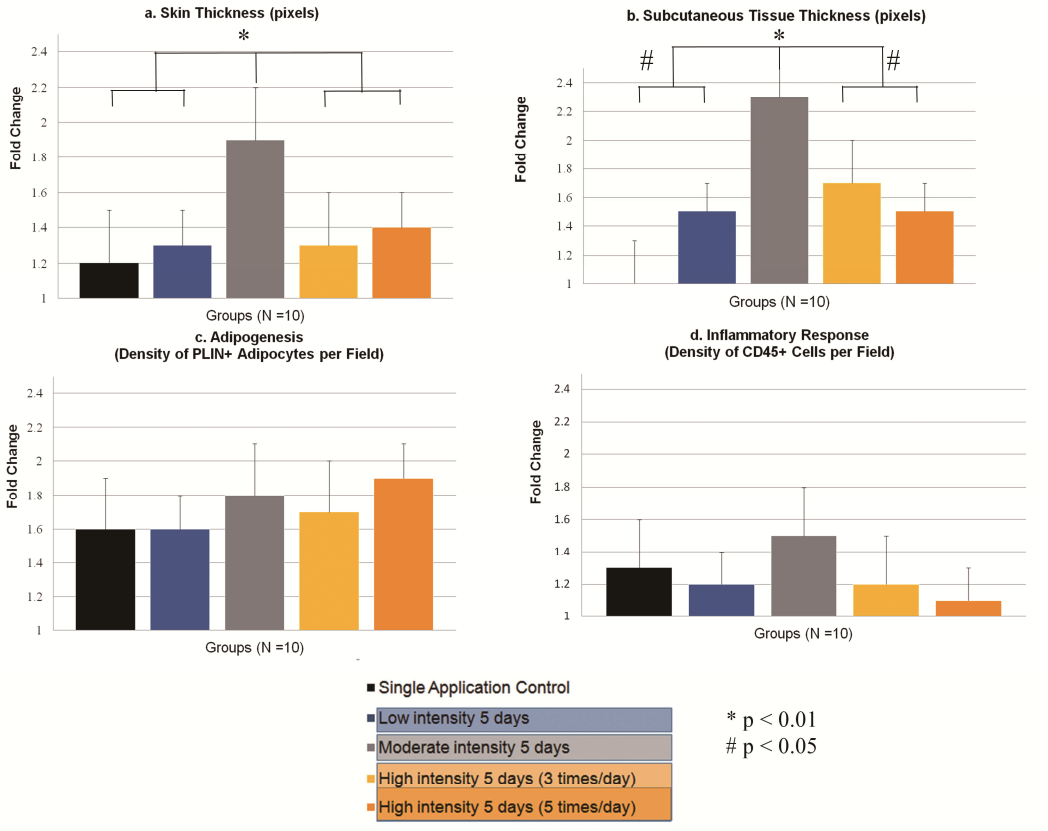


Figure 4: Outcomes of measurement on histological images. **a:** Skin thickness **b:** Subcutaneous tissue thickness. **c:** Perilipin staining for adipocytes. **d:** CD45 staining for inflammatory infiltrate. Single application control, Low-intensity EVE, Moderate-intensity EVE, High-intensity EVE.

Moderate-intensity EVE Effectively Induces Adipocyte Proliferation

The number of Perilipin+ adipocytes after EVE compared to internal controls increased from 1.6-fold to over 1.9-fold (Figure 4c, Supplemental Table 1). Moderate-intensity EVE more intensively increased the number of adipocytes (average fold increase over internal control: 1.8 ± 0.3; $p < 0.01$); High-intensity EVE increased this ratio (average fold increase over internal control: 1.9 ± 0.2; $p < 0.01$) (Figure 4c, Supplemental Table 1) but no significant differences between the groups and single application controls were found.

Kinetics of EVE Do Not Affect Tissue Inflammation in a Medium-term Follow Up

All experimental groups showed no difference in inflammatory CD 45+ cell density in stimulated tissue at PSD 5 (Figure 4d, Supplemental Table 1).

Discussion

Few methods to induce angiogenesis of soft tissues in reconstructive surgery have been successfully translated from research into clinical practice.[12,117–119] Mechanical stimulation provides a device-based approach that has led to the development of many innovative techniques, such as negative pressure therapy.[19,21,32,41–43] In this study we show that moderate-intensity intermittent EVE can induce new blood vessels that persist over a short-term follow up (5 days) that can resemble the time lag between preconditioning and graft surgery. Moderate-intensity intermittent kinetics likely allow the tissue to recover and remodel after a sub-critical ischemic stimulation, maximizing angiogenic drive. In particular, we observed that 5 days of moderate-intensity EVE, for only 3 hours of daily treatment, achieved the same outcome (1.9-fold increase in vascularity) as 28 days of continuous stimulation (shown in previous studies).[23] The results of our study suggest that moderate-intensity EVE can potentially reduce duration of treatment (from 3-4 weeks, as described in current clinical literature, to as little as 5 days) and the daily hours of application (from the current 6-10 hours to only 3 hours) and still achieve the same quality of recipient site preparation.[20,22,78] Moderate-intensity stimulation minimizes the likelihood of cutaneous complications, such as inflammation, blistering or ulceration, which have substantially limited the compliance of patients to treatment. [20,22,78,120,121] Furthermore, our study shows that EVE has the potential for a mild adipogenic effect in the subcutaneous tissue, confirming findings we recently described.[122] Since we analyzed samples at a five day follow up and not at shorter time periods after EVE (as in previous studies), we measured the consequences of the proliferative effect of EVE on endothelia (angiogenesis) and adipocytes (adipogenesis) by quantifying their differential density compared to controls.[23]

In summary, our animal model shows that intermittent moderate intensity EVE (0.5 hours, for 6 applications over 5 days at -25 mmHg suction) optimizes the angiogenic potential compared to high-intensity and low-intensity stimulations. Other parameters, such as the duration of treatment or the applied pressure generated from suction will need to be studied further and these findings will also need to be further confirmed in humans.

STUDY 2:

NON-INVASIVE INDUCTION OF ANGIOGENESIS IN TISSUES BY EXTERNAL SUCTION: SEQUENTIAL OPTIMIZATION FOR USE IN RECONSTRUCTIVE SURGERY

Summary of the study

Aim: Pre-surgical induction of angiogenesis in tissues (preconditioning) can limit post-surgical ischemic complications and improve outcomes but very few preconditioning strategies have successfully been translated to clinical practice due to the invasiveness of most proposed approaches, their suboptimal effects, and their challenging regulatory approval. We optimized a method that adopts non-invasive external suction to precondition tissues through the induction of hypoxia-mediated angiogenesis.

Materials and Methods: Using FDA-approved devices and a sequential approach in a rodent model we determined the parameters of application (frequency, suction levels, duration, and interfaces) that fine-tune the balance of enhanced angiogenesis, attenuation of hypoxic tissue damage, and length of treatment.

Results: The optimized repeated short-intermittent applications of intermediate suction induced a 1.7-fold increase in tissue vascular density after only 5 days of treatment ($p < 0.05$); foam-interfaces showed the same effectiveness and caused less complications. In a second separate experiment, our model showed that the optimized technique significantly improves survival of transferred tissues.

Conclusions: Here we demonstrate that non-invasive external suction can successfully, safely and promptly enhance vascularity of soft tissues: these translational principles can help design effective preconditioning strategies, transform best clinical practice in surgery and improve patient outcomes.

Materials and Methods

Research objective

The primary objective of this study was to optimize the therapeutic regimen of EVE in order to determine an effective balance of highest tissue angiogenesis to treatment time, lowest rate of complications, and shortest duration of treatment. We designed a sequentially optimization study to factor and ponder each parameter of treatment associated with these outcomes (frequency, suction, duration, and interface type). Our overarching goal was to determine translational biological principles that could help design clinically-relevant effective treatments.

Our secondary objective was to validate the in vivo effectiveness of our approach in a preclinical animal model of tissue transfer (adipose tissue grafting) closely resembling reconstructive surgeries routinely performed in a clinical setting. By doing so we aimed to confirm the feasibility of the strategy and support a safe and effective transition to its use for patient care.

The primary hypothesis of the study was that moderate cyclical-intermittent EVE with intermediate suction forces would have best enhanced angiogenesis while limiting tissue injury and complications: this outcome would relate to the induction of an angiogenic response by a sub-critical and sustainable ischemic stimulus. We also hypothesized that soft foam-shaped would further reduce the rate of complications (while retaining the pro-angiogenic effect) by harmful distributing compressing forces over the entire surface of the stimulated tissue.

Our secondary hypothesis was that the increased vascular density of tissues induced by EVE would have provided higher metabolic support to transferred tissues (grafts), increasing their survival at the follow up.

Animal model

Experimental animals were used under an approved protocol, in accordance with our Institutional Animal Care and Use Committee guidelines, and adhering to the NIH Guide for the Care and Use of Laboratory Animals. All experiments were performed in a clean environment inside the accredited animal facility of our Institution. Experiments were conducted at the same time of the day for all different groups. Animals were housed in pathogen-free facilities individually providing an enriched environment and standard bedding: animals had access to food and water ad libitum. Welfare of animals was monitored daily during experiments. We modified our previously described murine model of EVE [23–25,27,28]. Briefly, under mild anesthesia with isoflurane (induction 3%, maintenance 2%) we applied to the shaved dorsal skin of animals a dome-shaped silicone cup with an internal diameter of 1 cm. The device was applied in a standardized position, at the midline of a line going from the proximal portion of the neck to the proximal origin of the tail, 1 cm laterally to the spine. The cup was then connected to a suction pump (ActiVAC; Kinetic Concepts Inc., San Antonio, TX) through a pressure regulator and once proper sealing was assessed mice were promptly recovered from anesthesia. Mice were stimulated on the left dorsal side: during stimulation, no anesthesia is required and animals are free to move in their cages. For the F-EVE

group, we used as interface material a 1.0 cm^3 circular polyurethane foam with an interfacial layer (Prevena™ foam dressing; Kinetic Concepts Inc., San Antonio, TX) (Fig. 2c). Sealing was achieved using a transparent commercially-available adhesive dressing (Tegaderm™, 3M™, Maplewood, MN) placed above the foam. A silicone tube connected the foam to the pump passing through the sealing dressing.

Experimental design – Part 1: Optimization of EVE

The initial study involved 240 (n = 15 per group) female, 12-week old wild-type mice (C57BL/6J, Jackson Laboratories, Bar Harbor, ME). For sequential optimization of the treatment we designed an adaptive study involving four phases (Fig. 2a-b). Phase one aimed to assess the best frequency of stimulation (five experimental groups: Control = no stimulation; Continuous = 24 hours cycle a day; High-intensity = 12 hours cycle a day; High-intensity intermittent = 1.5 hours-long stimulations with 1 hour-long break cycles, 5 cycles each day; Moderate-intensity intermittent = 30 minutes-long stimulations with 1 hour-long break cycles, 6 cycles each day). Phase two aimed to optimize the suction levels applied and had four experimental groups (15 mmHg, 25 mmHg, 50 mmHg and 75 mmHg). Phase three aimed to assess the best duration of treatment (four experimental groups: 1 day; 5 days; 2 weeks; 4 weeks). In Phase four we used our best outcome from previous experimental phases to compare two different interfaces of EVE (EVE vs. F-EVE group) and a control group (sham) (Fig. 2c). In accordance with established methods of sequential optimization, the results of each phase were used to design later phases of the experiments. (Fig. 2a) Investigated variables were selected based on our previous studies and educated estimates of physiologically-relevant ranges of each parameter. Specifically, in order to determine investigated frequency of treatment we considered the known time of skin ischemia-resistance (~60 minutes) [123,124]; to determine investigated suction we considered the known capillary filling pressure (~32 mmHg) [123,124]; and to determine investigated duration we considered the time required for soft tissue preconditioning in surgery (~10-14 days) [11]. In phase one we chose to adopt a 25 mmHg suction level for five days of treatment: in phase two and phase three we sequentially changed parameters of treatment based on the best outcomes from previous phases (respectively: a moderate-intensity intermittent frequency for phase two and a moderate-intensity intermittent frequency at a suction level of 25 mmHg for phase three). On post-stimulation day five (PSD 5) a single investigator obtained full-thickness skin biopsies from stimulated areas with a 10-mm biopsy punch (euthanasia performed using overdose of isoflurane and vital tissue harvest): the size and shape (same as EVE device) of the collected biopsies allowed procurement of the entire stimulated tissue. Samples were cut along their midline in a standardized way to obtain comparable cross-sections (axis perpendicular to the animal's spine). The entire cross-section, which included all different portion of the non-uniform tissue strain field imposed by the EVE device, was analyzed with histology.

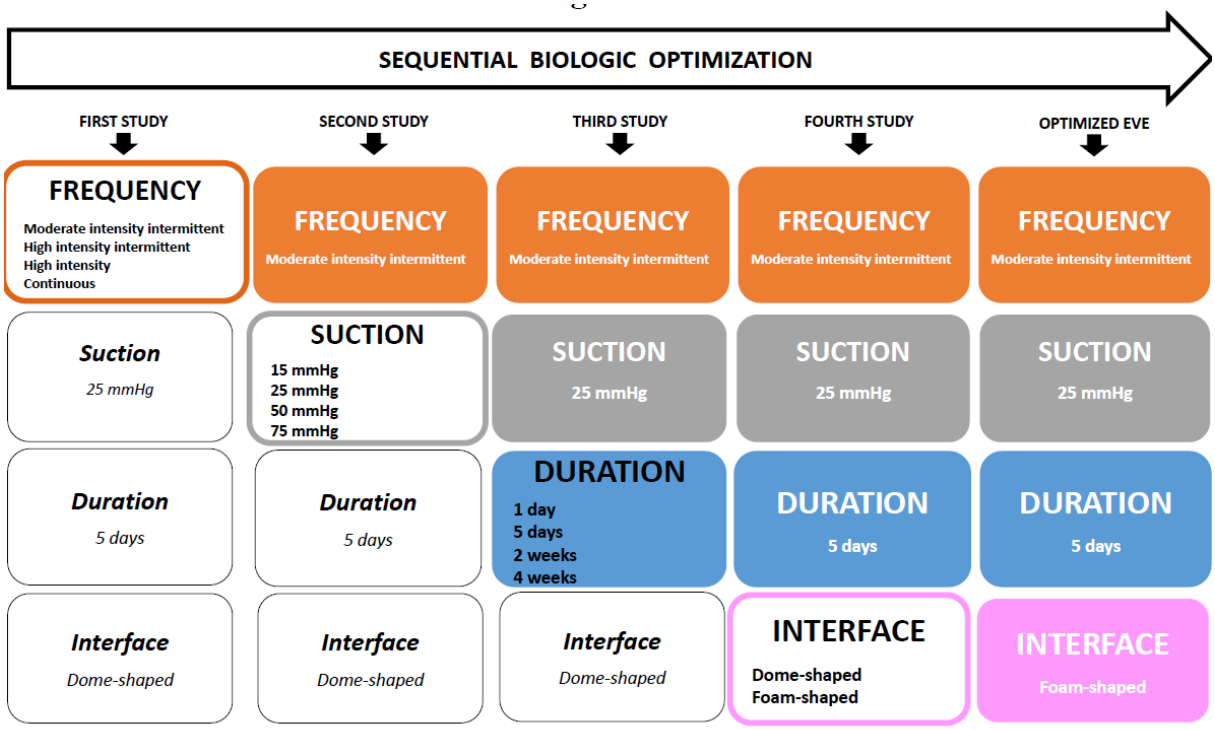
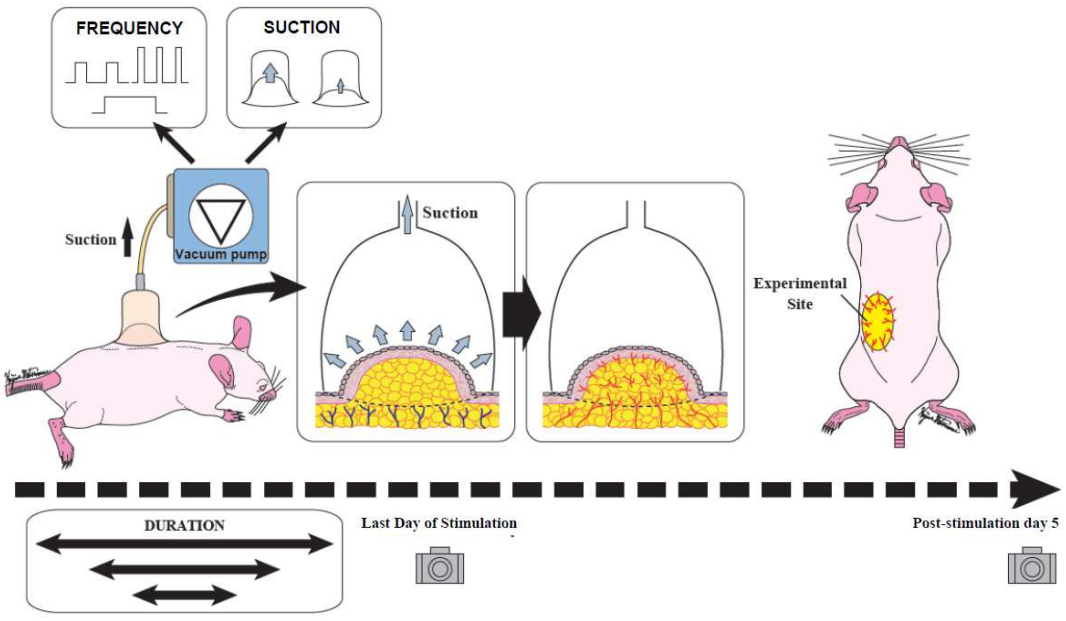
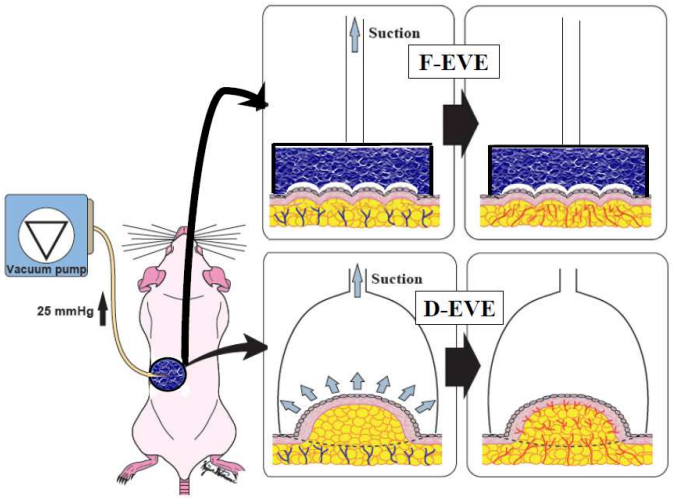


Fig. 2. Experimental study design, procedures and postulated mechanisms of action.

A: Experimental strategy using an adaptive study design with sequential optimization of parameters of treatment. Boxes with colored borders indicate the parameter under investigation in each study; black boxes indicate variables of other parameters yet to be studied but adopted in earlier studies; colored filled boxes indicate confirmed variables.



B: Study design for the optimization of frequency, suction levels and duration of External Volume Expansion (EVE). = Digital Photography



C: Study design for the optimization of treatment interfaces using dome-mediated EVE (EVE) or Foam-mediated EVE (F-EVE). Control animals received no stimulation.

Experimental design – Part 2: EVE preconditioning and adipose tissue grafting

The second study involved 34 female, 12-week old immune-deficient NOD SCID Gamma mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, Jackson Laboratories, Bar Harbor, ME). This mouse strain was selected based on the ability to tolerate xenografts without rejection. In this phase of our study we did not apply F-EVE in order to mimic techniques currently adopted in clinical practice (BRAVA is dome-shaped) and increase the immediate translational value of our findings (foam-shaped interface are not currently used in clinical practice). We obtained fat as lipoaspirate from discarded surgical panniculectomies from two non-smoking, non-diabetic patients with similar demographics (Fig. S1) and processed it according to the standard "Coleman's technique" [60]. Briefly in a clean environment (cabinet hood) and with sterile technique a manual liposuction was performed on the discarded abdominal specimen (subcutaneous adipose layer) to obtain 50 cc of lipoaspirate: this was centrifugated at 3,000 rpm (1,500 × g) for 5 minutes to separate the oil part (removed using sterile absorbent paper). The processed adipose tissue was then loaded in 1 cc syringes for subsequent subcutaneous injection in animals. Animals were randomly divided in two rounds of experiments (two human tissue donors): in each round animals received adipose tissue grafts from the same donor and the lipoaspirate was used fresh within 1 hour from procurement in the operating room (during this time it was preserved in an envelope covered by ice). Use of human tissue was approved by our Institutional Review Board. Animals underwent optimized EVE on the left dorsum (30 minutes-long stimulations with 1 hour-long break cycles, 6 cycles each day for a total of 5 days using a suction level of 25 mmHg). On PSD 5 we collected samples from 6 animals (Baseline) and grafted 2 cc of human fat (1 cc at the stimulated area and 1 cc at the contra-lateral non-stimulated control area) in the others (n = 28) using a previously reported "*tunnel technique*" and a 16 Gauge blunt lipo-injection cannula (Blunt Injector, Marina Medical Instruments Inc., Sunrise, FL) over a

length of 3 cm (Fig. 2d) [125]. On PSD 19 (Medium-term follow-up, n = 14) and PSD 47 (Long-term follow-up, n = 14) grafts were procured *en bloc* using standardized 2 x 2 cm biopsies (including the graft, the overlaying/surrounding full-thickness skin and the panniculus carnosus). Fresh samples were weighted with a precision scale (OHAUS Corporation, NJ).

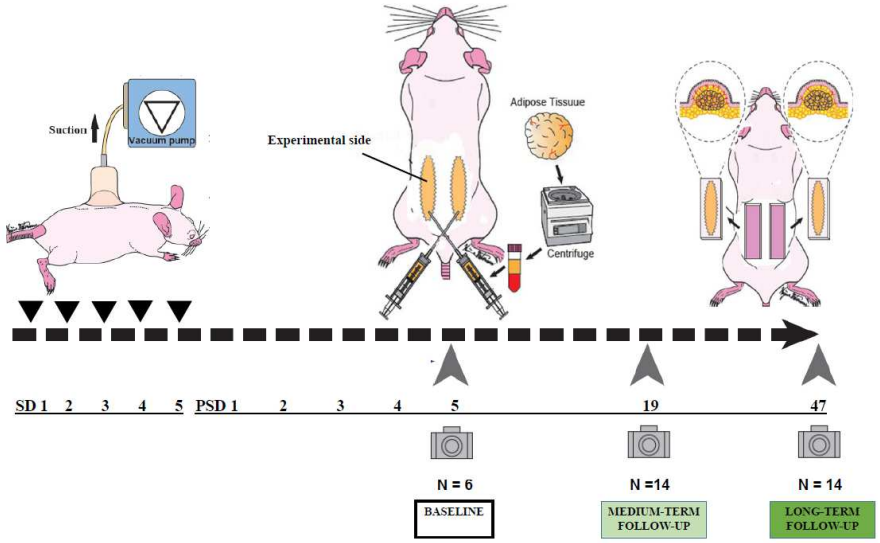


Fig. 2. Experimental study design, procedures and postulated mechanisms of action. **D:** Study design for the study validating the efficacy of EVE as preconditioning method before fat grafting (injection of human adipose grafts). SD = Stimulation Day; PSD = Post-Stimulation Day; ▼= Moderate-intensity intermittent EVE; 📷 = Digital photography and biopsy of samples; White box: Baseline analysis; Light Green box: Medium-term follow-up; Dark Green box: Long-term follow-up.

Parameter	Group	Value on PSD 5	Value on PSD 19	Value on PSD 47
Angiogenesis (blood vessels per 40X magnification field)	EVE	68 (± 4)	66 (± 6)	59 (± 7)
	Control	29 (± 2)	34 (± 4)	27 (± 6)
Graft weight (grams)	EVE	n/a	2.2 (± 0.2)	2 (± 0.2)
	Control	n/a	1.9 (± 0.2)	1.6 (± 0.2)
Graft cross-sectional area (μm^2)	EVE	n/a	8070 (± 1520)	4990 (± 1040)
	Control	n/a	6540 (± 1400)	3940 (± 650)
Graft thickness (μm)	EVE	n/a	580 (± 60)	486 (± 80)
	Control	n/a	320 (± 60)	270 (± 50)
Subcutaneous tissue thickness (μm)	EVE	420 (± 230)	480 (± 140)	620 (± 250)
	Control	270 (± 90)	340 (± 100)	430 (± 160)

Donor ID	Age	Sex	BMI	Donor Site	History of Diabetes	Smoking Habit
1	38	Female	29	Abdominal Wall (superficial adipose layer)	No	No
2	44	Female	33	Abdominal Wall (superficial adipose layer)	No	No

Fig. S1. Data for all measurements. Data is expressed as the mean ± SD; age is expressed in years. PSD = Post-Stimulation Day and clinical characteristics of donor patients for adipose tissue.

Macroscopic analysis

Digital photographs (Nikon Coolpix S4; Nikon Co., Tokyo, Japan) captured at the end of the last stimulation and on PSD 5 were evaluated for signs of skin damage. We designed a Complication Score to evaluate the overall impact of treatment on skin and animals. Complications were assigned an increasing value based on severity (No complications = 0, Erythema = 1, Blistering = 2, Ulceration = 4, and Cachexia = 8), and the cumulative score was calculated for each group. Erythema was described as skin redness with mild and temporary superficial inflammation but without any loss of integrity of the skin. Blistering involved presence of erythema and minor loss of skin integrity in the form of one or more blisters containing non-infected serum. Ulceration described complications in which blistering had evolved in a localized cutaneous injury without significantly affecting the animal well-being: this presented with loss of integrity in the epidermal layer and with areas of skin necrosis. Cachexia identified animal with severe necrosis in the areas stimulated with EVE, distress and impaired overall wellness of the animals (loss of weight, reduce food intake and mobility, lack of response to stimuli). All complications were treated in a conservative way (observation) to avoid altering their natural evaluation: no infection was observed in animals with cutaneous wounds. Animals showing signs of cachexia were euthanized. In the second part of the study digital photographs were also obtained on PSD 19 and PSD 47 to image the external appearance of adipose grafts.

Microscopic analysis

All samples were fixed in 10% neutral-buffered formaldehyde for 24 hours and stored it in 70% ethanol at 4 °C. We performed histology (Haematoxylin and eosin) and immunohistochemistry (platelet endothelial cell adhesion molecule-1 CD 31) of samples according to previously described standard protocols [23–25,27,28]. Briefly, histological sections were deparaffinized in xylene and rehydrated in graded ethanol series. Sections were treated with 40 µg/ml of proteinase K (Roche Diagnostics Corp., Indianapolis, IN) for 30 minutes at 37 °C. Primary antibodies were incubated at 4 °C overnight. Signal was intensified using the tyramide amplification system (Perkin-Elmer, Boston, MA), and positive staining was detected with 3, 3′-diaminobenzidine (Dako North America Inc., Carpinteria, CA). Slides were counterstained with hematoxylin. Image acquisition and analysis (density of CD 31+ blood vessels per magnification field, thickness of subcutaneous tissue, cross-sectional area of adipose grafts) was also performed according to previously established methods [23–25,27,28]. Three representative images in 10X fields were obtained from areas along the entire length of the sample: images were acquired using a Nikon E200 micro-scope (Nikon Corp., Tokyo, Japan). Vascular density was quantified as the number of CD 31 + vessels identified in each of the 10X fields. Blood vessels were measured in the dermal and the subcutaneous layers above the panniculus carnosus, and in between the panniculus carnosus and the skeletal muscle. Blood vessels counts from each of these areas were averaged to measure the overall increase in vascularity. Each slide was evaluated by three independent observers blinded to treatment. A qualitative analysis of the

distribution of adipocytes, inflammatory cells, and of the morphology of the grafts was also performed to evaluate presence of cystic-like areas.

Isolation of adipose-derived mesenchymal stem cells (ADSCs) and fluorescence activated cell sorting

Cells, including ADSCs, were isolated from a 100 ml aliquot of the lipoaspirate obtained as reported above from each patient. The lipoaspirate was washed three times with phosphate-buffered saline (PBS; Lonza), suspended in 500 ml of 0.1% I-type collagenase solution and gently shook on a water bath at 37°C for 1 hour. The supernatant was discarded and cells were collected. After collection, the cells were washed two times with sterile PBS and stained for CD 90 (+), CD 105 (+), CD 45 (-), CD 31 (-) as ADSCs markers (BD, Waltham, MA) and used according to manufacturer instructions. Potential leukocyte activation was explored by CD 3 (T cells), CD 11c (dendritic cells), CD 19 (B cells) and CD 56 (NK cells) staining (BD, Waltham, MA). Apoptotic and necrotic cells were measured with the same method according to established techniques.

Statistical analysis

Power analysis was used to calculate the sample size was required to detect meaningful differences between treated groups and controls with regards to the primary endpoint (angiogenesis measured as blood vessels density at histology). Samples size was not altered during the study. Animals were randomly allocated to groups and samples randomly processed, in both cases using a randomization sequence (known only to the main investigator). Animals were procured by the same vendor and identified with non-informative codes related to their random allocation number. Investigators in charge of data collection (macroscopic and histological) and analysis were blinded to treatment. Rules and criteria for data collection and analysis (including primary and secondary endpoints) were defined before starting the study and were not changed during the study. The primary endpoint was a 25% increase in angiogenesis in treated tissues compared to controls, measured as blood vessels density at histology; the secondary endpoint was a 25% decrease in complications in treated tissues compared to continuous static stimulations, measured with our complications score. All experimental data was included for analysis: no interim data analysis, ad hoc exclusion of data, or retrospective change of endpoints was performed. No outliers were identified in our results. Data was evaluated by three independent observers blinded to treatment. Normality tests were used to determine normal distribution of data before statistical analysis. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction (multiple groups comparison) was used to test differences among experimental groups (SPSS Statistics 20, IBM, NY, USA). A value of $p < 0.05$ was considered statistically significant. We express results as the mean ± SD in text and figures. For inter-group comparison outcomes were expressed as a fold increase over controls.

Results

In vivo sequential optimization of non-invasive mechanical preconditioning (EVE) using a rodent model

1) Frequencies of stimulation: Moderate-intensity intermittent EVE optimizes angiogenic response and limits tissue complications

We first tested the biological effect of different types of frequencies of stimulation on tissues. In particular, we wanted to evaluate whether cyclical-intermittent applications of external suction could show a higher angiogenic effect with lower complications compared to static-continuous regimens of treatment. In addition, we aimed to determine which frequencies would maximize effectiveness. Skin areas treated with moderate-intensity intermittent EVE (= 30 minutes-long stimulations with 1 hour-long break cycles, 6 cycles each day) showed a significant increment in dermal and subcutaneous tissue blood vessel density on post-stimulation day (PSD) 5 (average fold increase over control: 1.7 ± 0.1; $p < 0.05$). This outcome was significantly higher than static continuous suction (average fold increase over continuous stimulations: 1.3 ± 0.1; $p < 0.05$). All regimens of EVE, except for continuous suction, induced a statistically significant difference in vascularity compared to controls after five days of treatment (Fig. 3, Fig. S2-3).

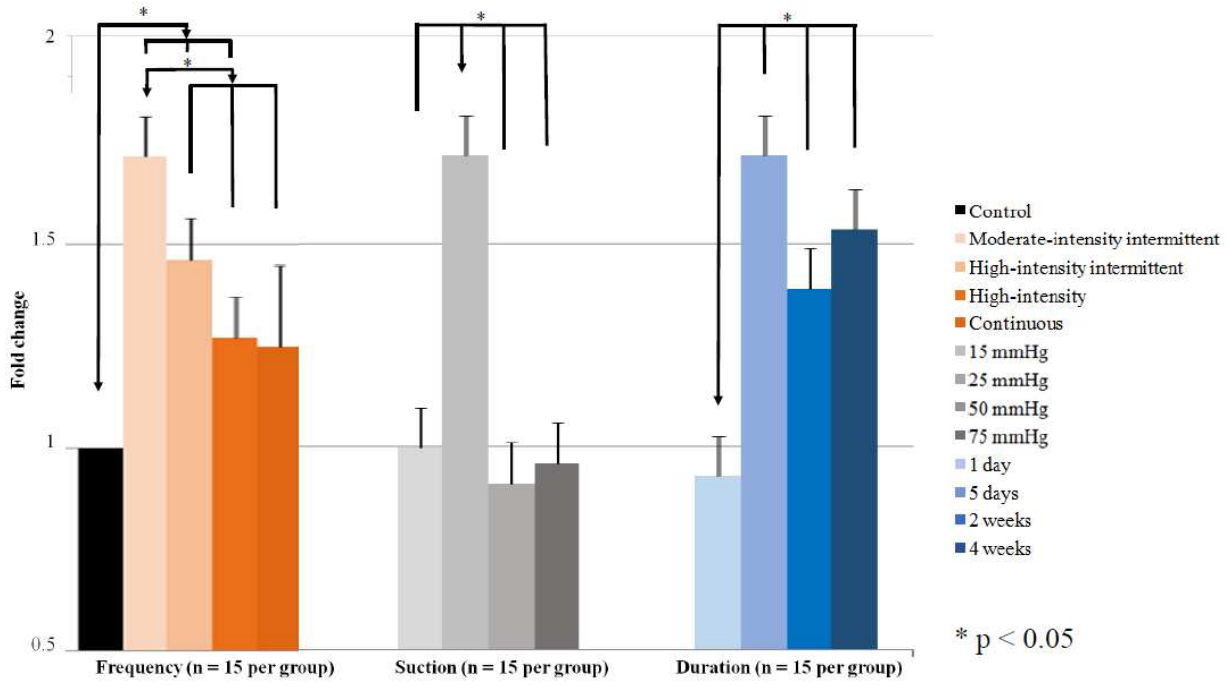


Fig. 3. Angiogenic effect of External Volume Expansion (EVE): outcomes of measurements (fold change) on histological images. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the fold change over control ± SD. (Control = no stimulation; Continuous = 24 hrs/ day; High-intensity = 12 hrs/ day; High-intensity intermittent = 1.5 hrs 5 times/ day; Moderate-intensity intermittent = 0.5 hrs 6 times/ day)

Parameter	Group	Angiogenesis (Blood vessels per 10X magnification field)
n/a	**Control**	24 (± 1)
Frequency	**Moderate-intensity intermittent**	41 (± 4)
	High-intensity intermittent	35 (± 4)
	High-intensity	31 (± 4)
	Continuous	30 (± 3)
Suction	**15 mmHg**	24 (± 5)
	25 mmHg	41 (± 4)
	50 mmHg	22 (± 6)
	75 mmHg	23 (± 6)
Duration	**1 day**	22 (± 5)
	5 days	41 (± 4)
	2 weeks	34 (± 7)
	4 weeks	37 (± 7)

Fig. S2. Angiogenic effect of External Volume Expansion (EVE). Outcomes of measurements (absolute number) on histological images. (Control = no stimulation; Continuous = 24 hrs/ day; High-intensity = 12 hrs/ day; High-intensity intermittent = 1.5 hrs 5 times/ day; Moderate-intensity intermittent = 0.5 hrs 6 times/ day)

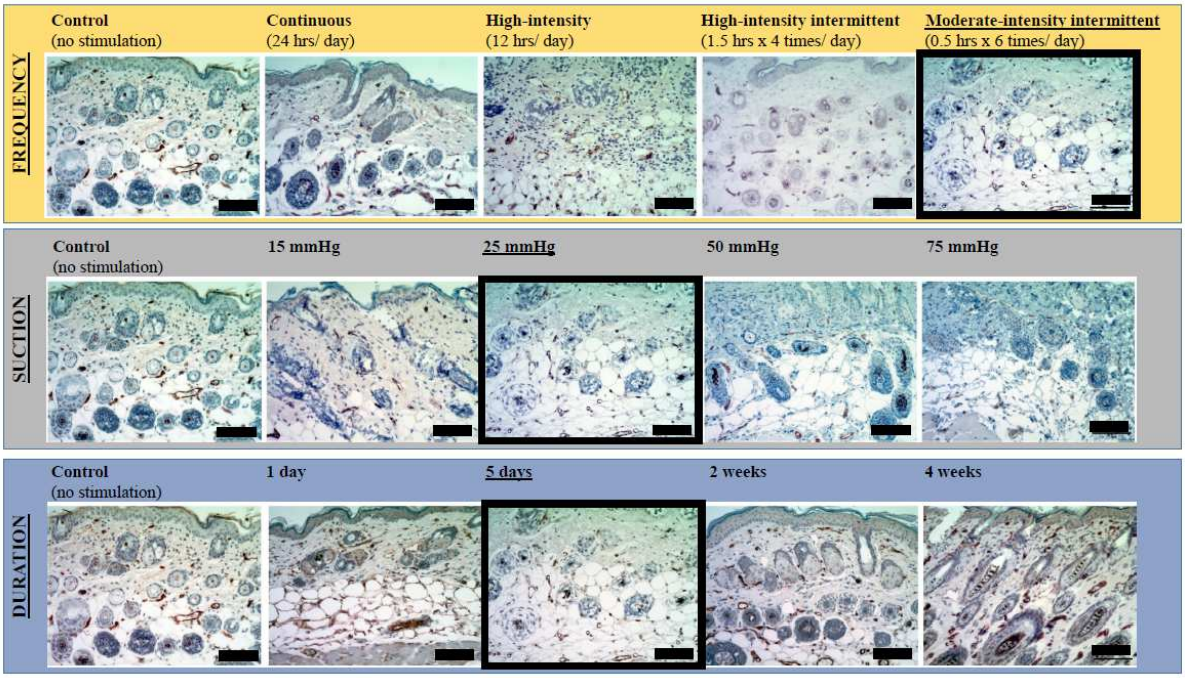
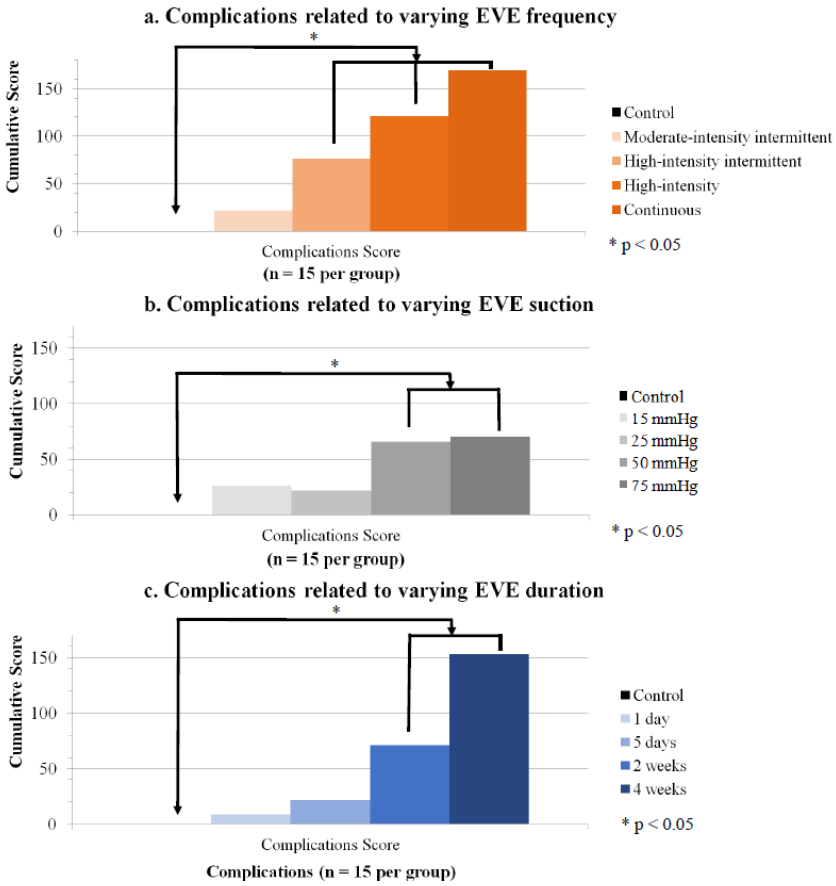


Fig. S3. CD 31 staining for blood vessels. (Magnification: 10X; Reference bar = 100 µm) Orange box: groups investigating varying frequency of External Volume Expansion (EVE). Gray box: groups investigating varying suction levels of EVE. Blue box: groups investigating varying duration of EVE.

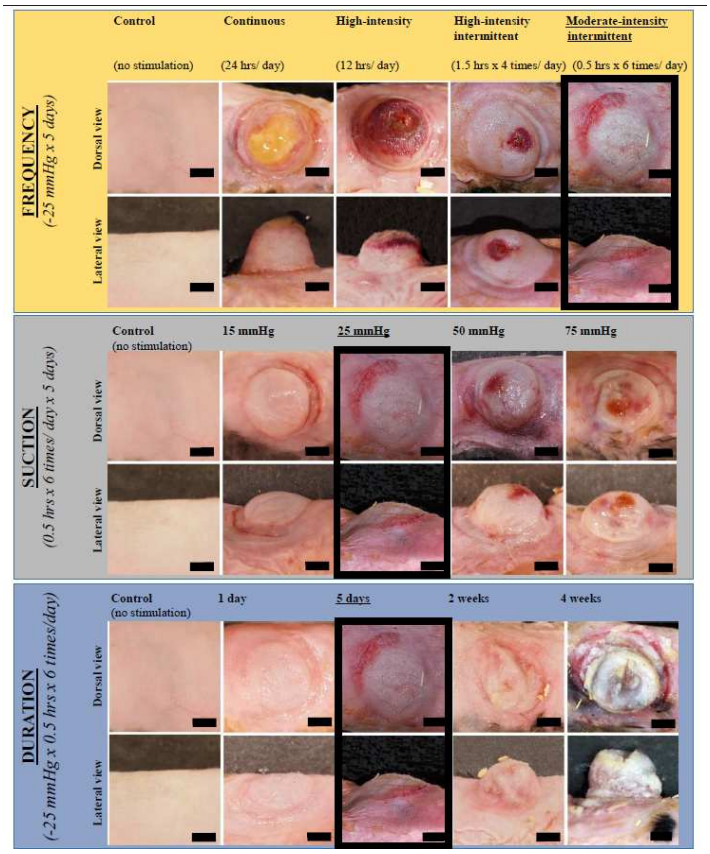
We then analyzed the rate of complications caused by the treatment and observed that moderate-intensity intermittent EVE limited the rate of cutaneous complications on the last day of stimulation with no statistically significant difference compared to controls (Complications Score: 22). Higher frequencies led to a substantial (Complications Score: 76; $p < 0.05$) number of major complications with blistering affecting over 90 % of animals, ulcerations occurring in at least 46 % of treated areas, and few animals requiring euthanasia (Fig. 4a, d; Fig. S4a, 5a). On PSD 5 all minor complications of the moderate-intensity intermittent group disappeared and significantly decreased in groups that underwent more frequent treatments (Fig. S4b, 6a).



d. Complications score

COMPLICATION	SCORE
Erythema	1
Blistering	2
Ulceration	4
Cachexia	8

Fig. 3. Cumulative score for cutaneous complications of External Volume Expansion (EVE) on last day of stimulation. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the cumulative score number. **A:** Complications related to varying frequency of EVE. (Control = no stimulation; Continuous = 24hrs/ day; High-intensity = 12 hrs/ day; High-intensity intermittent = 1.5 hrs 5 times/ day; Moderate-intensity intermittent = 0.5 hrs 6 times/ day) **B:** Complications related to varying suction levels of EVE. **C:** Complications related to varying duration of EVE. **D:** Score legend.



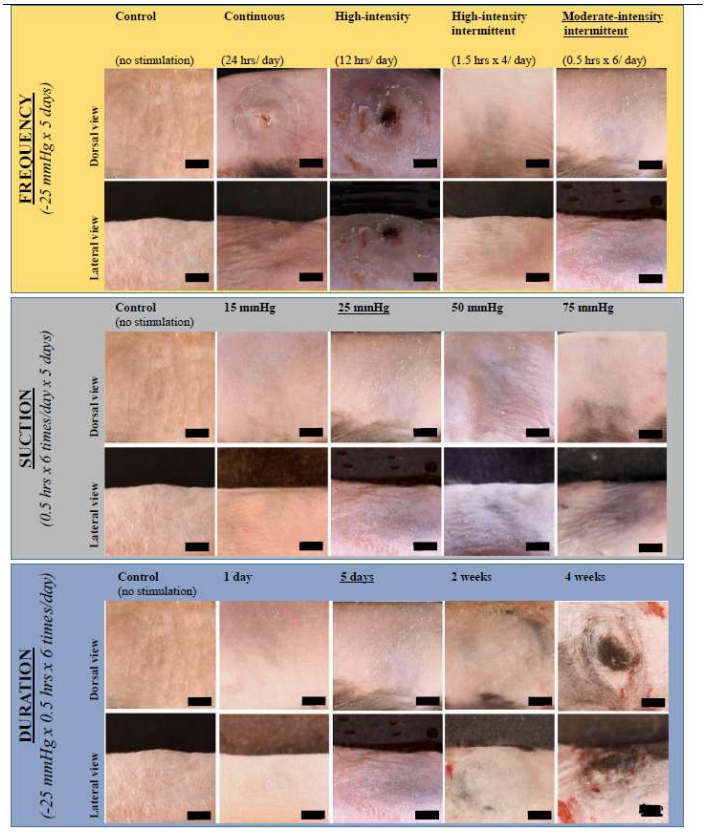


Fig. S4. A (ABOVE): Images of cutaneous complications of External Volume Expansion (EVE) on last day of stimulation. Orange box: Complications related to varying frequency of EVE. Gray box: Complications related to varying suction levels of EVE. Blue box: Complications related to varying duration of EVE. (Reference bar = 0.5cm) **b (BELOW):** Images of cutaneous complications of External Volume Expansion (EVE) five days after last stimulation (Post-stimulation Day 5, PSD 5). Orange box: Complications related to varying frequency of EVE. Gray box: Complications related to varying suction levels of EVE. Blue box: Complications related to varying duration of EVE. (Reference bar = 0.5cm)

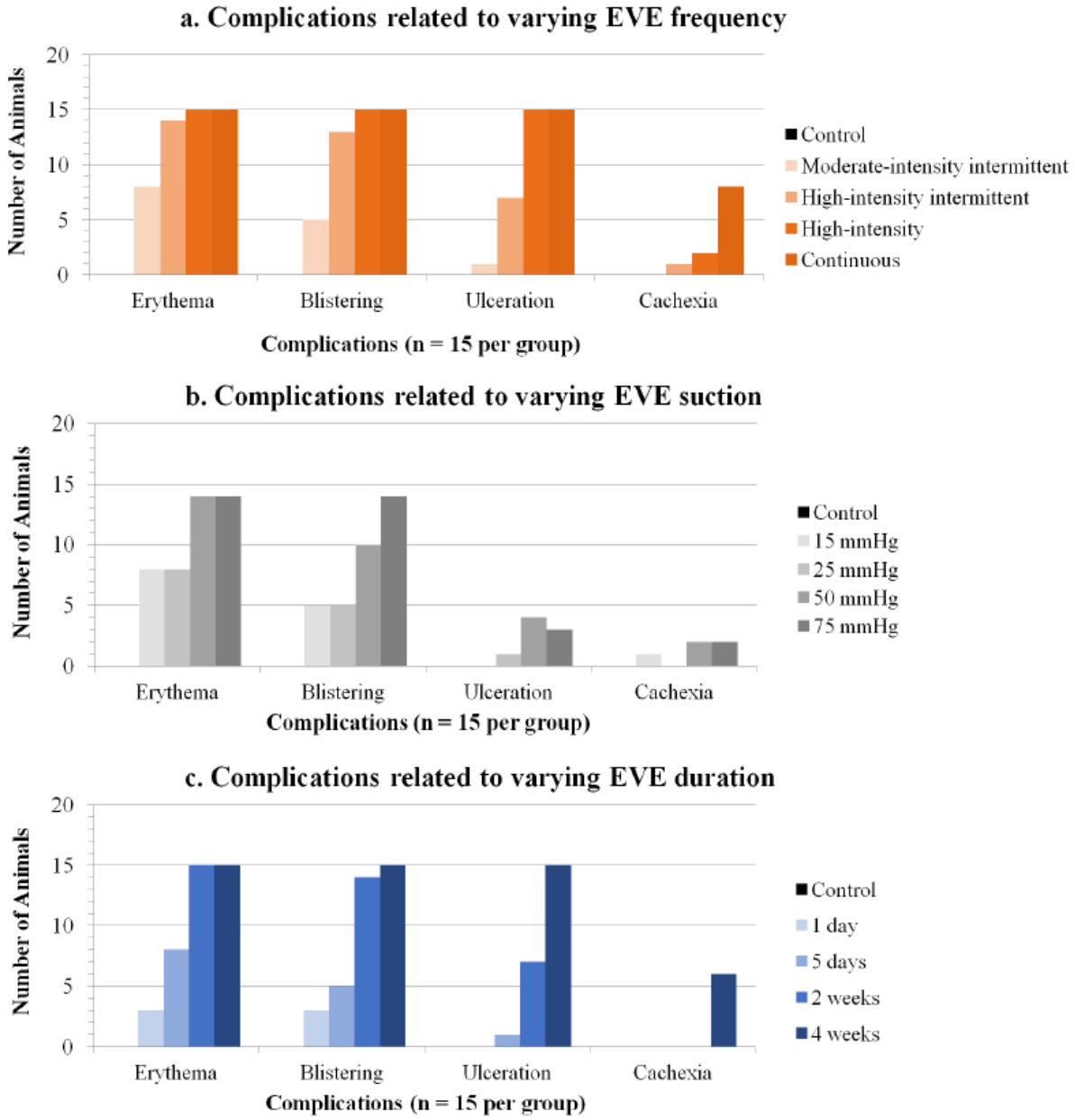


Fig. S5. Absolute number of cutaneous complications of External Volume Expansion (EVE) on last day of stimulation. **a:** Complications related to varying frequency of EVE. (Control = no stimulation; Continuous = 24 hrs/ day; High-intensity = 12 hrs/ day; High-intensity intermittent = 1.5 hrs 5times/ day; Moderate-intensity intermittent = 0.5 hrs 6 times/ day) **b:** Complications related to varying suction levels of EVE. **c:** Complications related to varying duration of EVE.

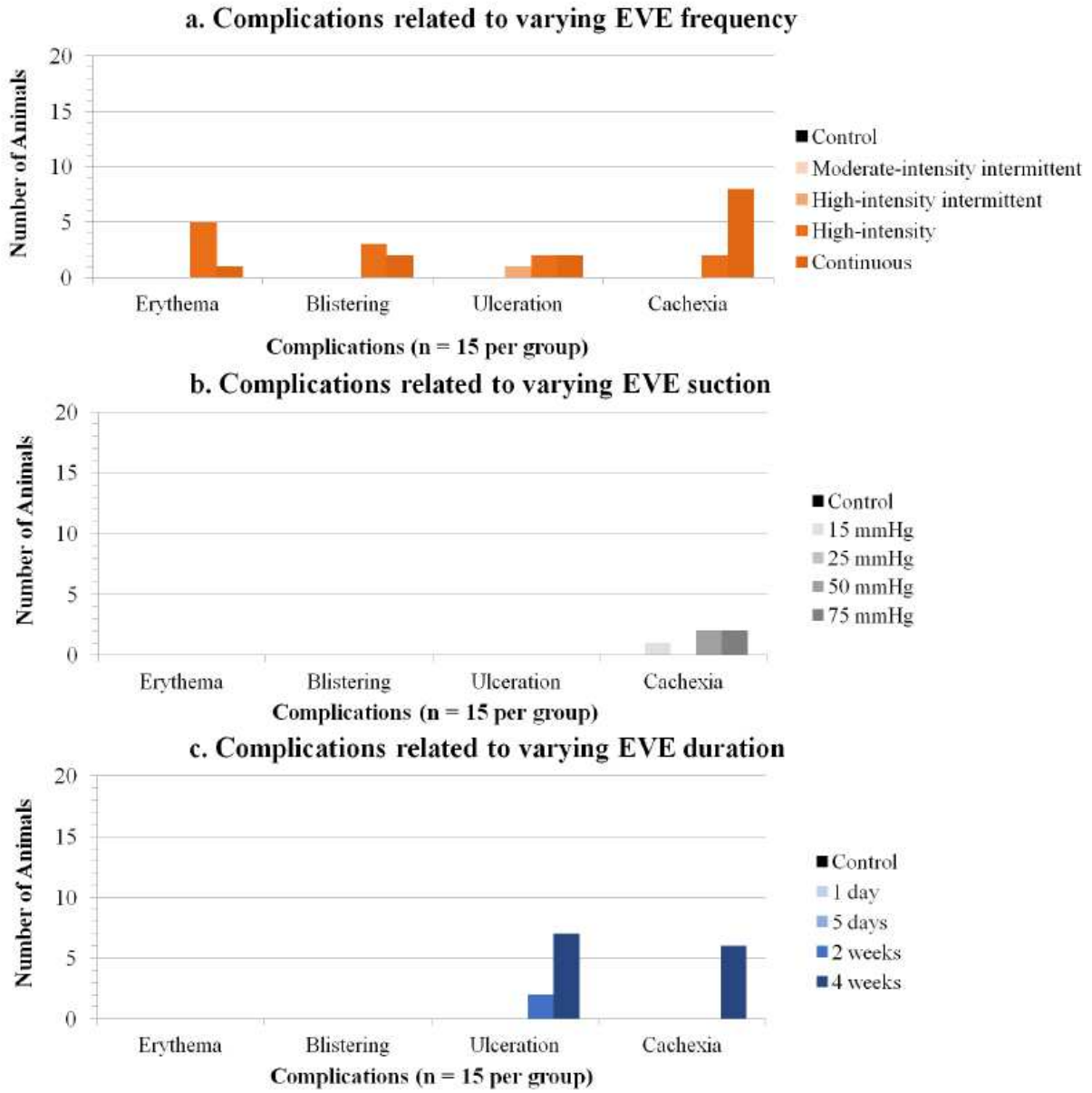


Fig. S6. Absolute number of cutaneous complications of External Volume Expansion (EVE) 5 days after last stimulation (Post-stimulation Day 5, PSD 5). **a:** Complications related to varying frequency of EVE. (Control = no stimulation; Continuous = 24 hrs/ day; High-intensity = 12 hrs/ day; High-intensity intermittent = 1.5 hrs 5 times/ day; Moderate-intensity intermittent = 0.5 hrs 6 times/ day) **b:** Complications related to varying suction levels of EVE. **c:** Complications related to varying duration of EVE.

2) Applied suctions: EVE selectively induces angiogenesis of skin only at suction level ranges close to 25 mmHg

In the second phase of our optimization study we aimed to define the optimal regimens of suction that could induce the highest angiogenic response while limiting tissue damage. To do so we tested several suction levels within those in a physiologically-relevant range. Obtained data demonstrated that only EVE at a suction level of 25 mmHg significantly induced angiogenesis in our samples. Neither lower nor higher suction levels (up to 75 mm Hg) showed any difference compared to controls (Fig. 3a, Figure S2-3). The rate of major complications (ulceration and cachexia) for suction levels at or below 25 mmHg was lower than 7 % (Complications score: 22-26; no statistical difference compared to controls) whereas for suction levels at or above 50 mmHg was higher than 40 % (Complications score: 66-70; $p < 0.05$ compared to controls), and significantly affected survival of animals (Fig. 4b, d; Fig. S4a, 5b). On PSD 5 almost all complications were resolved (Fig. S4b, 6b).

3) Duration of treatment: Five day-long treatments with EVE are sufficient to promote optimal angiogenesis and limit skin injuries

Once we had determined the ideal frequency of stimulation and suction level we aimed to assess how the duration of treatment would have impacted outcomes to define whether a plateau effect existed and what would have been the time range of effectiveness of the treatment. Our results indicated that five day-long or longer durations of treatment with EVE show a significant increment (average fold increase over control: 1.4-1.7 ± 0.1; $p < 0.05$) in dermal and subcutaneous tissue blood vessel density on PSD 5 compared to controls or to single day treatments (Fig. 3a, Fig. S2-3). Single day and five day-long treatments showed no significant differences compared to controls in terms of cutaneous complications on the last day of stimulation. Treatments longer than five days significantly affected the rate of major cutaneous complications compared to controls (Complications Score: 71 - 150; $p < 0.05$), in particular leading to blistering in + 93 % of animals, ulceration in 47-100 % of animals, and affecting animal survival in up to 40 % of cases (Fig. 4c-d; Fig. S4a, 5c). On PSD 5 animals that had undergone single day or five days-long EVE treatments showed not persistence of cutaneous complications: longer treatments led to persistent long-term complications and scarring (Fig. S4b, 6c).

4) Treatment interface: F-EVE retains the angiogenic capacity of EVE, causes less cutaneous complications

In the last phase of our optimization study we focused on the structure of the interface used to deliver suction to tissues. We compared the rigid dome-shaped interface that has been empirically used in patients as well as previously tested in animal models, to a soft and malleable foam-shaped interface (F-EVE). F-EVE significantly increased the density of blood vessels in stimulated areas compared to controls (average fold increase over control respectively: 1.8 ± 0.1; $p < 0.05$). No statistically significant difference was noted in angiogenesis between samples treated with EVE or F-EVE (Fig. 5a-b, Fig. S7a-b).

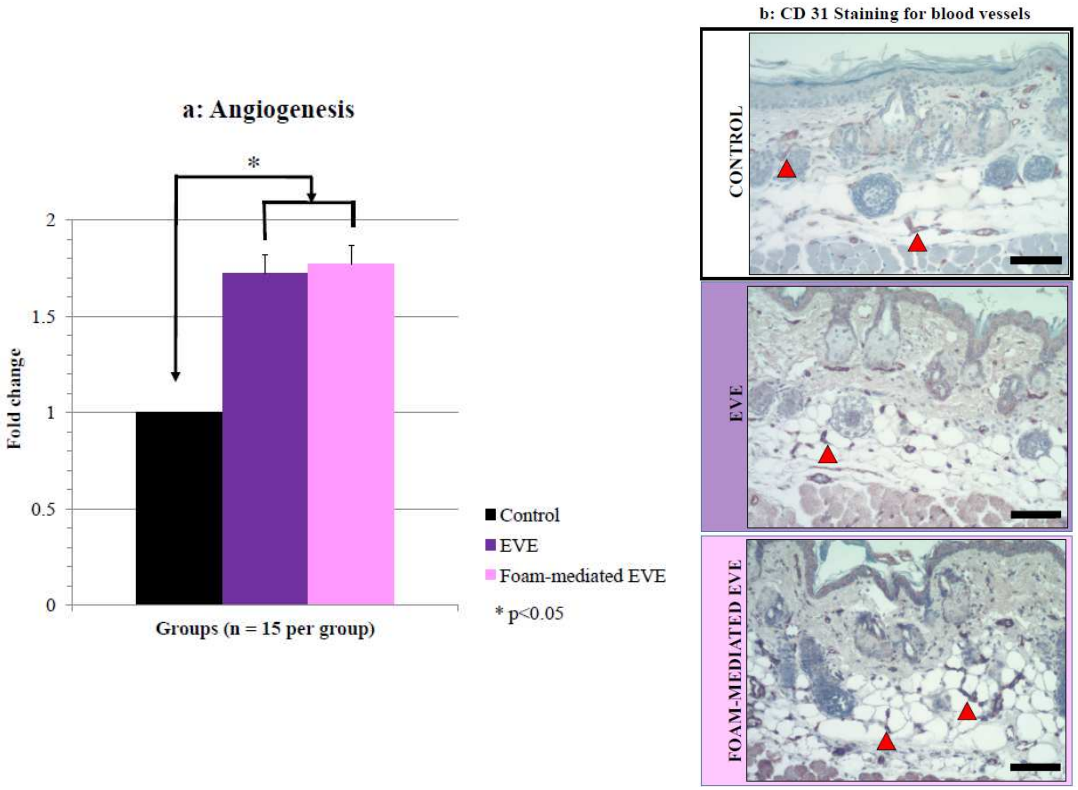
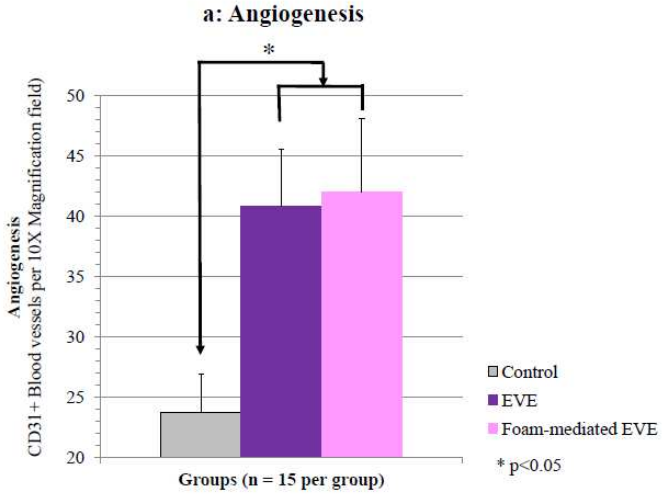


Fig. 4. Angiogenic effect of different interfaces of EVE. **A:** Outcomes of measurements on histological images. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the fold change over control ± SD. (Control = no stimulation; EVE = Dome-mediated EVE; F-EVE = Foam-mediated EVE) **B:** CD 31 staining for blood vessels. (Magnification: 10X; Reference bar = 100 µm) (Control = no stimulation; EVE = Dome-mediated EVE; F-EVE = Foam-mediated EVE; ▲ = CD 31+ blood vessel)

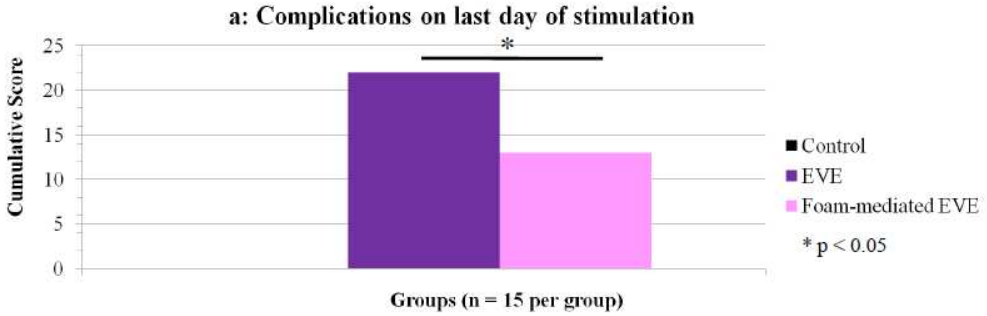


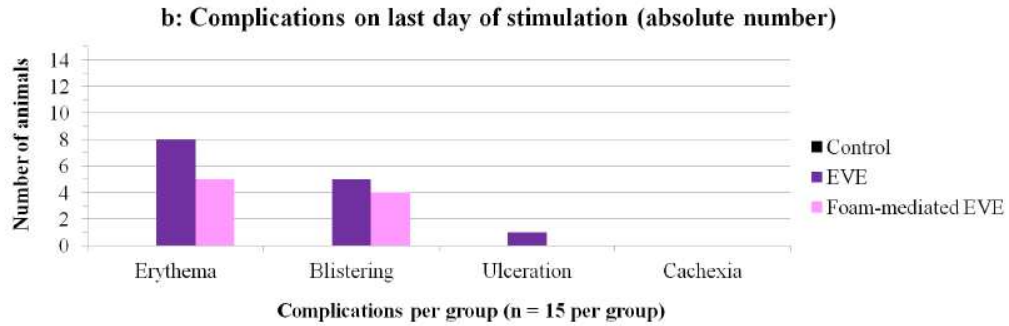
B: Data

Group	Angiogenesis (Blood Vessels per 10X magnification field)
Control	24 (± 3)
EVE	41 (± 5)
F-EVE	42 (± 6)

Fig. S7. a: Angiogenic effect of EVE and F-EVE. Statistical analysis was performed using One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the mean ± SD. **b:** Data for histological measurements. (Control = no stimulation; EVE = Dome-mediated EVE; F-EVE = Foam-mediated EVE) Data is expressed as the mean ± SD.

As we expected, F- EVE significantly limited the rate of cutaneous limitations compared to EVE: 6 % fewer animals treated with F-EVE suffered from skin blistering compared to EVE and 18 % fewer animals treated with F-EVE suffered from skin erythema compared to EVE (Complications Score F-EVE: 13 vs. EVE: 22; $p < 0.05$) (Fig. 6a-c, Fig. S8). At a qualitative analysis of macroscopic pictures, it was evident that most cutaneous complications in the EVE group occurred at the point of contact along the rim of the dome-shaped interface (Fig. S8): differently, the F-EVE group showed no specific site of injury. In addition, EVE led to macroscopic edema of stimulated skin in all samples on the last day of stimulation (but this was no more evident on PSD 5) whereas no edema was reported in the animals treated with F-EVE at all time-points (Fig. S8). On PSD 5 all complications for both groups were resolved (Fig. S8).





c: Complications score

COMPLICATION	SCORE
Erythema	1
Blistering	2
Ulceration	4
Cachexia	8

Fig. 5. Cutaneous complications related to treatment with different interfaces of EVE. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the cumulative score number. **A:** Cumulative score for cutaneous complications on last day of stimulation. **B:** Absolute number of cutaneous complications on last day of stimulation. **C:** Score for complications. (Control = no stimulation; EVE = Dome-mediated EVE; F-EVE = Foam-mediated EVE)

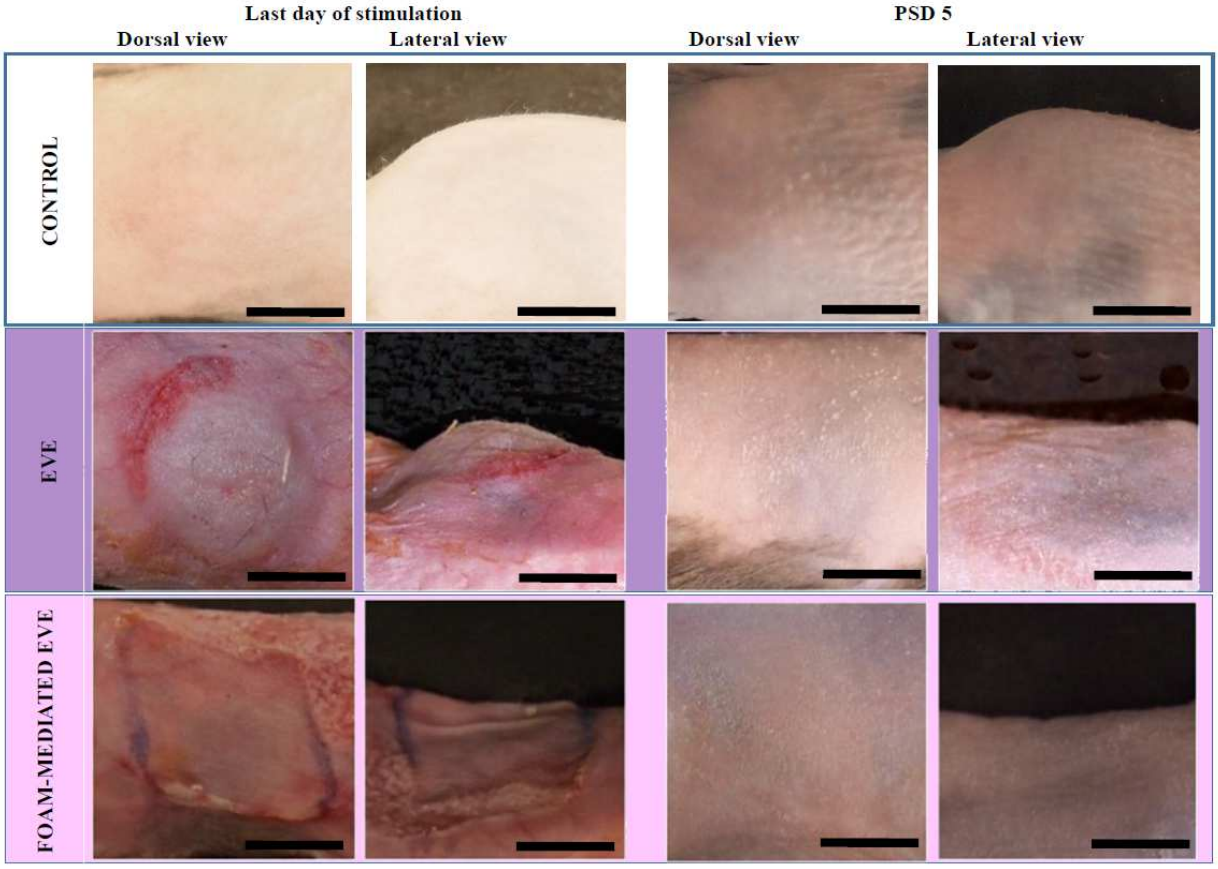


Fig. S8. Images of cutaneous complications on last day of stimulation and on post-stimulation day five (PSD 5). (Control = no stimulation; EVE = Dome-mediated EVE; F-EVE = Foam-mediated EVE) (Reference bar = 0.5 cm)

In vivo validation of effective EVE preconditioning using a free tissue graft rodent model

Recipient site preconditioning with optimized EVE enhances survival and quality of adipose grafts

After determining the optimal regimen of application of EVE we sought to validate the in vivo efficacy of such method as a preconditioning strategy in tissue reconstruction. This assessment is pivotal to confirm the translational feasibility of the strategy and establish the basis for its use in patients. To achieve this goal, we used an established murine model of adipose tissue grafting, a procedure widely used in clinical care to reconstruct soft tissue defects, to which we applied our optimized EVE treatment. Skin areas treated with (dome-shaped) optimized EVE showed a significant increment in blood vessel density in areas surrounding the adipose grafts at a medium-term follow-up (average fold increase over control: 2.0 ± 0.1; $p < 0.05$) and at a long-term follow-up (average fold increase over control: 2.2 ± 0.1; $p < 0.05$) (Fig. S8a-b, S9). Blood vessels more intensively infiltrated the fat grafts in areas treated with EVE. EVE-stimulated areas also showed a significantly expanded subcutaneous layer at long-term follow-up (average fold increase over control: 1.4 ± 0.3; $p < 0.05$) (Fig. S1, S10a-b).

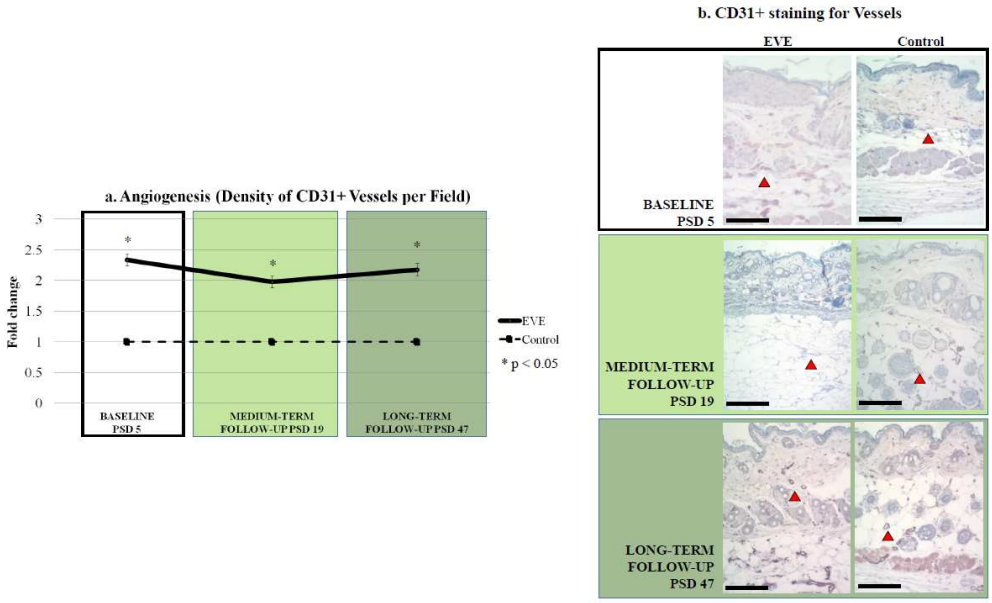


Fig. S9. Angiogenic effect of External Volume Expansion (EVE). **a:** Measurement of outcomes on histological images at different time-points. PSD = Post-Stimulation Day. Statistical analysis was performed using One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the fold change over control ± SD. **b:** CD 31 staining for blood vessels at different time-points. White box: Baseline analysis; Light Green box: Medium-term follow-up; Dark Green box: Long-term follow-up. PSD = Post-Stimulation Day. (Magnification: 10X; Reference bar = 250 µm; ▲ = CD 31+ blood vessel)

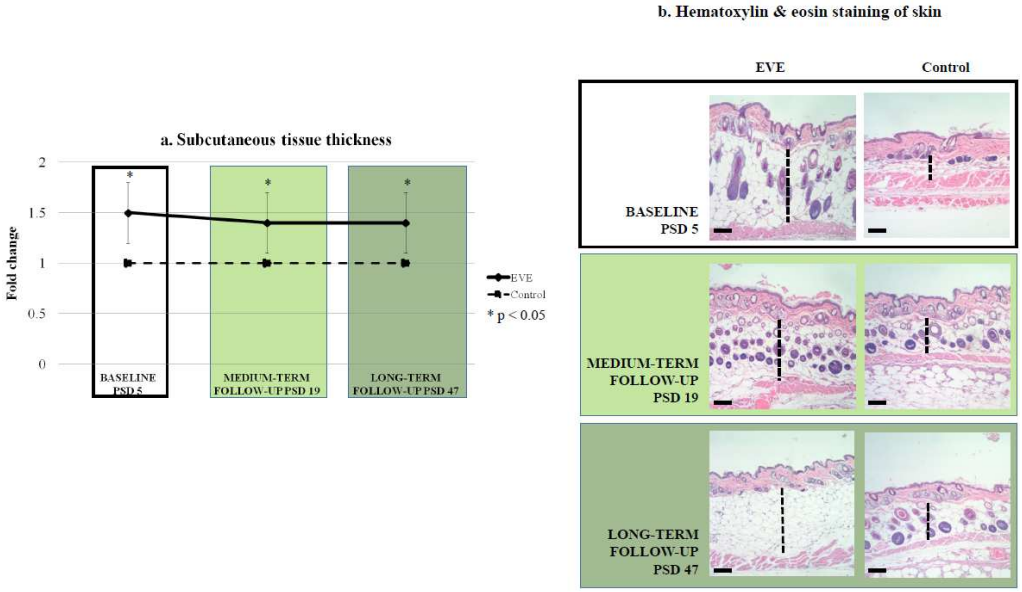


Fig. S10. Adipogenic effect of External Volume Expansion (EVE). **a:** Measurement on histological images of subcutaneous tissue expansion over time. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the fold change over control ± SD. PSD = Post-Stimulation Day. **b:** Histological staining (hematoxylin and eosin) of skin showing different thickness of the subcutaneous tissue (dotted line). White box: Baseline analysis; Light Green box: Medium-term follow-up; Dark Green box: Long-term follow-up. PSD = Post-Stimulation Day. (Magnification: 10X; Reference bar = 250 µm)

Macroscopic examination of grafts placed in EVE-treated areas showed a higher volumetric retention and vascularity (Fig. S11a-b). The weight of adipose grafts significantly increased in areas treated with EVE at a medium-term follow-up and at a long-term follow-up (average fold increase over control: 1.2 ± 0.1; $p < 0.05$) (Fig. 7a, Fig. S1). Grafts implanted in pre-treated areas had a significantly increased thickness and cross-sectional area at a medium-term follow-up (average fold increase over control: 1.2 ± 0.2; $p < 0.05$) and at a long-term follow-up (average fold increase over control: 1.3 ± 0.2; $p < 0.05$) (Fig. 7b-c, Fig. S1). Partial graft resorption was observed between medium-term and long-term follow-up in all measurements (weight persistence, graft cross sectional area, and graft thickness; $p < 0.05$). Grafts placed in EVE-treated areas showed a more homogenous morphology with less fibrosis and cystic-like degeneration (Fig. S12). We did not observe differences between adipose samples obtained from the two donors with regards to the quantity of ADSCs obtained, the percentage of necrotic or apoptotic cells, or the quantity of activated leukocytes.

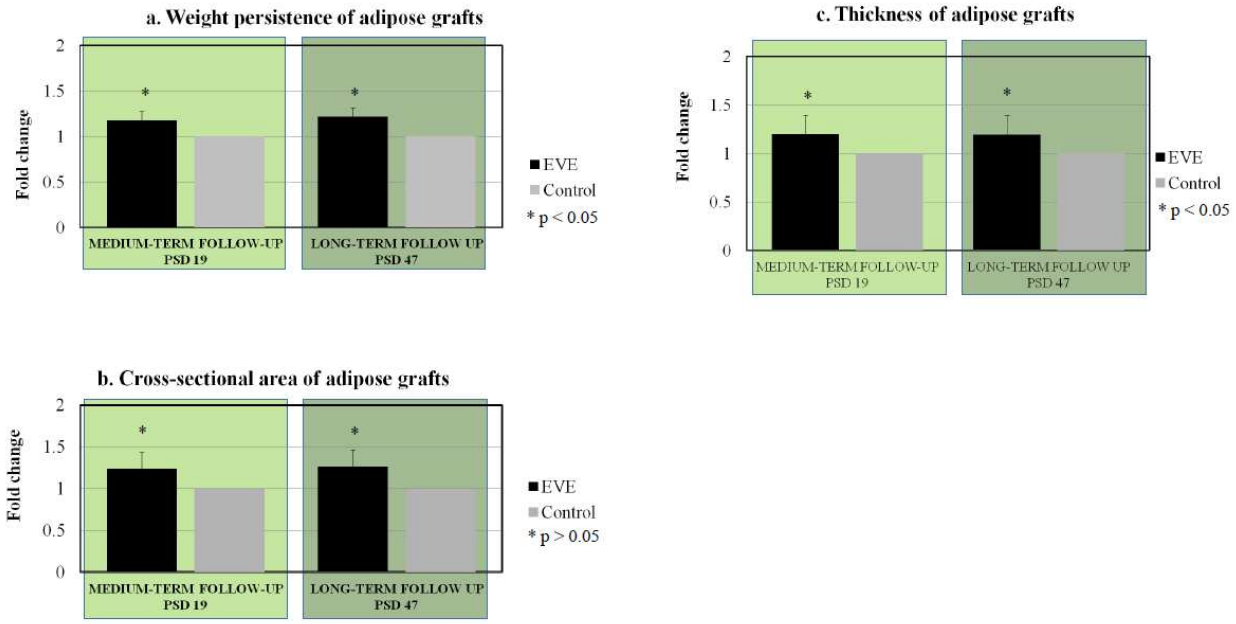


Fig. 6. Measurement of survival of adipose grafts. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the fold change over control ± SD. **A:** Weight persistence immediately after *en bloc* excision of a 2 x 3 cm standardized biopsy. PSD = Post-Stimulation Day. **B:** Cross sectional area of adipose grafts measured with histology on microscopic samples. PSD = Post-Stimulation Day. **C:** Thickness of adipose grafts measured with histology on microscopic samples. PSD = Post-Stimulation Day.

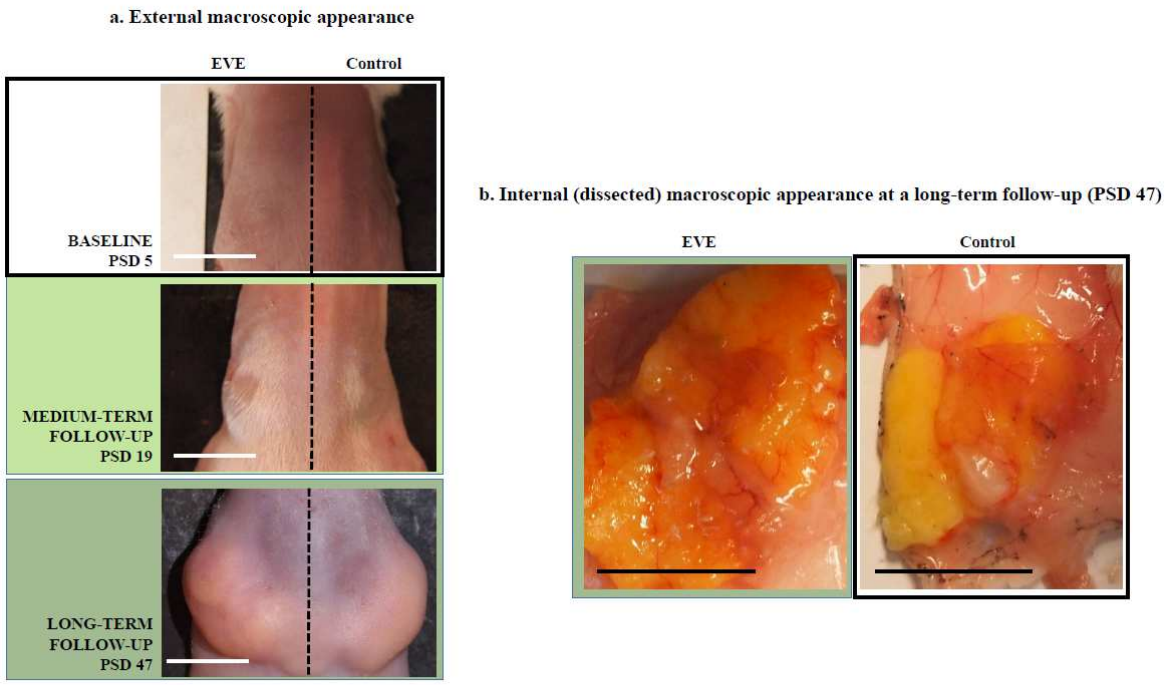


Fig. S11. Macroscopic appearance of adipose grafts. **a:** External macroscopic appearance of adipose grafts (pictures represent different animals) showing differential volume retention between the EVE-treated side (left) and the control (right). **b:** Internal (dissected) macroscopic appearance of adipose grafts on long-term follow-up showing higher re-vascularization of the grafts injected in EVE pre-treated areas (left) compared to controls. White box: Baseline analysis; Light Green box: Medium-term follow-up; Dark Green box: Long-term follow-up. PSD = Post-Stimulation Day. (Reference bar = 1 cm for both).

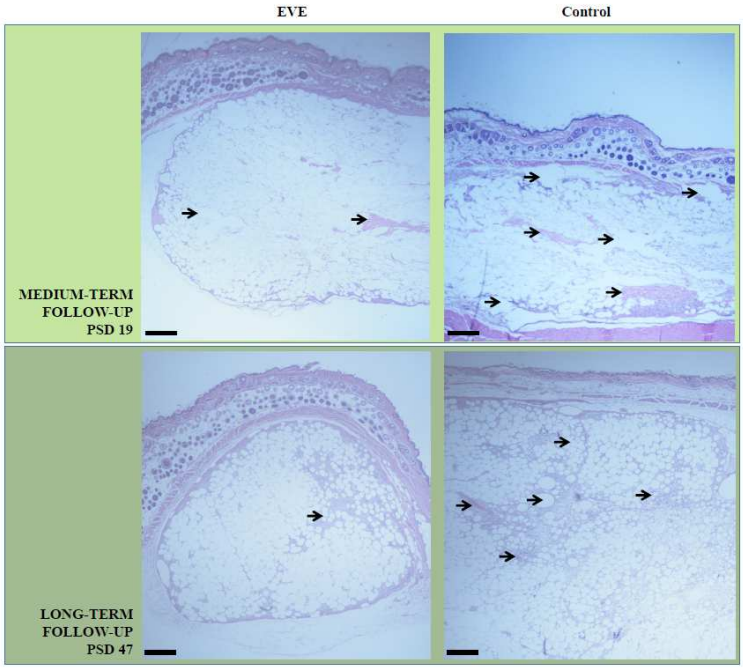


Fig. S12. Histological staining (hematoxylin and eosin) of adipose grafts showing their morphology (→= necrotic vacuoles or cystic-like areas). (Magnification: 4X; Reference bar = 300 µm) Light Green box: Medium-term follow-up; Dark Green box: Long-term follow-up. PSD = Post-Stimulation Day.

Discussion

In this study, we show that EVE has the potential to non-invasively and safely induce angiogenesis in soft tissues. Using clinically-approved devices we optimized the treatment with the goal of providing evidence-based knowledge that can support effective clinical application of EVE, the improvement of current best practice in surgery, and the development of novel preconditioning therapies [23–25,27,28,30,31,126,127].

Our data demonstrated that an eight hour-long daily treatment (30 minutes-long stimulations with 1 hour-long break cycles) for five days almost doubled vascular density of treated areas and significantly limited cutaneous complications associated to the treatment [28]. No other treatment, including invasive surgical approaches, has shown in a clinical or preclinical setting the potential to achieve a comparable increase in tissue vascular density in this relatively limited amount of time. Transition from continuous-static regimens of stimulation to cyclical-intermittent protocols not only improved outcomes but also supported translational application of EVE since shorter treatments and lower rate of complications improve patients' compliance to treatment. For example, a daily 8-hour long treatment could be done during sleep at night without affecting patients' normal life activities. The optimization of treatment interfaces represents another critical factor for translational application of EVE in patients. Current prototypes of EVE adopt a dome-shaped polymeric interface that has shown several limits: it cannot contour irregular surfaces, it cannot be customized in a specific shape by surgeons, it delivers excessive stress to skin at the point of contact along the rim, and it causes temporary edema in the tissue stretched inside the dome [20,22,35,36,38,39]. Although transient tissue edema is associated to adipogenesis, it seems to be less related to the increase of skin vascularity [25,28,30,31,128]. Micro-deformational Wound Therapy (MDWT) has shown to modulate angiogenesis in wounds using a polyurethane foam interface [18,19,21,43,129–133]. A similar approach has been used to develop a Closed Incision Negative Pressure Therapy to stimulate healing of surgical defects closed by primary suture [134–136]. We postulated that the same principles could apply to EVE: our outcomes confirmed this hypothesis and showed that by using a FDA-approved foam-shaped polyurethane interface (F-EVE) the distribution of mechanical forces (suction) among the multiple micro-domes of the foam (instead of a single rim) could further reduce EVE-induced cutaneous complications while retaining the same angiogenic effect [126]. We also confirmed effectiveness of EVE in improving surgical outcomes by using an established experimental model of adipose tissue grafting which closely resembles common clinical practice in reconstructive surgery. Volume retention of adipose grafts in patients commonly ranges from 30% to 60% over time, [60,64,137,138] with an inverse correlation to grafted volumes. Optimized EVE significantly increased (1.2-fold) the survival of grafts and better preserved the tissue reducing the areas of necrosis [126]. Although obtained in an animal study and with grafts of small volume, the increment in volume retention that we have observed is clinically relevant. These outcomes are consistent with those reported by Lee et al. (+ 24% graft survival) in rabbits and those reported by Reddy et al. in mice (+35% graft survival) [126,127].

Definition of the biological effect of each parameter of EVE treatment

Frequencies: Previous mechanistic studies on EVE investigated only a limited number of frequencies and did not directly compare the biological effects of continuous frequencies -which are those currently adopted in clinical practice with ineffective outcomes- to those of intermittent stimulations [18–20,22–28,126,127]. In this study, we compared a broad set of frequency regimens including high-intensity and continuous/static stimulations. Our outcomes confirmed and expanded previous findings related to moderate-intensity intermittent EVE [28]: application of a 25 mmHg suction level for five days best induces angiogenesis in skin, almost doubling initial vascular density (average fold increase over control: 1.7 ± 0.1; $p < 0.05$). High-intensity and continuous/static stimulations also induced angiogenesis but led to a significant rate of tissue injuries. Longer treatments exceed the ischemia-resistance time of skin (~60 minutes), resulting in excess inflammation and triggering apoptotic/necrotic processes. Macroscopic analysis of our samples corroborates this observation showing an increased rate of inflammation and tissue injury, including skin blistering and necrosis in animals subjected to those treatment regimens. Histological analysis was performed only on samples procured 5 days after the last stimulation, when most of the complications had resolved, and we did not quantify ischemic or necrotic/apoptotic cells. Yet, our conclusions are consistent with histological outcomes and observations of previous in vivo and in vitro models of EVE [29].

Suction levels: We observed that only suction levels close to 25 mmHg resulted in a potent angiogenic response. A suction level of 25 mmHg can partially occlude capillaries and establish a sub-critical hypoxic environment that can initiate pro-angiogenic stimuli [24,27,28]. Lower levels of suction may not be able to reduce the capillary blood flow by contrasting their filling pressure in skin (~32 mmHg) whereas higher levels of suction may completely occlude the vessels leading to critical ischemia, tissue injury and necrosis [123,139,140]. Myung et al. have recently confirmed the angiogenic effect of EVE at a suction level of 25 mmHg in patients even if adopting a longer duration of therapy (leading to a 40% complication rate) [39]. Similar outcomes have been shown by others [30,31]. Kao et al. also reported a robust vascular increase using EVE at a suction level of 50 mmHg in swine with 8 hour-long daily treatments for 10 and 21 days: their study however does not report the rate of complications observed [26]. EVE might also improve vascularity through a flow (shear) induced angiogenesis [139,140].

Duration of treatment: Our results show that EVE-mediated angiogenesis reaches a plateau after five days of treatment: longer treatments without recovery intervals (as currently implemented in clinical practice) may not be able to further increase angiogenesis due to a limited capacity of soft tissues to respond to an ischemic insult in a restricted time frame [29,119,141,142].

Interfaces: We also observed that F-EVE retains the angiogenic capacity of EVE. This finding implies that surface distribution of mechanical strain does not affect the effectiveness of treatment. Previous studies have demonstrated that foam interfaces can effectively transmit micro-mechanical stimulation to soft tissues in the form of strain [18,19,21,129,131]. A similar mechanism of action might apply to EVE. Lee et al. have recently reported a comparable angiogenic effect using suction-mediated preconditioning with a polyurethane foam interface and subsequent fat grafting model in rabbits' ears [126]. Despite differences in

experimental models, their outcomes are biologically consistent with ours and confirm our conclusions. F-EVE significantly decreased cutaneous complications compared to EVE: this result might be due to the parceling of mechanical forces along multiple points of contact (a “bed of nails” effect) and to the lack of excessive edema in tissues. On the contrary, in standard EVE the highest mechanical stress delivered to skin is condensed along the rim, at the point of contact between the dome-shaped interface and skin: this is the area where cutaneous injuries most frequently occur [20,24–28,35,36,38,39,126,127]. Consistently with our goal to improve usability of EVE and its application to different body areas the foam interface could be easily customized in sizes and shapes based on specific needs.

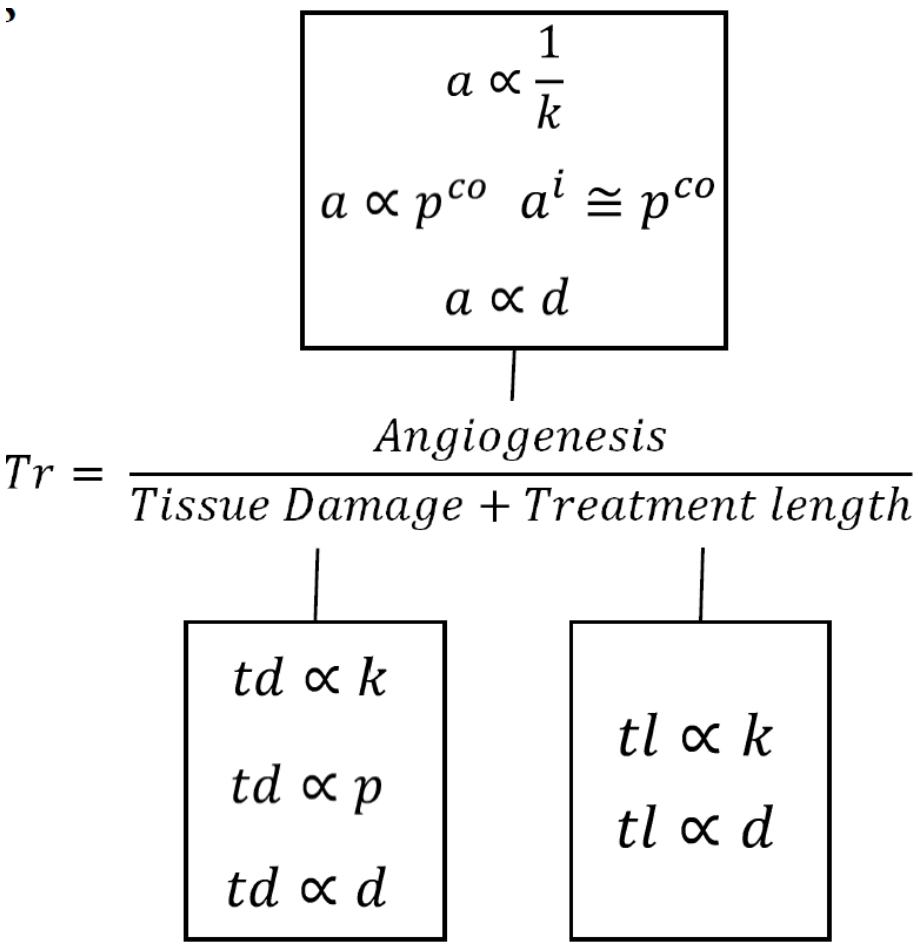


Fig. 7. Mathematical modeling of optimized EVE. *Tr* = Therapeutic ratio of EVE; a = Angiogenesis; k = Kinetics of treatment; p^i = Ideal pressure; p^{co}= Pressure of capillary occlusion; td = Tissue damage; d = Duration of treatment; p = Pressure; tl = Treatment length

EVE’s therapeutic ratio: The correlation among parameters of application of EVE can be simplified in a mathematical equation. (Fig. 8) Effectiveness of treatment improves as the therapeutic ratio

($Tr = \frac{Angiogenesis}{Tissue\ damage + Treatment\ length}$) increases. Angiogenesis (a, measured as blood vessel density in the target tissue) is inversely proportional to longer frequencies of stimulation ($a \propto \frac{1}{k}$), occurs within an ideal range of suction levels close to the capillary occlusion pressure ($a \propto p^{co}$ with $p^{i} \cong p^{co}$), and is directly proportional to the duration of treatment within a maximal limit ($a \propto d$). Tissue damage (td, measured as minor –inflammation/blistering- or major injury –ulceration and necrosis) is directly proportional to longer frequencies of stimulation ($td \propto k$), the suction level ($td \propto p$), and the duration of treatment ($td \propto d$). Treatment length (tl, measured in time) is directly proportional to longer frequencies of stimulation ($tl \propto k$), and the duration of treatment ($tl \propto d$). This therapeutic ratio is conceptual: its aim is to provide evidence-based biological principles to guide clinical application of EVE. The ratio should be adapted to each different scenario (characteristic of the tissue and of the patient) and should not be considered as an exact formula with specific units of measurement or constant dimensions of variables.

In summary, in this study we sequentially optimized parameters of application of EVE in support of its effective clinical application as non-invasive preconditioning strategy in surgery. Using FDA-approved devices and commercially available materials we confirmed that moderate-intensity intermittent treatments provide the best therapeutic ratio, almost doubling vascular density in target tissues without collateral damage. We also showed that F-EVE retains the angiogenic potential of EVE, further reduces cutaneous complications, and offers an easier method of application of EVE. Finally, we demonstrated that by increasing vascularity of the recipient site EVE promotes higher volume retention of adipose grafts over time. This knowledge of the biologic principles that regulate EVE will allow us to optimize the treatment in humans, guide better clinical application of EVE, and improve current best practice in reconstructive surgery. Future research should focus on the development of new interface materials (multi-dome sheet-shaped) and on the definition of patient-specific regimens of treatment using non-invasive imaging to measure tissue ischemia during EVE treatment.

STUDY 3:

DELAYED POST-CONDITIONING WITH EXTERNAL VOLUME EXPANSION (EVE) IMPROVES SURVIVAL OF ADIPOSE TISSUE GRAFTS IN A MURINE MODEL

Summary of the study

Aim: External volume expansion (EVE) improves the survival of adipose tissue grafts by pre-operatively pre-conditioning tissues that will receive the graft. EVE's mechanisms of action (induction of angiogenesis and of adipogenesis) could effectively improve graft survival also when applied post-operatively (post-conditioning). Here we test this hypothesis in an established murine model and optimize parameters of post-operative application of EVE.

Materials and Methods: Fifty-six 8-week-old athymic (nu/nu) mice received dorsal subcutaneous grafts of human lipoaspirate (0.3 ml each) bilaterally before undergoing EVE (left dorsum) or no treatment (right dorsum, controls). EVE was started either on the same day of (*Immediate group*), two days after (*Early group*), or one week after surgery (*Delayed group*). At follow-up, procured grafts were analyzed for volume retention, remodeling, adipogenesis, and angiogenesis using histology. Volume retention was also assessed by MRI. We subsequently assessed the effects of the *Delayed group* adopting a foam-shaped interface (F-EVE) to deliver treatment.

Results: At a 28 days follow-up delayed post-conditioning with EVE significantly improved the survival of grafts by 18% compared to controls (viable graft thickness ratio: 0.58 ±0.15 vs. 0.49 ±0.13) and increased the density of blood vessels within the graft (+63%: blood vessels/ 10x magnification field: 44 ±12 Vs. 27 ±11). Other groups did not lead to significant changes. Adoption of F-EVE similarly improved outcomes while further reducing fibrosis within the grafts.

Conclusions: Post-operative delayed (1 week) application of EVE modestly improves the survival of adipose tissue grafts by inducing adipogenis and angiogenis. Use of a foam-shaped interface decreases the fibrosis induced to the grafts.

Material and Methods

Study design

A total of 56 female (n = 14 /group), 8-week-old, *Foxn1*^{nu} (nude) mice (Jackson Laboratories, Bar Harbor, MA, USA) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care–certified facility and used in accordance with our Institutional Animal Care and Use Committee guidelines under an approved protocol. All animals received bilateral dorsal subcutaneous grafts of processed human adipose tissue and then underwent EVE stimulation using a previously optimized protocol.[28] *(Fig.1)* In the first phase of this study we tested different timings of application of EVE after surgery. Animals underwent post-conditioning with EVE either on the same day of graft surgery (*Immediate* group), 2 days after the surgery (*Early* group), or 1 week after the surgery (*Delayed* group). In the second phase of this study, we replicated the optimal group of phase 1 adopting Delayed post-conditioning with EVE using a previously described foam-shaped interface (F-EVE). Standardized full-thickness specimens including skin and fat grafts were procured by a single investigator from stimulated areas and control areas 28 days after surgery (n = 10 /group) and 56 days after surgery (n = 4 /group).

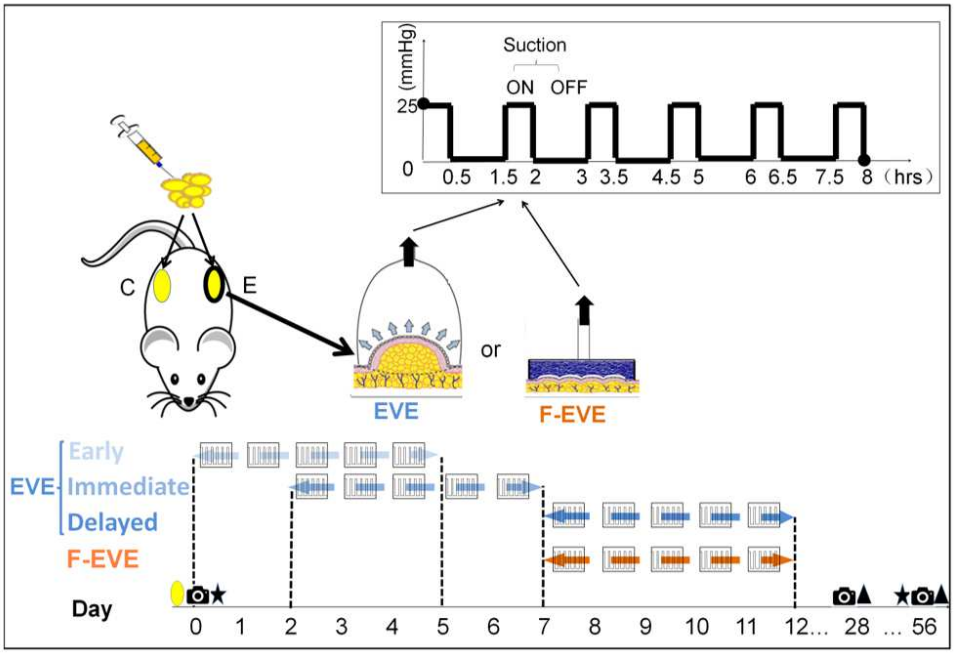


Fig.1 Study design. The yellow ovals on the dorsum of the mouse refer to the bilateral dorsal adipose grafts. The graft on the left side of the dorsum (E = Experimental) received post-conditioning with External Volume Expansion (EVE) （Phase 1, blue）or F-EVE (Phase 2, orange); whereas the grafts on the right side of the dorsum was used as contra-lateral internal control (C = Control) and did not receive additional treatment. The daily parameters of application of EVE are depicted in the top-right box (0.5 hours on / 1 hour off, 6 times a day, for 5 days).

📷 = Digital photography; ★ = Magnetic resonance imaging (n = 2 /group); ▲ = Sample procurement (Day 28: n = 10 /group; Day 56: n = 4 /group). EVE = External Volume Expansion; F-EVE = External Volume Expansion with a foam-shaped interface; hrs = Hours

Subcutaneous fat grafting

De-identified human adipose tissue was obtained from surgical discards under a protocol approved by our Institutional Review Board (IRB). The use of human-derived tissue was performed in accordance with existing regulations and ethical standards (including the World Medical Association Declaration of Helsinki of June 1964 and its subsequent amendments).

The tissue was processed under a sterile technique according to the technique described by Coleman et al. in order to obtain processed lipoaspirate (Table 1).[143] Hemi-spherical grafts (0.3 ml in volume, 1 cm in diameter) were injected in a subcutaneous pocket on both sides of the dorsum of animals in a standardized location positioned 5 cm cranially to the tail and 1 cm lateral to the spine. The tissue was injected using a standard 20 G needle. Grafts injected in the left dorsum underwent EVE treatment, whereas those injected in the right dorsum served as internal controls and received no further treatment.

	Date	Procedure	Age	Gender	BMI (kg/m^2)
EVE Batch1	11/2/2016	Panniculectomy	51	F	32.4
EVE Batch2	11/15/2016	Panniculectomy	45	F	31
F-EVE	3/24/2017	Abdominoplasty	36	F	18.9

Table 1 Clinical data relative to the human donors from which adipose tissue specimens were collected. EVE = External Volume Expansion; F-EVE = External Volume Expansion with a foam-shaped interface; BMI = Body Mass Index

External Volume Expansion

We adopted our previously described murine model of EVE. Briefly, under mild anesthesia with isoflurane (induction 3%, maintenance 2%) we applied to the dorsal skin of animals a dome-shaped silicone cup with an internal diameter of 1 cm (Fig.1). The device was applied in a standardized position, above the previously injected fat grafts and sealed using an adhesive semi-transparent dressing (Tegaderm™, 3M, Maplewood, MN, USA). The cup was then connected to a suction pump (ActiVAC; Kinetic Concepts Inc., San Antonio, TX, USA) through a pressure regulator and once proper sealing was assessed mice were promptly recovered from anesthesia. Mice were stimulated on the left dorsal side: during stimulation no anesthesia is required and animals are free to move in their cages. For the F-EVE group, we used as interface material a 1.0 cm^3 circular polyurethane foam (PrevenaTM foam dressing; Kinetic Concepts Inc., San Antonio, TX, USA). Stimulation was performed using previously optimized parameters (cycles of 0.5 hours on / 1 hour off, repeated 6 times /day for 5 days) and a suction of 25 mmHg.

Macroscopic analysis and Magnetic Resonance Imaging (MRI)

Digital photographs of animals were captured on the day of surgery, and 28 and 56 days after surgery: images were compared by two independent, blinded observers to qualitatively assess differences in volume retention of grafts. MRI scans of the grafts were performed on the day of surgery and 56 days

after surgery (n = 2 animals /group) using a 7-Tesla Bruker scanner (GE Medical Systems, Waukesha, WI, USA). Three-dimensional visualization of the grafts and calculation of the volume of the grafts was performed using T2-weighted images and the free software platform 3D-slicer (Brigham and women's hospital, Boston, MA, USA). Images were analyzed by two independent observers, blinded to treatment.

Histology and Immuno-histo-chemistry

Tissue specimens were fixed with a 10% neutral buffered formalin solution, embedded in paraffin, and cut into 5-µm sections for histological staining and analysis.

Cross-sections were stained with hematoxylin and eosin according to standard protocols. The haematoxylin and eosin-stained slides were examined under a light microscope (Olympus Inc., BX53F, Tokyo, Japan) and evaluated for inflammation (infiltration of lymphocytes and macrophages in the graft), the presence of cystic-like areas and vacuoles within the graft, and the quantity of fibrosis in the graft. Each parameter was classified using a previously validated scale with scores ranging from 0 to 5 (0 = absence; 1 = minimal presence; 2 = minimal to moderate presence; 3 = moderate presence;, 4 = moderate to extensive presence; and 5 = extensive presence)[102,144]. All the evaluations were performed by two independent observers, blinded to treatment.

To perform immuno-histo-chemistry, histological sections were deparaffinated in xylene and rehydrated in decreasing alcohol baths. Antigen retrieval for Perilipin (a marker of the cell membrane of living adipocytes) was accomplished by microwaving slides in 10 mM sodium citrate (pH 6.1). Antigen retrieval for CD 31 (pan-endothelial marker) were treated with 40 µg/ml proteinase K (Roche Diagnostics Corp., Indianapolis, IN, USA) for 30 minutes at 37 °C. The primary antibody for Perilipin (dilution = 1 : 2 50; ab3526, Abcam, Cambridge, MA, USA) was incubated at 37°C for 30 minutes whereas the primary antibody for CD 31 (dilution = 1 : 100; ab28364, Abcam, Cambridge, MA, USA) was incubated at 4 °C overnight. The signal was intensified using a tyramide amplification system (PerkinElmer, Boston, MA, USA) and the positive staining was detected using the Liquid DAB Substrate Chromogen System (Dako North America Inc., Carpinteria, CA, USA) before slides were counterstained with hematoxylin.

Survival of adipose tissue grafts was assessed by measuring the *viable graft thickness ratio* in each slide using Image-Pro Premier 9.3 (Media Cybernetics, Inc., Rockville, MD, USA). Briefly, on histological sections we measured the total thickness of the graft (Tw) and the thickness of the inner perilipin-negative layer (Tn) corresponding to the area of tissue necrosis (Fig.2a). The *viable graft thickness ratio* (VGTR) was defined as VGTR = (Tw - Tn) / Tw. For each graft, Tw and Tn were measured in three different sections and an average VGTR was calculated. Measurements were performed by two observers blinded to treatment, experienced in skin and fat graft histology, and trained in these specific methods.

Blood vessel density within the grafts was measured by counting CD 31+ blood vessel in 10× fields of histological sections using the Image J free software (National Institutes of Health, Bethesda, MD, USA). For each slide, blood vessels were measured in two different fields and an average density was calculated by two independent observers blinded to treatment.

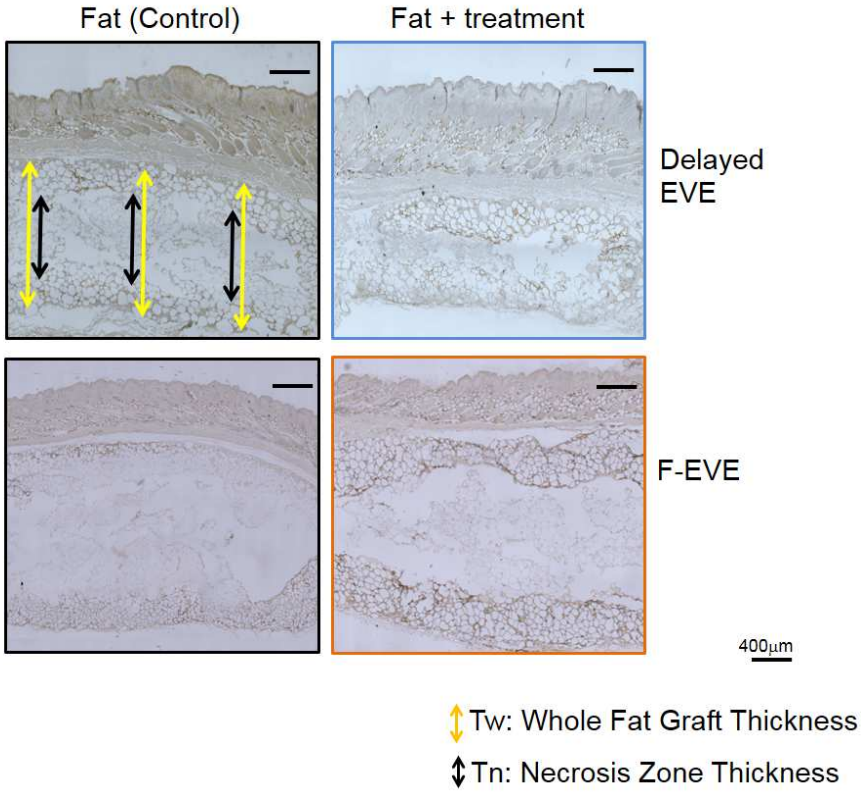


Fig.2 Effect of External Volume Expansion (EVE) on the survival of adipose tissue grafts as assessed by calculating the Viable Graft Thickness Ratio (VGTR) in Perilipin-stained histological slides at 28 days after surgery. (2a) Histological sections with Perilipin staining for adipocytes. Scale bar = 400 µm; Tw = Whole fat graft thickness; Tn = Necrosis zone thickness. EVE = External Volume Expansion; F-EVE = External Volume Expansion with a foam-shaped interface; Delayed = EVE started one week after surgery;

Statistical Analysis

Results were expressed as mean ± SD in text and figures. Two-way analysis of variance (Graphpad Prism 7.0, Inc., La Jolla, CA, USA) with Bonferroni post-hoc correction was used to detect significant differences among groups for outcomes obtained in the first phase of the study; for experiments of the second phase of the study, a paired two-tailed Student's t test was performed to compare data from the F-EVE group to controls. A value of $p < 0.05$ was considered statistically significant.

Results

Delayed External Volume Expansion Increases the Viable Graft Thickness Ratio

At a 28 days follow-up, delayed EVE significantly increased the Viable Graft Thickness Ratio (VGTR) of adipose tissue grafts compared to its internal control group (0.58 ±0.15 Vs. 0.49 ±0.13; p=0.014). On the contrary, neither Immediate nor Early EVE showed a significantly different outcome when compared to their respective controls (Fig2b). Comparable outcomes (0.52 ±0.11 Vs. 0.39 ±0.19; p=0.021) were obtained when analyzing grafts procured at a longer follow-up (56 days) (Supplemental Fig. 1).

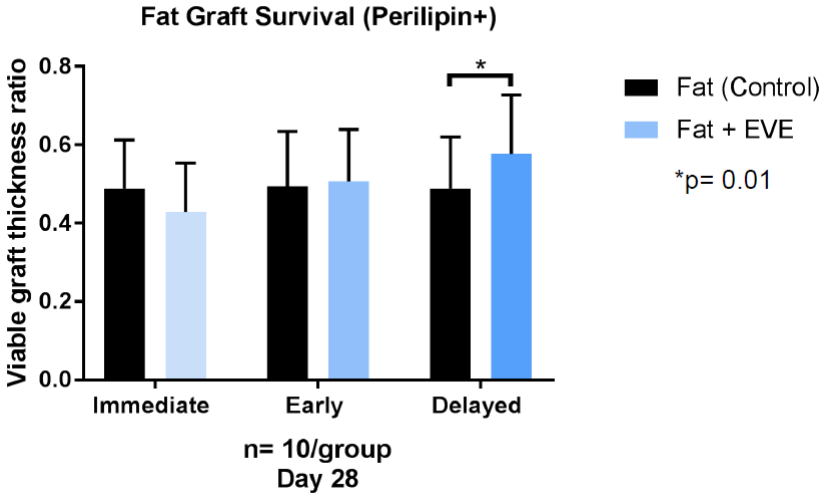
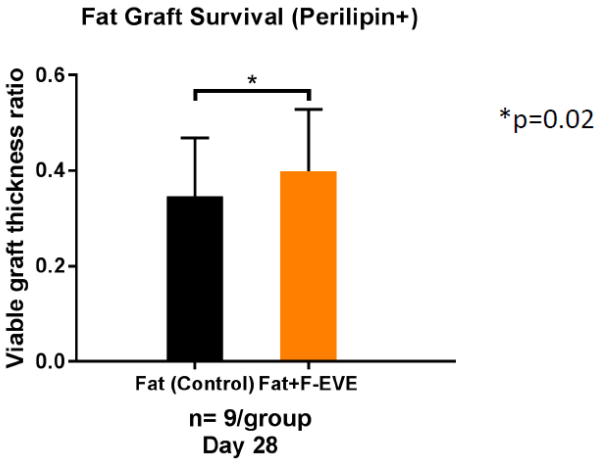
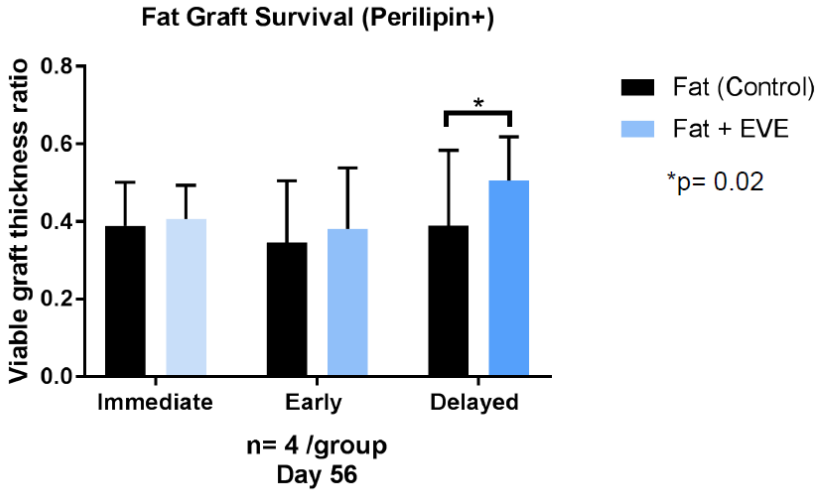


Fig.2 Effect of External Volume Expansion (EVE) on the survival of adipose tissue grafts as assessed by calculating the Viable Graft Thickness Ratio (VGTR) in Perilipin-stained histological slides at 28 days after surgery. (2b) Viable Graft Thickness Ratio (VGTR) as a measurement of the survival of fat grafts in Phase 1 of the study. * indicates p = 0.01. Immediate = EVE started on the same day of surgery; Early = EVE started two days after surgery; Delayed = EVE started one week after surgery; EVE = External Volume Expansion.



(2c) Viable Graft Thickness Ratio (VGTR) as a measurement of the survival of fat grafts in Phase 2 of the study. * indicates p = 0.02. F-EVE = External Volume Expansion with a foam-shaped interface

The F-EVE group also significantly increased the VGTR of treated samples when compared to their untreated control at a 28 days follow-up after surgery (0.40 ±0.13 Vs. 0.35 ±0.12; p=0.022) (Fig2c). No statistically significant differences were reported when comparing data obtained from samples procured at later time-points (56 days).



Suppl. Fig. 1 Effect of External Volume Expansion (EVE) on the survival of adipose tissue grafts as assessed by calculating the Viable Graft Thickness Ratio (VGTR) in Perilipin-stained histological slides at 56 days after surgery. * indicates p = 0.02. Immediate = EVE started on the same day of surgery; Early = EVE started two days after surgery; Delayed = EVE started one week after surgery; EVE = External Volume Expansion; F-EVE = External Volume Expansion with a foam-shaped interface

Delayed External Volume Expansion Increases the Density of Endothelial Vessels in Grafts

Our results showed that Delayed post-conditioning with EVE significantly increased the density of blood vessels within the adipose tissue grafts as compared to their untreated controls (44 ±12 Vs. 27 ±11; p =0.001). No statistically significant differences were observed among other groups (Immediate and Early post-conditioning) and their controls (Fig. 3a, above, left and right; Fig. 3b). Grafts treated with delayed F-EVE also showed a significantly higher density of blood vessels when compared with their untreated control (31 ±10 Vs. 28 ±12 ; p =0.025) (Fig.3a, below, left and right; Fig. 3c).

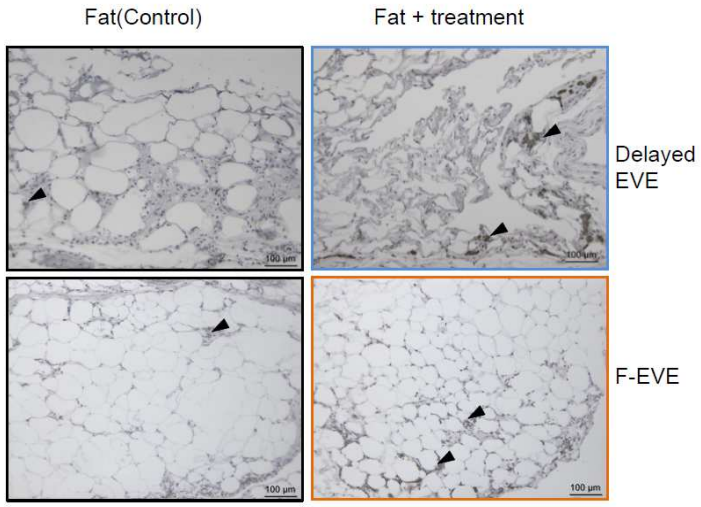
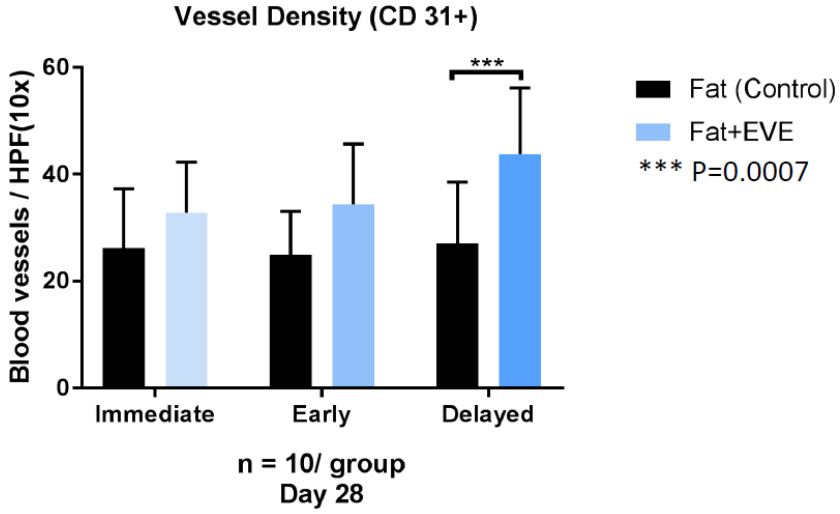
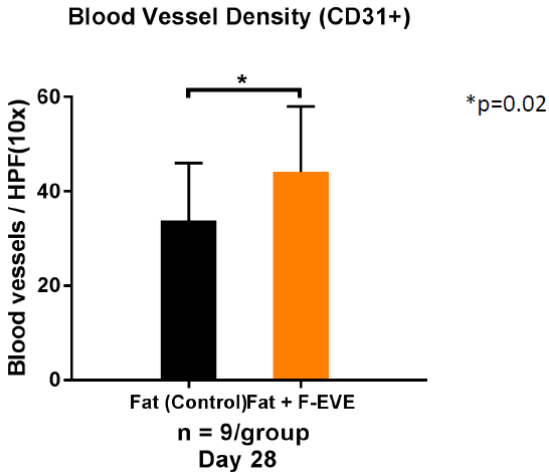


Fig.3 Effect of External Volume Expansion (EVE) on the vascular density of adipose tissue grafts as assessed by CD 31 staining at 28 days after surgery. (3a) Histological sections with CD 31 staining for endothelial vessels. Scale bar = 100 µm; ▲ = Blood vessels; EVE = External Volume Expansion; F-EVE = External Volume Expansion with a foam-shaped interface; Delayed = EVE started one week after surgery



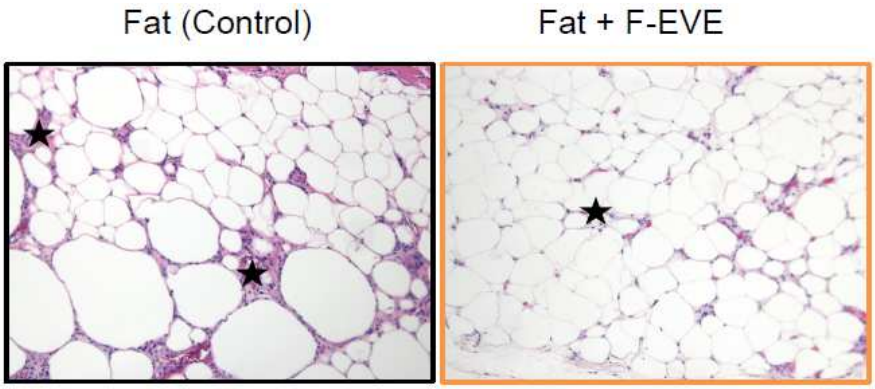
(3b) Density of blood vessels (CD 31+ positive vessels per 10x magnification field) as a measurement of angiogenesis within fat grafts treated with EVE or their untreated controls. * indicates p < 0.01. Immediate = EVE started on the same day of surgery; Early = EVE started two days after surgery; Delayed = EVE started one week after surgery; EVE = External Volume Expansion; HPF = High Power Field



(3c) Density of blood vessels (CD 31+ positive vessels per 10x magnification field) as a measurement of angiogenesis within fat grafts treated with F-EVE or their untreated controls. * indicates p = 0.02. F-EVE = External Volume Expansion with a foam-shaped interface; HPF = High Power Field

Delayed Foam-External Volume Expansion Decreases the Extent of Fibrosis in Fat Grafts

Histologic examination showed that amount of fibrosis in grafts that received post-conditioning with F-EVE was significantly lower than in their controls at a 28 days follow-up (1.4 ±0.4 Vs. 1.7 ±0.5; p =0.045) (Fig.4a, Fig.4b). No statistically significant differences were otherwise observed between standard delayed EVE and its control group or among the other groups and their respective controls. (Table 2)



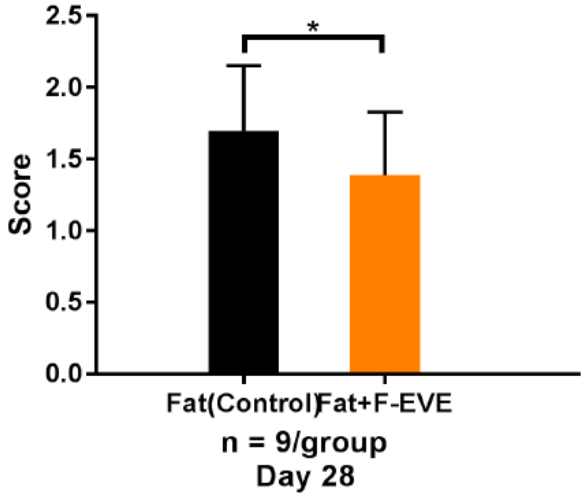


Fig.4 Remodeling effect of External Volume Expansion (EVE) on adipose tissue grafts as assessed by hematoxylin and eosin staining at 28 days after surgery. (4a) Histological sections hematoxylin and eosin staining to highlight tissue fibrosis. Scale bar = 100 µm; ★ = Fibrosis; F-EVE = External Volume Expansion with a foam-shaped interface;

(4b) Scoring of fibrosis in grafts treated with F-EVE and in their controls. * indicates p = 0.04. F-EVE = External Volume Expansion with a foam-shaped interface

	Immediate		Early		Delayed		P
	Fat	Fat+ EVE	Fat	Fat + EVE	Fat	Fat + EVE	
Inflammation	2.18± 0.70	1.75±0.83	2.06± 0.68	1.59± 0.58	2.30± 0.75	1.97± 0.69	ns
Presence of cyst/vacuoles	1.96±0.41	2.07±0.48	2.28±0.32	2.31± 0.63	2.13± 0.60	2.34± 0.44	ns

Table 2 Remodeling effect of External Volume Expansion (EVE) on adipose tissue grafts as assessed by hematoxylin and eosin staining at 28 days after surgery.

Scoring of inflammation and quantity of cystic-like areas/vacuoles in grafts treated with EVE and in their controls. Data is presented as mean ± SD. ns = $p > 0.05$; Immediate = EVE started on the same day of surgery; Early = EVE started two days after surgery; Delayed = EVE started one week after surgery; EVE = External Volume Expansion

	Fat	Fat+ F-EVE	P
Inflammation	2.18± 0.70	1.75±0.83	ns
Presence of cyst/vacuoles	1.96± 0.41	2.07± 0.48	ns

Table 3 Remodeling effect of External Volume Expansion with a foam-shaped interface (F-EVE) on adipose tissue grafts as assessed by hematoxylin and eosin staining at 28 days after surgery. Scoring of inflammation and quantity of cystic-like areas/vacuoles in grafts treated with F-EVE and in their controls. Data is presented as mean ± SD. ns = p > 0.05; F-EVE = External Volume Expansion with a foam-shaped interface

No statistical difference was reported between samples treated with EVE and its controls or between F-EVE and its controls when measuring the amount of inflammation within the grafts and the quantity of cystic-like areas or vacuoles at a 28 days follow-up. (Table 2-3).

Post-conditioning with External Volume Expansion Enhances the Retention of Shape by Grafts

At macroscopic examination, grafts treated with EVE appeared more well-defined in shape as compared to their controls on both 28 and 56 days after surgery. No signs of infection or tissue damage were detected in any of grafts (Fig. 5, *above*). MRI scans demonstrated that the presence of partial graft necrosis: grafted tissue had a homogeneous high-density T2 signal on the day of surgery (Fig.5, center, left), while dotted low-density T2 signal were evident in the inner core of grafts at 56 days after the procedure (Fig.5, center, right). A comparison of three-dimensional reconstruction of the volume of the grafts on the day of surgery and at 56 days of follow-up showed a higher volume retention in samples that received post-conditioning with EVE as compared to their controls; yet, this outcome was not statistically significant. (Fig.5, *below*)

Fig.5 Macroscopic assessment of the volume retention of grafts treated with F-EVE and their respective untreated control using digital imaging (top), MRI scan (middle), and three-dimensional digital reconstruction (bottom). E / Orange arrow = Experimental side; C / White arrow = Control side. (Above); Scale bar = 1 cm; F-EVE = External Volume Expansion with a foam-shaped interface

Discussion

Based on previous preclinical evidence on the positive effects of the preoperative (pre-conditioning) use of External Volume Expansion (EVE) before adipose tissue grafting on the survival of transferred tissues, in this study we hypothesized that the post-operative (post-conditioning) use of EVE could improve graft survival at follow-up through the same mechanism of action previously described. We proposed that post-conditioning with EVE could lead to an increase in vascularity of grafts (angiogenesis) and promote regenerative processes within the graft (adipogenesis) after surgical transfer. We also postulated that a delayed (one week) application of treatment would best improve outcomes by creating a pro-angiogenic sub-hypoxic stimulus at a post-acute time-point in which grafted tissues has recovered from the surgically-induced ischemia. On the contrary, delivery of EVE in an acute post-operative setting (< one week) would exacerbate the hypoxic condition of grafted tissue invariably leading to necrosis and reversing the beneficial effects of EVE.

Our outcomes confirmed these hypotheses and showed that delayed (1 week after grafting) post-conditioning with EVE can significantly increase the survival of adipose tissue grafts by providing angiogenic and adipogenic support to the surviving and regenerating zones of the graft (hence reducing the necrotizing zone). None of the other treatments improved the survival of adipose tissue grafts when compared to controls corroborating our theory that the use of EVE in already hypoxic tissues might limit its effectiveness although grafts treated with immediate or early EVE did not show significantly higher rates of necrosis as compared to controls.

The increment in graft survival observed was modest (<10%); yet, this represents a clinically-relevant outcome in a scenario in which a large percentage (30-70%) of grafted tissue is loss at follow-up due to necrosis and in absence of other techniques to facilitate the survival of grafts. The results achieved in this study are inferior to those observed when adopting EVE pre-operatively in a similar animal model (+20 % increase in volume retention of grafts) and might suggest that the use of EVE as a pre-conditioning method is more effective than its use as a post-conditioning method.[23,71] Yet, this study represents a first attempt to investigate the role and effects of EVE as a post-conditioning method and further evidence-based optimization could possibly enhance its therapeutic potential. In addition, the post-operative use of EVE is not opposed to its use pre-operatively and the two treatments could be combined to synergistically improve outcomes as suggested by preliminary empirical clinical experience. Alternatively, EVE could be used only post-operatively in all those cases in which its pre-operative use is not possible or not preferred by surgeons and patients.

In accordance with previous research in small and large animal models our results show that a key mechanism of action of EVE is the induction of angiogenesis in target tissues.[23,40,68,71–73,127,145,146] Here, the delayed application of EVE almost doubled the density of blood vessels within the graft at a 28 days follow-up as compared to untreated control grafts. Despite the different study design (post-grafting application vs. pre-grafting application) these outcomes are consistent with those previously reported.

Giatsidis et al. had also shown that the pre-operative adoption of EVE with a foam-shaped interface material (F-EVE) retains the biological effects of standard EVE while reducing the damage caused by the mechanical forces exerted by EVE on stimulated tissues. Here, we investigated whether the same principles would apply to the post-operative use of EVE and show that when compared to controls delayed post-conditioning with F-EVE achieves similar outcomes (increased survival of adipose tissue grafts, enhanced angiogenesis within the grafts) of EVE while reducing the amount of damage caused to grafts (measured as graft fibrosis). This evidence might support the use of more comfortable, less-invasive interface materials for EVE in a post-operative setting in which patients might still be healing from surgical incisions (e.g. access points for fat grafting): eventually these interfaces could be integrated in the dressings used for the treatment of the surgical incisions.

Our study provides initial evidence on the rationale for the use of EVE post-operatively to enhance the survival of adipose tissues and suggests preliminary criteria for its optimal use. Despite these outcomes have been obtained in immune-deficient small animal model with structural and mechanical properties of tissues different from those of humans, biologic phenomena observed in murine studies have shown to effectively predict outcomes reported in larger animal models (swine) and humans. In particular, mechano-transductive pathways have been reported to be preserved across species. Yet, we believe that our findings should be further optimized using immune-competent models and testing a broader set of variables, before being confirmed in larger animals and then in humans for clinical adoption.

In conclusion, we here demonstrate that delayed (one week) post-conditioning of adipose tissue grafts with EVE increases the density of blood vessels within the graft and modestly improves the survival of grafted tissue at follow-up. Foam-shaped interfaces reduce the damage caused to grafts and might represent a more comfortable solution for patients post-operatively. We hope that these insights might support the development of novel evidence-based strategies in fat grafting and lead to improved outcomes for patients, although additional studies will be required to better elucidate and optimize the biological mechanisms of action of graft post-conditioning with EVE.

STUDY 4:

DELIVERY OF EXTERNAL VOLUME EXPANSION (EVE) THROUGH MICRO-DEFORMATIONAL INTERFACES IMPROVES ANGIOGENESIS AND LIMITS COMPLICATIONS IN A MURINE MODEL OF DIABETIC SKIN

Summary of the study

Aim: External Volume Expansion (EVE) induces a sub-critical hypoxia that promotes angiogenesis in tissues while avoiding ischemic damage. Effectiveness of EVE depends on this delicate balance and its application to less-vascularized tissues with lower ischemia-resistance (e.g. diabetic skin) poses a risk for ineffectiveness or tissue damage. We investigated the effects of EVE on a murine model of diabetes type-2 and tested whether the adoption of micro-deformational interfaces of EVE optimizes its angiogenic properties while limiting complications to tissues.

Materials and Methods: Adult diabetic mice (Db/Db) received stimulation with EVE on their dorsal skin using the standard Cup-shaped silicone interface (C-EVE), a polyurethane Foam-shaped interface (F-EVE), or a silicone Micro-array chamber interface (M-EVE); controls received no treatment. Skin damage was visually assessed on the last day of stimulation and five days later. At a five-day follow-up, skin specimens (n = 5 /group) were procured and analyzed by histology to assess angiogenesis, adipogenesis, skin remodeling, and inflammation.

Results: All treatments significantly increased the density of blood vessels in skin compared to controls; F-EVE showed the most robust effect (+80 %). No relevant complications were observed using F-EVE or M-EVE but C-EVE lead to substantial skin damage and caused intense inflammation, fibrosis of the subcutaneous tissue, and dermal remodeling (increased thickness/collagen deposition). No adipogenic effect was assessed in any of the groups.

Conclusions: The adoption of EVE with micro-deformational interfaces allows the effective and safe preconditioning of less-vascularized tissues and could improve outcomes in diabetic patients at high-risk for surgical complications.

Material and methods

Study design and Animal model

A total of 10 female, 10-week-old, *BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J* (Db/Db) mice (Stock number: 00642, Jackson Laboratories, Bar Harbor, MA, USA) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care–certified facility and used in accordance with our Institutional Animal Care and Use Committee guidelines, under an approved protocol. Db/Db animals represented a well-established and widely adopted translational model to study the patho-physiologic effects of diabetes type 2.[147] This strain carries a homozygous spontaneous mutation (Leprdb) leading to obesity-induced chronic hyperglycemia, pancreatic beta cell atrophy and eventually by 4 to 8 weeks of age, to diabetes. Animals were individually housed with ad libitum access to food and water, and received environmental enrichment. Experiments were conducted following the ARRIVE guidelines.

We designed our study to have four groups (n = 5 samples/ group): one control group that received no treatment and three experimental groups receiving EVE treatment each with a different type of interface as described below. Each hemi-dorsum of animals was assigned to a separate group/treatment. Animals were assigned randomly to groups: 5 animals received EVE on their left dorsal skin using the standard Cup-shaped silicone interface (C-EVE) and on their right dorsal skin using a polyurethane Foam-shaped interface (F-EVE). The other 5 animals received EVE on their left dorsal skin using a silicone Micro-array chamber interface (M-EVE) and an occlusive dressing (Tegaderm™, 3M™, Maplewood, MN, USA) with no EVE treatment on their right dorsal skin as control. (Fig.1)

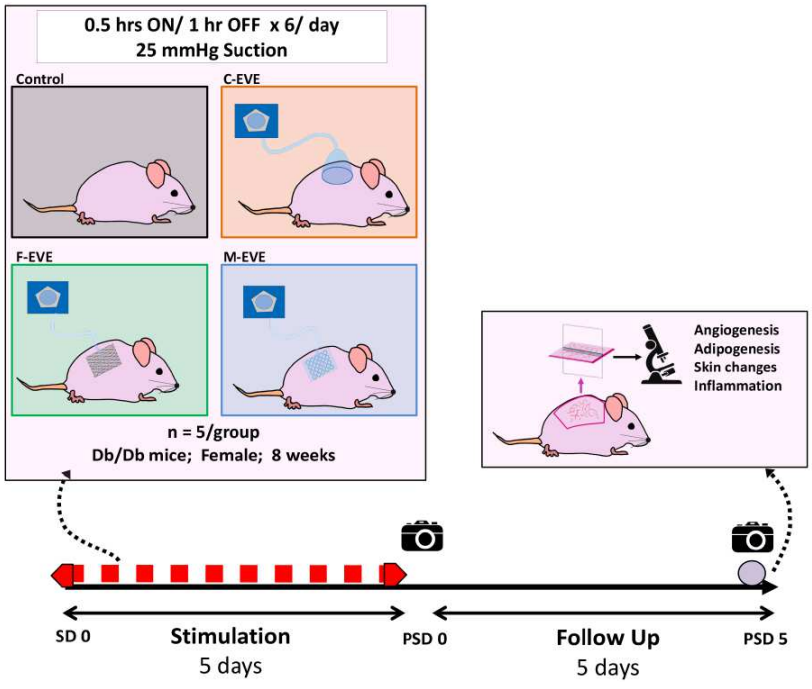


Fig. 1. Experimental study design and procedures.

Top left box: Parameters of treatment, experimental groups, and representation of the different interfaces of treatment adopted, all connected to a pump. C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; Db/Db = *BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J diabetic animals*

Bottom box: timeline of experiments. SD = Stimulation Day; PSD = Post-Stimulation Day; Dotted red line = EVE treatment; = Digital Photography; Grey circle = Samples procurement

Top right box: representation of histological analysis conducted on cross-sections of procured samples.

After the last stimulation with EVE, animals were followed-up for five days; on post-stimulation day 5

(PSD 5), a standardized 1 cm^2 *en bloc* excision biopsy was obtained by the same investigator from stimulated and control skin areas and processed for histology. (Fig.1)

EVE stimulation and Treatment Interfaces

EVE stimulation was conducted in accordance to a previously described optimized protocol that has shown to maximize the angiogenic stimuli to tissues while limiting the rate of cutaneous complications caused by the treatment.[28] Briefly, we adopted a moderate-intensity intermittent treatment using a 25 mmHg suction and 0.5 hours-long stimulation sessions repeated for 6 times a day with 1 hour - long breaks in between; the treatment was continued for 5 days (Stimulation Day 1 to 5, SD 1 – SD 5). Suction was delivered by a pump (ActiVAC; Kinetic Concepts, Inc., San Antonio, TX, USA) connected to the interface through a silicone tube. The three different treatment interfaces included:

a) The standard Cup-shaped silicone interface (C-EVE) with a diameter of 1 cm and an internal volume of 1 ml, as described in previous studies;[148,149]

b) A custom-made polyurethane Foam-shaped interface (F-EVE) with a diameter of 1 cm derived from a commercially-available dressing for Closed Surgical Wound Management (CSWM) (Prevena™, Kinetic Concepts, Inc., San Antonio, TX, USA);[73,150,151]

c) A flexible Micro-array chamber interface (M-EVE) made of two layers of patterned polydimethylsiloxane (PDMS) silicon rubber with open-faced hexagonally-shaped micro-chambers (each 1000 µm in width and 300 µm in height) distributed in an array configuration along the bottom surface of the interface. This design can achieve a uniform distribution of suction causing homogeneous tissue deformation as described in previous studies.[152] Each device was attached to the dorsum of animals, approximately 1 cm laterally to the spine, and sealed using a commercially available dressing (Tegaderm™, 3M™, Maplewood, MN, USA).[28]

Macroscopic Analysis

Digital photographs of the stimulated skin area were captured on Post-Stimulation Day 0 (PSD 0) and on Post- Stimulation Day 5 (PSD 5) and compared by two independent investigators, both blinded to treatment, to detect the cutaneous complications caused by the treatment. Cutaneous complications were scored using a previously described scoring system (1 = Erythema; 2 = Blistering; 4 = Ulceration; 8 = Cachexia). [21]

Histological Analysis

Procured tissue samples were fixed with a 10% buffered formalin solution, embedded in paraffin, and cut into histological sections of 5 µm for staining. Staining with Hematoxylin and Eosin (H&E staining) and with Masson trichrome were performed according to standard protocols and used to measure the effects of the different treatments on the extra-cellular matrix (ECM) and structure of skin. Digital images of the slides were acquired using a light microscope (Olympus Inc., BX53F, Tokyo, Japan) and the software

Image-Pro Premier 9.3 (Media Cybernetics, Inc., Rockville, MD, USA). H&E stained slides were used to measure the thickness of the dermal layer (as an end-point to assess the remodeling of the skin) and of the subcutaneous tissue (as an endpoint to assess adipogenesis) in each sample. Briefly, three measurements were randomly taken in each slide using a 4x histological magnification field and the software Image-Pro Premier 9.3. Slides stained with Masson trichrome were used to perform a qualitative assessment of the density and distribution of collagen fibers in the dermis of each sample. All the evaluations were performed by two independent investigators, both blinded to treatment. Immuno-histochemistry was performed to measure angiogenesis (using the CD 31 endothelial marker), tissue inflammation (using the CD 45 pan-leukocyte marker), and adipogenesis (using the Perilipin marker for the cell membrane of adipocytes). Histological sections were deparaffinated in xylene and rehydrated in sequential alcohol baths. Antigen retrieval for CD 31+ and CD 45+ stained slides was accomplished by incubating slides with 40 µg/ml of proteinase K (Roche Diagnostics Corp., Indianapolis, IN, USA) for 30 minutes at 37 °C. Antigen retrieval for Perilipin+ stained slides was obtained by microwaving slides in 10 mM sodium citrate (pH 6.1). The CD 31 (dilution = 1 : 100; ab28364, Abcam, Cambridge, MA, USA) and the CD 45 (dilution = 1 : 100; ab10558, Abcam, Cambridge, MA, USA) primary antibodies were incubated at 4 °C overnight; the Perilipin (dilution = 1 : 250; ab3526, Abcam, Cambridge, MA, USA) primary antibody was incubated at 37 °C for 30 minutes. Signal from the antibody was intensified using a Tyramide Amplification System (PerkinElmer, Boston, MA, USA). Positive staining was detected using the Liquid DAB Substrate Chromogen System (Dako North America Inc., Carpinteria, CA, USA) before slides were counterstained with hematoxylin. The density of CD 31+ blood vessels in skin was used as an end-point to quantify angiogenesis. CD 31+ blood vessels were counted in 10x fields of each stained slide using the free software Image J (National Institutes of Health, Maryland, USA). For each slide, three fields were evaluated by two independent investigators, both blinded to treatment. The degree of tissue inflammation was assessed for each sample by qualitative analysis of CD 45+ slides in both 4x and 20x histological magnification fields. To qualitatively analyze the rate of adipogenesis in samples belonging to different treatments, viable adipocytes and fibrosis in the subcutaneous adipose tissue of Perilipin+ stained slides were evaluated in 4x histological magnification fields by two independent investigators, both blinded to treatment.

Statistical Analysis

Sample size of experimental groups was calculated based on previous published data in order to obtain a significant statistical difference ($1-\beta = 0.85$; $\alpha = 0.05$) among experimental groups and controls with regards to the primary end-point (blood vessel density at histological analysis). Results were expressed as mean ± SD in text and figures. One-way analysis of variance (Graphpad Prism 7.0, Inc., La Jolla, CA, USA) with a Bonferrroni post-hoc correction test was used to determine the significance of differences among groups and with controls. A p value inferior to 0.05 was considered statistically significant.

Results

EVE increases the density of blood vessels in diabetic skin and the type of treatment interface adopted does not influence its angiogenic potential

Analysis of CD 31+ stained endothelial vessels in the skin samples procured 5 days after the end of EVE stimulation demonstrated that EVE significantly increases the number and density of blood vessels compared to controls (33-80% higher than controls, depending on groups; $p < 0.05$). (Fig.2a) This outcome was independent from the type of treatment interface adopted as all experimental groups statistically differed from controls; yet, samples treated with F-EVE and C-EVE showed a higher increment in vascularity than M-EVE (respectively: for F-EVE +80 % with 65 ±13 blood vessels/ 10x magnification field; for C-EVE +58 % with 57 ±18 blood vessels/ 10x magnification field; and for M-EVE +33% with 48 ±9 blood vessels/ 10x magnification field) when compared to controls (36 ±14 blood vessels/ 10x magnification field). A statistically significant increment in vascularity was also observed in the F-EVE group when compared to the M-EVE group (+35 %; $p < 0.05$). (Fig.2a)

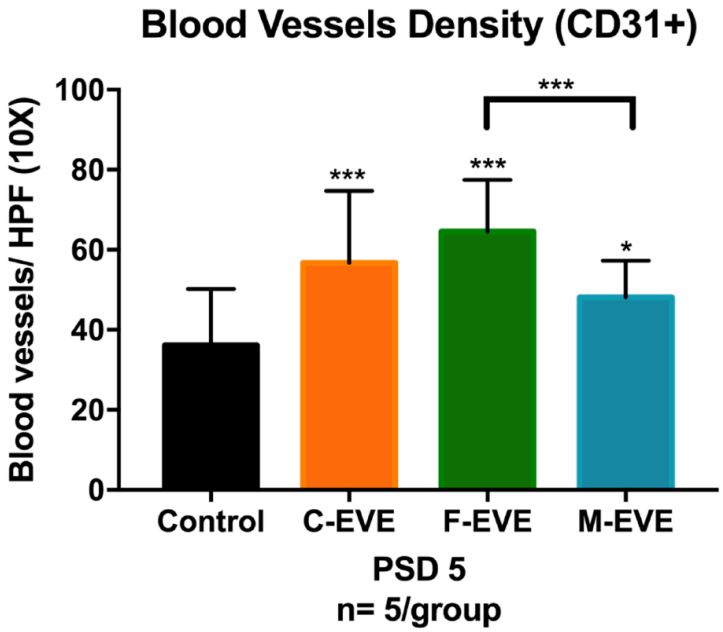


Fig. 2. a: Angiogenic effect of various interfaces of treatment of External Volume Expansion (EVE) at a 5-day follow-up after the end of treatment: outcomes of measurements on histological images. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the mean number of blood vessels / High power field (HPF, 10x) ± SD. C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; PSD = Post-stimulation day; * indicates $p = 0.04$; *** indicates $p < 0.001$

At a qualitative morphological analysis of CD 31+ stained histological images, samples in experimental groups clearly showed an increased density of endothelial vessels; compared to those of controls, these also appeared to be larger in diameter, especially in samples of the C-EVE and F-EVE groups. (Fig.2b)

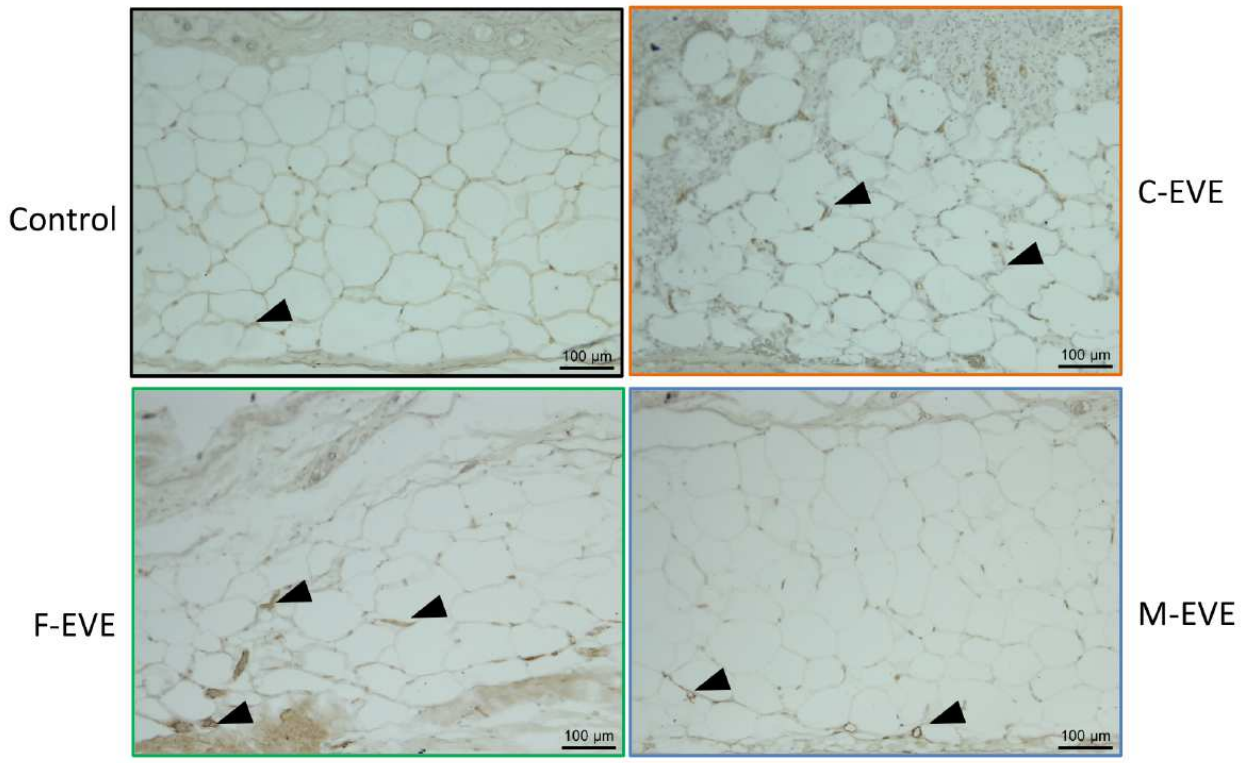
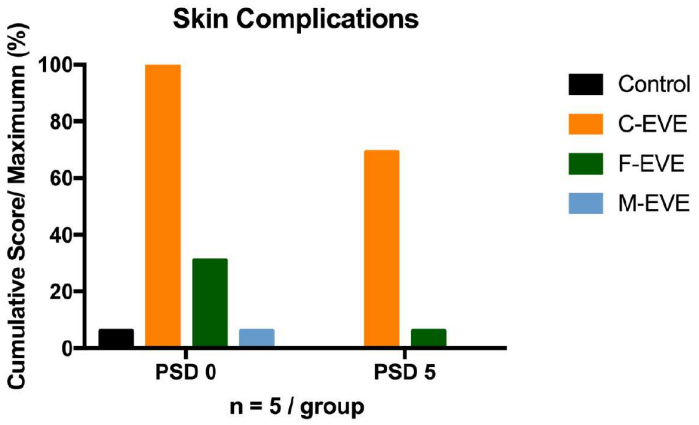


Fig. 2. b: CD 31 staining for blood vessels on samples procured at a 5-day follow-up after the end of treatment. (Magnification: 10X; Reference bar = 100 µm) C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; ▲ = CD 31+ blood vessel

Delivery of EVE using micro-deformational Interfaces limits treatment-induced cutaneous injuries in diabetic skin

Visual semi-quantitative evaluation of the cutaneous injuries on the skin of treated animals and controls on the last (fifth) day of stimulation with EVE showed a significantly higher rate of complications for animals treated with C-EVE as compared to those treated with F-EVE and M-EVE (respectively: cumulative score for C-EVE was used as 100 %; cumulative score for F-EVE = 31 % of the C-EVE maximum; cumulative score for M-EVE = 6 % of the C-EVE maximum; cumulative score for controls = 6 % of the C-EVE maximum). (Fig.3a, Supplemental Table 1) A difference was also observed between M-EVE and F-EVE with the latter causing a higher rate of complications; instead, no differences were reported between skin treated with M-EVE and controls. These observations were confirmed at visual analysis of digital images of skin obtained on the last day of EVE treatment and at a 5-day follow-up after the conclusion of the EVE treatment (Fig.3a). When analyzing the severity of observed complications, skin treated with C-EVE showed a predominance or moderately to highly severe cutaneous damage: in 60% of animals C-EVE causes skin ulceration and in 40% of cases it led to skin blistering at PSD 0. Due to the severity of these injuries, tissue damage was still present at a 5-day follow-up after the conclusion of the EVE treatment. (Fig.3b, Supplemental Fig.1a-b) On the contrary, cutaneous damage caused by F-

EVE was of moderate-low severity and mostly consisting of partial superficial erythema (60% of cases) and occasional blistering (20% of cases). Differently from the C-EVE group almost all cutaneous injuries caused by the F-EVE group were healed at a 5-day follow-up (Fig.3b, Supplemental Fig.1a-b). The M-EVE group and the control group caused only minimal cutaneous damage (erythema in 20% of cases for both groups) which was completely healed by PSD 5. (Fig.3b, Supplemental Fig.1a-b).



COMPLICATION	SCORE
Erythema	1
Blistering	2
Ulceration	4
Cachexia	8

LEFT: Fig. 3. a: Cumulative score for cutaneous complications caused by various interfaces of treatment of External Volume Expansion (EVE) and controls on the last day of stimulation (PSD 0) and at a 5-day follow-up (PSD 5) after the end of treatment. Data is expressed as a percentage of the cumulative score number. C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; PSD = Post-stimulation day

RIGHT: Supplemental Table 1: Score legend for the assessment of the cutaneous complications.

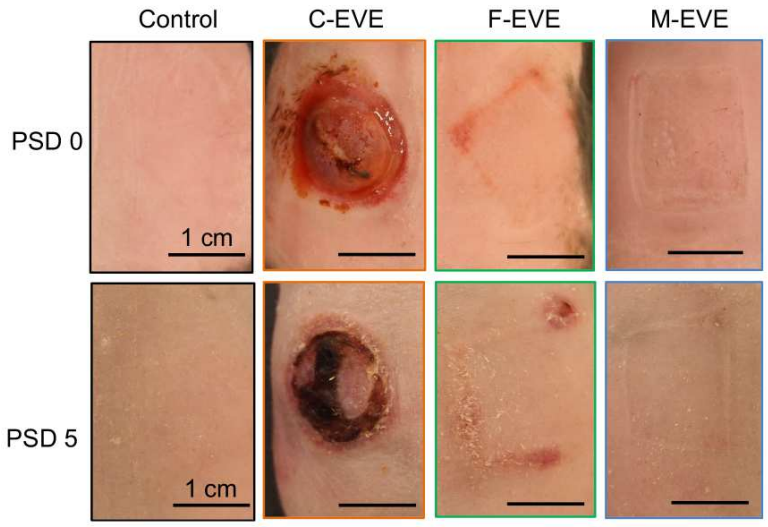
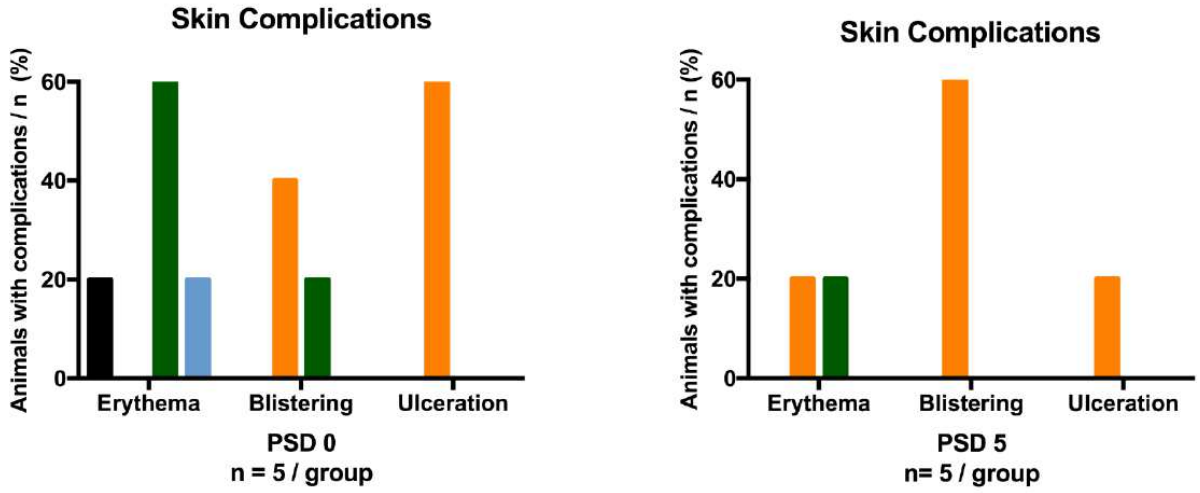


Fig. 3. b: Digital imaging for cutaneous complications caused by various interfaces of treatment of External Volume Expansion (EVE) and controls on the last day of stimulation (PSD 0) and at a 5-day follow-up (PSD 5) after the end of treatment. C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; PSD = Post-stimulation day; Scale bar = 1 cm



Supplemental Fig.1. a-b: Severity and distribution of cutaneous complications caused by various interfaces of treatment of External Volume Expansion (EVE) and controls on the last day of stimulation and at a 5-day follow-up after the end of treatment. Data is expressed as a percentage of the number of animals with complications in the group divided by the total number of animals in the group. C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; PSD = Post-stimulation day

EVE does not promote adipogenesis in diabetic skin

Measurement of the thickness of the subcutaneous tissue on histological sections stained for H&E did not show an increment in thickness for groups that had received treatment with C-EVE or F-EVE when compared to controls. Samples of skin treated with M-EVE showed a significant decrease in thickness when compared to those treated with C-EVE (respectively: 280 ±90 µm vs. 470 ±140 µm; p = 0.02) and those of controls (470 ±80 µm; p = 0.02). (Fig.4ab) Visual morphological analysis of histological slides confirmed the reported differences. (Fig.4b)

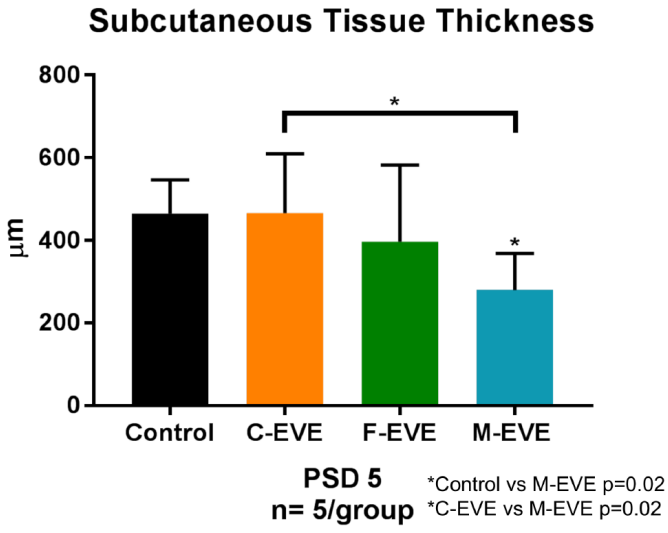


Fig. 4. a: Adipogenic effect on skin (thickness of the subcutaneous tissue layer) of various interfaces of treatment of External Volume Expansion (EVE) at a 5-day follow-up after the end of treatment (PSD 5): outcomes of measurements on histological images. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the mean number of µm ± SD. C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; PSD = Post-stimulation day; * indicates p = 0.02

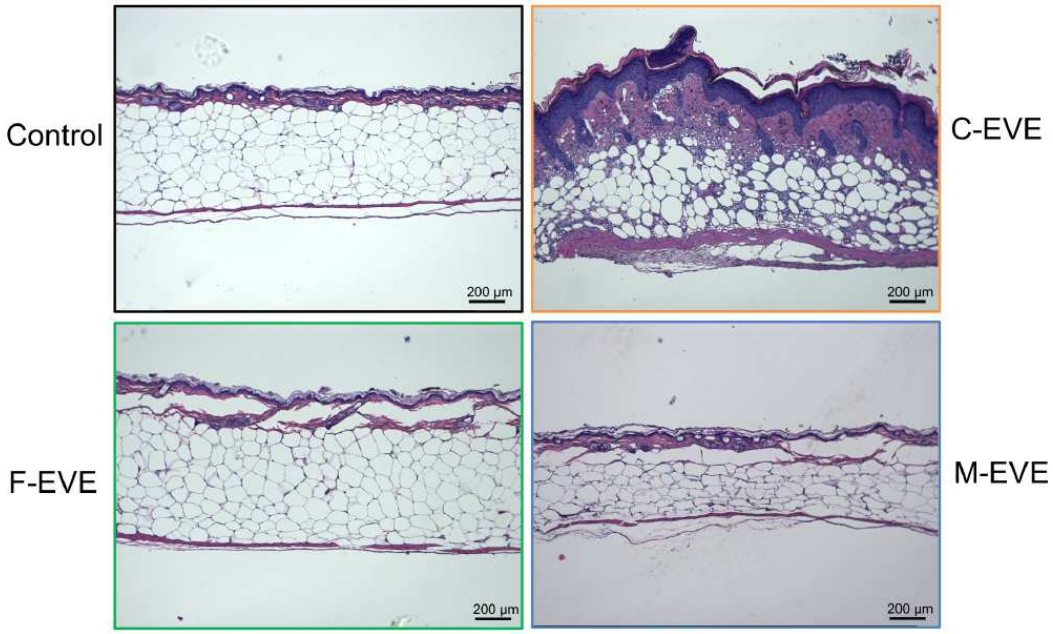
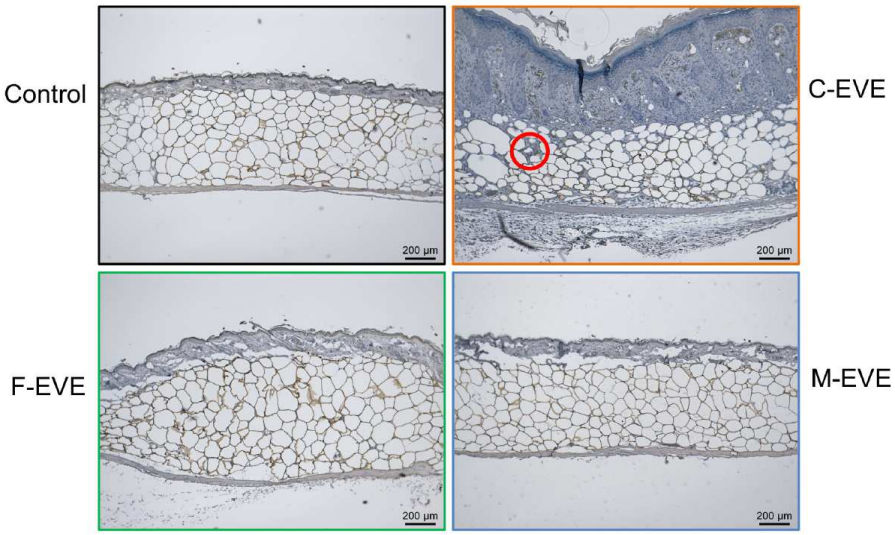


Fig. 4. b: Hematoxylin and Eosin (H&E) staining of skin on samples procured at a 5-day follow-up after the end of treatment. (Magnification: 4X; Reference bar = 200 µm) C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface

Immuno-histo-chemistry for the Perilipin, a marker of the cell membrane of adipocytes, was used to perform a qualitative analysis of the presence and distribution of adipocytes within histological sections of samples from all groups and controls: no significant differences were observed among groups and between experimental groups and controls. Adipocytes of samples that had been treated with F-EVE showed occasionally non-homogeneous shape with some fragmentation of the cell membranes; samples that had undergone treatment with C-EVE demonstrated a higher presence of interstitial fibrosis surrounding cells. (Supplemental Fig.2)



Supplemental Fig. 2. Adipogenic effect on skin (presence and distribution of adipocytes) of various interfaces of treatment of External Volume Expansion (EVE) at a 5-day follow-up after the end of treatment: Perilipin staining for adipocytes. (Magnification: 4X; Reference bar = 200 µm) C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; O = Fibrosis

C-EVE leads to significant remodeling of the dermal layer of stimulated skin

The thickness of the dermal layer of skin was measured on histological sections of specimens stained for H&E; samples that had undergone stimulation with C-EVE showed a significantly higher thickness compared to controls (+286 %, respectively: 260 ±120 µm for C-EVE vs. 70 ±20 for controls; $p < 0.05$) and to samples treated with F-EVE and M-EVE (+292-574 %; $p < 0.05$). (Fig.5a) No differences were observed among samples treated with F-EVE or M-EVE and controls (Fig.5a). Histological images clearly demonstrated these differences. (Fig.4b)

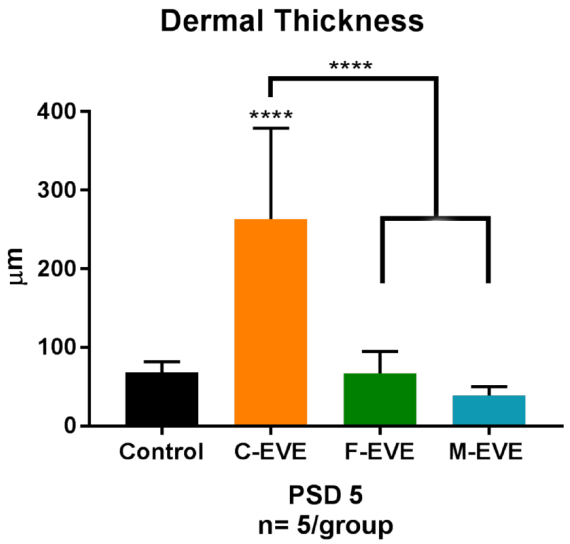
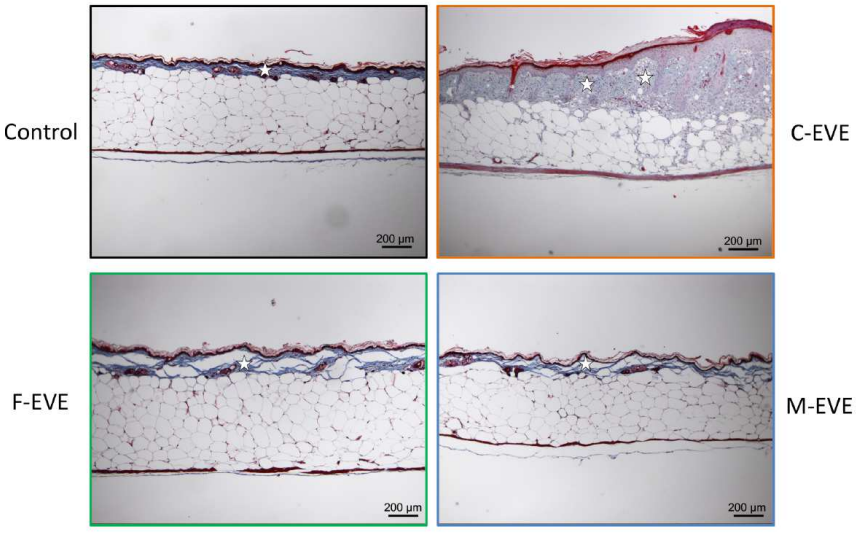


Fig. 5. Remodeling effect on skin (thickness of the dermal layer) of various interfaces of treatment of External Volume Expansion (EVE) at a 5-day follow-up after the end of treatment (PSD 5): outcomes of measurements on histological images. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the number of µm ± SD. C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; PSD = Post-stimulation day; *** means $p < 0.001$

Histological staining for collagen fibers (Masson trichrome) confirmed a significantly higher deposition of collagen in the dermis of skin treated with C-EVE as compared to controls or samples treated with other types of interfaces. Skin that had been stimulated with M-EVE and F-EVE showed a moderate fragmentation of the dermal layer. (Supplemental Fig.3)

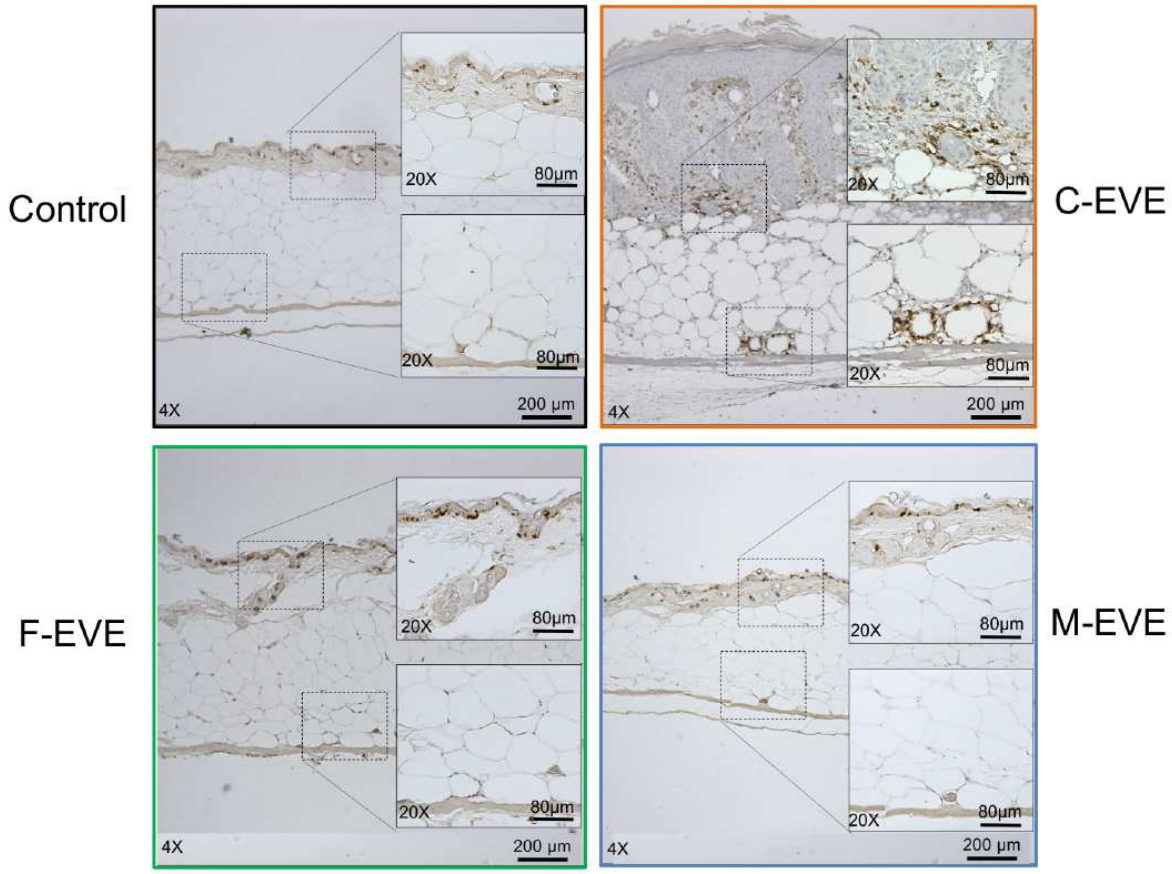


Supplemental Fig. 3. Remodeling effect on skin (deposition of collagen fibers) of various interfaces of treatment of External Volume Expansion (EVE) at a 5-day follow-up after the end of treatment: Masson trichrome staining for collagen fibers. (Magnification: 4X; Reference bar = 200 µm) C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface; ★ = Collagen fibers

C-EVE causes robust tissue

inflammation

Immuno-histo-chemistry for the CD 45 pan-leukocyte marker revealed an increased quantity and distribution of inflammatory cells in skin treated with C-EVE at a 5-day follow-up after treatment when compared to controls or samples that had been stimulated with F-EVE or M-EVE. Inflammation in the C-EVE group was localized in both the dermis and in the subcutaneous adipose tissue. (Supplemental Fig.4) Samples of skin treated with F-EVE and M-EVE showed an increase in the number of infiltrating inflammatory cells as compared to control still, although this difference was minimal (modestly higher in the F-EVE group compared to the M-EVE group).



Supplemental Fig. 4. Pro-inflammatory effect on skin (infiltration of inflammatory cells) of various interfaces of treatment of External Volume Expansion (EVE) at a 5-day follow-up after the end of treatment: CD 45+ staining for leukocytes. (Magnification: 4X and 20X; Reference bars = 200 µm and 80 µm) C-EVE = Cup-shaped silicone interface; F-EVE = Polyurethane Foam-shaped interface; M-EVE = Silicone Micro-array chamber interface

Discussion

In this study, we investigate whether External Volume Expansion (EVE) retains its pro-angiogenic properties when applied to diabetic skin, which is characterized by a lower micro-vascularity, a higher sensitivity to tissue ischemia, and an impaired ability to induce the proliferation of blood vessels in reparative or regenerative processes. We also tested whether the use of novel micro-deformational interfaces of treatment (similar to those that had shown to successfully promote angiogenesis and healing in experimental models of chronic wounds) might provide an angiogenic stimuli while limiting the mechanical-ischemic damage caused to stimulated tissues. The overarching goal of this research was to validate in an established murine model the biologic effects of EVE on diabetic skin and optimize its parameters of application to develop in a next stage of research a safe and effective method for preconditioning tissues in patients affected by diabetes and improve surgical outcomes.

Our results confirmed that the application of EVE using previously optimized parameters can promote angiogenesis and increase the density of blood vessels in stimulated diabetic skin;[28] all our experimental groups lead to a significantly higher (density of blood vessels 33-80 % higher) compared to untreated control skin. These outcomes are consistent with those previously observed and reported in similar non-diabetic animal models.[26,28,74,127,148,149,153,154] Interestingly, in both diabetic and non-diabetic skin the optimized use of EVE seems to double the vascularization of tissues independently from the original density of blood vessels in target tissues.[28,148] Diabetic skin is characterized by a lower vascularity and diffused micro-vascular damage: consistently, in our controls, the density of blood vessels was almost 25 % of that observed in control skin from non-diabetic in previous studies.[28,148] Yet, this baseline condition did not affect the rate of effectiveness of EVE. A 33-80 % increment in tissue vascularization -as observed here- is likely to be associated with substantially improved surgical outcomes such as a lower density to tissue (partial/total) necrosis, wound dehiscence, or infection. Today, no other clinically-available method has shown to be able to achieve this outcome in non-diabetic or diabetic patients. The fact that EVE adopts a cost-effective, non-invasive approach to tissue preconditioning based on FDA-approved devices further highlights the translational value of these findings and proposed strategy. In addition, besides its application as a preconditioning method in preparation of surgeries we postulate that EVE could possibly be used to preventively improve the vascularity of skin in diabetic patients at high-risk for the development of cutaneous complications related to an impaired vascularization (e.g. as a chronic, preventive therapy to reduce the rate of development of diabetic foot ulcers).[50,55,155] No significant differences were observed between the angiogenic potential of the standard C-EVE treatment and the F-EVE treatment: this outcome is consistent with what previously reported in the application of EVE to non-diabetic skin. Instead, the application of a novel M-EVE interface/treatment seemed to lead to a lower induction of angiogenesis in tissues (yet, still 33 % significantly higher than in controls). We believe that the sub-critical hypoxic stimulus provided by M-EVE is more moderate than the one exerted by other treatments, hence requiring longer stimulations (cycles or duration of treatment) to achieve the same outcomes of F-EVE or C-EVE.[152] The configuration we adopted in the M-EVE interface (micro-chambers

each 1000 μm in width and 300 μm in height) differed in size and shape from the one of the F-EVE interface (foam with pore sized approximately 400-600 μm). Previous research has shown that the geometry and size of the chambers in contact with tissue affect the delivery of mechanical stimulation and its biological effects;[18] the different configurations between M-EVE and F-EVE might have also affected outcomes.

The clinical application of EVE has been limited in part by the fact that its mechanical stimulation can cause cutaneous injuries at the contact point between the interface of treatment and patients' skin.[28,56] Previous studies on animal models has shown that the rate and severity of cutaneous injuries can be mitigated by optimizing the regimen of application of EVE (pressures, kinetics, and duration) and by using interfaces that distribute the mechanical stress exerted by EVE along the entire area of stimulated skin (vs. only along the rim of the cup-shaped silicone interface as in standard C-EVE).[28] Here we confirm these findings and show that in the less well-vascularized and more ischemia-sensitive diabetic skin, even an optimized regimen of EVE is not sufficient to avoid the induction of severe cutaneous injuries when adopting a standard cup-shaped silicone interface (C-EVE). Instead, the use of micro-deformational interfaces of treatment (F-EVE and M-EVE) could significantly limit the rate and the severity of complications caused by the treatment as noted in both the macroscopic analysis of skin specimen and the histological evaluation of infiltrating inflammatory cells. These results suggest that the use of micro-deformational interfaces of treatment (F-EVE and M-EVE) might be more beneficial and possibly mandatory when applying EVE to less well-vascularized soft tissues.

Contrarily from previous studies our results did not show a relevant adipogenic effect of EVE when applied to diabetic skin; C-EVE caused some interstitial fibrosis consistent with the other macroscopic and microscopic signs of tissue injury, whereas M-EVE led to a moderate reduction in thickness of the subcutaneous adipose layer.[28,148,156] The robust effect on the dermal layer of the skin demonstrated by C-EVE (thickening, cell proliferation and collagen deposition) might also be an indirect biologic response to the injury caused by the treatment to cutaneous tissues.

The clinical impact of this research translates to the possibility to develop a non-invasive, cost-effective, and patient-ready treatment to effectively and safely precondition soft tissues in diabetic patients in preparation of surgeries with the goal of reducing surgical complications (e.g. wound dehiscence or infections) and improving outcomes (e.g. tissue survival) in this high-risk, increasingly more high-volume category of patients. Diabetic patients could receive F-EVE or M-EVE treatment in preparation of any surgery requiring incisions prone to necrosis or dehiscence, a flap surgery, or a graft procedure. In our previous studies using non-diabetic murine models we observed a baseline vascular density 4-times lower than the one here observed in our control diabetic animals.[28,148] In another example, Choi et al. demonstrated in a rodent model that diabetes causes a 5-time higher failure rate and a significantly lower survival rate of adipose tissue grafts compared to non-diabetic controls; this result was in part due to the observed lower vascularity of both the tissues recipient of the graft and the graft.[157,158] Fat grafting is among the most popular techniques adopted by plastic surgeons in aesthetic and reconstructive

procedures: statistically, of the over 130,000 combined procedures reported in 2016 by the American Society of Plastic Surgeons at least 13,000 patients might have been diabetic and have likely incurred sub-optimal outcomes (lower rates of graft survival or lower volume retention at follow-up).[158,159] To-date this problem has been mostly neglected with the absence of studies assessing the clinical impact of diabetes on adipose tissue grafts and the lack of therapeutic strategies designed to overcome it:[66] The preventive use of EVE as described in this study might provide an effective solution to these challenges and other similar clinical problems.[61]

This study has some limitations. It was conducted on a genetically-induced diabetic murine animal model which has intrinsic differences in patho-physiology (diabetic micro-vascular damage) and cutaneous anatomy (e.g. thickness of the skin, density of the vascular network and sensitivity to ischemia) compared to those of human patients. Yet, we believe that, despite these differences, our results highlight the importance of developing therapeutic approaches specifically addressing the challenges posed by patients affected by diabetes.[160,161] In addition, they provide evidence-based principles obtained in a controlled animal model that can guide further clinical investigation to optimize an effective and safe use of EVE in diabetic patients.

In conclusion, in this study we demonstrate that EVE can robustly increase the vascularity of soft tissues in diabetic skin and could possibly be used as a preconditioning method before plastic and reconstructive surgeries in diabetic patients at high-risk of ischemia-related complications or as a preventive treatment to avoid the formation of ischemic wounds. In addition, we show that the intrinsic differences in sensitivity to ischemia of diabetic skin require the use of micro-deformational interfaces (F-EVE and M-EVE) of treatment that can safely retain the angiogenic effect of EVE while avoiding/limiting cutaneous complications. This evidence may establish the basis for further clinical studies on the use of EVE in diabetic individuals and we hope it might lead to novel therapeutic strategy to improve surgical outcomes in these patients.

STUDY 5:

TISSUE ENGINEERED SOFT TISSUE RECONSTRUCTION USING NON-INVASIVE MECHANICAL PRECONDITIONING AND A SHELF-READY ALLOGRAFT ADIPOSE MATRIX

Summary of the study

Aim: Soft tissues defects leading to severe functional (disability) and morphological (disfigurement) morbidity are a common consequence of trauma, surgery, chronic disease, or congenital malformations: yet, current surgical options for soft tissue reconstruction have shown limited efficacy, whereas tissue engineering strategies have failed to provide solutions ready to be translated to patient care. Adipose acellular scaffolds can provide the ideal bio-mimetic environment for in situ autologous soft tissue regeneration; synergistically, cell migration and proliferation (adipogenesis) can be enhanced by preliminarily increasing the vascularity (preconditioning) of the tissues recipient of the scaffolds.

Materials and Methods: Using an established small animal model we tested the potential of a human-derived, shelf-ready, injectable, acellular allograft adipose matrix (AAM) to reconstruct soft tissue defects when used in combination with non-invasive mechanical preconditioning of tissues.

Results: This strategy significantly improved long-term volume retention (50-80% higher at 12 weeks follow-up) and histological quality of reconstructed tissues compared to current standard of care (adipose grafts). The AAM induced both adipogenesis and angiogenesis. Combined use of the AAM and adipose grafts mitigated efficacy.

Conclusions: Our study suggests that the synergistic use of the AAM and non-invasive tissue preconditioning provide a more effective solution for soft tissue reconstruction: this strategy is ready to be translated to clinical trials and, if outcomes will be confirmed, it could establish the basis for a novel therapeutic paradigm in reconstructive surgery.

Materials and Methods

Research objective

The primary objective of this study was to determine whether subcutaneous graft of an Allograft Adipose Matrix (AAM) could provide a better soft tissue reconstruction compared to current standard of care (adipose tissue grafts) as assessed through macroscopic (graft weight and volume retention) and microscopic methods (graft adipogenesis and angiogenesis). The goal of this research is to establish the translational basis and evidence for clinical application of the AAM: by doing so we aim to improve current best practice in reconstructive surgery of soft tissues and provide better outcomes to a large number of patients undergoing these surgeries. Our secondary objective was to explore the differential effectiveness of a range of treatments that combined AAM grafts, adipose tissue grafts, and a clinically-ready non-invasive technique that uses external mechanical forces for recipient site preparation (preconditioning) before grafting (External Volume Expansion, EVE). The latter technique had previously shown the capacity to improve survival of adipose tissue grafts by increasing angiogenesis and adipogenesis in recipient tissues.[23,28,70,162] The primary hypothesis of the study was that -by providing the bio-physical cues (biological and structural components of the ECM) that support adipogenesis (adipocyte migration and proliferation) while lacking living components at risk for ischemia-induced necrosis after grafting- the AAM would provide a better reconstructive outcome (higher volume and weight survival, less necrosis and cystic-like areas) at follow-up in comparison to adipose tissue grafts. We also hypothesize that the ECM components of the AAM would provide inductive signals to promote infiltration of blood vessels within the grafts and angiogenesis. Our secondary hypothesis was that when considering combinations of treatments, the recipient site preparation provided by EVE (angiogenesis and adipogenesis) would have improved outcomes associated with grafts, whereas the combination of the AAM grafts with adipose tissue grafts would have mitigated and balance outcomes.

Animals

All animal experiments have been designed and performed in accordance with the ARRIVE. Experimental animals were used under an approved protocol, in accordance with our Institutional Animal Care and Use Committee guidelines, and adhering to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). All experiments were performed in a clean environment inside the accredited animal facility of our Institution which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were conducted at the same time of the day for all different groups. The study involved 54 female, 10-week old athymic nude mice (NU/J also known as nu/nu, strain 002019, Jackson Laboratories, Bar Harbor, ME). This mouse strain was selected based on the ability to tolerate xenografts without rejection. Animals were housed in pathogen-free facilities individually providing an enriched environment and standard bedding: animals had access to food and water ad libitum. Welfare of animals was monitored daily during experiments.

External Volume Expansion Model

We adopted our previously described murine model of EVE (Fig. 2b).[23,28,70,162] Briefly, under mild anesthesia with isoflurane (induction 3%, maintenance 2%) we applied to the shaved dorsal skin of animals a dome-shaped silicone cup with an internal diameter of 1 cm (Fig. 1). The device was applied in a standardized position, at the midline of a line going from the proximal portion of the neck to the proximal origin of the tail, 1 cm laterally to the spine. The cup was then connected to a suction pump (ActiVAC; Kinetic Concepts Inc., San Antonio, TX) through a pressure regulator and once proper sealing was assessed mice were promptly recovered from anesthesia. Mice were stimulated using our optimized protocol (0.5-hour long stimulations 6 times per day, each separated by 1-hour long intervals for 5 days,): during stimulation no anesthesia is required and animals are free to move in their cages.

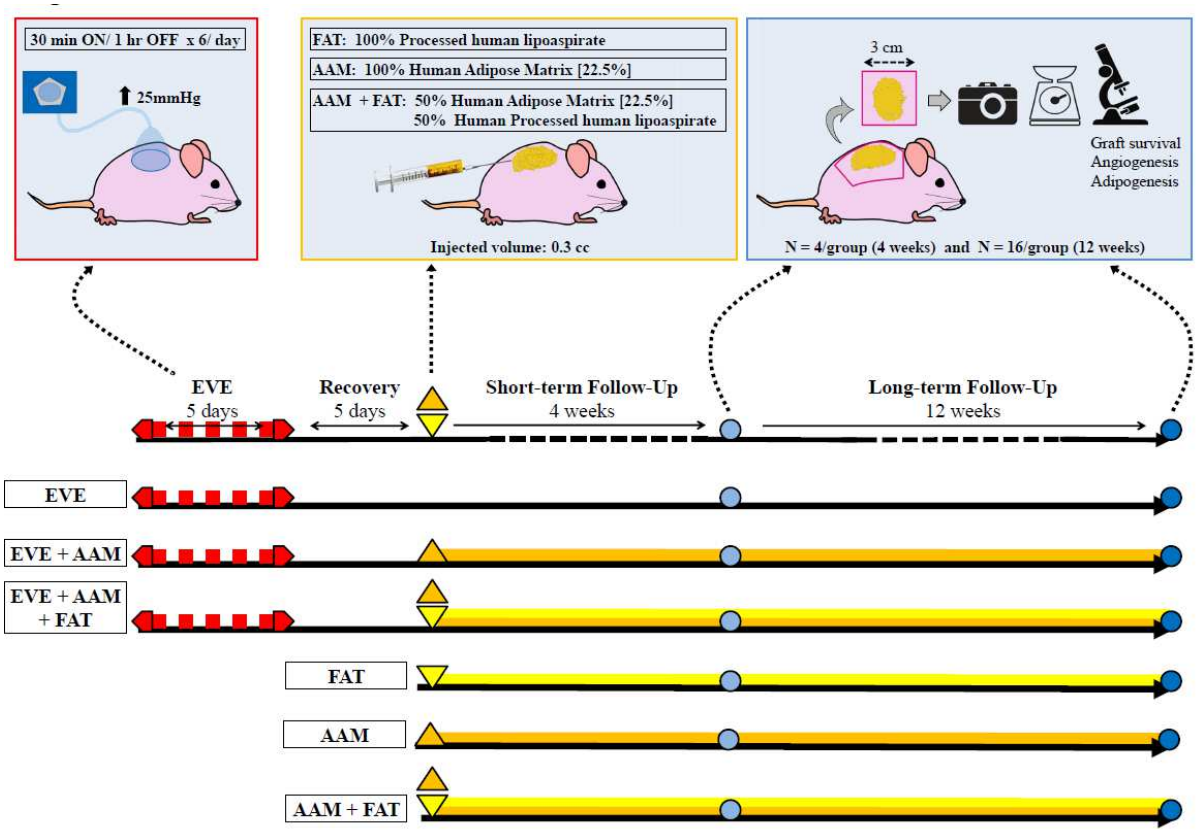


Fig. 1. Experimental study design and procedures. **Top-left box:** representation of tissue preconditioning with External Volume Expansion (EVE) using moderate-intensity intermittent kinetics (30 minutes ON/ 1 hour OFF, 6 times a day for 5 days) and a 25 mm Hg suction provided by a pump and delivered through a dome-shaped silicone interface. **Top-central box:** representation of the preparation of the different grafts and of the grafting technique in the subcutaneous lateral dorsum of animals. (FAT = Adipose Tissue; AAM = Allograft Adipose Matrix, ECM = Extra-cellular Matrix) **Top-right box:** representation of the analytic techniques adopted to assess outcomes and the number of samples analyzed at each time-point. (= Digital imaging; = Specimen weight; = Microscopic analysis through histology) **Central figure:** study design. (Red dotted line = EVE; Orange triangle and line = AAM graft; Yellow triangle and line = FAT graft; Blue dots = Time points for digital imaging and tissue procurement) (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix, ECM = Extra-cellular Matrix)

Subcutaneous injection model

Under general anesthesia, 0.3 cc of graft was injected on the lateral dorsum of animals through a distal surgical access (root of the tail) using a "tunnel technique" and a 16 Gauge blunt lipo-injection cannula (Blunt Injector, Marina Medical Instruments Inc., Sunrise, FL) over a length of 3 cm (Fig. 1). The choice of the cannula was determined by our previous studies and clinical experience.[125] In animals who had received EVE treatment the graft was centered with regards to the stimulated area.

Adipose tissue (FAT) grafts preparation

Lipoaspirate was obtained through manual liposuction from discarded human panniculectomies and processed according to the established Coleman's technique in a sterile fashion to obtain adipose tissue for grafting.[97] Briefly, the lipoaspirate was centrifuged at 3200 rpm for 3 minutes to separate the adipose tissue from the oily part and the stromal vascular fraction (SVF): both these two components were discarded to obtain the processed adipose tissue (FAT) (Fig. S41a-b). Human tissue was procured under a protocol approved by our Institutional IRB, in accordance with existing rules and regulations, and following all ethical standards. Samples were de-identified: non-identifying information related to the source of tissues was collected as allowed by the protocol. This data is listed in the supplemental material (Table S1). Animals were randomly divided in two rounds of experiments (two human tissue donors): in each round animals received adipose tissue grafts from the same donor and the lipoaspirate was used fresh within 1 hour from procurement in the operating room (during this time it was preserved in an envelope covered by ice).

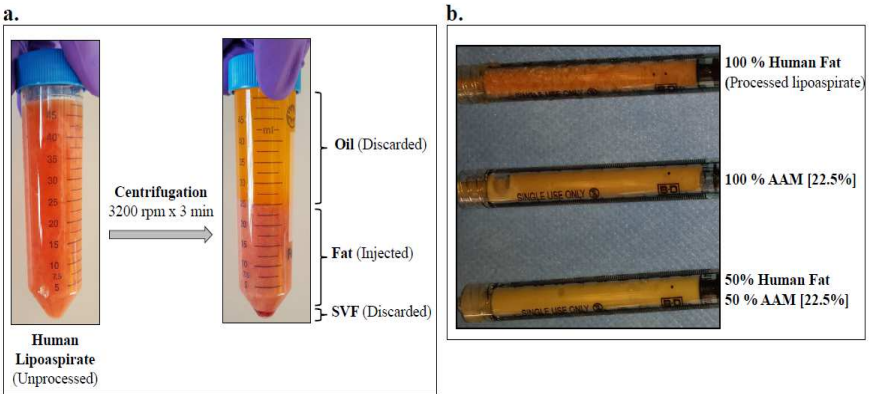


Fig. S1a-b. a: Preparation of processed adipose tissue grafts from human lipoaspirate through centrifugation. (SVF = Stromal vascular Fraction; rpm = rotations per minute) **b:** Appearance of the processed adipose tissue grafts, the rehydrated Allograft Adipose Matrix, and the combination of the two in 1 ml syringes before injection. (AAM = Allograft Adipose Matrix)

Donor ID	Age	Sex	Weight (lbs)	Height (Ft. In.)	BMI	Donor Site	History of Diabetes	Smoking Habit
1	53	Female	234	5'5"	38.94	Abdominal Wall (superficial adipose layer)	No	Yes (former)
2	50	Female	192	5'9"	28.34	Abdominal Wall (superficial adipose layer)	No	Yes (former)

Table S1. Clinical characteristics of donor patients for adipose tissue. Age is expressed in years. (lbs + pounds; Ft. In. = Feet and Inches; BMI = Body Mass Index)

AAM preparation

AAM was provided by the Musculoskeletal Transplant Foundation (Edison, NJ, USA) and obtained from processed human adipose tissue of cadaveric donors as previously described. Procurement of donor tissue was performed in accordance with existing regulations and under approved. The AAM was provided in a lyophilized and sterilized injectable powder containing small-size particles (~100-200 µm2 with a 2 : 1 aspect ratio). All procedures related to the procurement and processing of the AAM followed the requirements and criteria of "minimal manipulation" as regulated by the FDA. The AAM powder was reconstituted with sterile saline solution to obtain a 22.5% rehydration ratio (mass protein-to-volume saline ratio) prior to in vivo injection (Fig. S1b).

AAM + FAT (Adipose tissue) grafts preparation

The rehydrated AAM was mixed in equal parts (50% ratio, based on mass weight) with processed lipoaspirate using two Luer-lock syringes and a connector (Fig. 1, S1b).

Animal Study Design

Mice were randomly allocated to experimental groups undergoing subcutaneous injection of as described above. Animals receiving EVE treatment underwent stimulation from 10 days prior to surgery to 5 days prior to surgery. After surgery (dorsal grafting) animals were followed-up for 4 (n = 4/ group), and 12 weeks (n = 16 / group) before collecting samples of the grafts (skin only in the EVE group) for analysis. Grafts were procured en bloc using standardized 2 x 2 cm biopsies (including the graft, the overlaying/surrounding full-thickness skin and the panniculus carnosus) (Fig. 1).

Macroscopic Analysis

Qualitative analysis of the external and internal macroscopic appearance of grafts, before and after surgical dissection, was done with digital imaging captured on the day of sample harvesting (Nikon Coolpix S4; Nikon Co., Tokyo, Japan). Two independent observers blinded to the treatment compared digital photographs. Fresh samples were weighted (grams) with a precision scale (OHAUS Corporation, NJ).

Microscopic Analysis

All samples were fixed in 10% neutral-buffered formaldehyde for 24 hours and stored it in 70% ethanol at 4 °C before processing. Histology (Hematoxylin and Eosin) was used to measure graft thickness and cross-sectional area of the graft. The Aperio System (Leica Biosystems, Germany) was used for full-size slide scanning and the ImageJ software (NIH, Bethesda, MD, USA) was used for image analysis (measurement of the cross-sectional area of the graft). A qualitative analysis of the distribution of adipocytes, inflammatory cells, cystic-like areas, and of the morphology of the grafts was also performed according to established protocols.

Immuno-histo-chemistry was used to quantify graft and peri-graft angiogenesis using endothelial cell marker platelet endothelial cell adhesion molecule-1 (CD 31) and graft inflammation using a pan-leukocyte marker (CD 45). Briefly, histological sections were deparaffinized in xylene and rehydrated in graded ethanol series. Sections were treated with 40 µg/ml of proteinase K (Roche Diagnostics Corp., Indianapolis, IN) for 30 minutes at 37 °C. Primary antibodies were incubated at 4 °C overnight. Signal was intensified using the tyramide amplification system (Perkin-Elmer, Boston, MA), and positive staining was detected with 3, 3′-diaminobenzidine (Dako North America Inc., Carpinteria, CA). Slides were counterstained with hematoxylin. Images of the microscopy slides were acquired at a standard magnification (40 x) using a Nikon E200 microscope (Nikon Corp., Tokyo, Japan) and quantified using the ImageJ Software (NIH, Bethesda, MD, USA). Image acquisition and analysis (density of CD 31+ blood vessels per magnification field) was also performed according to previously established methods.[28,162] Three representative images in 40X fields were obtained from areas along the entire length of the sample. Vascular density was quantified as the number of CD 31 + vessels identified in each of the 40X fields. Blood vessel density was measured in the graft and in the tissue surrounding the graft. Each slide was evaluated by three independent observers blinded to treatment. A qualitative analysis of the distribution of adipocytes, inflammatory cells, and of the morphology of the grafts was also performed to evaluate presence of cystic-like areas. A qualitative analysis of images as performed to evaluate the presence and distribution of inflammatory cells.

Immuno-fluorescence was used to qualitatively analyze adipocyte proliferation/infiltration within the graft using the lipid droplet surface marker Perilipin.

Data and Statistical Analysis

Animals were randomly allocated to groups and samples were randomly processed. Animals were procured by the same vendor, and identified with non-informative codes. Investigators in charge of data collection and analysis were blinded to treatment. Sample size was calculated to detect meaningful differences (alpha: 0.05; power: 95%) between treated groups and controls with regards to the primary endpoint (graft cross-sectional area at a 12 week follow-up measured with histology). Samples size was not altered during the study. Secondary endpoints included histological outcomes from other measurements (graft angiogenesis, graft weight). All data was analyzed by three researchers blinded to the treatment. Rules and criteria for data collection and analysis (including primary and secondary endpoints) were defined before starting the study and were not changed during the study. All experimental data was included for analysis: no interim data analysis, ad hoc exclusion of data, or retrospective change of endpoints was performed. No outliers were identified in our results.

Differences between groups was quantified and expressed as a mean +/- SD in text and figures. The significance of differences was evaluated with one-way analysis of variance (ANOVA) and Bonferroni post-hoc correction (comparison of multiple groups). A $p < 0.05$ was considered statistically significant.

Results

Combining recipient site preconditioning with EVE and AAM grafting maximizes volume retention and soft tissue reconstruction

At a long-term follow-up (12 weeks) the group that had undergone a combined treatment using recipient site preconditioning with EVE and subsequent AAM grafting (EVE + AAM) showed a significantly higher graft volume retention in comparison to all other groups as measured though histology (graft cross-sectional area), specimen weight, and macroscopic observation of the dissected tissue. In the EVE + AAM group the cross-sectional area of the grafts at a 12 weeks follow-up was 82% higher than in the control (adipose tissue graft: FAT) group (6.22±2.80 mm^2 vs. 3.42± 2.76 mm^2; $p < 0.05$) (Fig.2a-b, Table S2). When compared to other combined treatments (combined adipose tissue and AAM graft: AAM + FAT; combined preconditioning with EVE and subsequent adipose tissue-AAM graft: EVE + AAM + FAT) the EVE + AAM group also showed a significantly better outcome; respectively the cross-sectional area was 56% higher than in the AAM + FAT group (4.00± 0.76 mm^2; $p < 0.05$) and 87% higher than in the EVE + AAM + FAT group (3.33± 1.80 mm^2; $p < 0.05$) (Fig.2a-b, Table S2). No statistically significant differences were observed between the EVE + AAM group and the AAM group; the latter showed a significantly higher cross-sectional area when compared to the EVE + AAM + FAT group but not when compared to the FAT control group. No statistically significant differences were observed among groups at an earlier time point (short-term follow-up, 4 weeks). Gradual graft re-absorption (58% loss) could be observed in the FAT group when comparing values at earlier (short-term follow-up, 4 weeks) and later (long-term follow-up, 12 weeks) time points; instead, both the AAM and the AAM + FAT group grossly retained the same values over time. Differently from all other groups, the EVE + AAM group showed a gradual increase (41% gain) in cross-sectional area between the two time points (Fig.2a-b, Table S2). Visual evaluation of whole-graft scanned histological images further provided evidence of the observed differences and the gradual recellularization of the AAM by infiltrating/proliferating cells (Fig.2a).

Analysis of the weight of specimens after procurement was also consistent with histological results. Specimens collected from the EVE + AAM group at 12 weeks follow-up showed a significantly higher weight compared to control FAT grafts (300% higher; $p < 0.05$) and all other treatments/groups. At 12 weeks follow-up differences between groups and outcomes resembled those observed at histology. Samples obtained from the EVE + AAM showed a mildly higher weight compared to the AAM group (0.7±0.1 grams vs. 0.6± 0.1 grams; $p < 0.05$) whereas those of the AAM group had a significantly higher weight compared to control FAT grafts (0.5± 0.1 grams; $p < 0.05$) (Fig.S2, Table S2). No statistically significant differences among groups were observed at an earlier time point: changes between earlier and later time points for each group mostly mirrored those described through histology with the FAT group showing the highest rate of weight loss over time. These results and changes were also evident at macroscopic evaluation of grafts through imaging before and after their dissection (Fig. S3).

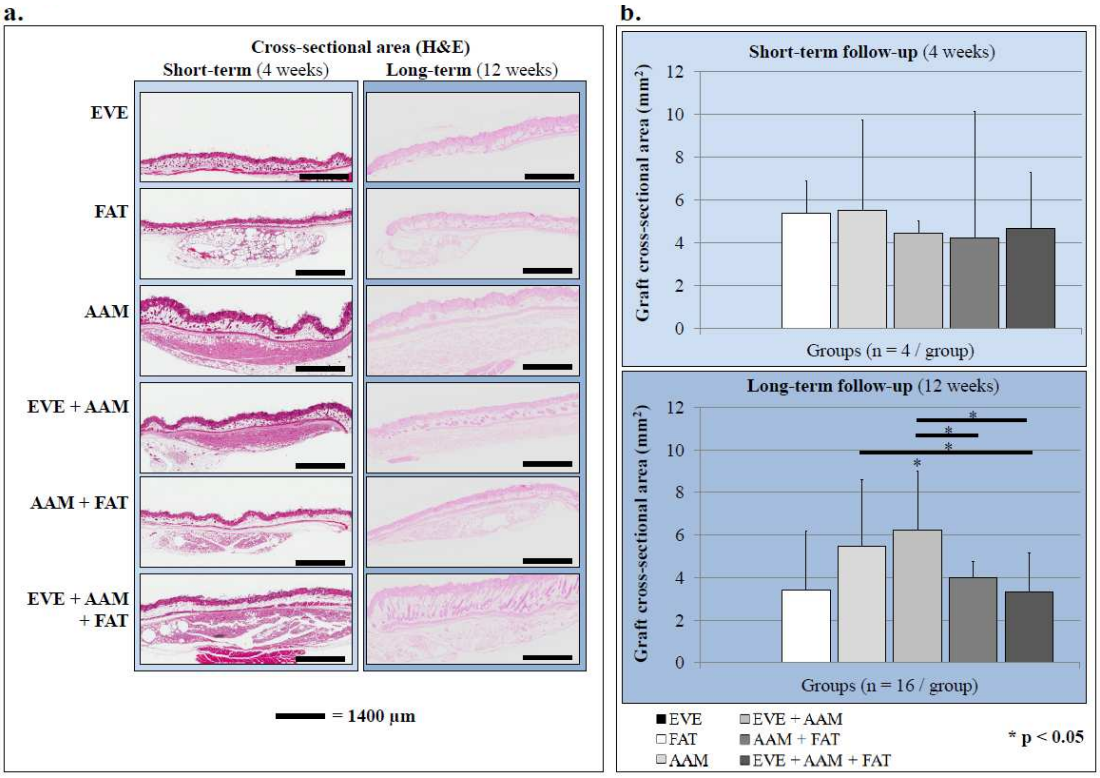


Fig. 2. Measurement of survival (volume) of grafts. **A:** Histological appearance (whole-graft scanning of hematoxylin & eosin stained slides) of grafts at a short-term, (4 weeks) and long-term (12 weeks) follow-up showing volume (cross-sectional area) retention of grafts as a method to assess their survival. (Magnification: 2X; Reference bar = 1400 µm) **B:** Cross sectional area of grafts measured with histology on microscopic samples. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the mean ± SD. (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix)

Parameter	Group	Value on short-term follow-up (4 weeks)	Value on long-term follow-up (12 weeks)
Graft weight (grams)	**EVE**	0.3 (± 0)	0.4 (± 0.1)
	Fat	0.4 (± 0.1)	0.5 (± 0.1)
	AAM	0.5 (± 0.1)	0.6 (± 0.1)
	EVE + AAM	0.6 (± 0.1)	0.7 (± 0.1)
	AAM + Fat	0.6 (± 0.1)	0.6 (± 0.1)
	EVE + AAM + Fat	0.6 (± 0.1)	0.6 (± 0.1)
Graft cross-sectional area (mm^2)	**EVE**	n/a	n/a
	Fat	5.4 (± 1.5)	3.42 (± 2.76)
	AAM	5.5 (± 4.3)	5.48 (± 3.13)
	EVE + AAM	4.4 (± 0.6)	6.22 (± 2.80)
	AAM + Fat	4.2 (± 5.9)	4.00 (± 0.76)
	EVE + AAM + Fat	4.7 (± 2.6)	3.33 (± 1.80)
Graft angiogenesis (blood vessels per 40X magnification field)	**EVE**	n/a	n/a
	Fat	33 (± 4)	43.8 (± 11.9)
	AAM	39.3 (± 6)	79.7 (± 24.9)
	EVE + AAM	47 (± 6)	69.2 (± 21.0)
	AAM + Fat	24 (± 13)	83.3 (± 27.0)
	EVE + AAM + Fat	41 (± 33)	84.8 (± 30.4)
Peri-graft angiogenesis (blood vessels per 40X magnification field)	**EVE**	58 (± 15)	62.8 (± 27.2)
	Fat	52 (± 9)	60.3 (± 18.3)
	AAM	42 (± 15)	59.4 (± 24.2)
	EVE + AAM	46 (± 6)	54.8 (± 24.2)
	AAM + Fat	59 (± 13)	70.5 (± 20.6)
	EVE + AAM + Fat	45 (± 9)	68.5 (± 20.1)

Table S2. Data for all measurements. Data is expressed as the mean ± SD. (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix)

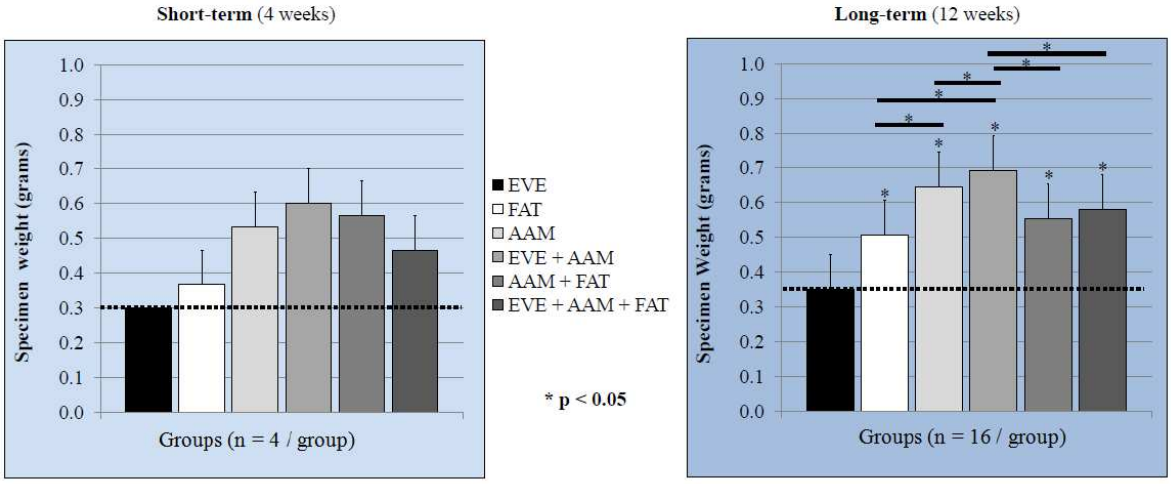


Fig. S2. Graft survival at follow-up. Statistical analysis was performed using One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the mean ± SD. Weight of graft specimens after procurement at a short-term (4 weeks) and at a long-term (12 weeks) follow-up. (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix) Data is expressed as the mean ± SD.

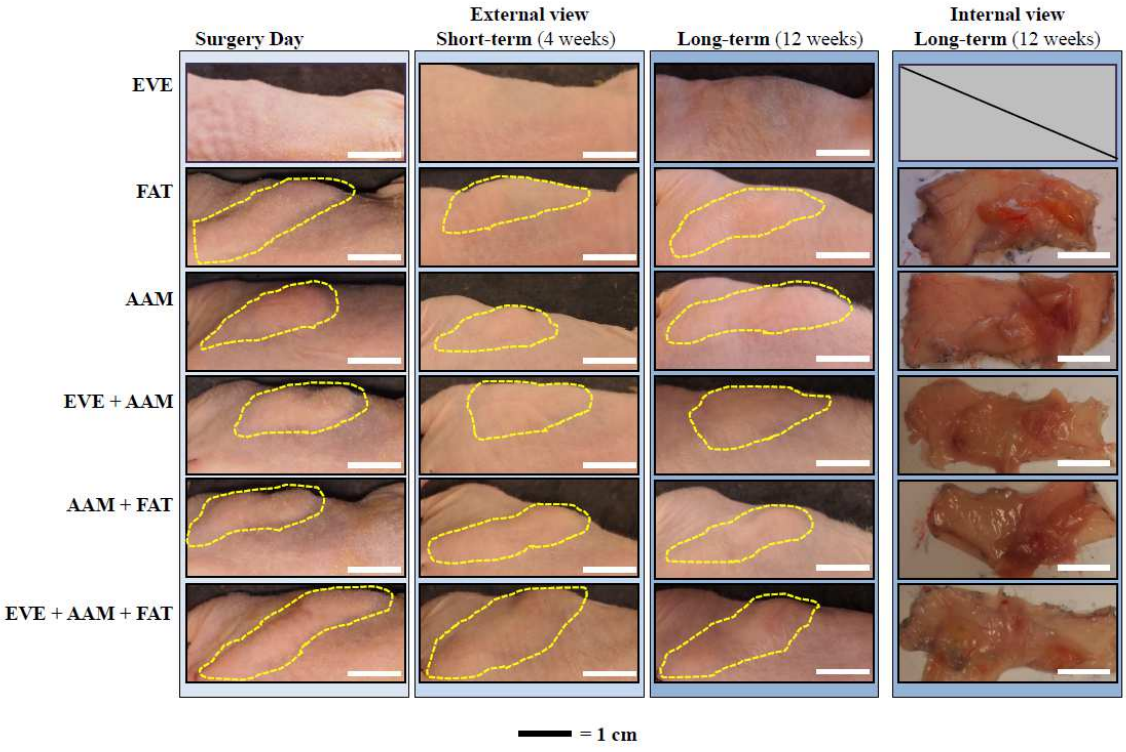


Fig. S3. Macroscopic appearance of grafts at follow-up. Digital imaging of the external macroscopic appearance of the grafts (pictures represent different animals) immediately after surgery, at a short-term (4 weeks) follow-up, and at a long-term follow-up (12 weeks) showing differential volume retention among groups. The right column shows the internal (dissected) macroscopic appearance of grafts after procurement at a 12 weeks follow-up and confirms observations drawn from the external macroscopic appearance. (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix) (Reference bar = 1 cm for both).

The AAM induces adipogenesis without formation of cystic-like necrotic areas

At a qualitative histological analysis of samples, we observed a gradual recellularization of the AAM over time (Fig. 3). In both the AAM and the EVE + AAM groups gradual migration and proliferation of adipocyte-like cells was evident along the graft margins at a short-term (4 weeks) follow-up whereas the inner core of the graft demonstrated presence of bio-inductive ECM components of the AAM with lack of cells (Fig. 3). At a longer-term follow-up (12 weeks) adipocyte-like cells appeared to have replaced most of the ECM core of the AAM graft. In the EVE + AAM group the clusters of migrating/proliferating adipocytes appeared higher in number as compared to the AAM group at both time points. In these two groups the histological architecture of grafts most closely resembled that of similar native adipose tissue (inguinal fat pads) as compared to other groups. Immuno-histo-chemical analysis using the perilipin marker (which targets the cell membrane of adipocytes) confirmed the adipocytic nature of these cells (Fig. 4).

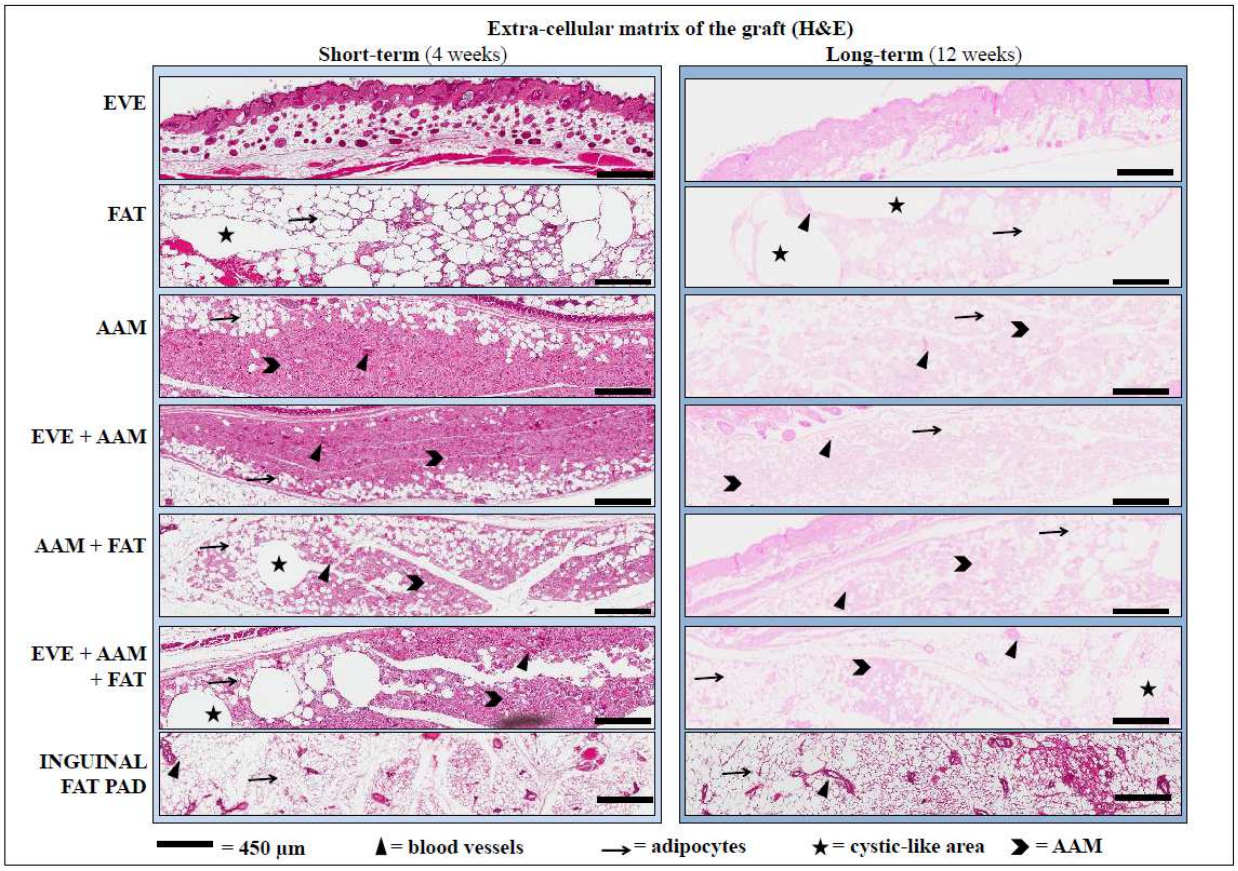


Fig. 3. Measurement of survival (quality) of grafts and adipogenesis. Histological appearance (whole-graft scanning of hematoxylin & eosin stained slides) of grafts at a short-term, (4 weeks) and long-term (12 weeks) follow-up showing the architecture of grafts, the presence of infiltrating/proliferating cells, and the presence of cystic-like areas as a method to assess adipogenesis and survival of grafts. (Magnification: 3X; Reference bar = 450 µm) (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix) (▲ = CD 31+ blood vessel; → = Adipocytes; ★ = Cystic-like areas; ➧ = AAM)

In groups containing adipose tissue grafts presence of survived living cells was observed along with the presence of multiple cystic-like areas (Fig. 3). Cystic-like areas representative of necrotic phenomena were higher in number and size in the FAT group and slightly less frequent in the AAM + FAT and the EVE + AAM + FAT groups: histology of the two latter groups presented features of both the AAM or EVE + AAM grafts (ECM core, marginal adipogenesis) and the FAT grafts (interspersed adipose cells, presence of cystic-like areas) (Fig. 3). Immuno-fluorescence (perilipin) further confirmed these outcomes and the presence of necrotic cystic-like areas in FAT grafts (more densely) and in the AAM + FAT and the EVE + AAM + FAT groups (less diffused) (Fig. 4).

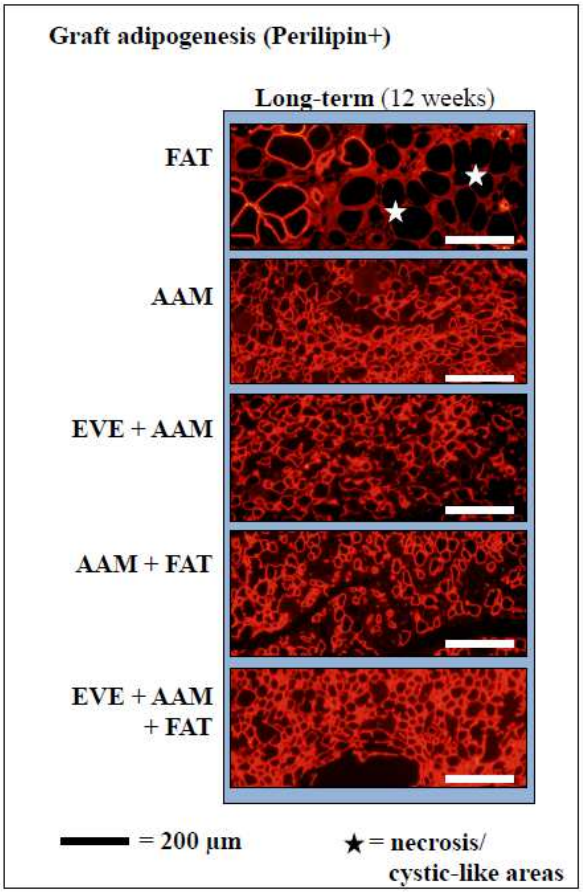


Fig. 4. Grafts survival and adipogenesis within the grafts. Histological staining (perilipin, immuno-fluorescence) of the grafts showing different presence of living adipocytes (red staining: cell membrane of adipocytes) and necrotic vacuoles or cystic-like areas (unstained areas). (Magnification: 40X; Reference bar = 200 µm) (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix) (★ = Necrosis / Cystic-like areas)

Further immuno-histo-chemical analysis using markers of inflammatory cells (CD 45, pan-leukocyte marker) showed the presence of numerous inflammatory cells close to clusters of adipocytes proliferating or infiltrating the graft in all groups containing the AAM (Fig. 5). Presence of inflammation was reduced in the groups in which the AAM was combined to FAT (AAM + FAT and EVE + AAM + FAT). Differently from other samples in the FAT group inflammatory cells aligned along the cystic-like areas of necrosis and did not cluster next to proliferating adipocytes (Fig. 5).

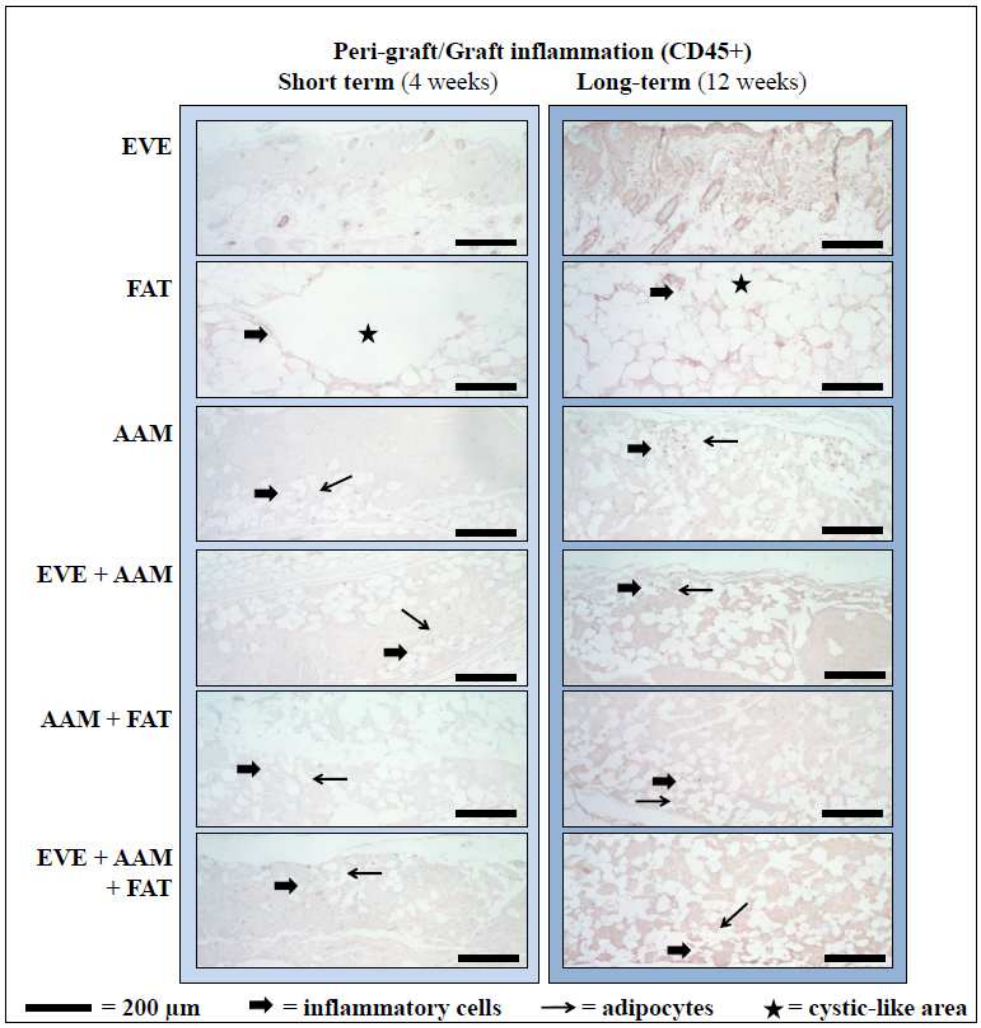


Fig. 5. Peri-graft and Graft inflammation. CD 45 (pan-leukocyte marker) immuno-histo-chemistry showing presence of inflammatory cells surrounding the clusters of proliferating adipocytes in the groups containing the AAM. In the FAT group inflammation surrounds cystic-like areas of necrosis. (Magnification: 40X; Reference bar = 200 µm) (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix) (→ = Adipocytes; ★ = Cystic-like areas; ➡ = Inflammatory cells)

The AAM promotes graft angiogenesis (infiltration of blood vessels)

Immuno-histo-chemistry for the endothelial marker CD 31 was used to measure the density of blood vessels surrounding the grafts and infiltrating it as a method to assess angiogenesis. No significant differences were observed in the density of blood vessels surrounding the grafts at both a short-term (4 weeks) and long-term (12 weeks) follow-up (Fig. S3a-b). In addition, no differences were noted in groups that received preconditioning with EVE and a subsequent graft (EVE + AAM or EVE + AAM + FAT) and those that did not received graft (EVE only).

With regards to blood vessels infiltrating the graft at a short-term follow-up (4 weeks) no significant differences were reported among groups (Fig. 6a-b, Table S2). At a later time point (12 weeks) groups containing the AAM (with or without combined treatments) showed a statistically significant higher density

of blood vessels inside the grafts (82-93% higher, depending on groups) when compared to control FAT grafts (for all groups: $p < 0.05$) (Fig. 6a-b, Table S2). The EVE + AAM group had a 58% higher density of blood vessels within the graft as compared to the FAT graft (respectively 69.2±21.0 vessels/40x magnification field vs. 43.8± 11.9 vessels/40x magnification field; $p > 0.05$) but this difference was not statistically significant (Fig. 6a-b, Table S2). No significant differences were observed among groups containing the AAM (AAM, AAM + FAT, EVE + AAM + FAT), including in comparison to the EVE + AAM group. At visual qualitative analysis of histological samples, no substantial differences were noted among groups with regards to the structure and morphology of infiltrating endothelial vessels (mostly small-diameter capillaries) (Fig. 6a). When compared to native adipose tissue (inguinal fat pad) the latter demonstrated a significantly higher density of blood vessels (>200% higher, data not reported) with the presence of larger-diameter capillaries and vascular structures (Fig. 6a).

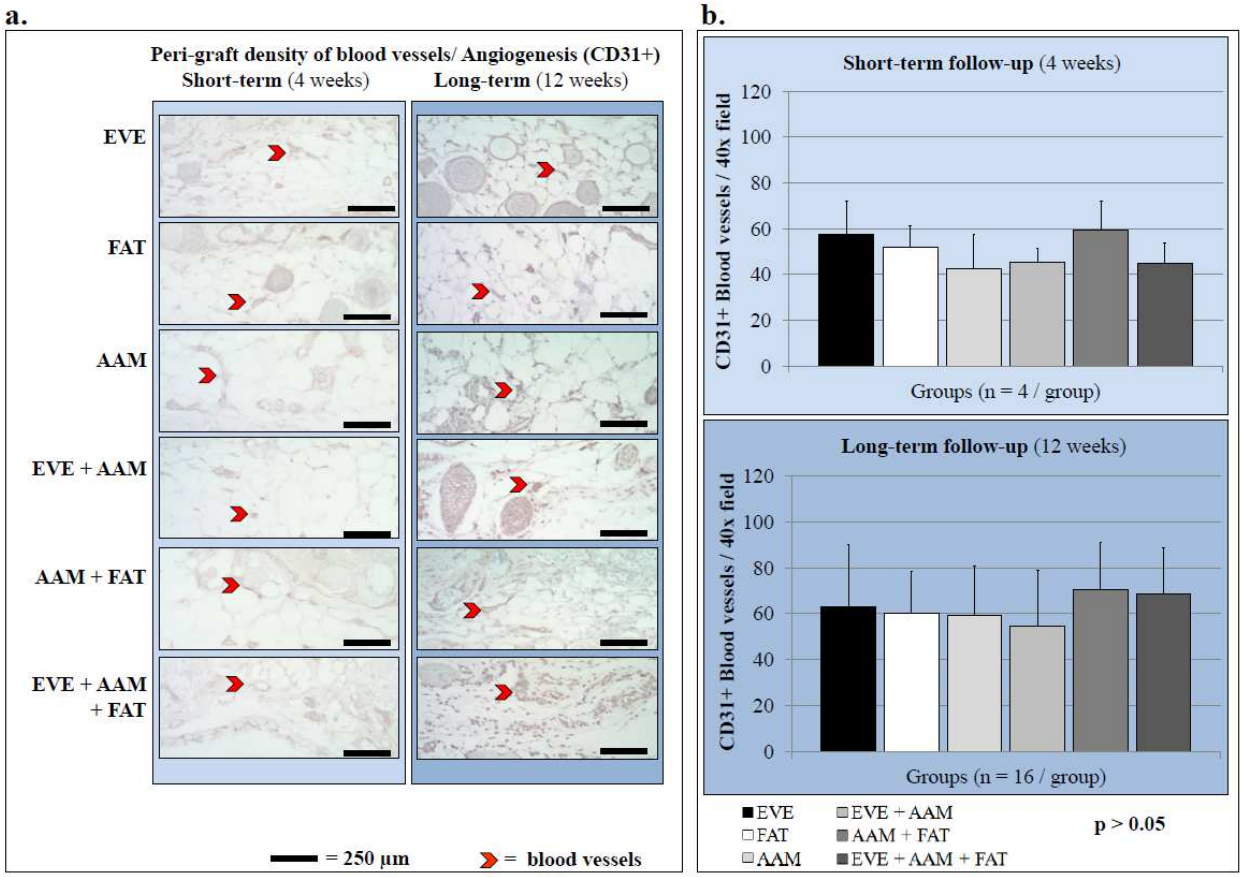


Fig. S4. a: Angiogenic effect (variations in blood vessels density) in the skin surrounding the grafts. Statistical analysis was performed using One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the mean ± SD.

b: Data for histological measurements. (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix) Data is expressed as the mean ± SD.

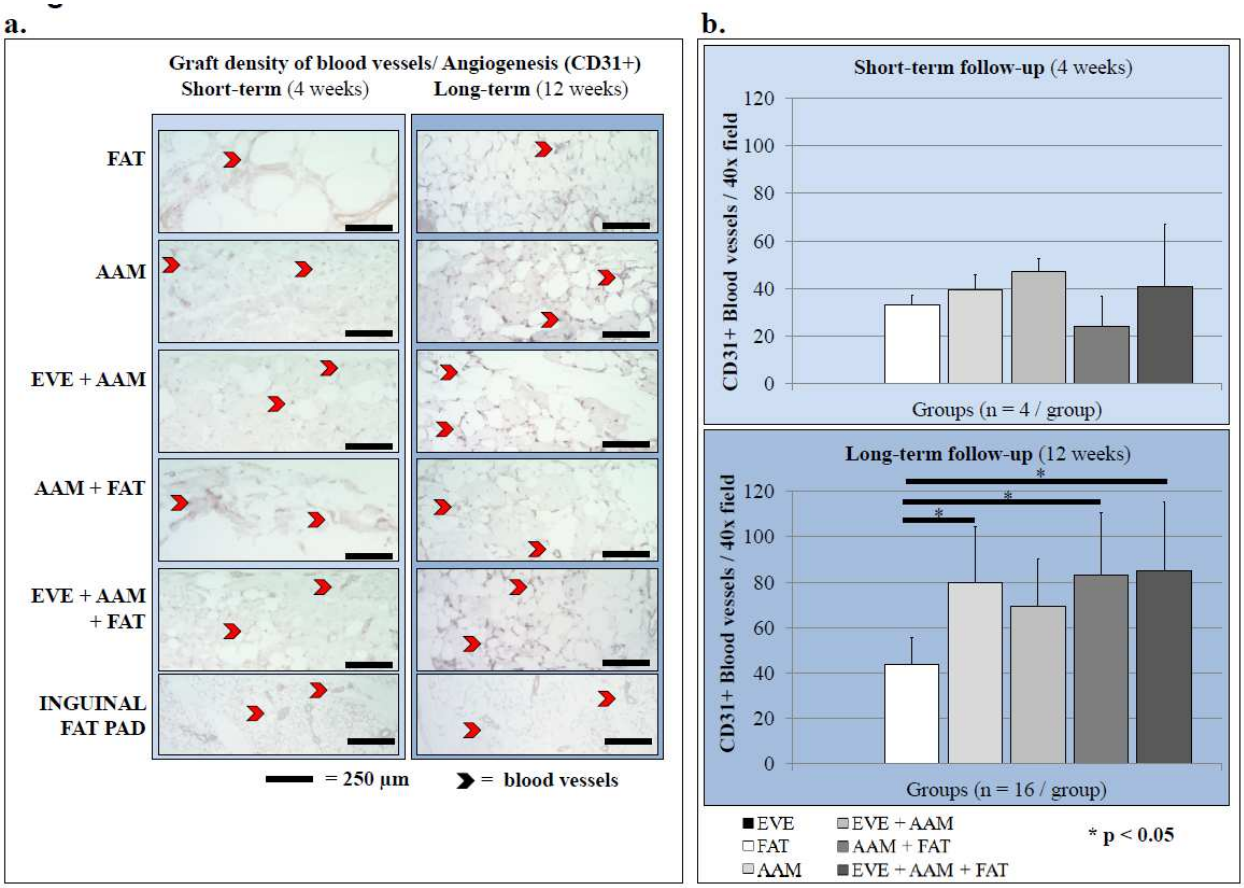


Fig. 6. Graft blood vessels density and Angiogenic effect of the AAM. **A:** CD 31 (endothelial marker) immuno-histo-chemistry of grafts at a short-term, (4 weeks) and long-term (12 weeks) follow-up showing presence of blood vessels within the grafts and their morphology as a method to assess angiogenesis. (Magnification: 40X; Reference bar = 250 µm) (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix) (➡ = CD 31+ Blood vessels) **B:** Outcomes of measurements of blood vessels density on histological images. One-way analysis of variance (ANOVA) with Bonferroni post-hoc correction. A value of $p < 0.05$ was considered statistically significant. Data is expressed as the mean ± SD.

Discussion

In this study, we show that an acellular Allograft Adipose Matrix, obtained from cadaveric donor tissues through a decellularization procedure that meets current FDA criteria of “minimal manipulation”, can create an ideal bio-physical inductive environment for soft tissue reconstruction and regeneration. Our results demonstrate that the bio-chemical and structural cued provided by the ECM of the AAM promote, once grafted in vivo, gradual migration and proliferation by both adipocytes (adipogenesis) and endothelial vessels (angiogenesis). As a consequence, AAM grafts used to repair soft tissue defects retain their original value (they actually gain volume) and restore the original morphology/histology of soft tissues (Fig.7a). When AAM grafts are combined to a non-invasive technique for recipient site preparation using mechanical forces (External Volume Expansion, EVE) these outcomes are further enhanced (Fig.7a-b). Compared to adipose tissue grafts (FAT), which represent current standard of care in patients and that can achieve only suboptimal outcomes (gradual re-absorption over time, loss of volume and shape, formation of cystic-like necrotic areas and vacuoles at histology), the combined strategy (EVE + AAM) provides substantially improved therapeutic opportunities ready to be translated to patient care. Furthermore, our studies show that when AAM grafts are combined to adipose tissue grafts (with or without EVE) positive outcomes associated with the AAM are partially mitigated (Fig.7a-b).

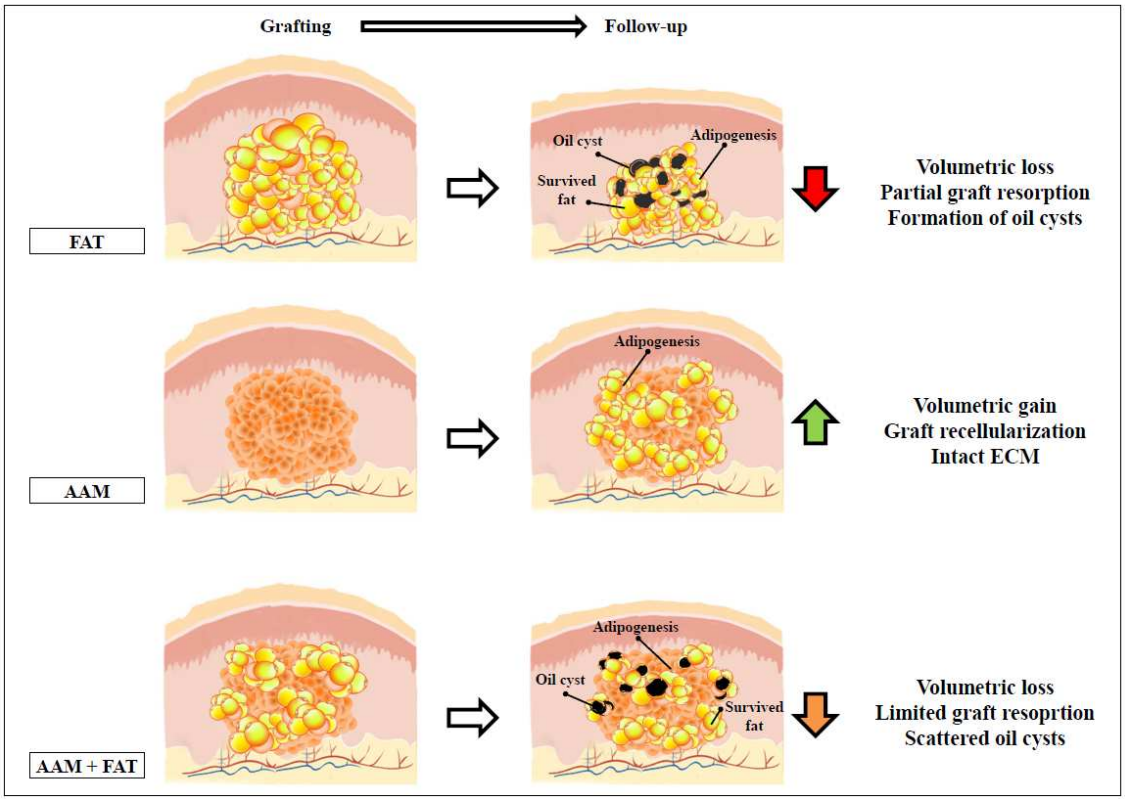
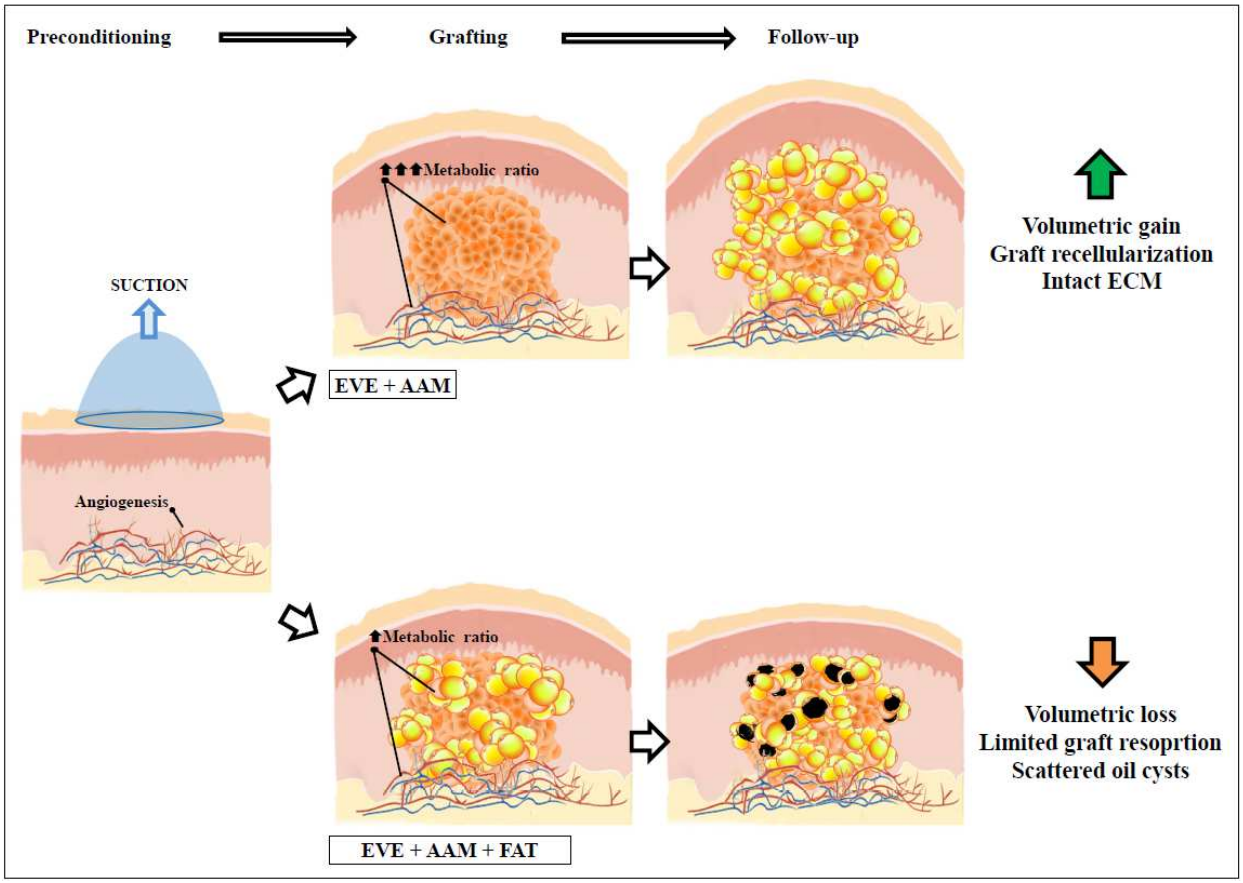


Fig. 7. Postulated mechanisms of action and conceptual representation of graft survival and soft tissue regeneration achieved using different strategies that combine adipose tissue grafts (FAT), an acellular Allograft Adipose Matrix (AAM) and non-invasive recipient site preconditioning with External Volume Expansion (EVE). **A:** Strategies and outcomes associated with FAT, AAM or combined AAM + FAT grafts. (FAT = Adipose Tissue; AAM = Allograft Adipose Matrix, ECM = Extra-cellular Matrix)



B: Strategies and outcomes associated with AAM or AAM + FAT grafts combined with EVE. (EVE = External Volume Expansion; FAT = Adipose Tissue; AAM = Allograft Adipose Matrix, ECM = Extra-cellular Matrix)

The overarching goal of this study is to use advances in bio-engineering, medical devices and biology to develop novel translational strategies in soft tissue reconstruction and regeneration: by doing so we aim to improve surgical outcomes and transform current therapeutic horizons for a large number of patients suffering from soft tissue defects.

Our data shows that when combined to preconditioning with EVE an AAM graft retains an 82% higher volume at a 12 weeks follow-up in comparison to an adipose tissue graft (Fig.1a-b). In addition, whereas the adipose tissue graft undergoes substantial re-absorption over time (58% volume loss from a 4 weeks follow-up to a 12 weeks follow-up) possibly due to ischemia-induced necrosis of grafted cells, the combined EVE + AAM treatment leads to a relevant increase of volume of the grafts (+ 41%), likely due to gradual graft recellularization of the acellular graft by migrating and proliferating adipocytes (Fig.2a-b). These findings are also confirmed by the analysis of the weight of specimens. No significant differences among the cross-sectional areas of grafts of different groups were observed at a 4 weeks follow-up whereas differences in weight were noted; this observation confirms the hypothesis that despite necrosis of adipose tissue begins early after grafting due to the ischemic insult, the graft retains initially retains its shape due to the accumulation of necrotic tissue in large cysts and vacuoles (which however have a

lower weight than living tissue) that undergo re-absorption at later time-points. The rate of re-absorption that we have observed in our samples of adipose tissue grafts is consistent with that reported in clinical practice: in patients, adipose tissue retains at a long-term follow-up (>3-6 months) only around 30-60% of the initially grafted volume.[61–64,96] Despite several strategies have been proposed to improve these outcomes by developing novel techniques to procure, process, and inject adipose tissue grafts, or by supplementing adipose grafts with autologous growth factors (e.g. Platelet Enriched Plasma, PRP) and stem cells (e.g. Stromal Vascular Fraction of adipose tissue, SVF) obtained at point-of-care, only very limited encouraging results have been achieved with modest increase in volume retention at follow-up.[65,101,104,163–166] Today, soft tissue reconstruction using adipose tissue grafting is still substantially limited by the tendency of grafted tissue to undergo ischemia-induced necrosis. To overcome this challenge, here we adopt a completely different approach avoiding the use of living tissue and providing uniquely a structural matrix to promote tissue regeneration in situ. Consistently with our hypothesis, we could observe that when grafted alone the AAM lead to a higher weight and volume retention (although the latter finding was not statistically significant possibly due to the chosen sample size of groups and the high intra-group variability) than adipose tissue grafts: addition of preconditioning with EVE further enhanced this result (Fig. 7a-b). When the AAM was grafted combined to living adipose tissue outcomes (volume and weight retention) did not significantly differ from those of pure adipose tissue grafts: this finding suggests that supplementation of the AAM might not be sufficient to mitigate the ischemia-induced necrosis of adipose tissue acutely (Fig. 7a-b). We postulate that the AAM is unable to prevent the lack of diffusion of metabolites to adipose tissue or might actually create an additional barrier for the survival of grafted cells. Despite in our previous studies we had confirmed that preconditioning of the recipient site with EVE is able to improve survival of adipose tissue grafts, in this study we observed that addition of EVE was not able to improve the survival of AAM + FAT grafts:[28,70,85,167] we suggest that this might also be related to creation by the AAM of an additional physical barrier to diffusion of metabolites to grafted adipocytes (Fig. 7a-b).

Our histological analysis confirmed that the bio-physical cues provided by the AAM can create a micro-environment for adipocyte migration and proliferation (adipogenesis) (Fig.3, 4). At a 12 weeks follow-up samples of AAM presented a histological morphology similar to that of native adipose tissue with complete re-cellularization of the graft by adipocytes. Preconditioning of the recipient site with EVE further improved this outcome. These results highlight that the adipogenic inductive properties of the AAM allowed not only a soft tissue reconstruction but beyond that the actual in situ regeneration of soft (adipose) tissue. [90,105–116,168] Infiltration of inflammatory cells along the external border of the scaffold might play a relevant role in the induction of adipogenic processes, as suggested by our results and previous research.[70,71,128] Furthermore, whether grafts containing adipose tissue led to the formation of cystic-like necrotic areas and vacuoles (as expected and known from both preclinical and clinical experience due to the ischemic insult that grafts receive after grafting), the lack of cellular components in

the AAM grafts prevented the formation of necrosis and associated complications such as cystic-like areas and vacuoles.

We also observed that the AAM promotes migration and proliferation of endothelial vessels (Fig.6a-b). At histology, all grafts containing the AAM (alone or combined to adipose tissue) showed a significantly higher density of blood vessels at a long-term follow-up as compared to control adipose tissue grafts (58-93% higher, depending on groups). This outcome seems to be unrelated to the adoption of preconditioning with EVE and might be linked to the presence of pro-angiogenic bio-chemical factors within the AAM as previously described for other decellularized matrices derived from adipose tissue.[116,169,170] Of note, the angiogenic increment in the density of blood vessels within the grafts induced by the AAM might be one of the biological mechanisms leading to improved graft survival and volume retention over time, as well as adipose tissue regeneration.

In summary, in this study we show that the combined use of EVE and an AAM graft for soft tissue reconstruction substantially improve outcomes in comparison to current standard of care (adipose tissue grafts) leading to a higher volume retention and better tissue preservation at follow-up. The bio-physical cues of the AAM promote tissue regeneration in the form of adipogenesis and angiogenesis within the graft. Combination of these two approaches, both of which are ready for clinical adoption, into a novel therapeutic strategy might transform best practice in the reconstruction of soft tissue defects for numerous patients suffering from the consequences of trauma, cancer, chronic disease, congenital malformations, or other disfiguring and disabling conditions.

DISCUSSION AND CLINICAL IMPACT

The use of EVE in Reconstructive Surgery

The results of our studies can be directly translated to a clinical scenario and will help provide an innovative method for non-invasive induction of angiogenesis in tissues in preparation of surgeries. We have optimized EVE to make it effective (increased vascular density and improved surgical outcomes such as graft survivals), safe (reduced complications) and suitable for clinical use (shorter duration of treatment, shorter daily use of EVE, and adoption of customizable foam interfaces). In order to further strengthen translation of our research to patients we have employed only clinically-available devices which are already FDA-approved for use in soft tissues under other indications (wound care) and that allow outpatient care or home care. The absence of other non-invasive, safe and effective clinically-available preconditioning strategy and the increasingly walloping need for methods to improve surgical outcomes in reconstructive surgery might make EVE an essential part of the standard of care if our results will be confirmed in a clinical scenario.

We envision that every patient scheduled to undergo a reconstructive surgery might preventively undergo EVE preconditioning: after consultation with their surgeon patient might be able to self-apply the device at home and receive treatment when most comfortable for them (possibly at night) for a brief period of time before their surgery. The importance of securing better surgical outcomes and limited complications might be even higher in the increasing number of patients with known co-morbidities affecting such results (e.g. diabetes, obesity, history of smoking, poor or severely poor health status due to cancer or chronic disease, previous or concurrent radiation therapy). As previously noted the number and variety of patients routinely undergoing reconstructive procedures after oncologic surgery, traumas to soft tissues, and consequences of chronic diseases or congenital malformations is enormous. For example EVE might be able to precondition a flap in diabetic patients before this is transferred to repair a chronic foot ulcer, a surgery which is very often burdened by necrosis or delayed healing due to the impaired vascularity of tissues in diabetes. EVE could also help improving vascularization in previously radiated chests –usually characterized by a lower vascular density leading to poorer surgical outcomes- in preparation of tissue transfer (grafts or flaps) in breast reconstruction after breast cancer removal. The number and types of other possible clinical applications of EVE as a precondition strategy is very broad and further fostered by its ease-of-use and non-invasiveness. In addition, EVE can be combined with other techniques to synergistically increase outcomes [60,103].

In addition, given the already known adipogenic effect of EVE (direct mechanical stimulation of the proliferation of adipocytes and indirect stimulation through inflammation and edema-induced adipogenesis), per se EVE represents an adjuvant strategy for soft tissue reconstruction. This effect has been extensively described in both clinical and preclinical studies.[36,67,78,80–84,171] Despite our study

does not focus on the adipogenic effects of EVE, our outcomes confirm these phenomena and are consistent with findings observed in previous literature. [36,67,78,80–84,171]

The combined use of EVE and an AAM in Regenerative Surgery

Several authors have extensively described techniques to obtain an acellular bio-inductive scaffolds derived from native adipose tissue through decellularization, and have investigated the biological effects of these scaffolds in vivo in animal models confirming their effectiveness in supporting adipose tissue regeneration.[90,105–116,168] In this studies we leverage this background knowledge and combine it to our own experience to develop a translational strategy ready to be applied to patients. The innovation of our approach originates from three factors.

In first place and differently from previous research we here report the development and use of an acellular AAM obtained from human cadaveric donors with a decellularization technique that meets current FDA criteria of "minimal manipulation". Previously published literature had investigated decellularized adipose tissue scaffolds derived from small animals, swine, or human surgical discards: whether the use of small animals-derived or human surgical discards-derived scaffolds can provide helpful preclinical experimental insights it cannot be translated to large-scale adoption in a clinical scenario.[90,105–116,168] Consistently, the lack of porcine-derived adipose scaffolds that have proven to not elicit immunological rejection currently limits this possible source of tissue. Today and similarly to what previously demonstrated with dermal scaffolds in skin repair and regeneration, human cadaveric donor tissue represents the best (and only) alternative for the development of an effective product ready to be adopted on a large-scale in patient care. In addition, despite several protocols and methods have been proposed to obtain decellularized adipose scaffolds, only few of these comply with the criteria of the FDA of "minimal manipulation", as our AAM does: meeting these criteria is pivotal to support an easier and faster translation of the scaffold to clinical care and commercialization while limiting the need of extensive trials.[90,105–116,168]

As a second point of innovation we here combine the use of acellular adipose scaffold grafts to an established, FDA-approved technique that uses non-invasive external mechanical stimulation (External Volume Expansion, EVE) as a preconditioning method of tissues that will receive the grafts. EVE has shown in several preclinical pre-clinical and clinical studies the capacity to induce angiogenesis and adipogenesis in soft tissues: these effects have been associated to the establishment of a densily vascularized and pro-regenerative environment that can increase the survival of subsequently grafted living tissues.[23,28,57,61,68–70,172,173] However, the adoption of EVE in preparation of the grafting of an acellular scaffold is novel. Our results demonstrate that the angiogenic and adipogenic properties of EVE not only retain their effectiveness when applied to acellular grafts but also improve the regenerative

performances of the grafts. This evidence might support a more widespread use of EVE in combination with the surgical grafting of cellular scaffolds, such as the AAM, in the reconstruction of soft tissues. The use of EVE in combination with the AAM (both of which are ready for clinical adoption) can synergistically enhance achievable outcomes.

Finally, in order to support the translational application of our research and improve clinical practice in this study we closely compared the performances obtained by the AAM (with/without EVE treatment) with the current standard of clinical care (human adipose tissue grafts obtained from processed lipoaspirate, with/without EVE treatment) and explored whether combined treatment might result equally or more effective. Previous studies on acellular adipose scaffolds have rarely compared the outcomes obtained with the proposed products to actual adipose tissue grafts (especially human-derived or processed from lipoaspirate).[90,105–116,168] We believe that the lacking of this evidence limits the translational value of reported outcomes and the capacity of such research to impact clinical practice by failing to provide a direct poof of superiority for the proposed products or therapeutic strategies. Out study directly compared the standard of care in soft tissue reconstruction with a proposed new strategy and provided evidence of the superior outcomes associated with the latter. These findings might help support the application of our strategy in patients and redesign existing clinical guidelines.

The major strength of our study lies in high potential for a prompt translation of these findings and of the proposed therapeutic strategy to patient care. Production of the AAM has been designed to meet current criteria and regulations for “minimal manipulation” dictated by the FDA: this allows a less complicated path to patient testing, clinical adoption, and commercialization following the successful examples set by other acellular scaffolds (e.g. dermal matrices) in the past. The use of cadaveric donor tissue also allows the possibility of producing and supplying the AAM on a large scale. Consistently, the clinical use of EVE has already been described and widely adopted to improve the outcomes of adipose tissue grafting. Other similar devices that delivery mechanical forces to tissues in order to promote wound healing (Negative Pressure Wound Therapy) or the closure of surgical incisions (Closed Surgical Wound Management systems) have been FDA-approved and have been routinely used on patients in the last two decades.[150,151,174–176] These premises suggest that, although the implementation of large animal studies might help optimize the treatment, the combined use of EVE and an AAM graft is potentially ready for clinical testing and adoption.

LIMITATIONS OF THE STUDIES

Despite our confidence in the translational potential of the optimized EVE our studies have limitations that should be addressed to further improve its implementation in clinical care. Our studies could not systematically test all possible combinations of variables related to treatment parameters; instead, it adopted a sequential optimization approach to define the biological principles that regulate a conceptual therapeutic ratio. Sequential optimization is a mathematical modeling method used in engineering that can be applied to the study of complex interactions within biological systems [43,129,177]. This strategy allows the screening of a plethora of different experimental conditions by evaluating the effects of a single varying parameter per time: the optimized variable is then sequentially implemented in the analysis of other parameters. An educated estimate of the physiologically-relevant ranges of variation for each analyzed parameter helped us further narrow the conditions to investigate. These studies have also been conducted on small animal models (rodents) which have intrinsic anatomic and physiologic differences in comparison to larger animal models (e.g. swine) and humans. In addition, some of our findings were obtained using immune-deficient animals receiving human grafts: these data should ideally be confirmed in an immune-competent animal model receiving autologous grafts. The adoption of small animal models limits the biological variability typically seen in patients (age, clinical history, anatomy, etc.) and provides a reproducible controlled environment to selectively highlight the biological effect of each varying parameter. Our studies aimed to define general principles that could later guide clinical application, more than exact parameters of treatment. Importantly, the angiogenic effects of EVE have been confirmed by others using large animal models (swine) and EVE has been already used on patients. We believe that validation of our results could be done in a large animal model or in a controlled clinical trial using non-invasive imaging techniques to assess the effect of EVE on tissue vascularity. In clinical care, the therapeutic ratio of EVE will need to be used to adapt treatment regimens to the individual characteristic of each patient (age, clinical history, anatomy, etc.). Furthermore, these studies were not aimed to provide extensive mechanistic insights on EVE. Proof of concept and definition of the cellular/molecular pathways induced by EVE have already been reported by our team and others in previous publications. Here, our analysis was targeted to the definition of optimized parameters of treatment that could support translational application of the technique. Since clinical relevance of EVE almost completely depends on its capacity to induce angiogenesis while limiting tissue damage our methods were focused to detect these phenomena.

Our choice to test the AAM and optimize its regenerative effects in a murine model allowed us to evaluate outcomes in a controlled and reproducible system with the goal of providing robust comparative evidence of the biological properties of different therapeutic strategies. In order to further support the translational value of our research we opted for the use of adipose tissue grafts obtained from human-derived processed lipoaspirate (as routinely occurs in clinical care) but this obliged us to choose an immune-deficient animal model to avoid rejection of the human xenografts. Despite this decision might have

masked the presence of inflammatory phenomena and their contribution to the regeneration of adipose tissue we believe that the removal of this variable allowed us a more direct comparison of treatments. With regards to the AAM, the lack of cellular components and of antigens in the ECM that might evoke an immune-reaction, allows its safe use as an allograft (human to human); albeit not expected, it is however possible that some rejection or foreign-body reaction might be observed in immune-competent animal models. In addition, although in this study the volume of grafted tissue (0.3 ml) is significantly lower than those adopted in clinical practice our and others' previous studies suggested that, when applied to a murine model, these volumes closely recapitulate the biologic phenomena observed in larger-scale volumes grafted in humans.[167,178] Overall, we believe that replication and confirmation of these outcomes in larger animal models (e.g. swine) using grafts of larger volume (>200 cc) or directly in humans in preliminary clinical trials would benefit the validation of the treatment and its optimization in preparation of a more widespread clinical use.

CONCLUSIONS

In summary, in this studies we sequentially optimized parameters of application of EVE in physiological and pathological animal models in support of its effective clinical application as non-invasive preconditioning strategy in surgery. Using FDA-approved devices and commercially available materials we confirmed that moderate-intensity intermittent treatments provide the best therapeutic ratio, almost doubling vascular density in target tissues without collateral damage. We also showed that micro-deformational interfaces of treatment retain the angiogenic potential of EVE, further reduces cutaneous complications, and offers an easier method of application of EVE. These outcomes and biological properties were also confirmed in an animal model of type-2 diabetes. We also demonstrated that by increasing vascularity of the recipient site EVE promotes higher volume retention of adipose grafts over time and that this effect can be achieved both using EVE as a pre-conditioning technique or as a post-conditioning technique. Finally, we showed that the combined use of EVE and an AAM graft for soft tissue reconstruction substantially improve outcomes in comparison to current standard of care (adipose tissue grafts) leading to a higher volume retention and better tissue preservation at follow-up. The bio-physical cues of the AAM promote tissue regeneration in the form of adipogenesis and angiogenesis within the graft.

This knowledge of the biologic principles that regulate EVE, combined to the previously described adipogenic effects of EVE (known to be elicited both through a direct mechanical stimulation and an inflammation/edema-driven stimulus), will allow us to optimize the treatment in humans, guide better clinical application of EVE, and improve current best practice in reconstructive surgery. Beyond this, the combination of EVE and AAM grafts, both of which are ready for clinical adoption, into a novel therapeutic strategy might transform best practice in the restoration of soft tissue defects, shifting our current capacities from the current reconstructive surgery to a future regenerative surgery of soft tissues. This innovation will significantly impact the life and therapeutic path of numerous patients suffering from the consequences of trauma, cancer, chronic disease, congenital malformations, or other disfiguring and disabling conditions.

Future research should focus on the translational application of these principles to clinical care and on the definition of patient-specific regimens of treatment using non-invasive imaging to measure tissue ischemia during EVE treatment. A broader set of molecular/cellular analysis will also help interpret the underlying signaling pathways involved and possibly further improve efficacy of EVE. In addition, we envision that the effectiveness of mechano-therapies might be beneficially extended to other areas of surgery, clinical needs and tissues (e.g. skeletal muscle regeneration).

REFERENCES FOR THE IMAGES USED IN THE TEXT

1. AIMS OF THE RESEARCH: Figures presented are part of a manuscript currently under review by *Angiogenesis*. (*Giatsidis G, Cheng L, Haddad A, Ji K, Succar J, Lancerotto L, Lujan-Hernandez J, Fiorina P, Matsumine H, Orgill DP. Non-invasive induction of angiogenesis in tissues by external suction: sequential optimization for use in reconstructive surgery. Angiogenesis)*
2. STUDY 1 - MODERATE-INTENSITY INTERMITTENT EXTERNAL VOLUME EXPANSION OPTIMIZES THE SOFT TISSUE RESPONSE IN A MURINE MODEL: Figures presented are part of a manuscript published by *Plastic and Reconstructive Surgery*. (*Giatsidis G, Cheng L, Facchin F, Haddad A, Lujan-Hernandez J, Lancerotto L, Nabzdyk CS, Matsumine H, Orgill DP. Moderate-intensity Intermittent External Volume Expansion Optimizes the Soft Tissue Response in a Murine Model. Plast Reconstr Surg. 2017 Apr;139(4):882-890. doi: 10.1097/PRS.0000000000003190.)*
3. STUDY 2 - NON-INVASIVE INDUCTION OF ANGIOGENESIS IN TISSUES BY EXTERNAL SUCTION: SEQUENTIAL OPTIMIZATION FOR USE IN RECONSTRUCTIVE SURGERY: Figures presented are part of a manuscript currently under review by *Angiogenesis*. (*Giatsidis G, Cheng L, Haddad A, Ji K, Succar J, Lancerotto L, Lujan-Hernandez J, Fiorina P, Matsumine H, Orgill DP. Non-invasive induction of angiogenesis in tissues by external suction: sequential optimization for use in reconstructive surgery. Angiogenesis)*
4. STUDY 3 - DELAYED POST-CONDITIONING WITH EXTERNAL VOLUME EXPANSION (EVE) IMPROVES SURVIVAL OF ADIPOSE TISSUE GRAFTS IN A MURINE MODEL: Figures presented are part of a manuscript currently under review by *Plastic and Reconstructive Surgery*. (*Wei S, Liu W, Gundogan B, Orgill DP, Giatsidis G. Delayed Post-conditioning with External Volume Expansion (EVE) Improves Survival of Adipose Tissue Grafts in a Murine Model. Plast Reconstr Surg)*
5. STUDY 4: DELIVERY OF EXTERNAL VOLUME EXPANSION (EVE) THROUGH MICRO-DEFORMATIONAL INTERFACES IMPROVES ANGIOGENESIS AND LIMITS COMPLICATIONS IN A MURINE MODEL OF DIABETIC SKIN: Figures presented are part of a manuscript currently under review by *Plastic and Reconstructive Surgery*. (*Wei S, Orgill DP, Giatsidis G. Delivery of External Volume Expansion (EVE) through micro-deformational interfaces improves angiogenesis and limits complications in a murine model of diabetic skin. Plast Reconstr Surg).*
6. STUDY 5: TISSUE ENGINEERED SOFT TISSUE RECONSTRUCTION USING NON-INVASIVE MECHANICAL PRECONDITIONING AND A SHELF-READY ALLOGRAFT ADIPOSE MATRIX: Figures presented are part of a manuscript currently under review by *Science Translational Medicine*. (*Giatsidis G, Succar J, Waters TD, Liu W, Rhodius P, Nilsen T, Chnari E, Orgill DP. Tissue Engineered Soft Tissue Reconstruction Using Non-invasive Mechanical Preconditioning and a Shelf-ready Allograft Adipose Matrix. Sci Transl Med).*

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