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Nanofiber polymeric scaffold for slow and steady release of VEGF and ECs to induce efficient angiogenesis and healing in a mouse model of hind limb ischemia

Settore scientifico-disciplinare: 06-scienze mediche

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Fortis vero animi et constanti est

non perturbari in rebus asperis,

sed praesenti animo uti et consilio

nec a ratione discendere

Cicero

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1. INTRODUCTION

Ischemic wounds are debilitating and distressing chronic conditions that can lead to infections, increased mortality and also to minor or major limb amputations[1].

Amputation is a major clinical problem especially in diabetic patients and the increasing rate of diabetes diagnoses worldwide is cause for alarm, as patients with diabetes have a 10-fold increased risk for lower extremity amputation compared with those who do not have diabetes. The recent development of novel bactericidal antibiotics against Gram+, Vancomycin-resistant organisms, together with arterial reconstruction and revascularization procedures, are the most efficient limb salvage strategies. However, the incidence of all forms of lower extremity amputation worldwide ranges from 46 to 96/100,000 in the population with diabetes compared with 5.8-31 /100,000 in the total population, reasonably because diabetic patients, which are often affected by various co-morbidities, present dysfunction not just of the main vessels – which are the targets of revascularization procedures - but also of the microcirculation[2,3].

Related healthcare costs are staggering, not only for the affected individual and his/her family, but also for the health authorities. As the diabetic epidemic expands worldwide, the disease takes an ever-increasing proportion of national health care budgets. For most countries, the largest single item of diabetes expenditure is hospital admissions for the treatment of long-term complications, mostly foot problems and amputation[4].

Multiple factors contribute to chronic wounds pathogenesis even though one considerable impairment is represented by lack of tissue neovascularization[5]. Neovascularization is crucial for wound healing as it restores damaged vessels and re-create the supply of oxygen and other molecules. The principal treatment for chronic ischemic wounds is represented by revascularization procedures in addition to local wound care management. In spite of the many innovations and improvements in endovascular procedures, vascular bypass approaches and wound healing treatment, ischemic non-healing wounds remain a significant clinical challenge and continue to be a serious unsolved problem[6].

Regenerative medicine is an emerging interdisciplinary branch of translational research in tissue engineering and molecular biology that cope with the process of replacing, engineering or regenerating human cells, tissues or organs and aims to re-establish or create normal function[7].

It uses a combination of several technological approaches that moves it beyond traditional transplantation and replacement therapies. These approaches may include, but are not limited to, the use of soluble molecules, gene therapy, stem cell transplantation, tissue engineering and the reprogramming of cell and tissue types [8].

Regeneration is used to describe the process in human where lost specialized tissue is replaced by proliferation of undamaged specialized cells. This mechanism is currently limited in humans in just a few tissues (i.e. liver, epidermis and intestinal mucosa) [9]. Therefore, the aim of regenerative medicine is to regenerate, by the delivery of different type of cells that can stimulate wider regeneration.

Classically the definition of repair is the replacement of lost tissue by granulation tissue formation that will mature and will lead to scar formation. Organ regeneration

is distinct and different from organ repair; repair is an adaptation to loss of normal organ mass and leads to restoration of the interrupted continuity by synthesis of scar tissue without restoration of the normal tissue. By contrast, regeneration restores the interrupted continuity by synthesis of the missing organ mass at the original anatomical site, yielding a regenerate. Regeneration restores the normal structure and function of the organ while repair does not [10].

The ultimate aim of regenerative medicine is to return the patient to full health with respect to the particular condition. Repair is invaluable but the consequences of repair can be unpleasant, for example, internal and external scaring. In most cases, the aim of regeneration will be to restore a function that has been impaired but it could also address congenital abnormalities, such as thalassemia, absence of corneas or so called 'hole in the heart' cases, where the normal functions were initially absent. This field holds the promise of engineering damaged tissues and organs by stimulating the body's own repair mechanisms to functionally heal previously irreparable tissues or organs[11].

Regenerative medicine also includes the possibility of growing tissues and organs in the laboratory and implanting them when the body cannot heal itself. If a regenerated organ's cells would be derived from the patient's own tissue or cells[12],this would potentially solve the problem of the shortage of organs available for donation, and the problem of organ transplant rejection[13].

Some of the biomedical approaches within the field of regenerative medicine may involve the use of stem cells[14]. Examples include the injection of stem cells or progenitor cells obtained through directed differentiation (cell therapies); the induction of regeneration by biologically active molecules administered alone or

as a secretion by infused cells (immunomodulation therapy); and transplantation of *in vitro* grown organs and tissues (tissue engineering)[15].

Tissue engineering is a novel interdisciplinary subject that incorporate material science, medicine end engineering notions to create biological substitute that could possibly repair, maintain or improve tissue function. Generally, a scaffolding material provides a three-dimensional support for cellular function. These engineered scaffolds can also supply mechanical and biochemical factors in order to facilitate cells to migrate, mature and integrate within the new tissue. Nowadays, a massive importance has been given to the development of scaffolds that can mimic the extracellular matrix (ECM) from a composition and organization point of view. The physiologic ECM is mad of different fibrillar protein structures, with diameter ranging from two to a hundred nanometers.

In general, tissue engineering includes three main stages: cells isolation and generally expanded in vitro, cells seeding on a 3D scaffold with or without growth factors and functional microenvironment creation.

The complex native human ECM is made by a fibrillar architecture that interacts with a range of bio-chemical signals, regulating the cellular function or tissue regeneration/recovery. A Few studies showed that nanotextured polymeric scaffolds are crucial for tissue engineering applications. Nanoscale polymeric scaffold, compared to the non-nano ones, have a higher surface-to-volume ratio making them more biomimetic and improving matrix-cell interactions, allowing to hold a larger number of cultured cells. The ideal wound healing scaffold should simulate the ECM properties with adequate porosity, hydrophilicity, geometry; it should also be biocompatible and biodegradable.

Different scaffolds are commercially available and among these, the Integra Dermal Regeneration Template, consists of a porous dermal component made of bovine type I collagen and shark chondroitin-6-sulphate glycosaminoglycan which is bonded to a silicone pseudo-epidermis. The dermal component of the bioconstruct becomes populated with the host cells, including fibroblasts, which contribute towards neodermis formation while the material's scaffold degrades and the pseudo-epidermal silicon component protects wounds from vapour loss and bacterial contamination. When Integra vascularization and neodermis formation are complete (usually within 15–20 days) the silicone layer is peeled off and the wound can be closed permanently with a skin graft. In attempts to achieve a single-stage surgical procedure, the product has been seeded with disaggregated cultured or non-cultured autologous keratinocytes, using in vivo experimental models.

Aside from the already commercially available acellular dermal matrix, many studies have been conducted on nanofiber polymeric scaffolds.

One of the most difficult achievement in tissue engineer is to develop a matrix that imitates the nanofiber structure of the human tissues. To date, to create a nanoscale matrix, several different techniques are described, however the electrospinning system seemed to produce fibers that are similar to the human ECM ones. In addition, the electrospun nanofibers (ENs) have satisfying porous structure and high surface-to-volume ratio, permitting to increase cell functionality.

Furthermore, modifying the process settings, solution, ambient factors different scaffolds can be obtained with aligned or non-aligned fibers. Due to the high surface-to-volume ration, the ENs can be biologically, chemically and physically customized

for the specific purpose to be obtained (i.e. continuous drug delivery, tissue filtration and colonization, etc.).

Based on the settings, the nanofiber scaffold can provide cell migration, adhesion, survival and proliferation as well as adequate mechanical function of the matrix (elasticity, tensile strength, etc.).

Electrospinning

The electrospinning is a technique that applies electric forced to a liquid polymer droplet in order to create a jet which is collected as fibers. Several settings can be customized starting from the high-voltage power, the number of the syrynges, the spinneret (that can be single, multi-jet or co-axial) and the collector (round, rotating or plate)[16].

When the electric force is applied, a high voltage is reached and the electrostatic force compensate the surface tension of the polymeric fluid allowing a spherical droplet of solution do change into a charged Taylor cone, forming a nanofiber jet.

The jet becomes solid as it is stretched to smaller diameter and it deposits on the collector [17] (Fig 1).

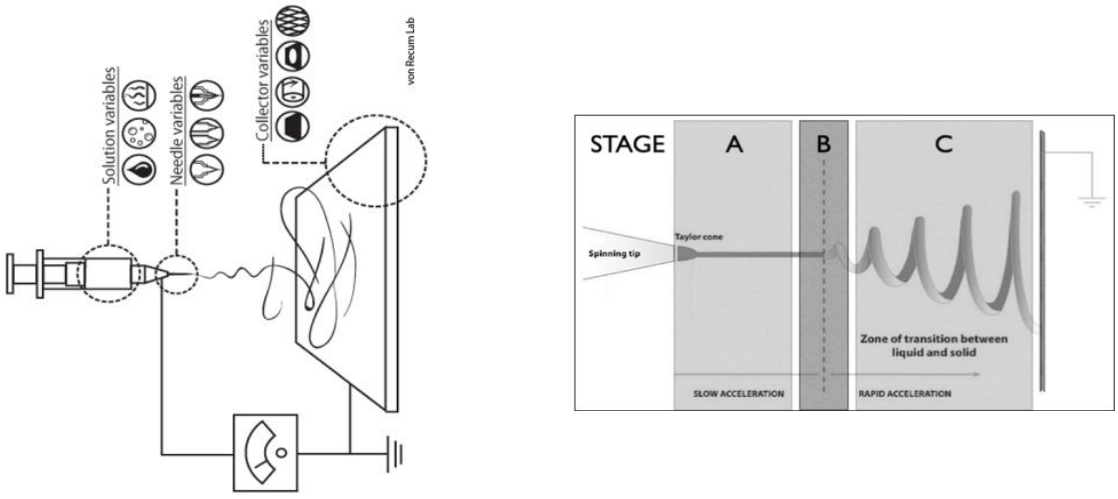


Fig 1: example of electrospinning procedure

Angiogenesis

Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels[18]. In precise usage this is distinct from vasculogenesis, which is the *de novo* formation of endothelial cells from mesoderm cell precursors[19], and from neovascularization). The first vessels in the developing embryo form through vasculogenesis, after which angiogenesis is responsible for most, if not all, blood vessel growth during development and in disease[20].

Angiogenesis is a normal and vital process in growth and development, as well as in wound healing and in the formation of granulation tissue.

In the angiogenetic process, the cell proliferation increase is one of the main actions required for the formation of new blood vessels. Normally endothelial cells present a turnover of about 1000 days but, in some conditions, both para-physiological and pathological they can undergo a rapid replication, with a turnover of only 5 days. The proliferation of vascular endothelial cells is associated with the degradation of the basement membrane: this allows the sprouting of pre-existing vessels[21]. The extracellular matrix does not undergo only proteolytic processes but it also changes its composition by exposing new epitopes to facilitate cell migration. The distribution of the factors involved leads to the formation of a chemotactic gradient thus allowing the proliferating cells to be guided through areas in which the extracellular matrix has been degraded.

Different molecules regulate this phase of the angiogenic process but can be divided into three classes:

- Factors that act specifically on endothelial cells (such as the VEGF family and angiopoietins);
- Molecules acting with a direct mechanism predominantly on other populations cellular (cytokines, chemokines and angiogenetic enzymes);
- Molecules acting indirectly mediating the release of other proangiogenic factors from macrophages, endothelial cells, inflammatory or neoplastic cells (such as TNF and TGF) [22]

Once activated to proliferate and migrate, endothelial cells form tubular structures that initially do not present a lumen. Only at a later time formation of the vessel lumen occurs through an intracellular canalization thanks to a fusion of cytoplasmic vesicles or through an alternative process of intercellular canalization in which the lumen is created through the apposition of two cell membranes[23].

Endothelial cells are the most quiescent and genetically stable cells in the body and they are typically bound to the basal membrane, which is composed of type IV collagen, laminin, proteoglycans, heparan-sulphate and other molecules. This indicates that the main signal originated from the basement membrane inhibits the proliferation and promotes an environment that facilitates cellular adhesions.

Endothelium

The endothelium represents the internal lining of blood vessels, it is a versatile and multifunctional tissue[24]:

- it regulates the migration of molecules (acting as a semipermeable membrane) and cells from the intravascular compartment to the peripheral tissues;
- it maintains a non-thrombogenic interface between blood and tissues while still ensuring a ready hemostasis in case of breakage, thanks to the processing of molecules procoagulants (vWf, tissue factor, plasminogen activator inhibitor) and anticoagulants, antithrombotic and fibrinolysis regulators (prostacyclin, trombomodulin, heparin-like molecules, plasminogen activator);
- it modulates the blood flow and vascular reactivity through the mechanism induced by vasoconstrictive molecules (endothelin, ACE) and vasodilators (NO, prostacyclins);
- it regulates inflammation and immunity by producing IL-1, IL-6, chemokines, adhesion molecules (VCAM-1, ICAM, E-selectin, P-selectin);
- it regulates cell growth (by stimulating factors: PDGF, CSF, FGF; and inhibitors: heparin, TGF-β);
- it is involved in the mechanism of hormones metabolism and in the LDL oxidation process.

The physiological endothelial function is characterized by a balance between vasoconstrictor and vasodilator factors and by the ability of the vessel to promptly respond to different stimuli. The endothelial disfunction is defined as an alteration that compromises the vascular reactivity or can lead to a thrombogenic condition. This situation can partially explain the typical vascular characteristic of certain pathologic conditions such as diabetes, hypertension, atherosclerosis[25].

For decades, the Vascular Endothelial Growth Factor (VEGF) has been considered one of the bases of the angiogenic process[26]. Many studies confirmed the role played by this specific molecule and they demonstrated that the loss of even one gene allele for VEGF leads to premature embryonic death[27].

The first description of VEGF dates back to the 80s, when this molecule was discovered as a vascular permeability factor (Vascular Permeability Factor, VPF). In 1989 Ferrara and Henzel identified a growth factor for endothelial cells in the medium conditioned by bovine pituitary follicular cells and they gave the factor the name of VEGF. Afterwards the VEGF was sequenced and proved to be coincident with the VPF.

The gene family of VEGF includes seven proteins designated as: VEGF-A, VEGF-B and the Placental Growth Factor (PlGF - involved in the angiogenic process), VEGF-C and VEGF-D (involved in recently isolated lymphoangiogenesis) and VEGF-E and VEGF-F (whose biological function is not

still been clarified)[28].

Neovascularization by VEGF gene/protein therapy has failed for inefficient delivery of the therapeutic factor. Based on the pioneering studies by J. Folkman, who identified a soluble tumor angiogenic activity that was mitogenic for ECs, in 1989 VEGF was identified as the most powerful factor sustaining such function. Identification of this factor led to expectation that it might stimulate therapeutic angiogenesis of ischemic limbs. Translational attempts in this respect, however, have been largely disappointing so far. Most likely, these negative results stem from a combination of reasons, including the very short half-life of the VEGF protein in vivo

and the de-sensitization of ECs to VEGF signaling in diabetic patients[29]. Gene therapy could overcome at least the first of these limitations and, quite encouragingly, a randomized, double-blind, placebo-controlled study based on the delivery of a plasmid encoding the 165 aa isoform of VEGF (VEGF165) in diabetic patients with Peripheral Artery Disease (PAD) and Critical Limb Ischemia (CLI) showed fewer amputations, enhanced skin ulcer healing and hemodynamic improvement[30].

VEGF is a multitasking growth factor. Beside its angiogenic potential, VEGF has more recently emerged as a pleiotropic factor, consistent with the widespread expression of its receptors. Relevant to the present project, we and others have shown that VEGF delivery significantly protects muscle tissue from damage and improves muscle repair by directly acting on both myogenic progenitors and mature muscle fibers[31,32].

Cell therapy for the induction of neovascularization suffers from rapid clearance of the injected cells in vivo[33]. Parallel to the development of gene therapy approaches, additional efforts have been recently devoted to the induction of neovascularization by transplanting various progenitor cells (mainly bone marrow mononuclear cells, mesenchymal stromal cells and endothelial progenitor cells), which possess the capability to differentiate into vascular cells, as well as to mediate paracrine effects through the release of pro-angiogenic growth factors[34]. However, in vivo imaging techniques indicate that the extent of cell retention was rather low in most experimental and clinical studies and thus novel strategies to improve cell survival and engraftment are absolutely warranted. Relevant to this proposal, transplantation of both endothelial progenitor cells (EPCs) and mature ECs for therapeutic vascularization or tissue engineering is a promising method for increasing tissue perfusion.

Based on these factors, considering the need to reduce the amputation rate in diabetic patients, the aim of the present study was to develop an innovative therapy based on the combination of VEGF, Endothelial Cells (ECs) and a polymeric scaffold in a hindlimb ischemia mouse model to promote neo-vascularisation and tissue survival in a Hindlimb Ischemia (HLI) mouse model.

The primary aim of the project is the application of a biocompatible polymeric nanofiber scaffold, and the comparison with micrometric biological scaffold, both combined with endothelial cells (ECs) and vascular endothelial growth factor (VEGF) in a hindlimb ischemia mouse model in order to stimulate the angiogenis and muscle protection.

2. MATERIALS AND METHODS

This project relies on the long-lasting collaboration between the Cardiovascular Biology Group and the Plastic Surgery Unit of the University of Trieste. We exploited the complementary expertise to develop new intervention strategies for the therapy of critical wounds in ischemic or diabetic patients, based on a polymeric scaffold able to sustain the slow and steady release of VEGF and Endothelial Cells (ECs) into the ischemic tissue, with the final, ambitious goal of promoting tissue survival and neo-vascularization.

All research activities adhered to fundamental ethics principles, as laid out in the Declaration of Helsinki, the Charter of Fundamental Rights of the European Union and the European Code of Conduct for Research Integrity. The project could not avoid the use of animal models of limb ischemia, consistent with its main scientific and translational objectives. For all experimental activities, the use of animals was conducted under control of qualified experienced personnel, having certified patent for the use of animals, in accordance with the recommendation of the Council Directive of the European Community of 24 November 1986 (86/609/EEC) and conforming to all regulations protecting animals used for research purposes, established under Directive 2010/63/EU. All animal experiments will be performed upon the approval by the competent Ethical Committee and Animal Welfare Board (AWB).

A clinical protocol was producted and submitted to the CEUR (Comitato Etico Unico Regionale) and the ethical board approval was obtained for the harvesting of human tissue to be performed at the Plastic Surgery Department of Trieste and the cell use

for in vitro and in vivo experiments (CEUR-2019-PR-01 con nota Prot. 15569/P/GEN/ARCS dd. 28/05/2019).

A systematic review was performed, until May 2018, using the following key words: ((nanofiber) AND scaffold) AND hindlimb ischemia; ((nanofiber) AND scaffold) AND ischemia; ((nanofiber) AND scaffold) AND vascularization; (scaffold) AND hindlimb ischemia; (bioengineering) AND hindlimb ischemia.

PCL nanofiber scaffold

In order to create the most suitable nanofiber scaffold, setting the parameters for the electrospinning was decisive. The polymer of choice was the polycaprolactone (PCL): a semi-crystalline, synthetic polymer, widely employed for biomedical applications due to its characteristics

The PCL nano scaffold used were electrospun in a horizontal spinning configuration, using a customized electrospinning unit (Holmarc Optomechatronics Pvt Ltd, India). Polymer solutions were supplied continuously to a syringe connected to a 21 gauge blunt end needle with a feeding rate of 1 ml/h for 72 h. A high voltage of 16 kV was applied between the needle kept at a distance of 20 cm from rotating mandrel set at 200 revolutions per minute.

Fig 2: jet coming out from the tip of the spinneret

In vitro experiments. ECs isolation:

Adult primary endothelial cells were isolated from the dermis using anti-CD31 MACS MicroBeads techology (Miltenyi Biotec). The tissue was collected and washed 2 times in PEB buffer (0,5% w/v BSA - Bovin Serum Albumin; 2 mM EDTA; pH 7,2; dissolved in PBS 1X). Subsequently using surgical scissors, it was cut in small pieces, which were then transferred in a 15 mL Falcon tube containing digestive enzyme mix (Dulbecco's Modified Eagle Medium – Gibco ThemoFisher Scientific) without Fetal Bovine Serum - FBS; 100 µL Enzyme D; 25 µL Enzyme P; 18 µL Enzyme A) as reported in Skeletal Muscle Dissociaton protocol. The sample was incubated for 1 hour at 37°C under continuous rotation using a magnetic stirrer. After the digestion step, the cell suspension was filtered using 70 µm nylon strainer in 50 mL Falcon tube and 3 mL of complete DMEM (20% FBS) were added to rinse the strainer and stop the enzymatic reaction. The sample was centrifuged at 300xg for 10 minutes and then the supernatant was aspirated completely using serological pipet. The cells were resuspended with a PBS/EDTA solution (PBS 1X; 250 mM EDTA) and incubated for 20 minutes on ice with mouse CD31 MicroBeads in proportion 1:10 with the added PBS/EDTA solution (per reaction: 200 µL PBS/EDTA; 20 µL CD31 MicroBeads). The adult CD31 positive endothelial cells were isolated using the MACS magnetic separator with MACS LS Column (CD31 MicroBeads – Miltenyi Biotec). In particular, after 20 minutes incubation, the cell suspension was applied drop by drop with P200 pipet onto MACS LS Columb, which was prepared by rinsing with 3 mL of PBS 1X and by full loading with PBS 1X beforehand. The column was filled with PBS 1X and when the volume of PBS/cell suspension reached half of the total load of the column until all of the sample was passed through it. Subsequently, Columb was washed with 5

mL of PBS 1X for 3 times in order to eliminate as much as possible the debris and to ensure an adequate ECs purification. At the end, the Columb was removed from the separator and placed it on 15 mL Falcon tube. The CD31 positive magnetically labeled cells were flushed out by 2 mL of EGM2 complete medium (Endothelial Growth medium-2 - Lonza).

2D culture for In vitro experiments – effect of VEGF (100 ng/mL) on ECs

Primary 24 well-plate (Falcon) was coated with a solution of Fibronectin (1 mg/mL Bovine Plasma Fibronectin – Invitrogen) and Gelatin (0,2 mg/mL Gelatin from bovine skin, type B – Sigma) in final proportion 1:200.

Immediately after the isolation, the adult primary ~~CD1~~ endothelial cells were counted using plastic counting chamber (Biosigma) and $2{,}0x10^5$ cells were seeded on pre-conditioned primary 24 well-plate. The cells were cultured for 7 days in incubator at 37°C, 5,0% pCO_2 using EGM-2 complete medium (Lonza). EGM-2 complete medium (Lonza) was changed the day after the isolation/seeding (day 1) and at day 4 and 100 ng/mL rhVEGFa (R&D Systems) were added.

3D culture for In vitro experiments – effect of VEGF (100 ng/mL) on ECs on Integra and nanofiber scaffold

Primary 24 well-plate (Falcon) was coated with a solution of Fibronectin (1 mg/mL Bovine Plasma Fibronectin – Invitrogen) and Gelatin (0,2 mg/mL Gelatin from bovine skin, type B – Sigma) in final proportion 1:200. Sterile a single layer INTEGRA Dermal Regeneration Template (Integra Life Science Corporation) and PCL scaffold were cut

in small pieces (6 mm x 5 mm) using sterile scalpel and placed with into previously coated primary 24 well-plate.

Immediately after the isolation, the adult primary CD1 endothelial cells were counted using plastic counting chamber (Biosigma) and $2{,}0x10^5$ cells were seeded on the INTEGRA and PCL scaffold. The cells were cultured for 7 days in incubator at 37°C, 5,0% pCO_2 using EGM-2 complete medium (Lonza). EGM-2 complete medium (Lonza) was chaged the day after the isolation/seeding (day 1) and at day 4 and 100 ng/mL rhVEGFa (R&D) were added.

The experiments were performed in duplicate. For the quantification analyses, the number of cells and the proliferating cells, defined as number of CD31 positive cells, were counted manually considering the same population density and using the Fiji software's cell counter in eight different fields per condition (20X magnification).

The tube formation analysis was executed counting numbers of the tubes in 3D consisting of two or more CD31 positive cells that formed a tube-like structure. The 3D reconstruction was made using Fiji software's 3D projection.

Immunofluorescence

The Endothelial Cells in 2D and in 3D culture were washed 2 times with PBS 1X and fixed using 4% v/v PFA solution (in PBS 1X) (paraformaldehyde – Santa Cruz). Subsequently, the cells were washed 2 times with PBS 1X and permeabilized by 0,5% v/v Triton X-100 in PBS 1X solution (Sigma) for 2 minutes. The blocking step was performed using 5% w/v BSA (Bovine Serum Albumin – Roche) in PBS 1X solution for 1 hour. The cells were incubated overnight at 4°C with anti-CD31 and Ki67 primary antibodies in proportion of 1:100 v/v with 1% v/v BSA, 0,1% v/v Tween-20 (Sigma) in

PBS 1X solution. Subsequently, the cells were washed properly 3 times with PBS 1X and the incubation with secondary antibody was performed for 1 hour at room temperature diluting the secondary antibody 1:500 v/v with 1% v/v BSA, 0,1% v/v Tween-20 in PBS 1X solution. The DNA-33342 Hoechst counterstaining (Invitrogen) was performed for 2 minutes after 3 washes with PBS 1X, diluting Hoechst 1:5000 v/v with PBS 1X. The images were acquired using Nikon A1 Plus Microscope for 3D and Leica Microscope for 2D.

In vivo experiments:

Adult endothelial cells were isolated from the dermis and maintained for 5 days in culture following the previously reported isolation protocol. All the isolated ECs were seeded with EGM2 complete medium on 10 cm primary dish, which was previously coated by FIG solution, and put in the incubator at 37°C and 5,0% pCO_2. After 24 hours the ECs were washed 2 times with PBS 1X, detached using Trypsin/EDTA 2X solution and incubated for 10 minutes at 37°C in incubator.

Subsequently, the cell suspension was transferred in 15 mL Falcon tube and the same volume of EGM-2 complete medium (20% FBS) was added to stop the reaction before the centrifugation (300xg for 5 minutes). The supernatant was aspirated completely and the pellet was resuspended using EBM-2 medium without factors and FBS (Endothelial Basal Medium-2 – Lonza). The endothelial cells were counted using plastic counting chamber (Biosigma) and $2{,}0x10^5$ cells (per experiment) were transferred and centrifuged (300xg; 5 minutes) in 15 mL Falcon tube. The supernatant was aspirated completely and the pellet was resuspended using 30 µL of EBM-2 medium without factors and FBS (Endothelial Basal Medium-2 – Lonza). The cell

suspension was injected in left leg (quadriceps, gastrocnemius and tibialis anterior muscles) of Balb/C mice with hind limb ischemia. The surgery was performed the same day of the injection.

All the isolated endothelial cells were seeded with EGM2 complete medium on 10 cm primary dish, which was previously coated by FIG solution, and put in the incubator at 37°C and 5,0% pCO_2. After 24 hours the cells were washed twice with PBS 1X, detached using Trypsin/EDTA 2X solution and incubated for 10 minutes at 37°C in incubator. Then the same cell suspension protocol described was applied.

3D culture for in vivo experiments

Primary 24 well-plate (Falcon) was coated with a solution of Fibronectin (1 mg/mL Bovine Plasma Fibronectin – Invitrogen) and Gelatin (0,2 mg/mL Gelatin from bovine skin, type B – Sigma) in final proportion 1:200. Sterile a single layer INTEGRA Dermal Regeneration Template (Integra Life Science Corporation) was cut in small pieces (6 mm x5 mm) using sterile scalpel and placed with into previously coated primary 24 well-plate.

Adult ECs were isolated, from discharged tissue after surgery, as previously described. Immediatel after the isolation the adult primary endothelial cells were counted using plastic counting chamber (Biosigma) and $1,5x10^6$ cells (per experiment) were seeded on INTEGRA without silicon layer in 24 well-plate. The cells were cultured for 5 days in incubator at 37°C, 5,0% pCO2 using EGM-2 complete medium (Lonza) and adding 100 ng/mL rhVEGFa (R&D). EGM-2 complete medium (Lonza) was changed the day after the isolation/seeding (day 1) and at day 3 and 100

ng/mL rhVEGFa (R&D) were added. INTEGRA and PCL scaffold were placed in the place of femoral artery of BALB/C mouse 5 days after isolation/seeding.

Hindlimb Ischemia Model (HLIM)

Surgical induction of unilateral hind limb ischemia on the left lower limb were be performed under general anaesthesia by Ketamine/Xylazine (100 mg/kg and 10 mg/kg body weight, i.p.). After hair removal from the hindlimb, mice were be placed in supine position over a draped heated pad, with the left limb extended and secured with a piece of tape. Using a dissection microscope, an incision of the skin, approximately 1 cm long, was made from the knee towards the medial thigh. The underlying femoral artery was dissected and separated from the femoral vein and nerve at the proximal location near the groin, to occlude the vessel using a 7-0 silk suture underneath the proximal end of the femoral artery. A similar occlusion was performed at the distal location close to the knee, followed by transection of the segment between the distal and proximal knots (Fig 3-4) . In the selected group, the matrix was placed on the site of the transected artery before skin closure.

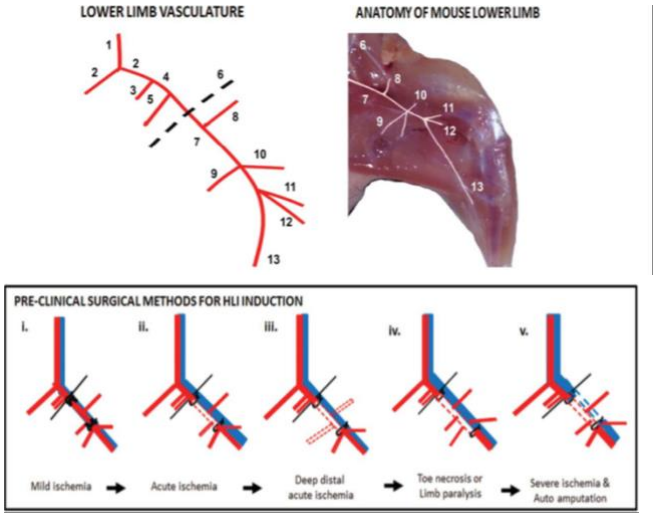


Fig 3: schematic femoral artery and types of ligatures [*Krishna SM et al*] [35]

After closure of the incision, the animal was kept on a draped heated pad in the recovery cage and monitor continuously until awake. Effective ischemia was confirmed by laser Doppler (Vevo 2100, Visual Sonics) 1 hour after surgery.

Different groups were identified:

Different group of HLIM were identified in two part of the experiments

First section:

Group A 10 HLIM control group

Group B 10 HLIMB with intramuscular ECs injection (in quadriceps, gastrocnemious and tibialis anterior muscle)

Group C 10 HLIM with subcutaneous Integra® positioning

Second section:

Group 1 10 HLIM control group

Group 2 10 HLIM with subcutaneous Integra® positioning

Group 3 10 HLIM with ECs seeded on Integra® 5 days before surgery and then subcutaneously positioned the day of surgery

Group 4 10 HLIM control group 2

Group 5 10 HLIM with subcutaneous PCL scaffold positioning

Group 6 10 HLIM with ECs seeded on PCL scaffold 5 days before surgery and then subcutaneously positioned the day of surgery

The postoperative clinical assessment was performed according to the Tarlov score (tab) and the Modified Ischemia score.

All hindlimb ischemia were assessed at day 1 and day 7. At day 7 mice were euthanized using cervical dislocation method.

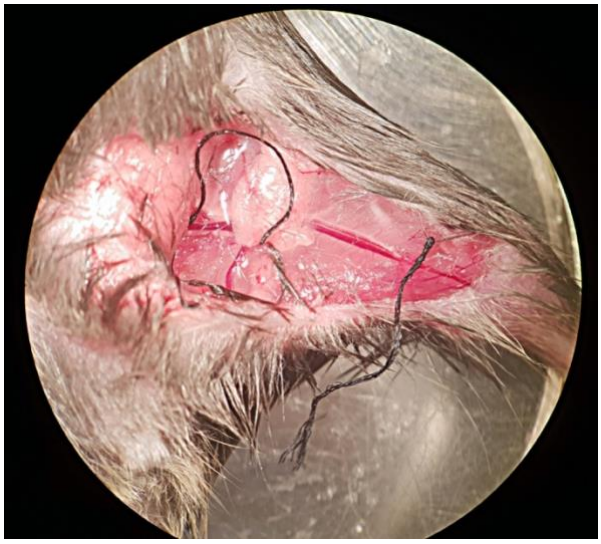


Fig. 4 – HLIM: femoral artery dissection and ligation performed under operating microscope

Immunofluorescence on frozen tissues

Seven days after surgery all the mice were sacrificed. The quadriceps, gastrocnemius and tibialis anterior muscles of ischemic hind limb were collected, transversely cut in half and fixed overnight using 2% v/v PFA in PBS 1X solution (Santa Cruz). The day after, PFA solution was removed and replaced with 20% sucrose cryoprotector solution (Sigma). The cryosections were stored at -20°C. The sections were thawed and dried at room temperature for 5 minutes. After a wash in PBS 1X for removing O.C.T. cryo gel, the samples were permeabilized using 0,5% v/v Triton X-100 (Sigma) in PBS 1X for 15 minutes. The blocking step was performed using 5% w/v BSA (Bovine Serum Albumin – Roche) in PBS 1X solution for 1 hour. The single section was separately incubated overnight at 4°C using a humid chamber with anti-CD31 (goat monoclonal CD31 antibody – R&D), anti-Isolectin beta 4 (biotinilated Isolectin B4

antibody – Sigma) and anti- aSMA primary antibody diluted in proportion of 1:200 v/v with 1% v/v BSA, 0,1% v/v Tween-20 (Sigma) in PBS 1X solution. The day after, the slides were washed with 0,02% v/v Tween 20 (Sigma) in PBS 1X solution for 5 minutes and with PBS 1X for 3 times for 3 minutes each. The incubation with secondary antibody (was performed for 1 hour at room temperature in a humid chamber diluting the secondary antibody 1:500 v/v with 1% v/v BSA, 0,1% v/v Tween-20 in PBS 1X solution. The tissues were washed with 0,02% v/v Tween 20 (Sigma) in PBS 1X solution for 5 minutes and with PBS 1X for 3 times for 3 minutes each. The DNA-33342 Hoechst counterstaining (Invitrogen) was performed for 15 minutes after 3 washes with PBS 1X, diluting Hoechst 1:5000 v/v with PBS 1X. Subsequently, slides were washed 3 times with PBS 1X and mounted using Mowiol mounting medium (Sigma). The images were acquired using Leica Microscope.

3. RESULTS

The ethical board approval was obtained for the harvesting of human tissue to be performed at the Plastic Surgery Department of Trieste and the cell use for in vitro and in vivo experiments (CEUR-2019-PR-01 con nota Prot. 15569/P/GEN/ARCS dd. 28/05/2019).

The human cells were obtained from discharged tissue, after surgery, anonymously. All patients filled and signed the informed consent form approved by the CEUR.

In vitro 2D culture:

The endothelial cells (ECs) cultured on monolayer for 7 days with and without VEGF (100ng/mL) showed an increased number of cells and proliferation rate. In particular both the Ki 67 and the CD 31 were more represented in the VEGF group (Fig 5).

The endothelial cells cultured on monolayer for 7 days in the presence of VEGFa (100ng/mL), showed an increase in the number of number of cells and proliferation rate compared to the non-treated cells (control). In particular the Ki 67 and the CD31 positivity was more represented in the VEGF group. Ki-67 is a nuclear protein that is expressed during various stages in the cell cycle, particularly during late G1, S, G2, and M phases. The correlation between low Ki-67 index and histologically low-grade tumors is strong. Ki-67 is routinely used as a marker of cell cycling and proliferation. In this work, Ki67 was used as proliferation marker.

CD31 (PECAM-1 – Platelet Endothelial Cell Adhesion Molecule) is a 140 kDa type I integral membrane glycoprotein. This cell adhesion and signalling molecule is normally found on endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, lymphocytes (T cells, B cells, and NK cells), megakaryocytes, and osteoclasts.

CD31 is a multifunctional molecule with diverse roles in modulation of integrin-mediated cell adhesion, transendothelial migration, angiogenesis, apoptosis, negative regulation of immunoreceptor signaling, autoimmunity, macrophage phagocytosis, IgE-mediated anaphylaxis and thrombosis. It is one of key regulatory molecules in vascular system.

~~In immunohistochemistry, CD31 is used primarily to demonstrate the presence of endothelial cells in histological tissue sections. This can help to evaluate the degree of tumor angiogenesis, which can imply a rapidly growing tumor. Malignant endothelial cells also commonly retain the antigen, so that CD31 immunohistochemistry can also be used to demonstrate both angiomas and angiosarcomas. In this work, CD31 was used as Endothelial Cells marker.~~

~~CD31 (platelet endothelial cell adhesion molecule 1, PECAM 1) is an inhibitory coreceptor involved in regulation of T cell and B cell signaling by a dual immunoreceptor tyrosine based inhibitory motif (ITIM) that upon associated kinases mediated phosphorylation provide docking sites for protein tyrosine phosphatases. CD31 is expressed ubiquitously within the vascular compartment and is located mainly at junctions between adjacent cells. N terminal Ig like domain of CD31 is responsible for its homophilic binding, which plays an important role in cell cell interactions. CD31 is a multifunctional molecule with diverse roles in modulation of~~

~~integrin-mediated cell adhesion, transendothelial migration, angiogenesis, apoptosis, negative regulation of immunoreceptor signaling, autoimmunity, macrophage phagocytosis, IgE-mediated anaphylaxis and thrombosis. It is one of key regulatory molecules in vascular system.~~

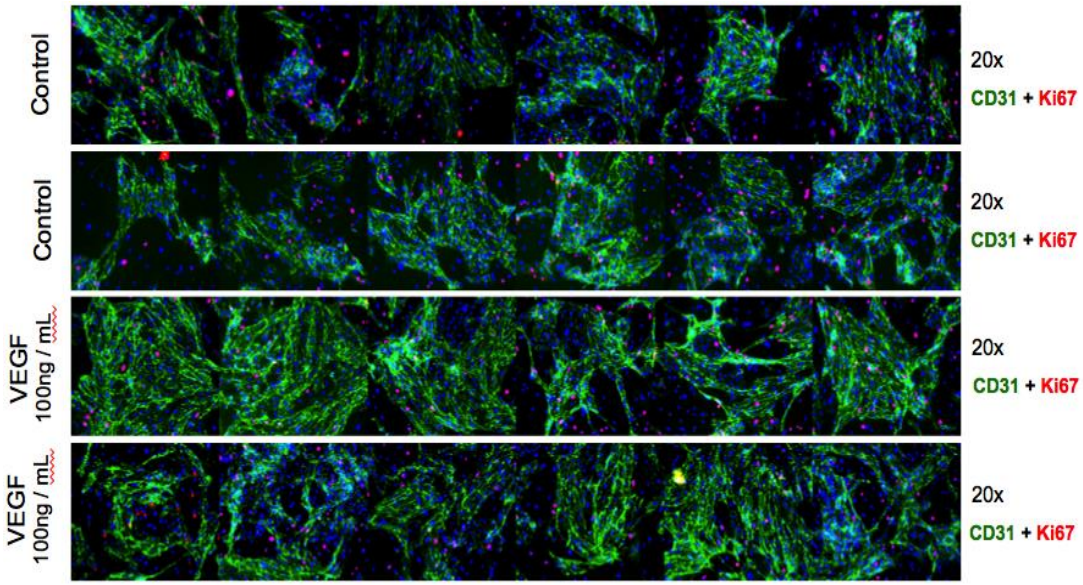
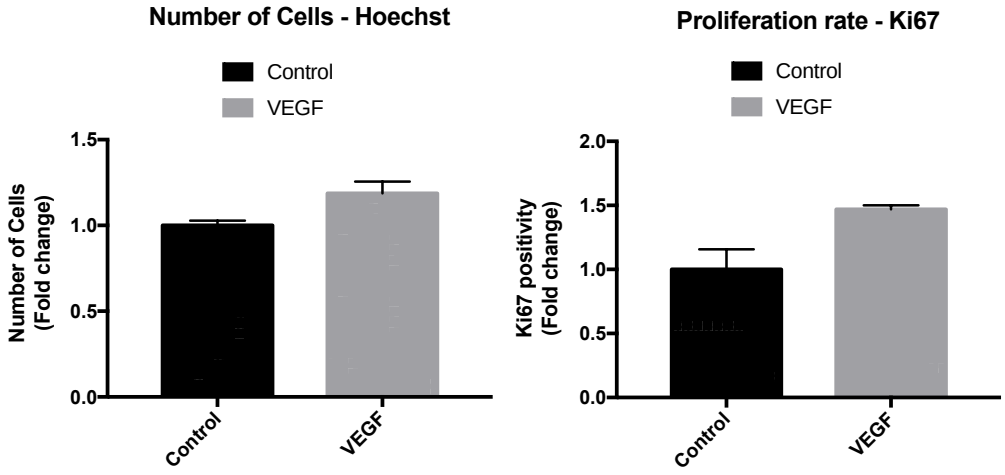


Fig 5: Effect of 100 ng/mL VEGF on 2D ECs culture. CD31-green; nuclei-blue; Ki67-red

The ECs are represented by CD31 signal, instead the other Hoechst positive cells represent fibroblasts (ordinary contamination). ~~The characteristic "cobblestone" like morphology of ECs can be seen.~~ The major presence of Ki67 signal into VEGF treated ECs compared to control confirms the pro-mitotic effect of VEGF on ECs. Moreover, the higher number of CD31 positive cells in treated samples compared to the control demonstrates the protective effect of VEGF on ECs.

In particular from the results of quantitative analysis, the treated ECs show a 15-20% increase of number of cells (CD31 positive cells). In addition, a gain of 45-50% of

proliferation rate in VEGF treated cells in comparison to control samples can be recorded (Ki67 positive ECs nuclei) (Graphic 1).



Graphic 1 - Effect of 100 ng/mL VEGF on adult primary Endothelial Cells – A) protective and B) pro-mitotic effect of VEGF on 2D ECs culture. X-axis: A-B) control-no treated; VEGF-100 ng/mL rhVEGFa. Y-axis: A) number of cells; B) ki67 positivity - proliferation rate. Data express as fold change (normalized on control).

The experiments were performed in duplicate and the data is expressed as average +/- SD of results, which were obtained from analysis of six 20X-fields per condition. A) Protective and pro-mitotic effect of 100 ng/mL VEGF. The VEGF treated ECs show an increase of 15-20% in term of number of cells compared to the control, by virtue of the fact that VEGF stimulates the ECs to survive and proliferate. B) Effect of 100 ng/mL VEGF on ECs proliferation rate. The proliferation rate of ECs increases by 45-50% in response of 100 ng/mL VEGF.

The results confirm the protective, anti-apoptotic and proliferative effect of VEGF, which was already demonstrated in literature, also for the primary ECs

Scaffolds

The literature review performed showed a total of 609 articles. The inclusion criteria were represented by articles written in English language dealing with the use of nanofiber scaffolds or different type of scaffolds in the ischemia/vascularization medical and biological field.

- ((nanofiber) AND scaffold) AND hindlimb ischemia: 1 article
- ((nanofiber) AND scaffold) AND ischemia: 6 articles
- ((nanofiber) AND scaffold) AND vascularization: 321 articles
- (scaffold) AND hindlimb ischemia: 56 articles
- (bioengineering) AND hindlimb ischemia: 225 articles

172 articles were found to be inherent with the and among these, 30 articles were strongly inherent with the purpose of the research project.

However, none of the selected articles exploited the additional use of primary Endothelial Cells (ECs) to improve the vascularization and soft tissue protection effect in a hindlimb ischemia mouse model

After a careful evaluation of the nanofiber scaffold used to improve the tissue vascularization, the most suitable nanofiber scaffold composition described to date seemed to be the one composed by Poly(lactic acid) (PLA) and polycarprolactone (PCL) since they are biodegradable, nontoxic and gave appropriate biocompatibility (they are degrade by enzymes and nonenzymatic hydrolysys and their monomers are eliminated)[36 37 38 39 40 41 42 43 44 45]. A valid alternative is represented by scaffolds composed of core–shell fibers fabricated using co-electrospinning with a core

solution composed of polyethylene oxide and mixed with rhVEGF. The shell solution was composed of PCL, with 0.25, 1, and 3% of polyethylene glycol (PEG) to manipulate pore size on the shell. Pore size and density as well as rhVEGF incorporation were affected by the PEG concentrations. Endothelial cell migration toward rhVEGFincorporated polymeric scaffold was 80-fold higher as compared to VEGF-free polymeric scaffold[46].

PCL is one of the most extensively used as a base of scaffolds: it is biodegradable, biocompatible, non-toxic, idrophobic and long lasting. It is degraded by enzymatic and non-enzymatic hydrosis and its monomers are eliminated by the hosting tissue cells. It is approved by the US FDA as a component of biomaterial-based scaffolds.

Based on these findings, the electrospinning parameters were set and the PCL nanofiber electrospun as previously described.

The fibrous mats were detached from mandrel, rinsed twice in deionized water to remove the solvent and air dried at 37° overnight. The scaffolds were sterilized by exposing to UV for 30 min prior to cell culture studies.The scaffolds were seeded with 2,0x105 cells on pre-conditioned primary 24 well-plate. Citotoxicity test by direct contact method were performed showing non-cytotoxicity effects when in direct contact with fibroblast monolayer. Cell detachment, lysis or extensive vacuolization were absent (Fig 6).

	Thickness (μm)
16 kV 60 min	215.6 ± 22.1

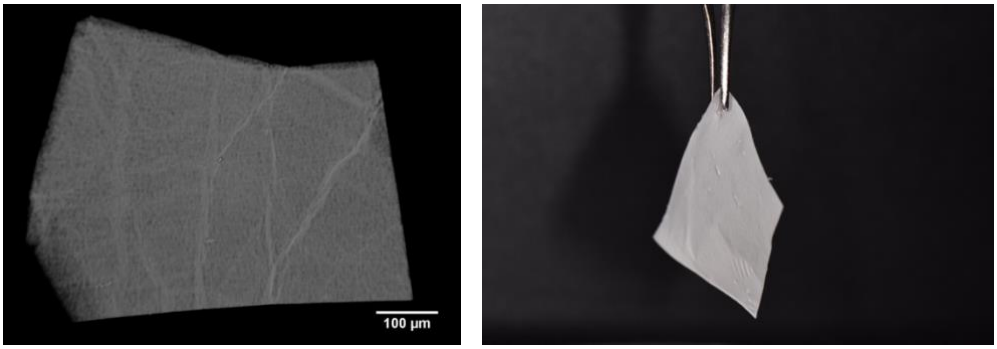


Fig 6: PCL nanofiber scaffold and macroscopical appearance.

In vitro 3D culture:

The ECs cultured on 3D scaffolds, Integra® and PCL with VEGF (100 ng/mL) showed the ability to migrate into the scaffold and organize forming tube-like structures.

Both scaffolds proved to be a suitable substrate to primary adult Endothelial cell culture. Indeed, the ECs attached on the scaffolds, survived for 7 days and formed tube-like structure in 3D culture (Figg 7-8). Furthermore, the presence of VEGFa (100 ng/mL) stimulated the ECs to survive, growth and form tube-like structures. In particular, the treated ECs showed a significant increase in the number of cells and tube formation compared to non-treated cells (control). The effect of VEGFa was confirmed by quantitative analysis (Graphic 2).

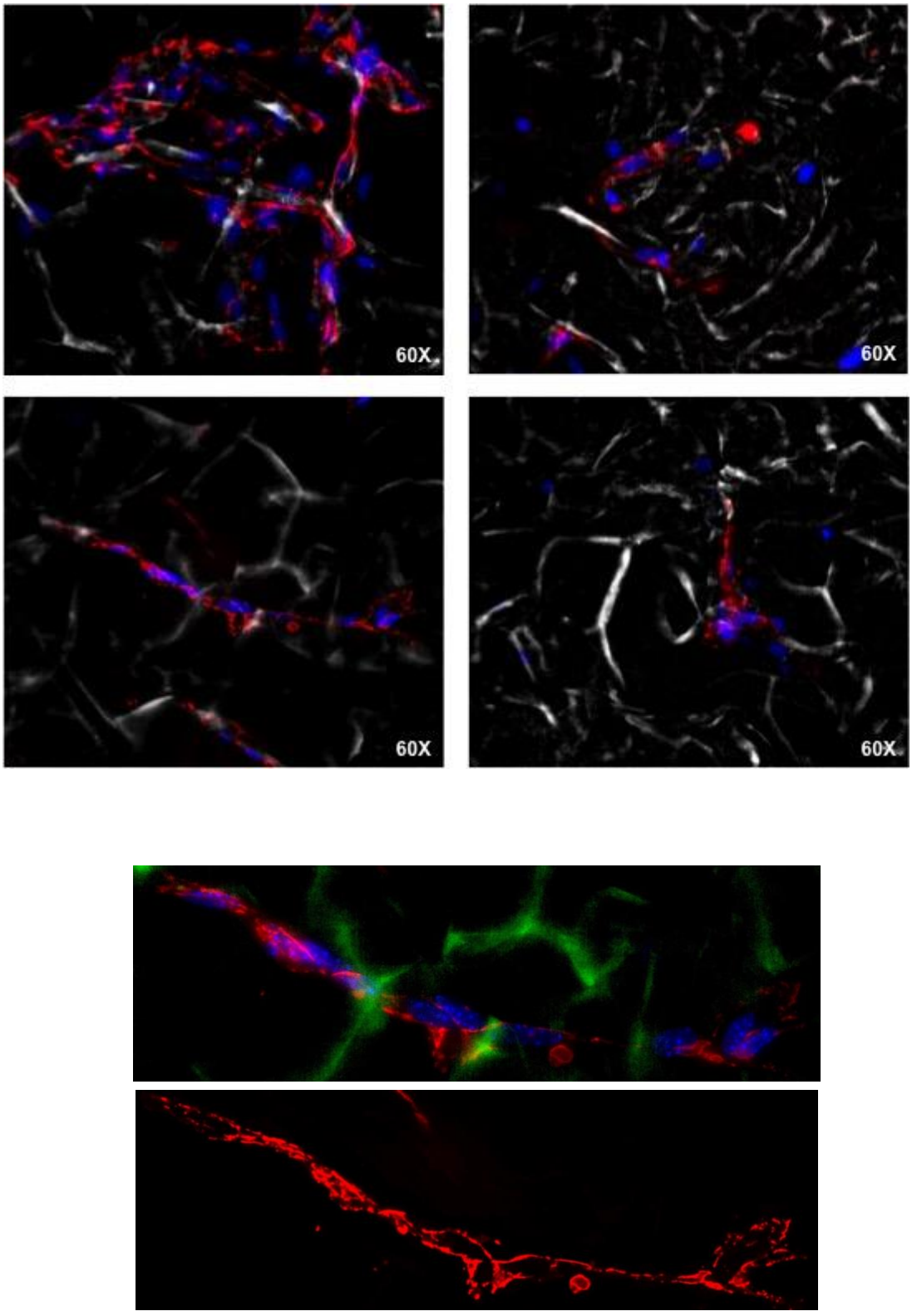


Fig 7: 3D culture of adult primary Endothelial Cells on INTEGRA. CD31-red; INTEGRA-white; nuclei-blue. INTEGRA demonstrates autofluorescence signal on green

wavelength (represented in white). Adult primary endothelial cells are able to attach, survive, growth and organize tube in INTEGRA 3D scaffold. The characteristic cobblestone-like morphology is not be observed at this cell density and in this substrate. However, the tube-like structure can be see through the final orientation of the cells ~~is very evident~~. As you can see from the figure, the ECs follow the structure of the scaffold and take contact each other inside the matrix forming tubes. 60X magnification; Z-projection, maximum intensity.

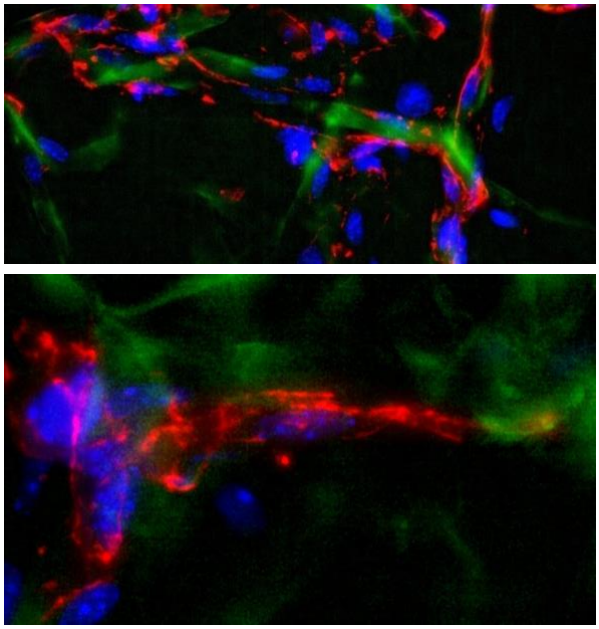
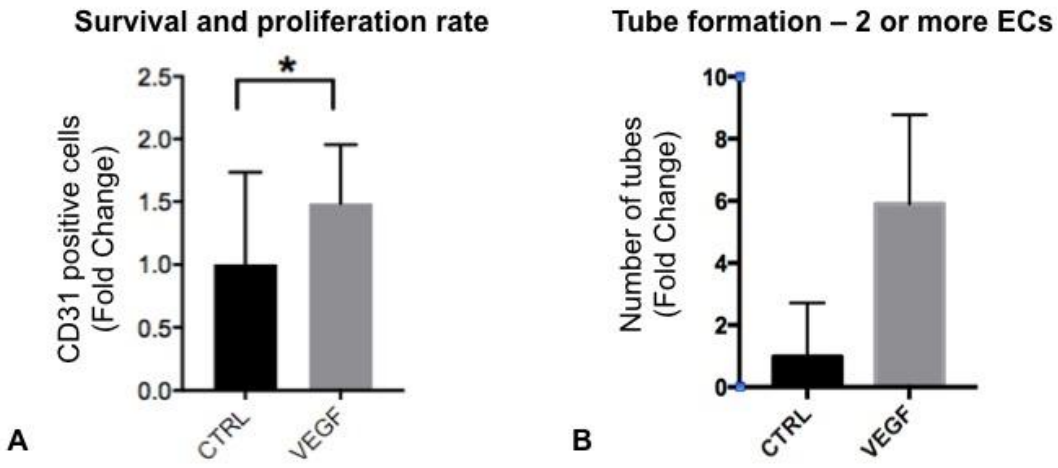


Fig 8: 3D culture of adult primary Endothelial Cells on PCL scaffold. CD31-red; scaffold-white; nuclei-blue. PCL demonstrates autofluorescence signal on green wavelength (represented in white). As per the Integra, the ECs were able to attach, survive, growth and organize tube. 60X magnification; Z-projection, maximum intensity



Graphic 2 – Quantification of proliferative and tube-forming effect of VEGF on primary ECs in 3D culture. X-axis: A-B) control-no treated; VEGF-100 ng/mL rhVEGFa. Y-axis: A) number of CD31 positive cells – survival and proliferation rate; B) Number of tubes – tube formation. Data express as fold change (normalized on control). A) Effect of 100 ng/mL VEGF on ECs proliferation in INTEGRA. The experiments were performed in duplicate and the data is expressed as average +/- SD of results, which were obtained from analysis of eight 20X-fields per condition. The VEGF treated ECs show an increase of 45-50% in term of number of cells compared to the control, by virtue of the fact that VEGF stimulates the ECs to survive and proliferate in 3D culture. Significant value confirmed by t test (p. values >0,05). B) Effect of 100 ng/mL VEGF on tube formation in INTEGRA. The VEGF treated ECs show an increase of 6 fold-higher in term of number of tube-like structures compared to the control, by virtue of the fact that VEGF induces the ECs to take a tube-like morphology and intercellular contacts.

Comparing the Integra and the PCL scaffold, the best cell adhesion was expected on the latter one and it was confirmed.

Histological analysis at 7 days revealed the presence of a massive number transplanted cells that colonized the Integra scaffold and also migrated into the muscle underneath, forming elongated vascular structures. Contrary, the small pores of the PCL scaffold layers prevented penetration of cells and enabled their growth mainly on the surface.

In vivo experiments:

~~Different group of HLIM were identified in two part of the experiments~~

~~First section:~~

- ~~Group A 10 HLIM control group~~
- ~~Group B 10 HLIMB with intramuscular ECs injection (in quadriceps, gastrocnemious and tibialis anterior muscle)~~
- ~~Group C 10 HLIM with subcutaneous Integra® positioning~~

~~Second section:~~

- ~~Group 1 10 HLIM control group~~
- ~~Group 2 10 HLIM with subcutaneous Integra® positioning~~
- ~~Group 3 10 HLIM with ECs seeded on Integra® 5 days before surgery and then subcutaneously positioned the day of surgery~~
- ~~Group 4 10 HLIM control group 2~~
- ~~Group 5 10 HLIM with subcutaneous PCL scaffold positioning~~
- ~~Group 6 10 HLIM with ECs seeded on PCL scaffold 5 days before surgery and then subcutaneously positioned the day of surgery~~

The clinical assessment of the first section of experiments, composed by 10 HLIM control group, 10 HLIMB with intramuscular ECs injection and 10 HLIM with subcutaneous Integra® positioning.

All mice were evaluated ad day 1 and 7 using the Tarlov score, the Modified Ischemia Score and the Clinical Evaluation of Hindlimb presentation (Tab 1- 2- 3).

The HLIM control group showed a slight heterogeneity in the final ischemia clinical presentation as well as the HLIM with subcutaneous Integra® positioning. However, the latter group presented a certain amount of clinical recover from ischemia, due to ECs and neoangiogenic factors migration in the scaffold and surrounding tissues (Fig 9).

The HLIM with intramuscular ECs injection group, however, showed a dramatic ischemic clinical presentation suggesting that the injection caused a high inflammatory response mediated by neutrophils and other inflammatory cells causing sever limb damage and sub-sequent limb amputation (Figg 10-11).

Histological samples collected from quadriceps, tibialis anterior and gastrocnemius muscles showed a (Hoest and Actin-GFP) an extremely higher inflammatory response, compared to the HLIM control group.

The second part of the in vivo experiments showed that the HLIM control group (group 1 and 4) presented signs of limb necrosis and disfunction that ranged from toe necrosis to limb amputation; in the HLIM with subcutaneous Integra®(group 2) without ECs the clinical aspect of the limb showed toe necrosis and a different range of discoloration and function impairment while in the HLIM with ECs seeded on Integra® group the lower limb seemed to have a slightly better function and aspect

(Fig 12). Group 5 showed a lower response in term of cell colonization and ischemia recovery compared to the group 2, however comparing group 3 ad group 6 (matrix and ECs previously seeded) appeared to be comparable (Fig 13)

SCALE	DESCRIPTION
0	No voluntary movement
1	Movement joints perceptible
2	Active movement but unable to sit without assistance
3	Able to sit but unable to hop
4	Weak hop
5	Complete recover of hindlimb funcion

Tab 1: Tarlov score

	MODIFIED ISCHEMIA SCORE
0	No necrosis
1	Discoloration of one nail
2	Discoloration of two or more nails
3	Discoloration of one finger
4	Discoloration of two or more fingers
5	Foot necrosis
6	Hindlimb necrosis
7	Hindlimb self-amputation

Tab 2: Modified Ischemia Score

CLINICAL EVALUATION OF HINDLIMB	
0	No differences from contralateral limb
1	Discoloration
2	Severe hypomobility and distal necrosis
3	Paw dragging and massive gangrene
4	Self amputation

Tab 3: Clinical evaluation of Hindlimb

The mean score showed a better recover and function in the ECs seeded on Integra group compared to the HLIM and the ECs injection; however the difference with the Integra only group was not clinically remarkably significant. Comparable results were obtained with the PCL scaffold. (Tab 4).

The mean final clinical evaluation score showed a better recover and function in the ESc seeded on the PCL nanoscaffold group compared to the HLIM and the nanoscaffold alone

The perfusion of treated limbs was quantified by Single Photon Emission Tomography (Fig 14)

	HLIM	ECs injection	Integra	ECs seeded on Integra
Tarlov score Average	0.7	0.5	3.5	3.8
Ischemia Modified Score average	5.2	5.25	4	3.5
Clinical score average	3.75	4	2	1.8

	HLIM	PCL scaffold	ECs seeded on PCL scaffold
Tarlov score Average	0.5	3	3.5
Ischemia Modified Score average	5	4.8	4
Clinical score average	3.5	2.7	2

Tab 4: mean score obtained

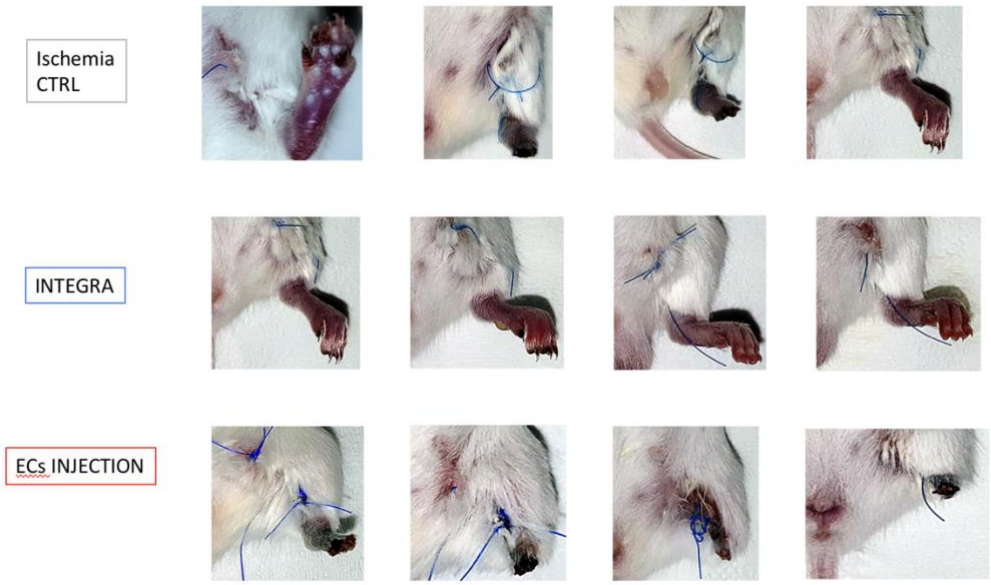


Fig 9: example of clinical presentation at 7 days

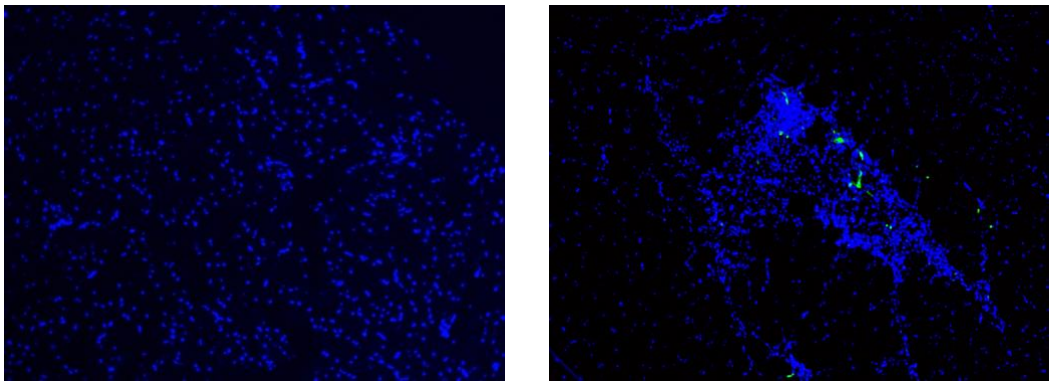


Fig 10: Hoechst and actin-GFP showing the highest rate of inflammatory infiltration in the ECs injection group

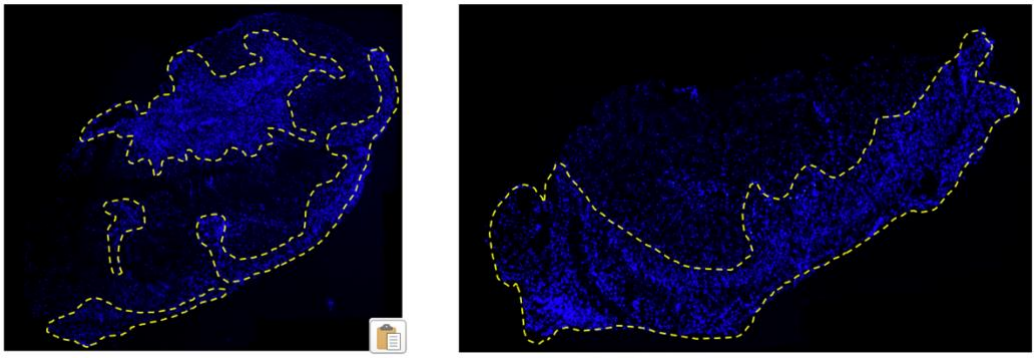


Fig 11: Areas of inflammation (Hoechst)

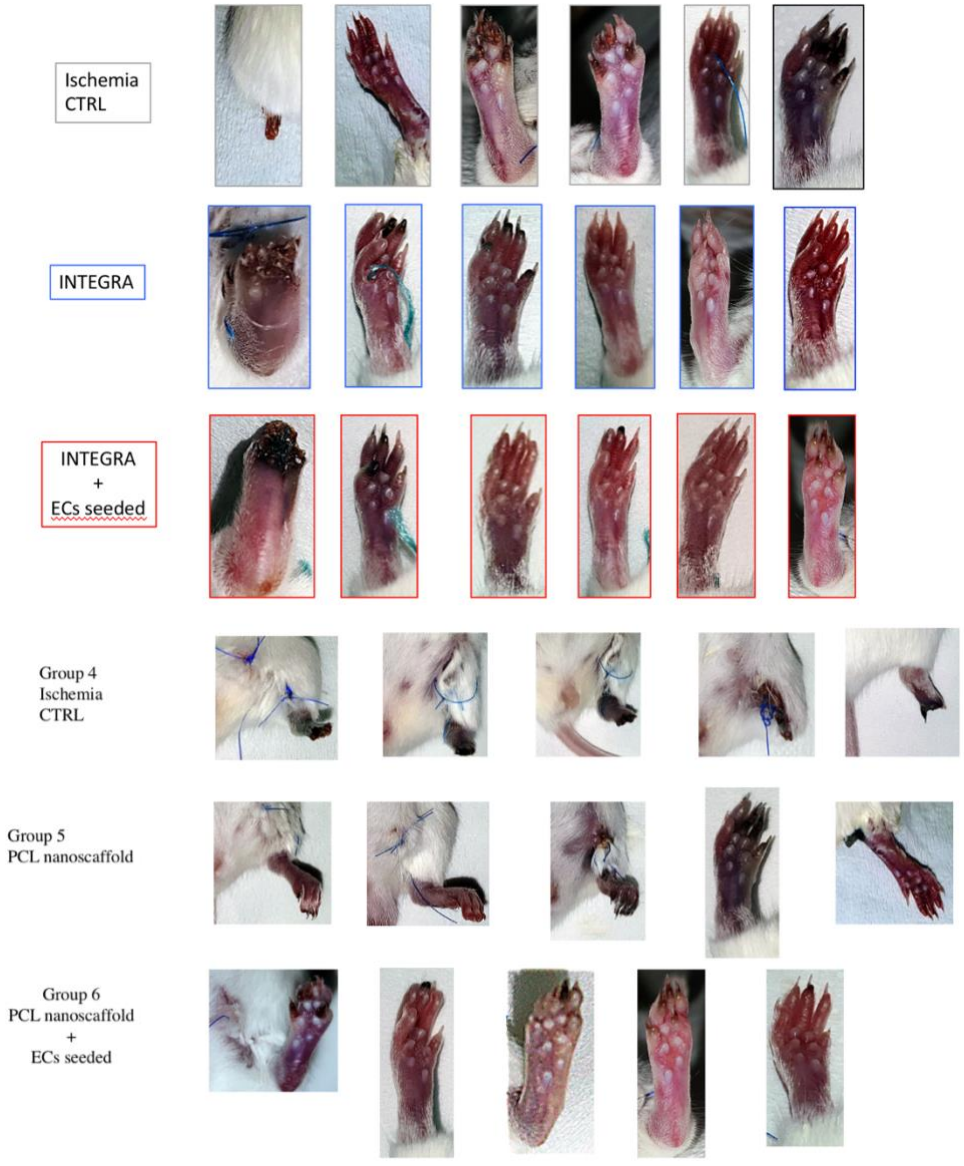


Fig 12: example of clinical presentation at 7 days

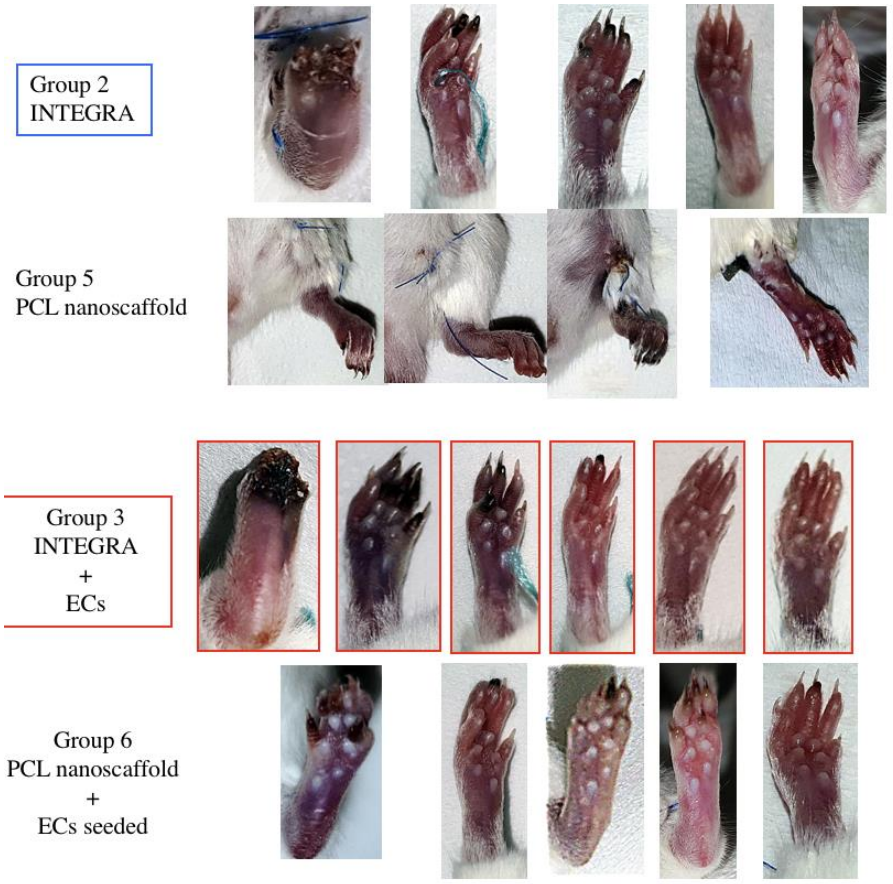


Fig 13 Comparison at 7 days

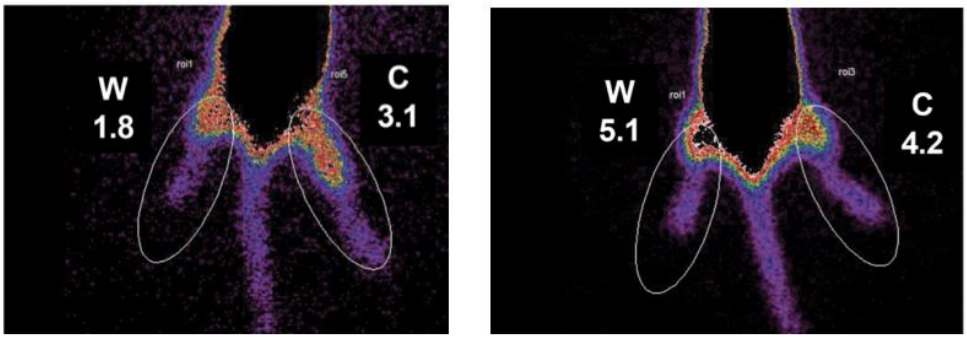


Fig 14 The perfusion of treated limbs was quantified by Single Photon Emission Tomography (SPECT)

Histological evaluation

Cross-section of quadriceps, tibialis anterior and gastrocnemius muscle of Balb/c one week after surgery was performed in all HLIM experiments.

The analysis of muscle section showed extensive necrosis, adipose substitution and marked infiltration by inflammatory cells in control animals and in HLIMB with intramuscular ECs injection.

In contrast the muscle of the HLIM treated with subcutaneous Integra® positioning and of the HLIM treated with ECs seeded on Integra® showed a reduction of the inflammatory infiltrate associated with smaller lesion area. Moreover, the number of ECs, regenerating vessel and fibers was slightly higher in animal treated with ECs seeded on Integra®. To assess the angiogenic potential of Integra® and ECs seeded on Integra®, muscles were triple labelled with Isolectin B4, antibodies against a-smooth muscle actin (a-SMA), which marks smooth muscle cells (SMCs) surrounding arterial vessels and CD31 which marks ECs and can help to evaluate the degree of angiogenesis. Histologically, numerous perfusable vessels were observed within the muscle in the ECs seeded on Integra® group and overall capillary density was increased in these groups in ischemic hindlimb muscle (Fig 15). The migration of host cells into the scaffolds was analyzed by the infiltration of host cells into scaffolds and sub-sequential migration of the ECs in the surrounding tissues.

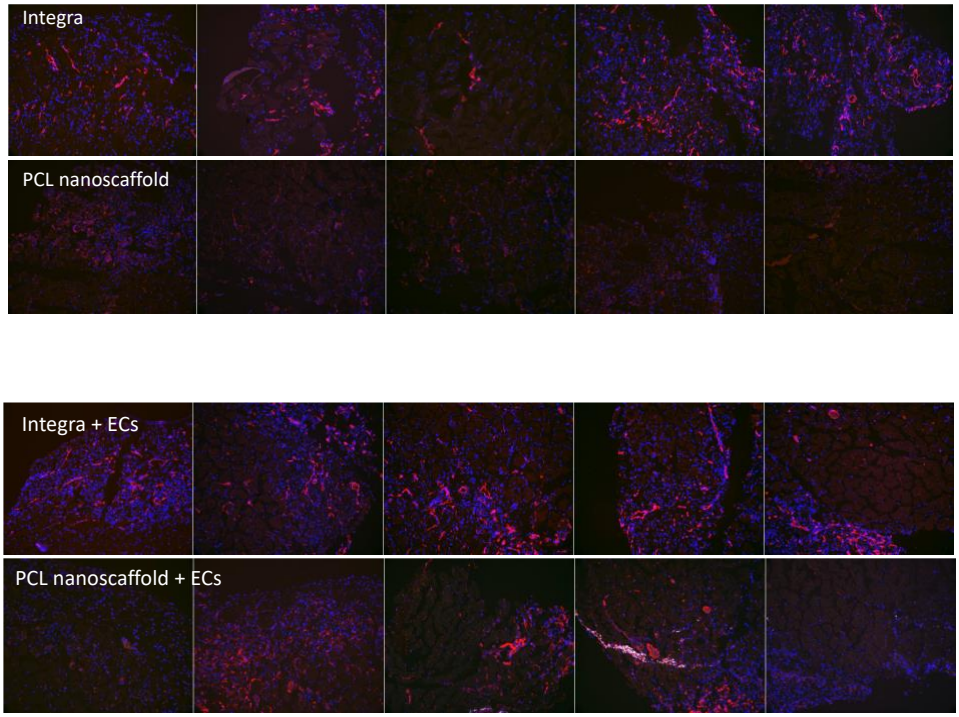


Fig 15: tibialis anterior 7 post op (CD31 and Hoechst)

The Cross-section of quadriceps, tibialis anterior and gastrocnemius muscle of Balb/c one week after surgery was performed in all HLIM experiments.

The muscle of the HLIM treated with subcutaneous PCL scaffold and ECs seeded showed a potential of vessels regeneration and muscle protection compared to the PCL scaffold alone.

The group 5 (PCL scaffold alone) showed a lower response in term of cell colonization and ischemia recovery compared to the group 2, however comparing group 3 ad group 6 (matrix and ECs previously seeded) appeared to be comparable.

Muscle were labelled as previously: Isolectin B4, antibodies against a-smooth muscle actin (a-SMA), which marks smooth muscle cells (SMCs) surrounding arterial vessels and CD31 which marks ECs and can help to evaluate the degree of angiogenesis. From

the histological point of view, the capillary density was equally increased in these groups.

Histologically, numerous perfusable vessels were observed within the muscle in the ESc seeded on Integra® group and overall capillary density was increased in these groups in ischemic hindlimb muscle.

The migration of host cells into the scaffolds was analyzed by the infiltration of host cells into scaffolds and sub-sequential migration of the ECs in the surrounding tissues.

4. DISCUSSION

Lower extremity muscle ischemia activates development of new blood vessels – capillaries and collaterals. Importantly, however, only arteries (generated by arteriogenesis), but not capillaries (generated by angiogenesis) can substitute an occluded artery. Apparently, the *de novo* formation of an artery by arterialized capillaries is a very slow and rare event and therefore its contribution to tissue perfusion after occlusion of a main artery is rather questionable. Moreover, the function of capillaries generated by angiogenesis in ischemic tissue is mostly to remove cell debris and as long as the distal feeding artery is occluded, they cannot contribute to tissue perfusion. Thus, the role of adult primary endothelial cells in post-ischemic blood flow restoration seems to be rather limited.

Animal models are indispensable tools in exploring the pathological processes underlying Critical limb Ishcemia (CLI), as well as in preclinical testing of potential therapeutics. However, lack of standardization of these experimental methods leads to variations in the severity of ischemia affecting not only VEGF expression, but also revascularization and reperfusion potential.

Apparently, detection of VEGF in the ischemic tissue is not always possible and can be time- and region-dependent. The production of this protein is stimulated by hypoxia and, at least according to some literature data, its level in ischemic muscles increases shortly after acute ischemic episode and decreases soon thereafter. Level of hypoxia, inflammatory cells infiltration, muscle necrosis, type of analyzed muscle, upregulation of VEGF inhibitors, age and associated diseases – all of these factors

may influence the measurement and should be considered while planning experiments and data interpretation.

In addition, in immunohistochemistry, CD31 is used primarily to demonstrate the presence of endothelial cells in histological tissue sections. This can help to evaluate the degree of tumor angiogenesis, which can imply a rapidly growing tumor. Malignant endothelial cells also commonly retain the antigen, so that CD31 immunohistochemistry can also be used to demonstrate both angiomas and angiosarcomas. In this work, CD31 was used as Endothelial Cells marker.

CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1) is an inhibitory coreceptor involved in regulation of T cell and B cell signaling by a dual immunoreceptor tyrosine-based inhibitory motif (ITIM) that upon associated kinases-mediated phosphorylation provide docking sites for protein-tyrosine phosphatases. CD31 is expressed ubiquitously within the vascular compartment and is located mainly at junctions between adjacent cells. N-terminal Ig-like domain of CD31 is responsible for its homophilic binding, which plays an important role in cell-cell interactions. CD31 is a multifunctional molecule with diverse roles in modulation of integrin-mediated cell adhesion, transendothelial migration, angiogenesis, apoptosis, negative regulation of immunoreceptor signaling, autoimmunity, macrophage phagocytosis, IgE-mediated anaphylaxis and thrombosis. It is one of key regulatory molecules in vascular system.

The hind limb ischemia model (HLIM) involves acute interruption of arterial supply and remains the most commonly used pre-clinical in vivo method of assessing the angiogenic and arteriogenic potential of agents and cells[47]. The HLIM has been used

extensively as an in vivo assay to gain pre-clinical mechanistic and therapeutic insights into revascularization of ischemic muscle.

The rodent is most frequently used although rabbit, porcine, canine and primate models have also been described[48,49]. The use of small animals such as rodents (in particular the mouse) has the advantage of a wide availability of transgenic strains and lower cost of experimentation, whereas larger animals, such as the pig, more closely mimic the size and haemodynamics of the human vasculature allowing easier identification of blood vessels and the ability to assess blood flow within individual vessels[50]. Although the HLIM is considered to be most clinically relevant to peripheral arterial disease, and especially Critical limb Ischemia (CLI), it does not entirely reproduce the complex human condition and there are important limitations that must be taken into consideration when interpreting results.

Ligation of the femoral artery at two points and excision of the intervening segment seems to be the most appropriate method for induction of ischemia, producing signs in the mouse that are similar to those observed in human CLI. Some investigators routinely ligate both the femoral vein and femoral artery to induce hind limb ischemia. This cannot be considered representative of human CLI since these patients do not generally present with concurrent arterial and venous occlusions. Simultaneous ligation of the femoral vein may also confound any results observed as this significantly inhibits collateral vessel formation in the mouse when compared with femoral artery ligation alone. The femoral vein is in close proximity to the femoral artery in the rodent and dissecting one away from the other can be technically challenging but most investigators who are carefully assessing the contribution of recruitment of collateral arteries do not ligate both vessels[51].

Hind limb ischemia is induced by acute ligation or excision of the artery of interest. In man, however, CLI generally occurs as a result of a chronic process associated with the build-up of atherosclerotic plaque over many years leading to arterial stenosis. Studies comparing acute induction of hind limb ischemia (by femoral artery excision) with gradual occlusion using a constrictor have found very different responses in the animal[52]. In acute ischemia blood flow recovery peaked at 84% of normal after 35 days and the gastrocnemius muscle showed significant necrosis. This form of ischemia appears to be more representative of acute limb ischemia in man because it is more severe and the pattern of the ulceration and necrosis observed is different from the tissue loss seen in human CLI, which is usually confined to the foot[53].

While some studies assume ischemic repair is essentially an inflammatory response with muscles showing damage, we and others, who have avoided significant venous trauma or neuropathy, find little evidence of changes in the muscle (macrophages, edema, centrally located nuclei etc.)[54]. It is also feasible that inflammatory cytokines such as interleukin-6 that are abundant after acute induction of ischemia may blunt the angiogenic response in an otherwise pro-angiogenic environment[55]. This has important implications for the pre-clinical testing of novel therapeutics, the majority of which are carried out using the standard method of acute femoral artery ligation.

In a nut shell, we have observed that rhVEGFa stimulates a) surviving, b) proliferation, c) tube-formation of primary adult endothelial cells in a 2D and 3D culture. In particular, the treated ECs showed a significant increase in the number of cells and tube formation compared to non-treated cells (control). The combinatorial therapy that provides for the exploitation of Integra skin substitute matrix/PCL scaffold ,

primary adult endothelial cells and VEGFa could bring significant benefits for the revascularization of necrotic and ischemic tissue using tissue engineering.

There is considerable variation in the distribution of collateral arteries in the lower limbs of healthy humans and the response of this collateral circulation following obstruction to arterial flow as a consequence of atherosclerotic disease varies between individuals[56]. This is thought to be the result of environmental and genetic factors that are as yet poorly understood. Mice appear to exhibit similar inter- and intra-strain differences in collateralization. The two most common strains of mouse used for the HLIM, BALB/c and C57BL/6, are highly inbred strains with minimal genetic heterogeneity among animals, which would suggest that experiments using the same strain are reliable and reproducible[57]. There is, however, some variability between mice within the same strain; for example, BALB/c mice demonstrated considerable inter-animal variation when stratified with regard to functionality of their pre-existing collaterals 24 h after femoral artery ligation[58] . One possible cause of variation in the endpoint measures observed and comparisons between the results of different research groups may be genetic drift, which is known to occur in highly inbred strains of mice.

The BALB/c mouse is considered the most appropriate strain in which to model CLI as it demonstrates slower recovery after induction of hind limb ischemia and is more susceptible to tissue necrosis and limb loss after arterial ligation[59]. BALB/c mice exhibit poorer collateral growth in response to femoral artery ligation, but show a greater increase in capillary density (angiogenesis) within the ischemic hind limb muscle compared with C57BL/6 mice[60].

Angiogenesis produces relatively small, thin-walled capillaries that are less robust than the collateral network that forms by arteriogenesis and are less effective in revascularizing the ischaemic hind limb[61]. On the basis of collateral reserve, BALB/c mice seem to be a more appropriate strain to use for simulating CLI, whereas C57BL/6 may be better suited as a model for intermittent claudication. Claudication is a milder sequela of PAD in man where a transient arterial insufficiency occurs in the limb only when oxygen demand is increased by walking and not at rest.

The majority of patients with Peripheral Artery Disease (PAD) and CLI are older and their regenerative capacity and dynamic response to stimuli is accordingly depressed. A 3-year-old mouse would be the equivalent of an 80-year-old human making it more appropriate to use older mice to model CLI[62]. The husbandry costs may, however, make such experiments prohibitively expensive. Mice that are aged demonstrate slower recovery rates after femoral artery ligation compared with young mice and are less likely to recover completely without therapeutic interventions. Young, adult (3-month-old) mice had a 50% better functional recovery compared with aged (18-month-old) mice 14 days after induction of hind limb ischemia[63]. Arteriogenesis appears to be diminished in the ischemic hind limb of older mice and therefore revascularization occurs predominantly through angiogenesis, which is less effective in this model. Collaterals in aged mice are less able to remodel and enlarge in response to femoral artery ligation because of a deficiency in eNOS production and increased susceptibility of their endothelial and smooth muscle cells to apoptosis [64]. In general, young, wild-type mice do not have any of the co- morbidities commonly seen in CLI patients, such as diabetes mellitus, hypertension and

hypercholesterolaemia [65]. A common sequela of these conditions is induction of inflammation and oxidative stress, leading to endothelial dysfunction[66] and impaired arteriogenesis, in part through a decrease in both flow-mediated dilatation and outward vascular remodeling [67]. Diabetes appears to impair arteriogenesis by blunting the response to shear stress, decreasing monocyte activation, and inhibiting the mobilization and integration of progenitor cells in the endothelium[68].

To date, the clinical utility of gene therapy using the VEGF gene has been reported for the treatment of critical limb ischemia and myocardial ischemia [69,70]. A novel therapeutic strategy using angiogenic growth factors to expedite and/or augment collateral artery development has recently entered the realm of treatment of ischemic diseases, although there is no pharmacological treatment for patients with critical limb ischemia. The present study raises the possibility of a newstrategy, therapeutic angiogenesis using adult primary ECs, in addition to VEGF, for the treatment of patients with critical limb ischemia.

The in vitro experiments, both 2D and 3D cultures showed a promising ability of the ECs, in presence of VEGF, to grow and form tube-like structures.

The in vivo experiments showed promising results in the HLIM group treated with Integra® and PCL scaffold and with ECs seeded regarding the clinical presentation.

Beside the physical and chemical differences between the two scaffolds, taking into consideration that the Integra matrix is the scaffold most used in reconstructive surgery worldwide, it is burdened by high costs. One 5x5 cm of Integra matrix roughly costs 3.000 euros while a 5x5 cm of PCL scaffold costed less than 100 euros. However, the less than 100 euros of the PCL does not include all the post-production costs before the commercial use.

Cells live in a multi-layered and composite native ECM, bordered by a 3D fibrillar architecture and respond with a range of biological and chemical signals that control their functions, including damaged tissue recovery. The ECM is mainly composed by nanometer structure oh well organized particles.

Investigation of electrospinning is extremely challenging due to the interaction of distinct parameters on the resulting polymeric nanofibers. The polymer jet interacts with the external electric field. Whenever a high voltage is applied to a polymer solution held together by surface tension, it generates a charge on the liquid's surface. At a critical voltage, a spherical polymer droplet deforms into a Taylor cone and forms ultrafine ENs as a current flowing from a high-voltage power source into a polymer solution through a metallic syringe needle.

Higher voltage promotes the growth of thinner (5–40 nm) fibers but it may also allow more fluid to be expelled, resulting in fibers with thicker (0.4–1.4 μm) diameters[71].

A detailed awareness of the process parameters is of paramount importance for understanding the nature of electrospinning along with the transformation of a charged polymer solution into nanofibers. The different factors (voltage, viscosity, polymer concentration, spinneret to tip distance, humidity, temperature) have a major effect on the formation of a multifunctional electrospun nanofibers [72] and a considerable impact on bead-free fibers[73].

Numerous studies shown that nanofiber polymeric scaffold are becoming fundamental for tissue engineering practice[74]. Their high surface-to-volume ratio makes them more interactive and biomimetic, enabling stronger cell-matrix interactions. In addition, electrospun nanofiber scaffolds have long-term mechanical

strength, “cell-friendly” microenvironments and the ability to capture different molecules without changing the surface characteristics.

In skin regeneration, electrospun systems have received special attention. They could represent the ideal scaffold when dealing with wound healing thanks to their biocompatibility, biodegradability, ability to simulate ECM’s morphological properties (porosity, hydrophilicity, cellular signaling, antibacterial activity, etc.)[75]. For these reasons, they could make ideal candidates for several biomedical application, such as skin tissue scaffolds, drug carriers, wound dressing materials.

Further Perspective

Currently available tissue-engineered products for skin substitution, including dermal and epidermal constructs, although not perfect, occupy a specific niche within a complex approach to treat full-thickness extensive burns, improving patients’ survival rates and their quality of life after injury. Such products target only limited specific roles in the wound-healing process. Predominantly, they serve as temporary biologically active dressings, donators of cytokines and structural molecules necessary for wound healing while the patient’s own skin regenerates to be used for serial autografting. Products based on autologous cultured keratinocytes and fibroblasts are more likely to contribute to actual skin substitution and results of clinical trials are encouraging; however, no one will agree that these products at the current level of sophistication can fully replace damaged tissues[76].

There are many challenges faced by bioengineers supplying live cell products that should also be taken into account. There are long, complicated and expensive cultivation procedures, specific (and expensive) transport and storage conditions, a

limited shelf-life, the friable nature of cell-containing biomaterials, especially for products based on live cells, a need for precise coordination between the tissue culture facility and the clinic if autologous cells are used. All the above reasons as well as an unattractive cost-effectiveness of cell-based biomaterials which are only partially effective at fulfilling skin functions make it also very difficult for any cell-based skin substitute product to reach the clinic.

The simultaneous combination of different skin cell types including keratinocytes, melanocytes, fibroblasts and endothelial cells derived from postnatal skin is aiming to create a functional skin replacement[77,78]. Attempts are being made to restore skin appendages, such as hair follicles and sebaceous glands to maximally functionalize skin replacement bioconstructs[79]. Bone marrow-derived cells have also been looked at as a potential cell source for skin-substitute products. Another approach to even further functionalize artificial scaffolds for skin substitution is the addition of signaling molecules for the regulation of cell–cell and cell– matrix interactions either to accelerate biointegration [80,81] or to adjust it according to the phases of the wound-healing process. Such 'intelligent' biomimetic hybrid materials are termed 'smart' with the aim of producing a more natural skin restoration.

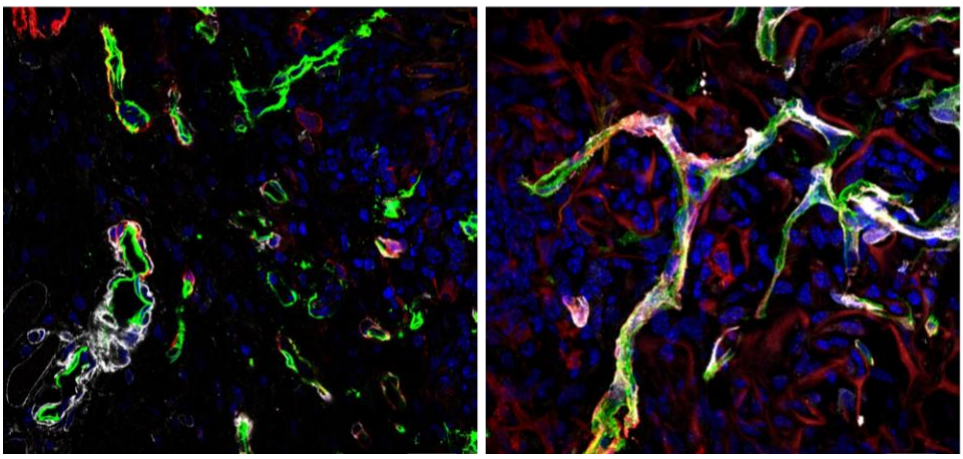
Apart from function restoration, accepted by our ancestors as appropriate results of wound healing, issues of cosmesis, improved functionality and quality of life are now of great importance during skin-restoration treatment. The inflammatory response, the production of cytokines to promote fibrovascular tissue proliferation, is considered a normal process to cope with wounds to deliver their *repair*. However, in the light of current advances in medical science, these are now more likely to hinder optimal tissue *regeneration* as they nearly always result in scarring. Any

bioengineered product based on *natural* mechanisms of wound healing will result in scarring as well as limited functionality, rather than giving fully functional skin regeneration

The restoration of the vascular system of the skin is a complex cascade of cellular, humoral and molecular events in the wound bed to reconnect to the nutritive perfusion. Initiators are growth factors, e.g. VEGF, PDGF, bFGF and the serine protease thrombin. The first step in new vessel formation is the binding of growth factors to their receptors on the endothelial cells of existing vessels, thereby activating intracellular signaling cascades. The activated endothelial cells secrete proteolytic enzymes which dissolve the basal lamina. Thus, the endothelial cells are now able to proliferate and migrate into the wound, a process also known as *sprouting*. The endothelial cells orientate themselves at superficial adhesion molecules e.g. integrins. Furthermore, they release matrix metalloproteinases at the front of proliferation, lysing the surrounding tissue for the ongoing endothelial proliferation. The newly built sprouts form small tubular canals which interconnect to others forming a vessel loop. Thereafter, the new vessels differentiate into arteries and venules and mature by a further stabilization of their vessel wall via the recruitment of pericytes and smooth muscle cells. Finally, the initial blood flow completes the angiogenic process. Within full dermal thickness wounds the neovascularization process follows a distinct pattern in time and shape. At the beginning the vessels form an inner ring of circularly arranged vessels at the wound margin followed by outer radially arranged vessels supplying the inner ones. Because the design of the vessels is similar to the sun, this has also been called *sola cutis se reficientis'*[82]. As the wound closure proceeds, the inner vascular ring shrinks, resulting

in the complete disappearance of the vessel ring. The radially arranged vessels, however, interconnect with each other in time, forming a new dermal vascular network.

Parallel to these experiments, we applied the same protocol seeding adult Adipose Cells. The results were more encouraging than with the ECs as the regenerative potential found in the SVF appeared to be even more promising in the promoting the microvascularization (Fig 16).



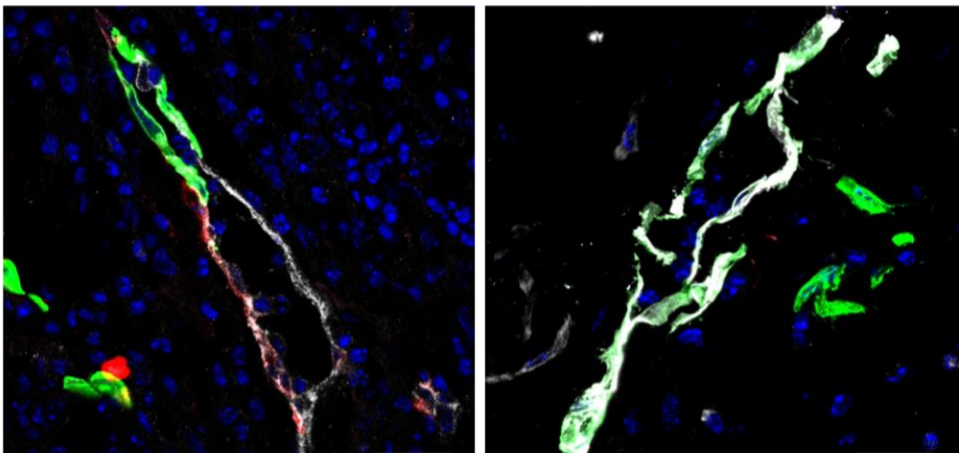


Fig 16: SVF seeded on scaffolds

5. CONCLUSIONS

The incidence of CLI is rising as risk factors in an increasingly elderly population become more prevalent. There is considerable demand for novel treatment modalities such as therapeutic neo- vascularization and consequently each new discovery that appears to hold promise is greeted with much excitement. An imperative of the translational research approach is to remain objective about the potential of each treatment before use in man. The limitations of pre-clinical assays such as the hind limb ischemia model must be recognized and addressed in an effort to discover treatments that are likely to be equally as effective at the bedside as they are at the bench. The operative technique, endpoint measures and characteristics of the mice used have not been standardized by investigators that work within this field and there is a danger that researchers from different groups may not be comparing like for like.

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