A double chamber rotating bioreactor for enhanced tubular tissue generation from human mesenchymal stem cells

"A promising tool for vascular tissue regeneration"

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Abstract:

Cardiovascular diseases represent a major global health burden, with high rates of mortality and morbidity. Autologous grafts are commonly used to replace damaged or failing blood vessels, however such approaches are hampered by the scarcity of suitable graft tissue, donor site morbidity and poor long-term stability. Tissue engineering has been investigated as a means by which exogenous vessel grafts can be produced, with varying levels of success to date, a result of mismatched mechanical properties of these vessel substitutes and inadequate ex vivo vessel tissue genesis. In this work, we describe the development of a novel multifunctional dual-phase (air/aqueous) bioreactor, designed to both rotate and perfuse small

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diameter tubular scaffolds and encourage enhanced tissue genesis throughout such scaffolds. Within this novel dynamic culture system, an elastomeric nanofibrous, microporous composite tubular scaffold, composed of Poly(caprolactone) and acrylated Poly(lactide-co-trimethylene-carbonate) and with mechanical properties approaching those of native vessels, was seeded with human mesenchymal stem cells and cultured for up to 14 days in inductive (smooth muscle) media. This scaffold/bioreactor combination provided a dynamic culture environment that enhanced (compared to static controls) scaffold colonisation, cell growth, extracellular matrix deposition, and in situ differentiation of the human mesenchymal stem cells into mature smooth muscle cells, representing a concrete step towards our goal of creating a mature ex vivo vascular tissue for implantation.

Keywords: Bioreactor, Dynamic culture, Mesenchymal stem cell, Composite scaffold, Vascular grafts

1. Introduction:

Over the last 60 years, transplantation of a wide variety of tissues and organs, re-constructive surgical techniques and replacement with artificial devices has significantly enhanced patient life expectancy and quality. Unfortunately, these solutions still suffer from many limitations, including donor shortage and lifelong immunosuppression, increased risk of infections, unwanted side effects and, in some cases, finite durability (Nasseri BA et al. 2001, Fuchs JR et al. 2001). This situation has led to an increasing interest in tissue engineering as a possible solution to develop *in-vitro* cellularized functional substitutes able to restore or improve tissue and organ function (Peloso A et al. 2015, Vacanti JP 2010, Martin I et al. 2014).

Significant progress has already been made in the field and examples of successful clinical implementation of *in vitro* tissue-engineered products include skin substitutes (Catalano E et

al. 2013, Gauvin R et al. 2013), bone grafts, cartilage, operational bladders and trachea (Atala A et al. 2006, Asnaghi MA et al. 2009, Jungebluth P et al. 2011, Fulco I et al. 2014).

Whilst this progress offers significant promise, besides appropriate preclinical models to investigate the host response (e.g., neovascularization, re-modelling) (Piola M et al. 2013a) and the behaviour of the produced substitute once grafted, more effort is still required in terms of elucidating basic mechanisms regulating cell responses to these scaffolds under dynamic versus static culture conditions, and further, the behaviour of the engineered constructs during *ex vivo* maturation (Couet F and Mantovani D 2012, Martin I et al. 2014). Moreover, it is acknowledged that each tissue target and each pathologic condition will likely require a specific approach for optimal results, and hence having a modular and flexible approach to *ex vivo* tissue engineering will be important.

Tissue engineered tubular tissues are seen as an alternative source of tissues for the replacement of small diameter damaged hollow organ structures (< 6 mm ∅), such as blood vessels and urethra (Atala A et al. 2012). There is a critical demand for the production of tissue-engineered vascular substitutes capable of meeting the functional requirements of the vessel without inducing immune or inflammatory responses, or losing function over time (Martin I et al. 2014).

The tissue engineering (TE) approach is based on the well-established triad comprising cells, scaffolds (synthetic or natural), and stimuli (Martin I et al. 2014, Wendt D et al. 2009). Several studies have highlighted the important roles of mechanical and biochemical stimulation in driving the development of various cell phenotypes from various cellular starting points in order to create a mature and functional tissue-substitute (Seifu DG et al. 2013, Tresoldi C et al. 2015, Prandi F et al. 2015, Moore M et al. 2012). Bioreactors thus have the potential to play a critical role in TE, having the ability to provide such stimulation in a dynamic but controlled manner to create the appropriate environment for tissue growth,

remodelling and maturation, prior to implantation (Asnaghi MA et al. 2009, Piola M et al. 2013b, Flanagan TC et al. 2007, Piola M et al. 2015, Uzarski JS et al. 2014).

To address this potential, numerous groups of researchers have developed dynamic culture systems suitable for applying, in a controlled manner, single or combined biomechanical stimuli to seeded scaffolds, such as shear stress, pressure, axial stretching or torsion loads (Sivarapatna A et al. 2015, Syedain ZH et al. 2011, Uzarski JS et al. 2015, Nieponice A et al. 2008). However, these devices still have their limitations, mainly associated with cumbersome handling and assembly procedures, and an inability to simultaneously produce different stimuli during culture that are required for the successful production of suitable tissue engineered grafts (Seifu DG et al. 2013, Fishman JM et al. 2014, Mantero S et al. 2007).

The present study aimed to redesign and validate an innovative multifunctional bioreactor, previously used for large hollow organs engineering (Asnaghi MA et al. 2009), for the generation of small diameter tubular tissues. The whole dynamic culture system was redesigned to suit small diameter tubular constructs and two new important features were added, namely pre-tensioning and luminal perfusion. Furthermore, as adaptability is considered nowadays a key element for tissue engineering (Morgan KY and Black LD, 3rd 2014), great importance was given to modularity and scalability during the development process, in order to allow regeneration of patient-specific graft, always considering as mandatory sterility maintenance during long term culture (Mollet BB et al. 2015).

The functionality and the benefit of the bioreactor system were assessed in the context of small calibre vessel engineering, using human mesenchymal stem cells and a promising elastomeric composite Polycaprolatcon and Poly(lactide-co-trimethylene-carbonate) (PCL/aPLA-TMC) based electrospun (Stefani I and Cooper-White JJ 2016), having first determined an optimized medium composition to induce mesenchymal stem cells (hMSCs)

differentiation into mature smooth muscle cells. Under dynamic culture within the bioreactor system for 14 days, the small diameter tubular scaffold and the novel optimized differentiation media resulted in substantial improvements in scaffold colonisation, cell growth, smooth muscle cell extracellular matrix secretion, and differentiation of hMSCs into mature smooth muscle cells (SMC) throughout the tubular scaffold as compared to static controls.

2. Materials and Methods:

2.1. hMSC 2-dimensional culture and elucidation of optimal smooth muscle cell differentiation media

hMSC were purchased from Lonza (donors: 8006, 7219, 0956). hMSC were seeded at 4000 cells/cm^2, expanded in growth media and subsequently, when needed, exposed to differentiation media for 7 or 14 days before collecting samples for analysis. Growth medium (GM) consisted of low-glucose DMEM supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL) (Gibco, Australia). Four different myogenic media were tested and consisted of GM supplemented with: 1 ng/mL TGF-β1, 30 μM ascorbic acid; 10 ng/mL TGF-β1, 30 μM ascorbic acid; 1 ng/mL TGF-β1, 30 μM ascorbic acid, 10% FBS; 10 ng/mL TGF-β1, 30 μM ascorbic acid, 10% FBS. The different media are coded as (X Y) were X is the % of FBS and Y is TGF-β1 ng/mL added to low-glucose DMEM. Media were changed every two days.

2.2. Quantitative real-time reverse transcription polymerase chain reaction

Total RNA was isolated using an RNeasy Mini Kit with on-column DNAse treatment (QIAGEN VWR, Stockholm, Sweden) according to the manufacturer's protocol. The concentration and purity of RNA was determined by using a Nano-Drop spectrophotometer

(NanoDrop Technologies, Inc., Rockland, DE. Data not shown). cDNA was synthesised from 100 ng of RNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Life Technologies) in a total volume of 21 μL, as per manufacturer's instructions. An equivalent volume of DNase and RNase-free water (Sigma) was used in place of RT Enzyme Mix for no-RT controls.

qPCR reactions were set up in triplicates with each reaction having a total volume of 10 μL containing 1X Platinum SYBR Green qPCR SuperMix-UGD (Invitrogen), 0.2 μM forward and reverse primers and 1 μL cDNA. A CFX Connect™ Real-Time PCR Detection System (Bio-rad, USA) was used at standard cycling parameters: 50 °C for 2 min, 95 °C for 2 min and then 95 °C for 15 s and 60 °C for 30 s for a total of 40 cycles. Results of qPCR were analysed using the Pfaffl model to normalise gene expression to the reference gene GAPDH (Pfaffl MW 2001). Analysed genes (Table 1) were chosen due to their previous use within literature as SMC differentiation markers (Murrell M et al. 2011, Ball SG et al. 2004, Narita Y et al. 2008, Cameron AR et al. 2011, Cameron AR et al. 2014, Tang AT et al. 2012).

2.3. Cell morphology and differentiation analysis

Cells seeded on 48 multi-well plates (triplicate samples, 1, 7 and 14 days post-seeding) were fixed in 4% paraformaldehyde (in PBS) for 20 min and rinsed with PBS before being permeabilized with a 0.1% (w/v) Triton 100-X solution for 5 min. After PBS rinsing, samples were blocked in 3% BSA in PBS solution for 1h.

The following staining solutions (in 3% BSA in PBS) were used (150 μl/well): 1/200 Anti-alpha smooth muscle Actin [1A4] antibody and 1/100 Anti-Calponin antibody [CALP] (abcam,UK) overnight (4°C) followed by 1/500 AlexaFluor 568 (Life Technologies) and 1/400 AlexaFluor 633 (Life Technologies) 60 min (Room Temperature, RT); finally 1/200

Phalloidin 488 (actin staining)(Life Technologies, Australia) 30 min (RT) and 1/1000 Hoechst 33342 (nucleus staining, Life Technologies, Australia) 5 min (RT).

Wells were then rinsed in PBS three times and left immersed at 4° C until imaging. High-resolution images were obtained using an LSM Zeiss 710 confocal microscope.

Pictures of cell populations for chosen treatment conditions (more effective myogenic medium and control) were processed with a self-made method implemented with cell-image analysis open source software *CellProfiler* (Broad Institute, USA) (Carpenter AE et al. 2006) to identify, count and measure individual cells. The method recognized nuclei first, in order to generate a cytological profile, containing a collection of measurements of features of each cell, like fluorescence intensity for all the different channels.

CellProfiler Analyst system then presented individual cells for classification, sampled randomly from the screen-wide population (Jones TR et al. 2009). After fifty cells were classified by the operator as α-SMA and Calponin positive/negative, the iterative machine-learning phase, in which the computer generates a tentative rule based on the classified cells and presents the researcher with cells classified according to that rule, began. After correcting classification errors and retraining for several rounds, the automatic classification became more accurate. When the accuracy was considered sufficient, the trained method was used to classify all cells in the experiment in order to calculate the number of double positive cells in each sample.

2.4. PCL/aPLA-co-TMC scaffold

A previously developed PCL/aPLA-co-TMC tubular electrospun porous scaffold (SF- 1) was chosen due to the promising chemical and mechanical feature for vascular tissue engineering. The three-dimensional (3D) support is composed of fibers with diameter □= 2.0 ± 0.6 μm, presenting a spontaneous in-process phase separation: the inner core is formed from a stiffer

material (PCL), and the outer layer is crosslinked aPLA-co-TMC. The structure of the scaffold is porous, but tightly packed (pore size = 12.5 ± 0.8 μm). The outer surface presents axially aligned structures (SF-1D) (Stefani I and Cooper-White JJ 2016). Five centimetre long scaffolds with internal diameter of 4 mm and a wall thickness of 0.75 mm were sterilized with 70% ethanol wash and rinsed in 1% antibiotic-antimycotic (Gibco, Thermo Fisher Scientific Inc, USA) solution in PBS. Before cell seeding, scaffolds were kept in GM without FBS overnight.

2.5. Bioreactor Design

The bioreactor design and development focused on achieving the following criteria for the system: 1) simple cell seeding procedures on both sides of a tubular matrix, to permit a homogeneous cell distribution; 2) the ability to culture different cell types on either side of the tubular support, with the possibility to supply different media; 3) the ability to enhance oxygenation of the culture medium and mass transport (oxygen, nutrients and catabolites) between media and adhering cells; 4) the ability to perfuse the internal lumen, and connect the bioreactor to different perfusion systems; 5) the ability to introduce scaffold tensioning in order to assure a pervious lumen; 6) the achievement and maintenance of sterility and other criteria of Good Laboratory Practice (GLP).

In order to ensure applicability of the device for different purposes (i.e., different scaffold sizes (diameter up to 1 cm and length up to 10 cm) and cell types), the major design principles were high versatility and modularity of the whole system. Furthermore, two perfusion systems were developed to bench-test the device under laminar and pulsatile (P= 120-80 mmHg) flows.

2.6. Bioreactor dynamic culture

After sterilization (autoclave), the culture chamber was assembled in a biosafety cabinet. The sterilized scaffold was positioned on the developed shafts (see Figure 2A). Approximately 2 to 3 mm linear motion of the pre-tensioning system was required, depending on the experiment, to maintain the used scaffold straight and pervious. The electrospun scaffold was not visibly modified: no dimensional change was visible; no modification in porosity or fiber alignment is expected. hMSCs (Donor 9056, Lonza – Passage 5) were harvested from culture flasks; a cell density of $2x10^6$ cells/cm was calculated, considering 4 cm of scaffold as 'seedable' (0.5 cm each side were used to block the scaffold with the developed pinches). The entire cell pellet was re-suspended in 320 µL of GM, and 10 µL drops were pipetted on the external surface of the tube positioned in the bioreactor, slowly rotating the scaffolds during the seeding phase (Figure 1).

The bioreactors were then placed in the incubator (37°C, 5% CO2) and left for 3.5 h, setting a rotation speed of 2 rpm (Arrigoni C et al. 2008), to encourage more even cell distributions. The static controls (1 cm each), positioned in an ultra-low attachment 24 well-plate, were seeded, under sterile conditions, rotating the matrix of 90° every 30 min. After 3.5 h, the bioreactors were stopped and filled with myogenic media (scaffold submerged), and left in the controlled environment of the incubator overnight. At the same time myogenic media was also applied to the static controls. The following day, 15 ml of media was removed from the main chambers and the bioreactors were turned on with a speed of 3 rpm exposing the scaffold to double-phase (Zeisberg EM et al.) (Figure 1). Optimal myogenic media was changed every two days. After 14 days of culture, scaffolds were harvested and samples were collected for SEM analysis and Histology imaging.

2.7. Histological and scanning electron microscopy analysis

After 14 days, samples cultured in the bioreactor and the static controls were processed for histological analysis and SEM observation. Briefly, they were fixed with 4% PFA for 6h at 4°C, left overnight in a 30% sucrose (w/v) solution, then immersed for 12 h in OCT embedding media and finally frozen in fresh OCT. Samples were stored at -80°C prior to cutting. Sections (10µm thick) of cultured tubes were collected on SuperFrost Plus slides (Thermo Scientific), washed with PBS, rinsed with MilliQ water, and left to dry overnight. Lillie's allochrome stain was performed and the slides scanned with an Aperio AT slide-scanner (Leica Biosystems, Australia) to analyse the cell distribution and extracellular matrix (ECM) deposition in the tube cross-sections. Lillie's allochrome method was adapted from *Culling* (Culling CFA 1974).

As for immunofluorescence staining, a 0.05% Trypsin solution in 0.1% Calcium chloride was used to unmask the antigens and epitopes for protein staining (20 min, RT). After PBS rinsing, sections were blocked in 3% BSA in PBS solution for 1h. The following staining solutions (in 3% BSA in PBS) were used: 1/400 Anti-collagen IV antibody, 1/300 Anti-fibronectin antibody, 1/300 Anti-laminin antibody, 1/400 Anti-collagen I antibody (abcam,UK) 60min (RT) followed by 1/500 AlexaFluor 488 (Life Technologies60 min (Room Temperature, RT); finally fluorescence mounting media was used to preserve the sections. Sides were analysed using a LSM Zeiss 710 confocal microscope.

Furthermore, samples were analysed using a scanning electron microscopy Stereoscan 360 (Cambridge Instruments, UK). Before analysis, the samples were dehydrated, with an increasing ethanol scale, and coated with 20 nm layer of gold (SC7620, Quorum Technologies, UK).

3. Results:

3.1. Differentiated mesenchymal stem cells gene expression (qPCR) and CellProfiler Analysis

The cells cultured with TGF-β1 increased the gene expression level of early SMC-specific markers in a dose dependent manner (9056 Donor Figure 3; other donors SF- 2), consistently with previous publications [27, 28]. A significant increase in gene expression from an increase in TGF-β1 from 1 ng/ml to 10 ng/ml was only seen across all the three donors for Calponin F (CNN1) and Collagen IV. Throughout the time course, Desmin (DES) and SM1 expression levels remained similar to day 1; DES is an early marker of a SMC phenotype, whilst SM1 is expressed later in more mature SMCs [14]. It is therefore probable that at day 7 and 14 the cells that were analysed were in an intermediate stage, past the high DES expression, but still not in a final differentiation stage, expressing SM1. The up-regulation trend is still visible at day 14 of exposure.

Whilst there was an alteration in gene expression profiles between the conditions of 10% vs 20% FBS, this was not considered significant enough to justify the increase of concentration of FBS for further experimental studies; with 10% FBS selected due to an improved cost to performance ratio, whilst reducing potential confounding elements from such an undefined serum. The following media was thus chosen as our optimal media for all work using the bioreactor: low-glucose DMEM supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL), plus 10 ng/mL TGF-β1 and 30 μM ascorbic acid.

Further investigations were carried out to quantify the efficiency of differentiation along the time course with the chosen medium. *CellProfiler* was utilized to analyse 20 images (examples reported in Figure 4), coming from each tested donors, for each time point. At day 7, 48% ± 10 positive cells were detected, while at day 14 the number increased to 90% ± 4 positive cells, the control didn't show any double positive staining. Confocal imaging also allowed to study cell morphology: mesenchymal stem cells gradually acquired an elongated

shape, reaching a smooth muscle-like morphology, after 14 days of myogenic media culture in the optimal media.

3.2. Bioreactor final design and bench validation

A rectangular based bioreactor structure (40x190x45 mm) was manufactured, out of polycarbonate (PC), with access from the top, to allow easy insertion and removal of the scaffold construct and facilitating cell seeding of the external surface. The lid design mimicked a Petri dish configuration to permit gas exchange, while preserving sterility of the culture environment. Two orifices for the insertion of the shafts were created on two of the chamber walls, in order to define the structure that allows rotational motion. Viton® dynamic seals (highlighted in green in Figure 2/A), positioned into slits properly manufactured on chamber orifices, guaranteed isolation and sterility of the culture milieu in correspondence of critical interfaces.

The tubular scaffold is positioned on two Polyether ether ketone (PEEK) supports, developed to securely connect with the rotating shafts, in an easy and reliable way; inside the supports, two O-rings prevent medium infiltration. Along the axis of the shafts, a 2 mm wide conduit was made, to allow perfusion of the inner lumen by connecting it to a hydraulic circuit; the choice of two rotating shafts instead of a single chuck maximizes the exposure of the scaffold-lumen of small calibre constructs to medium flow, during perfusion. Connection between chamber and actuator were designed to allow easy positioning and extraction of the device from the incubator. The bioreactor rendering and prototype are shown in Figure 2. Thanks to the modularity of shafts and scaffold-supports, together with their scalability, it is possible to host scaffolds of different sizes (both length and diameter) by simply changing the scaffold supports. The pre-tensioning system (Figure 2/A-2) allows extension of the scaffold, after securing in the culture chamber, to avoid any geometrical change (due to bending) of the scaffold, maintaining its lumen pervious. The pre-tensioning mechanism allows for a

maximum increase in length of 10mm. All the described components can be autoclaved in order to reduce contamination risks. All the described components can be autoclaved in order to reduce contamination risks.

Rotation of the scaffold is provided by a DC brushless motor AXHM230KC-GFH (Oriental Motor Ltd., UK,), with a fine speed regulation (AXHD30K, Brushless DC Speed Controller, Oriental Motor) even at low revolutions per minute, and ensuring reliability inside the incubator.

All prototypes were manufactured by Zerotre Meccanica Srl (Costa di Mezzate, Italy) using in-house technologies, a parallel lathe (OPTI D 240x500 G-Vario, Optimum, Bamberg, Germany) and a numerical control machine (Roland MDX-40, Roland DG Mid Europe s.r.l, Peschiera Borromeo, Italy).

A custom-made hydraulic circuit was developed to supply pulsatile pressure stimuli in the physiological range of 80 - 130 mmHg, as we wanted to test critical points like the connection between shafts and supports and the luer-lock joints for high-pressure resistance. The system was assembled and left in working condition (rotation and pulsatile perfusion) for 7 days and connection stability assessed for absence of leakages within perfusion circuit and main chamber and within bioreactor and the external environment. The validity of the pulsatile system was ensured using two pressure transducers PX600F (Edwards Lifescience LLC, USA) connected in line with the bioreactor shafts.

3.3. Bioreactor culture outcomes

Scaffolds from Static controls (24 well-plate) and dynamic experiments (bioreactor) were first analysed with SEM imaging (Figure 5). The external surfaces of each scaffold were scanned to visualize differences in extracellular matrix production and distribution. Differences at 14 days of culture are clear. Bioreactor samples showed complete coverage of

the scaffold outer surface with significant production of ECM. It was possible to observe the underlying morphology of the fibers, but the surface was homogenously covered. The static cultured scaffolds, on the other hand, presented covered zones as well as matrix free areas, typical of the cell-suspension drop seeding technique. The comparison allowed us to prove that the rotation conditioning, starting from the seeding time point, in the bioreactor, during the adhesion timeframe for the MSCs, permitted improved cell seeding and distribution and hence a more favourable starting point for more even matrix production and distribution throughout the scaffold (Wendt D et al. 2006), whilst in the controls cells were not exposed to the whole scaffold outer surface and settled to a large extent where they were initially seeded. The production of ECM was also lower in the static condition, confirming previous reports suggesting dynamic culture promote protein production and deposition, and cell proliferation (Scotti C et al. 2012, Au P et al. 2008, Wendt D et al. 2009).

Histological analysis using Lillie's Allochrome (Figure 6), a connective tissue stain, permitted further analysis of the developed tissue structure, as it clearly distinguishes between nuclei (black), collagen (blue), muscle reticulin (reddish purple) and fibrin (red to pink), highlighting differences in cell and protein distributions across the two conditions tested. Circumferential and axial sections of the bioreactor cultured samples gave important information showing how cells were colonizing more than half of the scaffold thickness. Matrix deposition was relevant at the outer surface and it was possible to see, at the highest magnification (20X), tissue developing around fibers. The contrast between the bioreactor samples (Figure 6 C-L) with the static control (Figure 6 A-B) was clear: only an outer and non-continuous layer of cells was present in the static control, probably due to the highly packed structure (pore size $\leq$ 30μm) of the matrix, which does not facilitate spontaneous cell migration in the absence of intra-mural flow - further encouraged by the air-aqueous rotating interface in the bioreactor. Static cultured scaffolds contained considerably fewer cells,

mainly producing collagen, whereas a complete network of proteins, with collagen, reticulin and fibrin, was present in the samples cultured in the bioreactor. A clear homogenous distribution of cells and ECM could be seen on the outer half of the tubular scaffold. It was possible to recognize differently stained structures also in the inner part of the scaffold thickness, confirming that nutrient and growth factors could reach the inner zone of the structure easily with the rotation stimuli.

4. Discussion:

Vascular tissue engineering aims to develop a vascular construct that demonstrates biological and mechanical properties as close as possible to those of a native vessel (Couet F and Mantovani D 2012), with many different approaches having already been reported in literature. More than 40 reviews on this topic, plus countless primary research articles, were written in 2014, and given that cardiovascular diseases account for most non-communicable diseases deaths (17.3 million people annually) (Lim Ss VT et al. 2012), the need of a flexible, functional and patient-specific solution is clear.

Bioreactors are a useful tool in regenerative medicine, building a suitable environment for functional tissue growth and maturation. These devices can provide a standardized and reproducible range of stimuli, confined in a safe sterile environment, presenting a possible regulatory-compliant approach to reach the desired clinical substitute (Couet F. MD 2012, Autors V 2013). Many vascular-bioreactors have been reported in literature, mainly due to the necessity to discover an alternative to small-diameter synthetic prostheses that currently lead to the development of anastomotic intimal hyperplasia, coagulation and thrombogenesis (Graham LM et al. 1989, Pasic M et al. 1995).

Niklason et al. developed and patented, in 1999, a bioreactor applying cyclic pulsatile strain. The device, connected to a closed flow system equipped with a peristaltic pump, provide

cyclic pulsatile strain to cell-seeded scaffolds. The main idea was to reproduce the cyclic circumferential stretching imposed on native vessels by the heart (Niklason LE et al. 1999). The device allowed them to create a decellularized scaffold starting from a polyglycolic acid tubular mesh seeded with porcine smooth muscle cells and cultured for 10 weeks in a perfusion bioreactor system. The constructs were then decellularized, leaving behind the mechanically robust extracellular matrix of the graft wall. After seeding endothelial progenitor cells on the lumen, the graft was then implanted (Quint C et al. 2011), with promising results, but it is important to underline how 10 weeks of culture plus decellularizing and re-seeding significantly enhance the probability of bacterial or fungal contamination.

Mironov et al. thereafter suggested that longitudinal strain is a necessary stimulus to produce functional grafts. Their bioreactor included two media perfusion systems: one for the luminal side of the tubular construct and the other for the watertight main chamber. The device allowed for a manually-invoked change of longitudinal strain of the construct during culture, from 0% to 200%. The prototype was also equipped with pressure transducer and a digital TV camera, to analyse culture evolution. The bioreactor was tested using a native bovine carotid artery as a tubular construct, but never exploited for graft culture and tissue regeneration (Mironov V et al. 2003).

Bilodeau et al. describe a bioreactor that allows for the possibility of applying an oscillatory/constant strain utilizing a pneumatic cylinder controlled by a microcontroller, reproducing torsion generally induced by heart beating. The internal and external surfaces of the seeded scaffold are in contact with culture medium to feed seeded cells. Two perfusion systems were then developed to avoid contamination between different cell types (Bilodeau K et al. 2005). Although the system was considered of great interest, no biological results were published.

Finally, *Tranquillo et al*. developed a transmural flow bioreactor allowing for different flow configurations and for measurement of changes in transmural dissolved oxygen concentration. It consisted of a glass external housing with a polyetheretherketone internal housing. Culture medium, directed through the internal lumen of a scaffold, was supplied by syringe pumps. The device allowed for the introduction of transmural perfusion, lumen perfusion and a combination of the two. SMCs culture was performed, enhancing production of extracellular matrix (Bjork JW and Tranquillo RT 2009). The system allowed seeding of two different cell types, as the structure allowed one to connect two different hydraulic circuits. However, perfusion and transmural flow are the only two stimuli exploited, limiting the potential of this device.

Taking into account these published results we report in this paper the development of a device that could reproduce the most promising stimuli for vascular tissue regeneration (perfusion, rotation, tensioning) never presented before in literature in a single bioreactor. Considering modularity and scalability as one of the main goals, the produced prototype can house scaffolds with a variable length from 5 to 10 cm (just by changing one of the shafts) and internal diameter varying from 3 to 10 mm (just by producing a set of supports). This modular design permits the application of the bioreactor to constructs of varied dimensions as required, depending on the targeted tissue structure and function. Furthermore, we focused our design on easy assembly procedures after sterilization and ease of handle.

Taking into consideration the current state of art in vascular TE practices as reported in the literature (Pashneh-Tala S et al. 2015), we believe that bone marrow derived hMSCs show potential as a practical cell source (in particular for SMCs) for a less invasive way of obtaining an appropriate number of cells prior to implantation. Little is known about the mechanisms causing the differentiation of hMSCs into SMCs (Narita Y et al. 2008, Wang D et al. 2004, Gong Z et al. 2009). TGF-β_1 is recognized as fundamental signalling protein,

playing important roles in many aspects of embryonic development and adult homeostasis (Wu MY and Hill CS 2009). This factor has different effects on cells, mainly depending on the concentration of TGF-β_1, the environment, or cellular phase. TGF-β_1 has been shown to up-regulate some smooth muscle markers in hMSCs (Narita Y et al. 2008, Wang D et al. 2004) and embryonic cells (Sinha S et al. 2004). We thus decided to investigate four media combinations using low glucose DMEM and 30μM Ascorbic Acid with two different concentrations of FBS (10/20 %), and two concentrations of TGF-β_1 (1/10 ng/mL) across three different MSC donors. Higher FBS was tested as SMC primary cultures are usually performed with 20% of serum (Arrigoni C et al. 2008). Real Time-PCR analysis was performed and whilst different magnitudes of gene up-regulation across the three hMSC different donors investigated were observed, they all displayed similar trends. Late differentiation markers were still low at day 14, while early differentiation genes were activated. Calponin was significantly up-regulated in comparison to other genes only in cultures exposed to 10ng/mL of TGF-β_1, guiding the decision to choose the 10 ng/mL + 10 % FBS media combination. Since no significant differences were noticed with the higher FBS concentration, the FBS concentration was kept at 10%. Our results are in line with previous works demonstrating the important of TGF-β_1 in mesenchymal stem cells differentiation into smooth muscle cells lineage (Narita Y et al. 2008, Gong Z et al. 2009).

Further analyses were conducted using confocal imaging to quantitate the amount of cells undergoing differentiation at day 7 and 14 and to study changes in cell morphology. After building a method on CellProfiler, images were processed (as described in Methods) with results indicating that at day 14 in the (10 10) media, 90% of cells were positive for a smooth muscle cell phenotype ([α-SMA+ calponin positive/phalloidin positive] *100). Cells also appeared elongated with less randomly (in terms of direction) attached filopodia, a phenotype characteristic of SMCs (Patel S et al. 2000, Galvin DJ et al. 2002).

The experiments that utilised our novel bioreactor produced outstanding results, compared to the static culture: SEM analysis showed a non-homogenous distribution of cells on static culture, due to drop seeding, with cells seemingly proliferating where they were positioned without migrating consistently across/throughout the scaffold surface; on the other hand, scaffolds cultured in the multifunctional bioreactor presented a continuous layer of extracellular matrix that completely incorporated and covered scaffold fibres. Histological analysis of these two culture methods (bioreactor versus static) confirmed clear differences between static and dynamic culture: scaffold colonization across a significant fraction of the scaffold thickness was consistent within the bioreactor environment, while cells survived only on the outer surface in the static condition. Moreover, the stimuli given by rotation and exposure to the air-aqueous phases appeared to facilitate cell migration into a scaffold with small pore-size like the one produced.

Matrix deposition was also enhanced in the case of the bioreactor dynamic culture, as shown from Lillie's allochrome staining; different proteins were deposited by differentiating MSCs. Collagen, muscle reticulin, and fibrin were all detected. Proteins were arranged in a complex network, incorporating the scaffold structure. Nuclei staining demonstrated cell infiltration exceeding half way through the scaffold thickness. In stark contrast, matrix deposition in the static condition was minimal and uneven, and cell penetration minimal, underlying the importance of a dynamic culture environment. After *only* two weeks of culture in our bioreactor, the obtained amount of matrix is comparable with most tissue engineered vessels reported in literature, but in these cases, 6 to 10 weeks of cell culture was needed (Quint C et al. 2011, L'heureux N 2012, L'heureux N et al. 2006, Syedain ZH et al. 2011).

This work using the described bioreactor focused on vascular tissue engineering, however it is clear that the approach detailed herein could be applied for all tubular organs that have a SMC muscularis and an epithelium or endothelium covering the internal lumen.

Whilst the obtained results are promising, further analysis is now required on the impacts of scaffold rotation and pulsatile perfusion rates, along with simultaneous cell seeding on both the outer surface and in the internal lumen, in order to comprehensively examine the full functionality of the bioreactor in terms of creating a constitutionally-correct (in terms of both cell types and ECM) vessel. We believe that the coexistence and right combination of these two stimuli will lead to further increases in matrix deposition, giving rise to a mature muscular layer, facilitating cell differentiation and tissue cohesion.

5. Conclusions:

In this paper, we have presented an innovative bioreactor specifically designed for the regeneration of small diameter tubular structures, allowing rotation and perfusion of the entire lumen of cylindrical grafts. With particular focus on small diameter vascular regeneration, we tested the developed device in combination with a previously developed PCL/a-PLA-co-TMC electrospun tubular matrix, proving promising mechanical properties and excellent biocompatibility. Human mesenchymal stem cells were chosen to prove the bioreactor's ability to create a suitable environment for *in vitro* tissue generation, as we strongly believe that hMSC are a valuable source of cells for our purpose as they can be differentiated in SMC and, in a future prospective, they are easily harvestable form patients. The combination of our novel polymeric matrix and dynamic culture system created an environment that promoted hMSC growth and differentiation into applicable cell types to generate a functional vessel, result confirmed by histological staining showing a considerable amount of the targeted extracellular matrix produced. In conclusion, we have developed an efficient, versatile and valuable tool that in synergy with the produced scaffold is a concrete step towards our goal of creating a mature tunica media for vascular tissue engineered constructs.

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References

Arrigoni C, Chitto A, Mantero S & Remuzzi A. 2008, Rotating versus perfusion bioreactor for the culture of engineered vascular constructs based on hyaluronic acid. *Biotechnol Bioeng,* 100: 988-97.

Asnaghi MA, Jungebluth P, Raimondi MT, Dickinson SC, Rees LE, Go T, Cogan TA, Dodson A, Parnigotto PP, Hollander AP, Birchall MA, Conconi MT, Macchiarini P & Mantero S. 2009, A double-chamber rotating bioreactor for the development of tissue-engineered hollow organs: from concept to clinical trial. *Biomaterials,* 30: 5260-9.

Atala A, Bauer SB, Soker S, Yoo JJ & Retik AB. 2006, Tissue-engineered autologous bladders for patients needing cystoplasty. *Lancet,* 367: 1241-6.

Atala A, Kasper FK & Mikos AG. 2012, Engineering complex tissues. *Sci Transl Med,* 4: 160rv12.

Au P, Tam J, Fukumura D & Jain RK. 2008, Bone marrow–derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood,* 111.

Autors V. 2013. The Xi'an papers. In *World Summit on Regenerative Medicine*, 72. Xi'an, China.

Ball SG, Shuttleworth AC & Kielty CM. 2004, Direct cell contact influences bone marrow mesenchymal stem cell fate. *Int J Biochem Cell Biol,* 36: 714-27.

Bilodeau K, Couet F, Boccafoschi F & Mantovani D. 2005, Design of a perfusion bioreactor specific to the regeneration of vascular tissues under mechanical stresses. *Artificial Organs,* 29: 906-912.

Bjork JW & Tranquillo RT. 2009, Transmural flow bioreactor for vascular tissue engineering. *Biotechnol Bioeng,* 104: 1197-206.

Cameron AR, Frith JE & Cooper-White JJ. 2011, The influence of substrate creep on mesenchymal stem cell behaviour and phenotype. *Biomaterials,* 32: 5979-93.

Cameron AR, Frith JE, Gomez GA, Yap AS & Cooper-White JJ. 2014, The effect of time-dependent deformation of viscoelastic hydrogels on myogenic induction and Rac1 activity in mesenchymal stem cells. *Biomaterials,* 35: 1857-68.

Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P & Sabatini DM. 2006, CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol,* 7: R100.

Catalano E, Cochis A, Varoni E, Rimondini L & Azzimonti B. 2013, Tissue-engineered skin substitutes: an overview. *Journal of Artificial Organs,* 16: 397-403.

Couet F & Mantovani D. 2012, Perspectives on the advanced control of bioreactors for functional vascular tissue engineering in vitro. *Expert Review of Medical Devices,* 9: 233-239.

Couet F. MD. 2012, Perspectives on the advanced control of bioreactors for functional vascular tissue engineering in vitro. *Expert Rev. Med. Devices,* 9(3): 6.

Culling CFA. 1974. *Handbook of Histopathological and Histochemical Techniques: Including Museum Techniques*. Butterworth-Heinemann.

Fishman JM, Wiles K, Lowdell MW, De Coppi P, Elliott MJ, Atala A & Birchall MA. 2014, Airway tissue engineering: an update. *Expert Opin. Biol. Ther. ,* 14: 1477-1491.

Flanagan TC, Cornelissen C, Koch S, Tschoeke B, Sachweh JS, Schmitz-Rode T & Jockenhoevel S. 2007, The in vitro development of autologous fibrin-based tissue-engineered heart valves through optimised dynamic conditioning. *Biomaterials,* 28: 3388-3397.

Fuchs JR, Nasseri BA & Vacanti JP. 2001, Tissue engineering: A 21st century solution to surgical reconstruction. *Annals of Thoracic Surgery,* 72: 577-591.

Fulco I, Miot S, Haug MD, Barbero A, Wixmerten A, Feliciano S, Wolf F, Jundt G, Marsano A, Farhadi J, Heberer M, Jakob M, Schaefer DJ & Martin I. 2014, Engineered autologous cartilage tissue for nasal reconstruction after tumour resection: an observational first-in-human trial. *Lancet,* 384: 337-46.

Galvin DJ, Watson RW, Gillespie JI, Brady H & Fitzpatrick JM. 2002, Mechanical stretch regulates cell survival in human bladder smooth muscle cells in vitro. *Am J Physiol Renal Physiol,* 283: F1192-9.

Gauvin R, Larouche D, Marcoux H, Guignard R, Auger FA & Germain L. 2013, Minimal contraction for tissue-engineered skin substitutes when matured at the air-liquid interface. *Journal of Tissue Engineering and Regenerative Medicine,* 7: 452-460.

Gong Z, Calkins G, Cheng EC, Krause D & Niklason LE. 2009, Influence of Culture Medium on Smooth Muscle Cell Differentiation from Human Bone Marrow-Derived Mesenchymal Stem Cells. *Tissue Engineering Part A,* 15: 319-330.

Graham LM, Brothers TE, Darvishian D, Harrell KA, Vincent CK, Burkel WE & Stanley JC. 1989, Effects of thromboxane synthetase inhibition on patency and anastomotic hyperplasia of vascular grafts. *J Surg Res,* 46: 611-5.

Jones TR, Carpenter AE, Lamprecht MR, Moffat J, Silver SJ, Grenier JK, Castoreno AB, Eggert US, Root DE, Golland P & Sabatini DM. 2009, Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc Natl Acad Sci U S A,* 106: 1826-31.

Jungebluth P, Alici E, Baiguera S, Le Blanc K, Blomberg P, Bozóky B, Crowley C, Einarsson O, Grinnemo K-H, Gudbjartsson T, Le Guyader S, Henriksson G, Hermanson O, Juto JE, Leidner B, Lilja T, Liska J, Luedde T, Lundin V, Moll G, Nilsson B, Roderburg C, Strömblad S, Sutlu T, Teixeira AI, Watz E, Seifalian A & Macchiarini P. 2011, Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a proof-of-concept study. *The Lancet,* 378: 1997-2004.

L'heureux N. 2012, Developing a completely biological tissue-engineered blood vessel for clinical use. *Faseb Journal,* 26.

L'heureux N, Dusserre N, Konig G, Victor B, Keire P, Wight TN, Chronos NA, Kyles AE, Gregory CR, Hoyt G, Robbins RC & Mcallister TN. 2006, Human tissue-engineered blood vessels for adult arterial revascularization. *Nat Med,* 12: 361-5.

Lim Ss VT, Flaxman Ad, Danaei G, Shibuya K & Al. A-RHE. 2012, A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet,* 380: 2224-2260.

Mantero S, Sadr N, Riboldi SA, Lorenzoni S & Montevecchi FM. 2007, A new electro-mechanical bioreactor for soft tissue engineering. *Journal of Applied Biomaterials & Biomechanics,* 5: 107-116.

Martin I, Simmons PS & Williams DF. 2014, Manufacturing Challenges in Regenerative Medicine. *Sci. Transl. Med,* 6: 3.

Mironov V, Kasyanov V, Mcallister K, Ovliver S, Sistino J & Markwald R. 2003, Perfusion Bioreactor for Vascular Tissue Engineering with Capacities for Longitudinal Stretch. *The Journal of craniofacial surgery,* 14: 340-47.

Mollet BB, Bogaerts ILJ, Van Almen GC & Dankers PYW. 2015, A bioartificial environment for kidney epithelial cells based on a supramolecular polymer basement membrane mimic and an organotypical culture system. *Journal of Tissue Engineering and Regenerative Medicine*.

Moore M, Sarntinoranont M & Mcfetridge P. 2012, Mass transfer trends occurring in engineered ex vivo tissue scaffolds. *Journal of Biomedical Materials Research Part A,* 100A: 2194-2203.

Morgan KY & Black LD, 3rd. 2014, Investigation into the effects of varying frequency of mechanical stimulation in a cycle-by-cycle manner on engineered cardiac construct function. *J Tissue Eng Regen Med*.

Murrell M, Kamm R & Matsudaira P. 2011, Substrate viscosity enhances correlation in epithelial sheet movement. *Biophys J,* 101: 297-306.

Narita Y, Yamawaki A, Kagami H, Ueda M & Ueda Y. 2008, Effects of transforming growth factor-beta 1 and ascorbic acid on differentiation of human bone-marrow-derived mesenchymal stem cells into smooth muscle cell lineage. *Cell Tissue Res,* 333: 449-59.

Nasseri BA, Ogawa K & Vacanti JP. 2001, Tissue engineering: An evolving 21st-century science to provide biologic replacement for reconstruction and transplantation. *Surgery,* 130: 781-784.

Nieponice A, Soletti L, Guan J, Deasy BM, Huard J, Wagner WR & Vorp DA. 2008, Development of a tissue-engineered vascular graft combining a biodegradable scaffold, muscle-derived stem cells and a rotational vacuum seeding technique. *Biomaterials,* 29: 825-33.

Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R & Langer R. 1999, Functional arteries grown in vitro. *Science,* 284: 489-93.

Pashneh-Tala S, Macneil S & Claeyssens F. 2015, The Tissue-Engineered Vascular Graft-Past, Present, and Future. *Tissue Eng Part B Rev*.

Pasic M, Muller-Glauser W, Odermatt B, Lachat M, Seifert B & Turina M. 1995, Seeding with omental cells prevents late neointimal hyperplasia in small-diameter Dacron grafts. *Circulation,* 92: 2605-16.

Patel S, Shi Y, Niculescu R, Chung EH, Martin JL & Zalewski A. 2000, Characteristics of coronary smooth muscle cells and adventitial fibroblasts. *Circulation,* 101: 524-32.

Peloso A, Dhal A, Zambon JP, Li P, Orlando G, Atala A & Soker S. 2015, Current achievements and future perspectives in whole-organ bioengineering. *Stem Cell Res Ther,* 6: 107.

Pfaffl MW. 2001, A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acid Research,* 29: 5.

Piola M, Prandi F, Bono N, Soncini M, Penza E, Agrifoglio M, Polvani G, Pesce M & Fiore GB. 2013a, A compact and automated ex vivo vessel culture system for the pulsatile pressure conditioning of human saphenous veins. *J Tissue Eng Regen Med*.

Piola M, Prandi F, Fiore GB, Agrifoglio M, Polvani G, Pesce M & Soncini M. 2015, Human Saphenous Vein Response to Trans-wall Oxygen Gradients in a Novel Ex Vivo Conditioning Platform. *Ann Biomed Eng*.

Piola M, Soncini M, Cantini M, Sadr N, Ferrario G & Fiore GB. 2013b, Design and functional testing of a multichamber perfusion platform for three-dimensional scaffolds. *The Scientific World Journal,* 2013: 9.

Prandi F, Piola M, Soncini M, Colussi C, D'alessandra Y, Penza E, Agrifoglio M, Vinci MC, Polvani G, Gaetano C, Fiore GB & Pesce M. 2015, Adventitial vessel growth and progenitor cells activation in an ex vivo culture system mimicking human saphenous vein wall strain after coronary artery bypass grafting. *PLoS One,* 10: e0117409.

Quint C, Kondo Y, Manson RJ, Lawson JH, Dardik A & Niklason LE. 2011, Decellularized tissue-engineered blood vessel as an arterial conduit. *Proc Natl Acad Sci U S A,* 108: 9214-9.

Scotti C, Osmokrovic A, Wolf F, Miot S, Peretti GM, Barbero A & Martin I. 2012, Response of Human Engineered Cartilage Based on Articular or Nasal Chondrocytes to Interleukin-1 beta and Low Oxygen. *Tissue Engineering Part A,* 18: 362-372.

Seifu DG, Purnama A, Mequanint K & Mantovani D. 2013, Small-diameter vascular tissue engineering. *Nat Rev Cardiol,* 10: 410-21.

Sinha S, Hoofnagle MH, Kingston PA, Mccanna ME & Owens GK. 2004, Transforming growth factor-beta1 signaling contributes to development of smooth muscle cells from embryonic stem cells. *Am J Physiol Cell Physiol,* 287: C1560-8.

Sivarapatna A, Ghaedi M, Le AV, Mendez JJ, Qyang Y & Niklason LE. 2015, Arterial specification of endothelial cells derived from human induced pluripotent stem cells in a biomimetic flow bioreactor. *Biomaterials,* 53: 621-33.

Stefani I & Cooper-White JJ. 2016, Development of an in-process UV-crosslinked, electrospun PCL/aPLA-co-TMC composite polymer for tubular tissue engineering applications. *Acta Biomaterialia,* 36: 231-40.

Syedain ZH, Meier LA, Bjork JW, Lee A & Tranquillo RT. 2011, Implantable arterial grafts from human fibroblasts and fibrin using a multi-graft pulsed flow-stretch bioreactor with noninvasive strength monitoring. *Biomaterials,* 32: 714-722.

Tang AT, Campbell WB & Nithipatikom K. 2012, ROCK1 feedback regulation of the upstream small GTPase RhoA. *Cell Signal,* 24: 1375-80.

Tresoldi C, Pellegata AF & Mantero S. 2015, Cells and stimuli in small-caliber blood vessel tissue engineering. *Regen Med,* 10: 505-27.

Uzarski JS, Cores J & Mcfetridge PS. 2015, Physiologically Modeled Pulse Dynamics to Improve Function in In Vitro-Endothelialized Small-Diameter Vascular Grafts. *Tissue Eng Part C Methods,* 21: 1125-34.

Uzarski JS, Van De Walle AB & Mcfetridge PS. 2014, In Vitro Method for Real-Time, Direct Observation of Cell-Vascular Graft Interactions under Simulated Blood Flow. *Tissue Engineering Part C-Methods,* 20: 116-128.

Vacanti JP. 2010, Tissue engineering and regenerative medicine: from first principles to state of the art. *J Pediatr Surg,* 45: 291-4.

Wang D, Park JS, Chu JS, Krakowski A, Luo K, Chen DJ & Li S. 2004, Proteomic profiling of bone marrow mesenchymal stem cells upon transforming growth factor beta1 stimulation. *J Biol Chem,* 279: 43725-34.

Wendt D, Riboldi SA, Cioffi M & Martin I. 2009, Potential and bottlenecks of bioreactors in 3D cell culture and tissue manufacturing. *Adv Mater,* 21: 3352-67.

Wendt D, Stroebel S, Jakob M, John GT & Martin I. 2006, Uniform tissues engineered by seeding and culturing cells in 3D scaffolds under perfusion at defined oxygen tensions. *Biorheology,* 43: 481-8.

Wu MY & Hill CS. 2009, Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell,* 16: 329-43.

Zeisberg EM, Ma Q, Juraszek AL, Moses K, Schwartz RJ, Izumo S & Pu3 WT. 2005, Morphogenesis of the right ventricle requires myocardial expression of Gata4. *The Journal of Clinical Investigation,* 115: 1522–1531.

Table 1: Primer sequences used in qPCR analysis

Gene	Forward	Reverse	Reference or NCBI sequence
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC	(Murrell M et al. 2011)
α-SMA	ACGTGGGTGACGAAGCACAG	GGGCAACACGAAGCTCATTGTA	(Narita Y et al. 2008, Ball SG et al. 2004, Cameron AR et al. 2011)
CNN1	TCGGCAACAACTTCATGG	GCCTGGCTGCAGCTTATT	(Ball SG et al. 2004)
DES	GAGACCATCGCGGCTAAGAAC	GTGTAGGACTGGATCTGGTGT	NM_001927.3
SM1	ATTCGCCAAGCCAGAGAC	GAAGCCCTCCAAAAGCAA	(Ball SG et al. 2004)
Col1A1	CCTGCGTGTACCCCACTCA	ACCAGACATGCCTCTTGTCCTT	(Tang AT et al. 2012, Cameron AR et al. 2014)
Col4A1	GCCGTGGGACCTGCAATTACT	CGTAGGCTTCTTGAACATCT	NM_001845

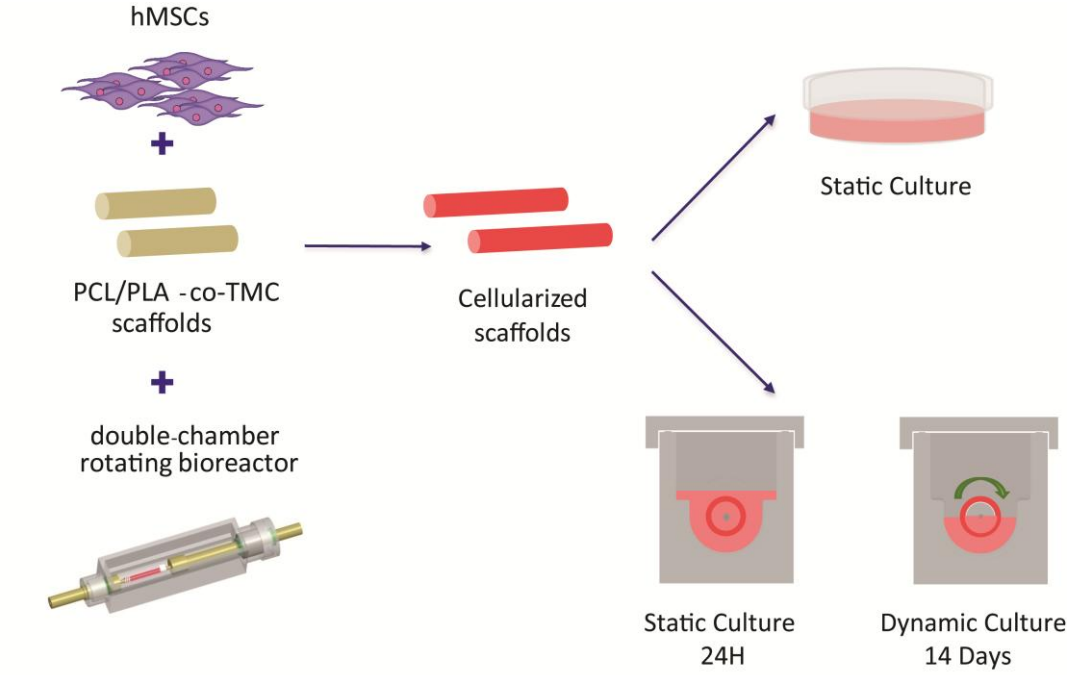

Figure 1 Experimental scheme for Tissue engineered vascular grafts (TEVG) study. A summary of the approach we followed to cellularize a synthetic scaffold: seeding and culture conditions are reported to simplify the understanding of the set-up of the study. hMSC were seeded dropwise on the developed synthetic scaffold and cultured under dynamic condition for 14 days. Static culture was carried out as control Figure 1

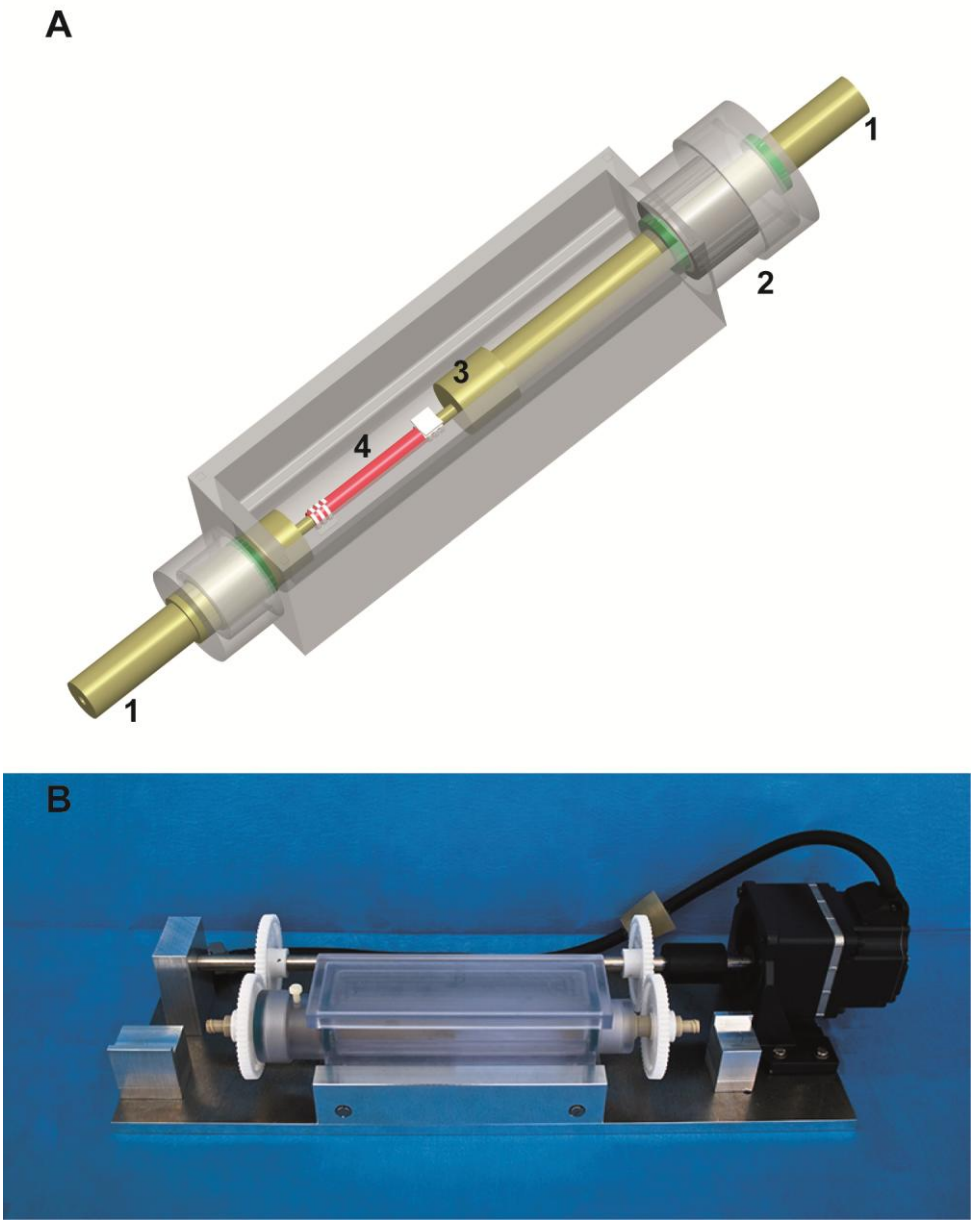

Figure 2 A) 3D rendering of the developed bioreactor. 1) Shafts for rotation and pre-tensioning system, produced in different sizes in order to house scaffolds of different lengths; 2) The pre-tensioning system (in green Viton® dynamic-seals, in dark grey the ball bearing holder); 3) Scaffold supports. They are interchangeable and can be inserted in the chamber after the scaffold housing outside of the chamber; 4) Scaffold and custom-made clips, specifically designed to stably hold the scaffold in place. B) Picture of the developed bioreactor coupled with the motion unit: the whole base can be easily positioned in a standard incubator and left in it for all the time of the culture, while the culture chamber can be independently moved for medium change procedures or samples collection.

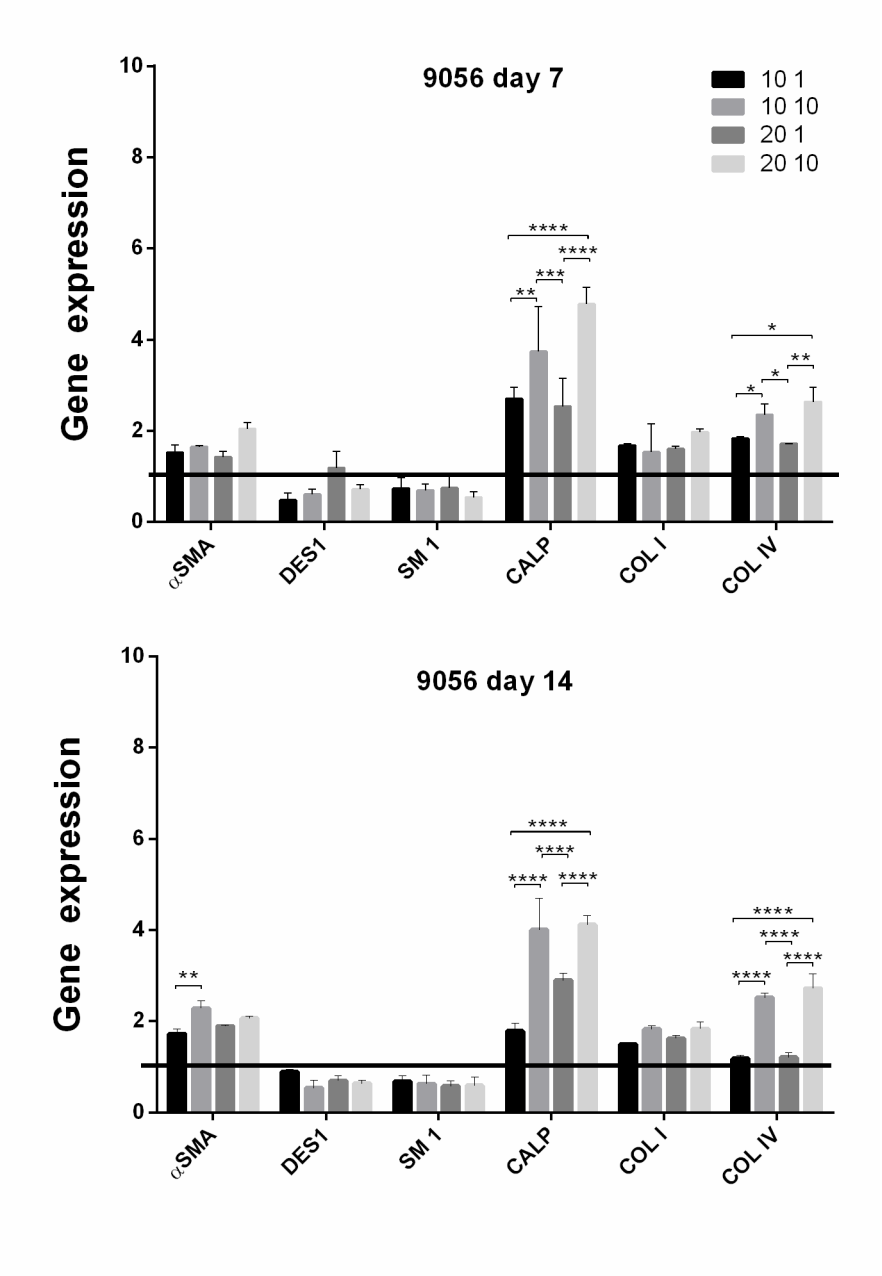

Figure 3 Gene Expression in hMSCs (donor 9056 used for dynamic experiment later in this paper) exposed to four different myogenic media for 7 (LHS) and 14 (RHS) days. Samples number represents the % of Fetal bovine serum (10 or 20) and the concentration of TGF-β1 (1 or 10 ng/mL). Data are normalized on GAPDH and growth media exposed hMSCs gene expression data (black-line). Data were not combine due to similar trends, but different expression values due to donor variability.

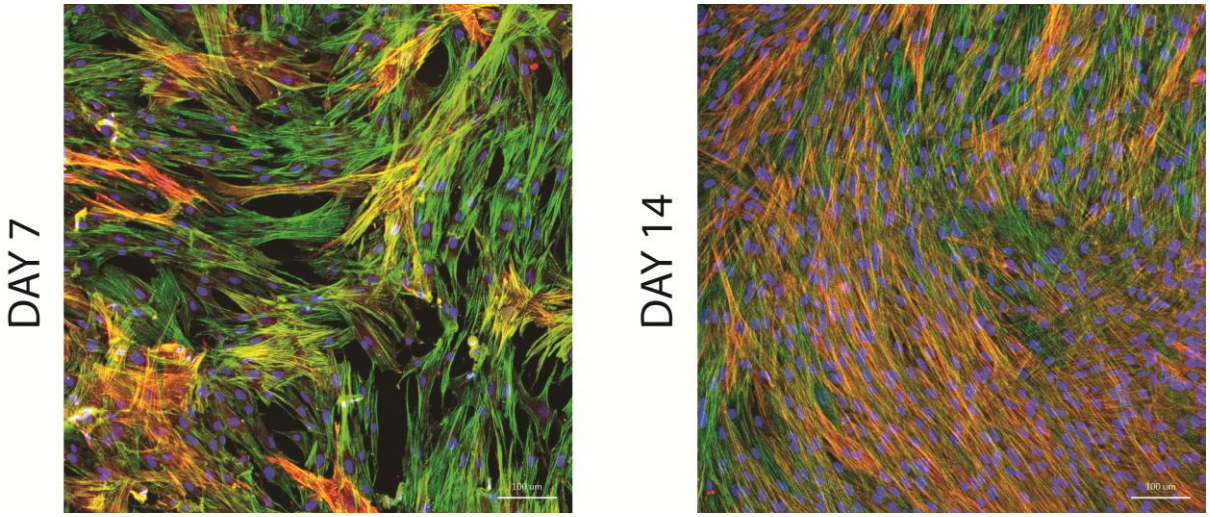

Figure 4: Confocal pictures of MSCs after 7 and 14 days of exposure to 10-10 differentiation medium. Nuclei (blue), actin (green), smooth muscle actin (α-SMA) (red) and calponin (yellow) were stained. Images were loaded into CellProfiler method as separate channels, as required from the software.

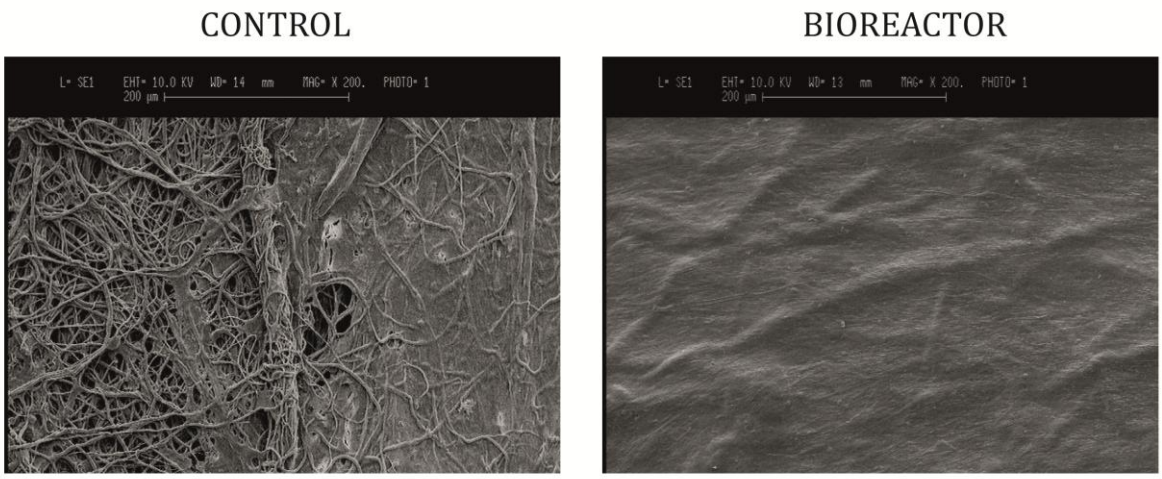

Figure 5: Scanning electron microscopy of PCL/PLA-TMC scaffold seeded with hMSCs and cultured in myogenic media for 14 days. Outer surfaces of static (CONTROL) and dynamic (BIOREACTOR) cultured samples were analysed to investigate cells morphology and extra cellular matrix deposition. Scale bar = 200 µm

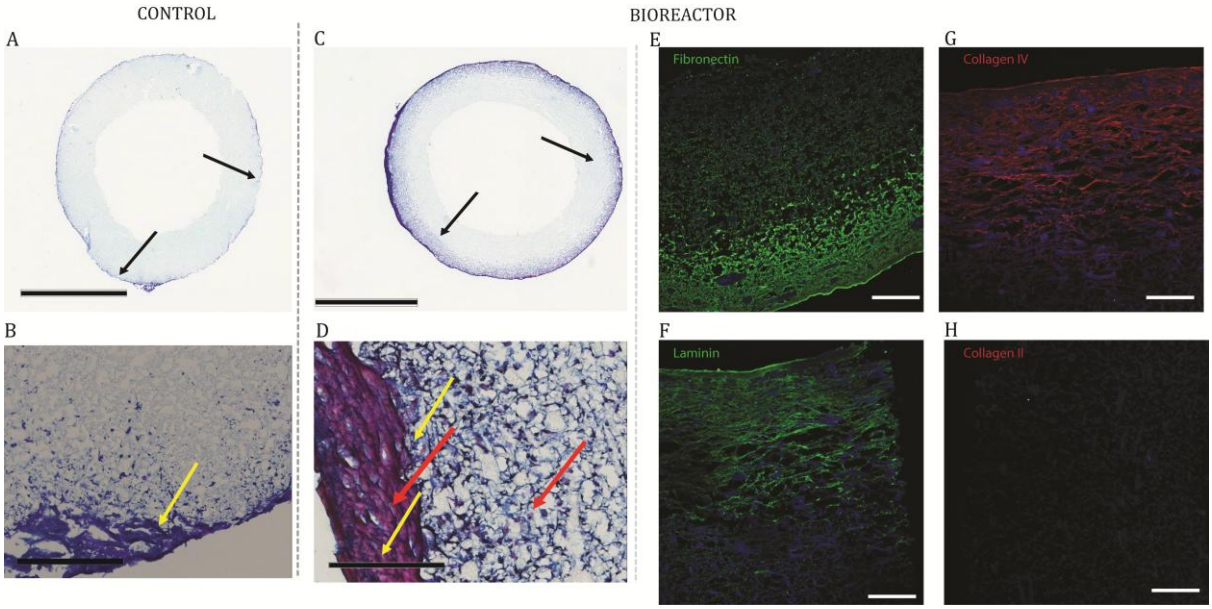

Figure 6: A-D Lillie's allochrome staining. Control and bioreactor scaffolds were analysed circumferentially. Black arrows in image A and B present the extent of cell penetration. Deposition of different matrix proteins was investigated throughout the scaffolds. Nuclei are stained in black, collagen in blue (Yellow arrows), muscle reticulin reddish purple (red arrows), fibrin red to pink. (Scale-bar top=3 mm, bottom=200 μm). EH) Immunohistochemistry was performed on histology sections from the bioreactor samples in order to highlight the main components of the produced extracellular matrix (E - Fibronectin; F - Laminin; G -Collagen IV; H - Collagen II). Scaffold is visible in blue (Scale-bar=200 μm)