A compact and automated *ex-vivo* vessel culture system for the pulsatile pressure conditioning of human saphenous veins

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Running title
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Abstract

Saphenous vein (SV) graft disease represents an unresolved problem in coronary artery bypass grafting (CABG). After CABG, a progressive remodeling of the SV wall occurs, possibly leading to the lumen occlusion, a process termed intima hyperplasia (IH). The investigation of cellular and molecular aspects of IH progression is a primary endpoint toward the generation of occlusion-free vessels that may be used as 'life-long' grafts. While animal transplantation models have clarified some of the remodeling factors, the human SV pathology is far from being understood. This is also due to the lack of devices able to reproduce the altered mechanical load encountered by the SV after CABG. The manuscript describes the design of a novel *ex vivo* vein culture system (EVCS) capable to replicate the altered pressure pattern experienced by SV after CABG and reports the results of a preliminary biomechanical conditioning experimental campaign on SV segments. The EVCS applied a CAGB-like pressure (80-120 mmHg) or a venous-like perfusion (3 ml/min, 5 mmHg) conditioning to the SVs, keeping the segments viable in a sterile environment during 7-day-culture experiments. After CABG-like pressure conditioning, SVs exhibited a decay of the wall thickness, an enlargement of the luminal perimeter, a rearrangement of the muscle fibers, and a partial endothelium denudation. Considering these preliminary results, the EVCS is a suitable system to study the mechanical attributes of SV graft disease, and its use, combined with a well-designed biological protocol, may be of help in elucidating the cellular and molecular mechanisms involved in SV graft disease.

Keywords: *ex vivo* vessel culture system, coronary artery by-pass grafting, saphenous vein remodeling, pulsatile pressure stimulation, mechanical conditioning, saphenous vein

graft disease

1. INTRODUCTION

Coronary artery bypass grafting (CABG) using autologous vessels is a widely used procedure to recover myocardial perfusion in patients with coronary artery disease (de Waard *et al.,* 2006; Parang and Arora 2009; Wallitt *et al.,* 2007). The saphenous vein (SV), due to its length and its superficial anatomical position, represents a preferred natural bypass conduit, especially when the implantation of multiple bypasses is required (Dashwood and Loesch 2009; Muto *et al.,* 2010; Severyn *et al.,* 2004; Surowiec *et al.,* 2000). Compared with artery-made bypasses (radial and mammary arteries) SV grafts show lower long-term patency; in fact, approximately 15-30% of vein grafts fail during the first year and more than 50% patients require re-intervention within 10 years after implantation (Goldman *et al.,* 2004; Tsui and Dashwood 2002). The major cause of SV graft failure is an over-proliferation of smooth muscle cells (SMCs) into the vessel intima layer. This process, named intimal hyperplasia (IH) can be detected between 1 month and 1 year after graft implantation (Lemson *et al.,* 2000; Motwani and Topol 1998). However, the beginning of the pathology occurs at much earlier stages after implantation (one week), with the activation of biomechanical- and inflammatory-driven cascades priming vessel remodeling (Mitra *et al.,* 2006; Muto *et al.,* 2010). The new hemodynamic conditions experienced by vein after implantation in arterial position are postulated to be an important cause for SV remodeling. In particular, in the coronary artery, the blood flow is characterized by a high pulsatile pressure (80 - 120 mmHg) and a pulsatile flow (mean flow rate of 250 ml/min), which results in an elevated shear stress (1 - 7 Pa) (Bouten *et al.,* 2011; Dummler *et al.,* 2011). Such forces may have antagonistic effects on disease progression. Indeed elevated shear stress may have an atheroprotective role due to

haemodynamic-related increase of nitric oxide (NO) release by endothelial cells while a non-physiologic mechanical loading of the vein wall may have a pro-pathologic effect due to mechanical ruptures in the endothelial layer and abnormal strain of SMCs sheets (John 2009).

Various animal models have been devised to address the pathologic evolution of the arterialized veins. These studies have highlighted the relevance different cell types and signal transduction pathways involved in the initiation of phenomena leading to IH (Hoglund *et al.,* 2010; Torsney *et al.,* 2005). Despite these approaches, however, the establishment of IH in human arterialized vein is still far from being understood, thus posing the need for novel experimental models to be set up in order to: *i)* tightly reproduce the altered hemodynamic conditions, especially the raise in wall strain, *ii)* obtain *ex vivo* arterialized SV segments for investigating the mechano-biological basis of the early events leading to IH at global molecular level, and *iii)* in perspective attempt therapeutic strategies by pharmacological conditioning of the dynamically cultured vein segments. As we have discussed recently, various devices, tailored to perform *ex-vivo* culture of human SVs for a period of time spanning from 4 to 14 days and under dynamic conditions, have been devised (Piola *et al.,* 2012).

In this manuscript we propose the design of a compact and automated *ex-vivo* vessel culture system (EVCS) able to artificially produce the effects of the arterial pressure-related cyclic wall distention, one of the major biomechanical causes of IH in venous CABGs together with the pulsatile wall shear stress (Anwar *et al.,* 2012; Berceli *et al.,* 1990; John 2009; Muto *et al.,* 2010; Owens 2010; Stigler *et al.,* 2012). This aim is achieved by the development and functional assessment of a low-volume, reliable and user-friendly device, capable to replicate automatically the pulsatile pressure patterns of

the physiological coronary environment. In perspective, the present EVCS could be used as tool to carry out molecular and cellular studies in order to better understand the impact of modified hemodynamic conditions on *in vivo* SV remodeling.

2. MATERIALS AND METHODS

2.1 Design of the EVCS

Design specification

The design of the EVCS took into account the general specifications of a bioreactor for tissue engineering application (Martin *et al.,* 2004), with particular emphasis on the ease of assembly under laminar flow hood, and the safety of use in a cell culture laboratory. Specifically, the following basic requirements were addressed: *i)* biocompatibility of materials, *ii)* transparency, to ensure visual inspection for air bubble and/or medium color changes, *iii)* compatibility with sterilization processes, e.g., via autoclaving and/or ethylene-oxide (EtO), *iv)* minimization of priming volume, in order to limit the cost of soluble culture medium compounds, and finally *v)* easiness of vessel accommodation and handling during the EVCS assembly.

Architecture of the ex-vivo vessel culture system

The EVCS is designed to apply a CABG-like pressure stimulation (CABG-PS), *i.e*. a pulsed pressure oscillating between a diastolic minimum and a systolic maximum (*e.g*., 80-120 mmHg), or a steady flow perfusion, *i.e.*, a physiological venous perfusion condition (VP, *e.g.,* 5 mmHg) within a controlled and strictly reproducible mechanical environment. A schematic representation of the system's layout is shown in Figure 1C. During culture, SV grafts are hosted in a culture chamber accommodated inside an

incubator. The culture chamber is connected to a hydraulic circuit and actuators (pump and solenoid pinch-valve) to apply pressure stimulation to the human vessels or to allow the medium recirculate within the vessel. The hydraulic actuators are managed by a programmable monitoring and control (M/C) system, which operates via a pressure-based feedback loop.

SV culture chamber

The culture chamber (Figure 1A) includes a commercial reservoir and a purpose-developed vessel housing which is integrated with the reservoir cap. All the culture chamber parts built in house were designed with 3D-CAD Pro/Engineer Wildfire 4.0 (PTC, Needham, MA), and manufactured by laser cutting (Versalaser VSL2.30, SK Laser, Germany), and/or computer numerical control machining (Modela MDX-40, Japan) from polymethylmethacrylate blocks (Plasting S.r.l., Segrate, Italy). All the utilized materials are suitable for EtO sterilization. The vessel housing allows hosting SV samples up to 5.5 cm in length. The hosted vessel segment is cannulated at both ends using polypropylene (PP) barbed fittings (Cole Parmer, IL, USA), and secured using an extensible vessel loop (Esafarma S.r.l., Italy) as an elastic tourniquet. A standard 50-ml falcon tube (International PBI S.p.A, Italy) acts as a medium reservoir. The reservoir and the housing are coupled trough a silicone O-ring.

Five ports through the cap ensure the chamber's connection to the outside. Two ports ensure injection/removal of the culture medium to/from the vessel (Figure 1A, port a and b). Two other ports provide connection for recirculation of the reservoir medium, *i.e*. the medium external to the hosted SV (Figure 1A, port c and d). One additional port provides communication with the incubator environment through a HEPA filter, to guarantee a

sterile gas exchange while keeping the pressure inside the culture chamber atmospheric (Figure 1A, port e).

The hydraulic circuit

The hydraulic circuit consists of silicone tubing (Platinum Cured, Cole Parmer, IL, USA) and PP-based pump tubing (PharMed BPT®, Carlo Erba Reagenti, Milano, Italy). PP luer connectors (Cole Parmer, Cole Parmer, IL, USA) are used to guarantee leak-free connections, and facilitate circuit assembly under laminar flow hood. A 5-ml syringe, filled with 1.6 ml of culture medium and 3.4 ml of air, acts as a compliance chamber for damping pump disturbances.

The tubing length was dimensioned in order to minimize the head loss along the hydraulic circuit during the vessel stimulation period. Further, the silicone tubing length was dimensioned according to an analytical model (Orr and Burg 2008) based on the balance between oxygen depletion (vessel cell metabolism) or replenishment (silicone tubing) in order to guarantee appropriate vessel oxygenation. Briefly, the oxygen consumption of native vascular tissue was set equal to $3.57\ 10^{-3}$ mlO_2/min, assuming an oxygen demand of $3.57\ 10^{-6}$ mlO_2/(min kg) and considering the mass of a SV graft to be about 1 g (Hoenicka *et al.,* 2010). In this condition, the necessary silicone tubing size resulted to be at least 250 cm long.

Monitoring and control system

The M/C system is able to automatically apply the CABG-PS to the SV segments or the SV perfusion by automated control of a peristaltic pump (Watson Marlow 323D with 314D pumping head, Watson Marlow Group, UK) and a solenoid pinch-valve (S305-09,

SIRAI® Elettromeccanica, Italy) (Figure 1C). The solenoid pinch-valve enables switching between the vessel stimulation loop and the recirculation loop (Figure 1D). The hydraulic actuators are connected to a PC equipped with a I/O board (NIDAQCard-6036E, National Instruments Corp.) and are managed via a customized LabView software (National Instruments Corp., TX, USA) adapted from Vismara and colleagues (Vismara *et al.,* 2009). The pressure sensor Press-S-000 (PendoTECH, NJ, USA) provides the intraluminal pressure feedback signal to the software (Figure 1C).

2.2 Functional experiments for testing the performance of the EVCS

SV sample preparation

In the present investigation, the use of human SV segments was authorized by the local Ethical Committee with the approval of an informed consent. SV segments were obtained from the Department of Cardiovascular Surgery of the Centro Cardiologico Monzino. Briefly, surplus segments of SV were obtained from 19 patients undergoing CABG surgery (mean age of 66.7±7.4). SV samples were harvested with a "no touch" technique avoiding venous spasm and dilatation, and ensuring endothelial, medial and adventitial integrity (Dashwood and Tsui 2013). The distal end of each SV segment was cannulated in the operating room, thus allowing the identification of the valve direction, side branches were ligated, and SVs were immediately stored at 4°C in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, and 1% Penicillin/Streptomycin. All the SV segments were maintained at 4°C for up to 48h before their use. Six SV samples were used for the compliance measurements, two samples were used for the functional validation of the EVCS, and eleven samples were used for the remodeling study. In all the experiments described in the following

paragraphs, the distal end of each sample was mounted onto the inlet connector of the devices after gently removing the existing cannula.

Pressure-volume measurements of SV segments

Pressure-volume relationship of human SV segments was obtained in order to dimension the fluid volume that the pump has to process for obtaining the desired pressure increments within the vessel. To this aim, a custom-made system was developed to measure the pressure-volume relationship during SV filling. A Schematic diagram of the custom-made system is reported in Figure 2A. For each SV specimen (n=6, length of the sample > 35 mm), the inner diameter (D_i) was measured using a caliper. Then, each SV segment was mounted in the custom-made system and pre-tensioned by imposing a 10% axial strain, corresponding to a length named $l_{10\%}$. Table 1 reports the geometrical characteristics (D_i and $l_{10\%}$) of the SV and the volume at zero pressure (V_0), calculated as the volume of a straight cylinder of diameter equal to D_i and length equal to $l_{10\%}$. SV segments were pre-conditioned by imposing five cycles of loading (0 to 120 mmHg) and unloading (120 to 0 mmHg), as described in the literature (Costantino *et al.,* 2004). Thereafter, the vessel lumen was exposed to incremental pressure (0 to 120 mmHg, step 10 mmHg) and the associated inner volume increment was measured (ΔV, Figure 2A) using an interposed graduated pipette. For each sample, this procedure was repeated three times. The volume increment was normalized to V_0.

Functional assessment of the EVCS

Preliminary tests were performed using a SV sample, mounted within the EVCS, in order to verify the robustness and the reliability over time of the system. The EVCS was placed

in the incubator and the performances were evaluated by changing the pulse frequency and the pressure stimulation range. The tested pulse frequency was 0.5 Hz, 0.75 Hz, 1 Hz and 1.2 Hz, while the pressure range was 60 – 90 mmHg (Hypotension), 80 – 120 mmHg (Normal) and 100 – 140 mmHg (Hypertension).

For the following tests the EVCS components were sterilized by EtO or autoclaving, where appropriate. The EVCS sterility maintenance was verified with two tests using a sterile silicone vessel substitute and a SV sample. In both conditions, the system was assembled under laminar flow hood and filled with 42 ml Roosvelt Park Memorial Institute medium 1640 (RPMI, Lonza Group LTD, Switzerland) without antibiotics, and placed in the incubator (37°C and 5% CO_2) for 7 days with a partial medium replacement at day 3, reproducing standard culture conditions. As a control, a Petri dish containing the same medium was also incubated. At every time step (day 0, day 3, and day 7) the culture medium was added to the Thioglycollate broth (BD, Maryland, USA) for microbiological analyses.

Mechanical conditioning of human SV within the EVCS

Generally, SV samples were divided into two segments (only for one sample, the length of the sample was sufficient for cutting the vein into three segments). One portion was immediately stored and used as control (native segment); the second portion (length > 5.5 cm) was cultured in the EVCS. SV samples were cultured under CABG-PS conditions (luminal pressure: 80 - 120 mmHg; pulse frequency (f): 0.5 Hz with a stimulation interval (Ts) of 10 minutes; recirculation flow rate (Q_R): 1 ml/min, with a luminal pressure between 1-2 mmHg, and recirculation interval (T_R) of 2 min), or VP conditions (steady flow with luminal pressure: 5 mmHg; flow rate: 3 ml/min). After the sterilization of the

EVCS, SV segments were mounted as previously described, and then the EVCS was filled with 42 ml DMEM with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% streptomycin-penicillin. Then, the EVCS was kept in the incubator at 37° C and 5% CO_2 for a culture period of 7 days. The culture medium was partially changed at day 3. At the end of the mechanical conditioning period, SV samples were disassembled, vein ends were discharged (thus avoiding any edge effects induced by the mounting onto rigid connectors), and the central portion of the SV was processed for the following analyses.

Tissue viability evaluation

Rings of cultured and native (positive control) SV samples were stained with methylthiazol tetrazolium (MTT, Sigma Aldrich) (Miyakawa *et al.,* 2008). MTT powder was dissolved in PBS, obtaining a final concentration of 0.5 mg/ml. SV rings were incubated at 37°C for 1 hour in PBS/MTT solution. As a negative control, a SV ring was fixed with 4% formaldehyde overnight and then incubated with MTT staining. After 1 hour, pictures were taken using a stereo microscope (STEMI 2000-C, Carl Zeiss®, Germany).

Morphological and immunofluorescence assessment of the mechanically conditioned human SV segments

Cultured (n = 6 VP and n=6 CABG-PS treated samples) and control (n=11) SV samples were fixed with 4% formaldehyde overnight, paraffin embedded, and cut in 5-µm-thick sections using a microtome. Sections were stained with Masson's trichrome staining (Bio-Optica Milano SpA, Italy), according to manufacturer's protocol. Six slices of each SV sample were observed for qualitative inspection, and digital images were acquired using a

light microscope (AxioVision Bio Software, Carl Zeiss®, Germany) at a magnification of 10x.

For immunofluorescence (IF) analysis, 3 samples for each group (VP, CABG-PS and related controls) were observed (6 slices for each sample). Briefly, 4-µm-thick sections were obtained from formalin-fixed/paraffin-embedded SV specimens. Sections were dewaxed and hydrated, and antigen retrieval was performed with 10mM Tris-HCL/1mM EDTA for 10 minutes in microwave. Therefore, sections were blocked with 3% bovine serum albumin (room temperature, 1 hour), and incubated (4°C, overnight) with mouse anti human-αSMA (1:500; cat. M-0851, Dako) for labeling SMCs, goat anti human-CD31 (1:200; cat. sc-1506 Santa Cruz Biotechnology, Inc.) and rabbit anti-human vWF (1:200; cat. A-0082 Dako) for labeling endothelial cells (ECs). Slides were incubated with Alexa Fluor 488 anti-mouse, Alexa Fluor 546 anti-goat, and Alexa Fluor 633 anti-rabbit (1:200; Invitrogen) secondary antibodies for 1 hour at room temperature, and nuclei were counterstained with DAPI (Vector Laboratories, CA, USA). Finally, digital images were obtained using a multicolor detection protocol in a LSM-710 confocal scanning microscope (Carl Zeiss®, Germany).

Morphometric measurements

Morphometric analyses were performed on Masson's trichrome stained sections in order to measure thickness and luminal perimeter. One section for each sample was used. Thickness measurements were manually processed on digital images taken with AxioVision Bio Software (Carl Zeiss®, Germany) at 10X magnification. At least 40 measurements per sample were made. The inner perimeter was calculated using Image-J software (Version 1.47f-sofware for Java, National Institutes of Health, USA).

Statistical analysis

Statistical comparison was performed using Graph-Pad 5 (Prism) statistical software. All data were initially analysed with the Kolmogorov-Smirnov normality test and then compared using a non-parametric Mann Whitney test. A p value <0.05 was assumed as statistically significant in all statistical tests.

3. RESULTS

Pressure-volume measurements of SV segments

Figure 2B reports the pressure-volume relationship obtained by progressively inflating fluid volume in human SV segments. The results showed an initial rapid volume rise with the first pressure increment step from 0 to 10 mmHg (a physiologic pressure range for veins), followed by a decreasing slope of the pressure-volume relationship, indicating that a considerable stiffening of the SV tissue progressively occurred when shifting towards the arterial pressure range (Figure 2B).

Figure 2C reports the luminal volume increment within the vein after imposing a pressure in the range of 0 – 10 mmHg, 10 – 80 mmHg and 80 – 120 mmHg. According to these results, the mean inflation fluid volume necessary to generate a pressure increment from 80 mmHg to 120 mmHg, was 0.06±0.025ml (Figure 2C). This value was used as rough estimate for selecting the dimensions of the relevant hydraulic components, such as the volume of the compliance chamber and the inner diameter of the pump tubing.

Preliminary functional assessment and setting of EVCS stimulation program

An overview of the EVCS during the assembling phase under laminar flow hood is shown in Figure 3 (Video_1_SuppInf.avi in the Supporting information on-line section). The time

necessary to fix the vein to the housing (Figure 3A, B, C and D) was less than 15 minutes; during this period the SV was constantly kept hydrated by pipetting culture medium on the SV surface. Finally, the housing was inserted within the reservoir, and connected to the hydraulic circuit (Figure 3E). Once assembled, the EVCS was placed in the incubator and the culture under mechanical conditioning started imposing either a VP condition or a CABG-PS.

The CABG-PS program consists of a cyclical alternation of a pulsatile stimulation period and a recirculation period. Particularly, the single cycle is composed of four steps (Figure 1B): *i)* the loading step, in which the culture medium is delivered through the vessel until the intraluminal pressure reaches a lower pressure limit (P_{min}); *ii)* the pulsatile stimulation step, during which the vessel is inflated and deflated in order to apply a controlled CABG-PS within the pre-defined pressure ranges (P_{min} - P_{max}) and for a pre-defined time and number of pulses per minutes; *iii)* the unloading step, in which the intraluminal pressure within the vessel is lowered again to zero (P_0) by inverting the medium flow direction; and finally, *iv)* the recirculation step, characterized by a constant flow rate allowing a metabolic supply to the vessel for a predefined recirculation period. The user can set all the specific parameters via the software interface, namely P_{min} and P_{max}, the pulse frequency and the number of pulses for the pulsatile stimulation period; the duration and the medium flow rate for the recirculation period.

Figure 4A reports representative screen printouts of the M/C software of the EVCS, showing an example of pressure tracing during the CABG-PS cycle. The outcomes of the functional tests indicated a good reliability of the M/C system. Figure 4B shows the system's response to different pressure conditions reproducing hypertension (100 – 140 mmHg), normal (80 – 120 mmHg) and hypotension (60- 90 mmHg) conditions; while the

system's response to different values of frequency (0.5, 0.75, 1 and 1.2 Hz) is shown in figure 4C. In both configurations, the stimulation pressure tracings are fairly regular, repeatable and compliant to the user's settings. Finally, microbiological tests showed no contamination in the culture medium after 7 days of culture in either tested configurations (silicone vessel or SV sample). Absence of any apparent contamination was also observed in the subsequent CABG-PS and VP culture tests.

Validation of the EVCS during culture under mechanical conditioning.

The culture of SV segments for 7 days under VP and CABG-PS conditions was finally performed in the EVCS. At the end of the mechanical conditioning period, the vessels were un-mounted and immediately processed for viability, histological and IF investigations.

To assess tissue viability MTT staining was used (Figure 5). This showed that after 7 days under VP (Figure 5A) or CABG-PS (Figure 5B) conditions the vessel viability was maintained similarly to freshly harvested SV rings, used as a positive control (Figure 5C). To assess vessel integrity and structure after VP/CABG-PS periods, Masson's trichrome staining of transversally cut sections was performed. Results showed a relatively good integrity of the vessel structure with a good preservation of SMCs and the adventitia layers, and without signs of tissue degeneration and swelling (Figure 6). Changes in cellular arrangement were instead observed in the VP (Figure 6B) vs. CABG-PS (Figure 6C) segments. In fact, in VP-cultured vessel segments (Figure 6B), the arrangement of medial SMC layers appeared similar to that present in non-cultured vessels (Figure 6A); by contrast, the application of CABG-PS caused an almost complete SMCs disarrangement (Figure 6C). To better evaluate the organization and arrangement of

cellular components of the SV following *ex vivo* culture, IF analysis for SMC marker αSMA was performed (Figure 6, *right column*). Low magnification imaging of the vessels confirmed a difference in the medial layer of CABG-PS *vs.* VP vessels again suggesting a loss in circumferential SMCs sheets due to the modified pressure conditions (Figure 6C, *right column*).

The observation of the vessel structure by histochemistry and IF showed that vessels cultured under CABG-PS conditions had relatively thinner wall and larger lumen compared to VP or native vessels (Figure 6 A-C). To obtain quantitative data both parameters were evaluated by computer-aided measuring. As shown in Figure 6D, the quantitative evaluation of wall thickness and luminal perimeter revealed major morphometric changes in CABG-PS *vs.* native vessels. Interestingly, veins perfused with a venous pressure did not undergo remodeling, confirming that application of a CABG-like wall strain for 7 days was sufficient to induce structural changes in the SV. Histological analyses did not allow to evaluate whether the SV endothelium is affected by change in pressure conditions in CABG-PS stimulated vessels. To assess this, an IF analysis for endothelial markers vWF and CD31 was set up, followed by low and high power confocal imaging (Figure 7). Panels A, B and C show, respectively, low and high power views of the EC layer stained in the same vessels presented in Figure 6 A, B and C. While at low magnification the appearance of the endothelial layer appeared similar in the three conditions, the high power views showed the loss of ECs integrity and the partial detachment of the EC layer in CABG-PS *vs.* native and VP-stimulated veins (compare panels 7C with 7A and 7B).

4. DISCUSSION AND CONCLUSION

In the present contribution, we described the design of a novel and versatile platform to perform dynamic stimulation of SV segments mimicking the physiological pressure patterns of the coronary circulation. This is indicated as one of the major insults received by the SV segments following their CABG implantation (Anwar *et al.,* 2012; Berceli *et al.,* 1990; John 2009; Muto *et al.,* 2010; Owens 2010). Hence, the rationale of its inclusion as a controlled factor for conditioning SV segments in our culture device is to enable *ex vivo* campaigns for elucidating the role of a major biomechanical factor in SV arterialization. The EVCS was verified to reliably apply the desired pressure patterns to SV segments and to maintain vessels viability at least for 7 days in a controlled and sterile environment.

Enhanced versatility and automation of an ECVS for ex vivo vessel conditioning

Regarding the EVCS design, the specifications of an easy assembly and handling were satisfied thanks to the technical solutions adopted. First, the entire system is very compact through the adoption of an integrated medium reservoir, which makes the device easy to be used into a standard cell culture incubator, minimizing the need for dedicated culture instrumentation. In addition, compactness allowed abating the overall priming volume to 42 ml, substantially lower than the 5000 ml reported by Voisard and colleagues (Voisard *et al.,* 2010) and the 300 ml stated by Clerin (Clerin *et al.,* 2002), and comparable with the overall priming volume of 20 ml declared by Dummler and colleagues (Dummler *et al.,* 2011). Finally, working in vertical conditions facilitates the air de-bubbling through the HEPA-port during the assembling and filling of the EVCS and during the culture experiments. This configuration avoids the air bubbles accumulation within the vessel during the mechanical conditioning culture.

Compared to other EVCS reported in literature (Piola *et al.,* 2012), with particular reference to the systems by Dummler and colleagues (Dummler *et al.,* 2011) and Voisard and colleagues (Voisard *et al.,* 2010), our EVCS is designed to ensure the perfusion of the hosted SV segments, or to impose controlled physiologic CABG-PS conditions, resulting in a cyclic wall circumferential strain. To this purpose, the EVCS is equipped with dedicated control hardware and software, which, from the user's standpoint, implies interaction with a programmable and user-friendly graphical interface. In this way the device is suitable to automatically manage the generation of the pressure pulse or the medium recirculation for fluid refreshing and tissue oxygenation.

The strategy we adopted for the pressure stimulation consists of inflating and deflating the SV by forcing small volumes of medium into and out of the vessel through both ends. Thus, a recirculation phase is mandatory in order to re-establish the correct amount of nutrients within the luminal region of the vein. In addition, pulse generation is based on the commands of a robust programmable M/C system, which operates via a pressure-based auto-tuning feedback loop. A main feature of the M/C system is the possibility to freely set different stimulation parameters such as pressure range, pulse frequency, and number of cycles in order to modulate properly the stimulation pattern. In this manner, a versatile system is envisaged with the capability to perform simultaneous stimulation of SV segments in different conditions to perform more stringent paired biological observations on SVs segments or for mechanical conditioning of tissue-engineered blood vessels.

Our EVCS suffers from some limitations. The system does not allow for the application of a coronary-mimicking flow through the vessel, hence the shear stress stimulus deriving from the arterial transposition of the vein is not replicated. Specifically, being the net flow approximately zero during the stimulation period, the resulting shear stress experienced by

the ECs in the EVCS is negligible with respect to arterial shear stress. On the one hand, this allowed us to selectively study the early remodeling effects caused by wall strain alone as a biomechanical stimulus. On the other hand this did not represent a fully biomimetic condition, because in real CABG pulsatile strain is always associated to pulsatile wall shear. The system, however, is prone to a fully biomimetic upgrade; this will require minimal changes to the culture chamber design (such as, changes in conduit diameters), whereas appropriate refinements will be needed for the hydraulic circuit layout and for the M/C system. In addition, the system is not suitable for replicating the artery/vein compliance mismatch, hampering the possibility of study its contribution on the early remodeling events associated with IH.

Pressure-volume relationship of human SVs, obtained experimentally, was used in order to determine the volume of medium to be processed within the vessel to obtain the desired intraluminal pressure oscillation within CABG-PS range. In addition, the results indicated that the pressure-volume curves are non-linear and revealed a considerable hardening of the SV tissue occurring at arterial pressures. These data fully comply with data reported in literature (Stooker *et al.,* 2003). It is worth to notice that, as attested in our functionality tests as well as in the subsequent 7-day device use experience, pulse generation stability was not affected at all by the strong nonlinearity of the treated samples. From a strict engineering viewpoint, this is an index of the system's robustness as an automated tool for biomechanical SV sample stimulation.

Validation of the ECVS using human SV tissues reveals profound changes in vessel structure due to arterial-like wall strain.

A major interest for vascular biologists in developing devices to perform vessel biomechanical conditioning, consists in the possibility to investigate the specific contribution of hemodynamic forces involved in vessel pathologies. In particular, platforms able to apply arterial-like pressure patterns to vein segments may be crucial to resolve the timely issue of graft patency reduction following CABG surgery, a very frequent side effect of arterialization in patients receiving venous grafts (Owens 2010; Piola *et al.,* 2012).

Two major biomechanical components are believed to contribute to vein arterialization; these are the flow-related shear stress, which is increased in CABG due to a raise in flow velocity, and the wall stress/tension, which is also increased due to a switch from venous to arterial pressures (John 2009). Flow shear stress and wall strain may have antagonistic functions in vein arterialization process. In fact, arterial shear stress enhances the EC release of vaso-relaxing molecules such as Nitric Oxide which is also known to inhibit neointima formation (Berard *et al.,* 2013; Gusic *et al.,* 2005). By contrast the wall strain is involved in the remodeling of the venous wall, causing the reduction of the SV thickness, and the mechanical rupture of the endothelial layer and the underlying SMC sheets (John 2009; Owens 2010; Saucy *et al.,* 2010).

In a wider view of addressing the role of the different mechanical components involved in IH, the approach followed in the present report confirms an important contribution of arterial-like wall strain in SV structural changes. In fact, our data indicated a clear thinning of the SV wall and a marked enlargement of the luminal perimeter, two parameters which have been found significantly changed during arterial positioning of vein segments in patients and animal models ((Owens 2010) and reference therein). In addition, we observed a contribution of the CABG-PS condition to determine microscopic ruptures in

the endothelial layer and a striking disarray in the SMC layers in the medial tissue, two factors known to predispose the vessel to pathologic remodeling (Motwani and Topol 1998)

In summary, the compact and automated EVCS appears to be a well-suited system able to reproduce the wall strain conditions typical of the coronary circulation. The system maintains an optimal tissue viability, operates under sterility and performs vessel stimulation in a low-volume culture chamber. For these enhanced design characteristics this platform is therefore a novel laboratory-oriented tool that, will be useful to carry out *in vitro* culture campaigns under strictly controlled hemodynamic conditions and dissect the contribution of different biomechanical factors involved in the early IH priming in vein CABGs.

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Disclosure Statement

The authors declare no conflict of interest to disclose.

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Tables

Sample	D_i [mm]	$l_{10\%}$[mm]	V_0 [ml]
SV01	3.2	59	0.47
SV02	3.1	35	0.26
SV03	3.8	60	0.68
SV04	3.4	35	0.27
SV05	3.2	35	0.28
SV06	3.8	60	0.68
Mean	3.4	46.5	0.44

Table 1. Geometric values of the SV samples (n=6) used for the pressure-volume measurements. D_i represent the inner diameter; $l_{10\%}$ is the length of the sample hosted within the custom-made device, and V_0 was calculated as the volume of a straight cylinder of diameter D_i and length $l_{10\%}$.

Figures

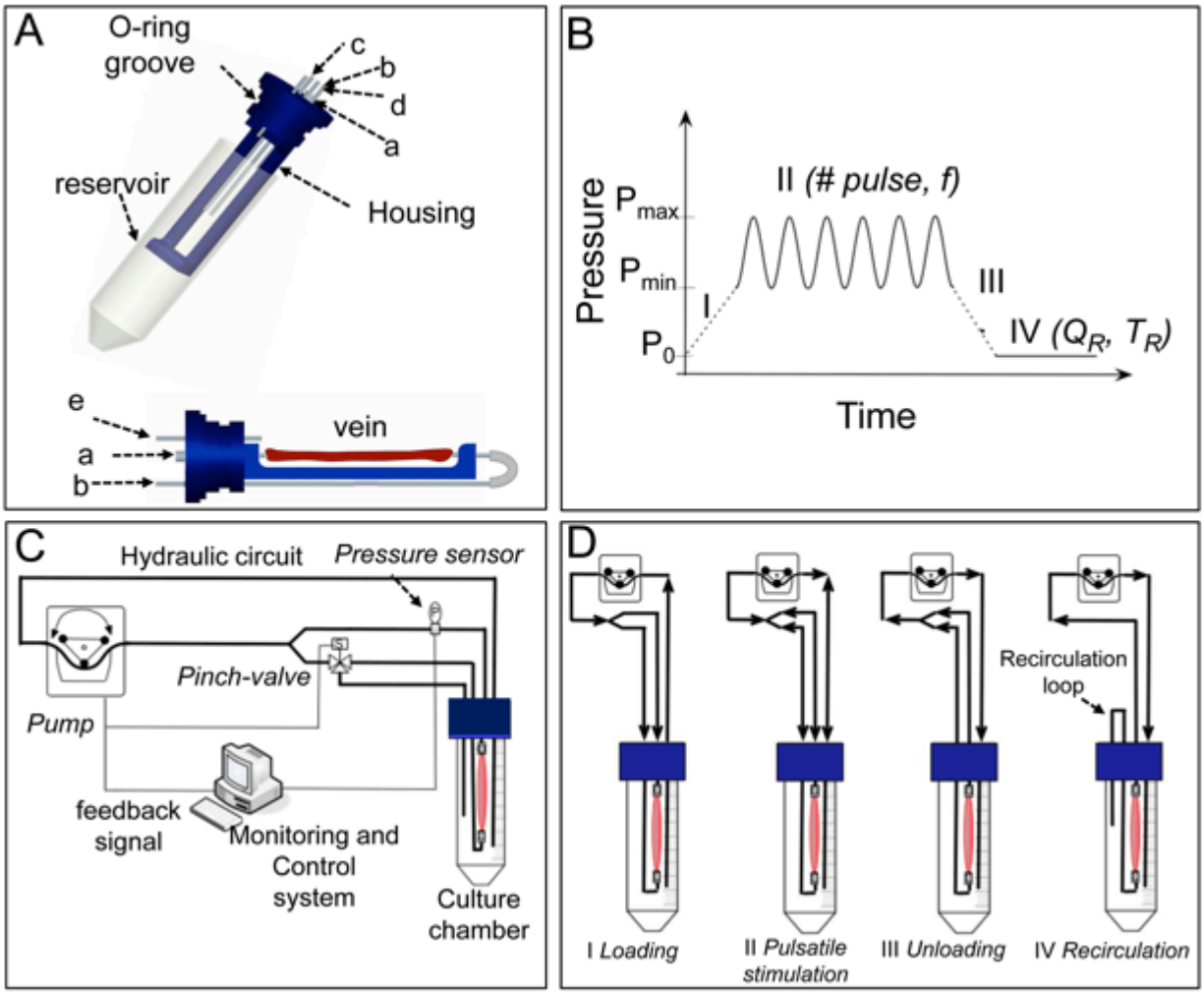

Figure 1. Design of the EVCS. (A) 3D CAD model of the SV culture chamber. The chamber consists of the SV housing inserted into a 50-ml falcon tube which acts as a reservoir; *a* and *b* are the ports for the vessel connection sites; *c* and *d* are the ports for connecting the culture chamber to the hydraulic circuit; *e* is the port for HEPA filter. (B) Schematic diagram of the CABG-PS program. The CABG-PS consists of: I) a loading phase up to the lower limit of a pre-defined pressure range, II) a pulsatile stimulation in a pre-defined pressure range, III) an unloading phase, and IV) a recirculation phase. (C) Layout of the EVCS: thick lines represent the hydraulic circuit; thin lines represent the monitoring and control (M/C) signals. In particular, the M/C system manages the hydraulic actuators (a pump and a solenoid pinch-valve) via a pressure-based signal registered by the pressure sensor. (D) Simplified layout of the flow paths within the EVCS during the CABG-PS cycle. During pulsatile stimulation the medium is forced into and out of the sample through both ends.

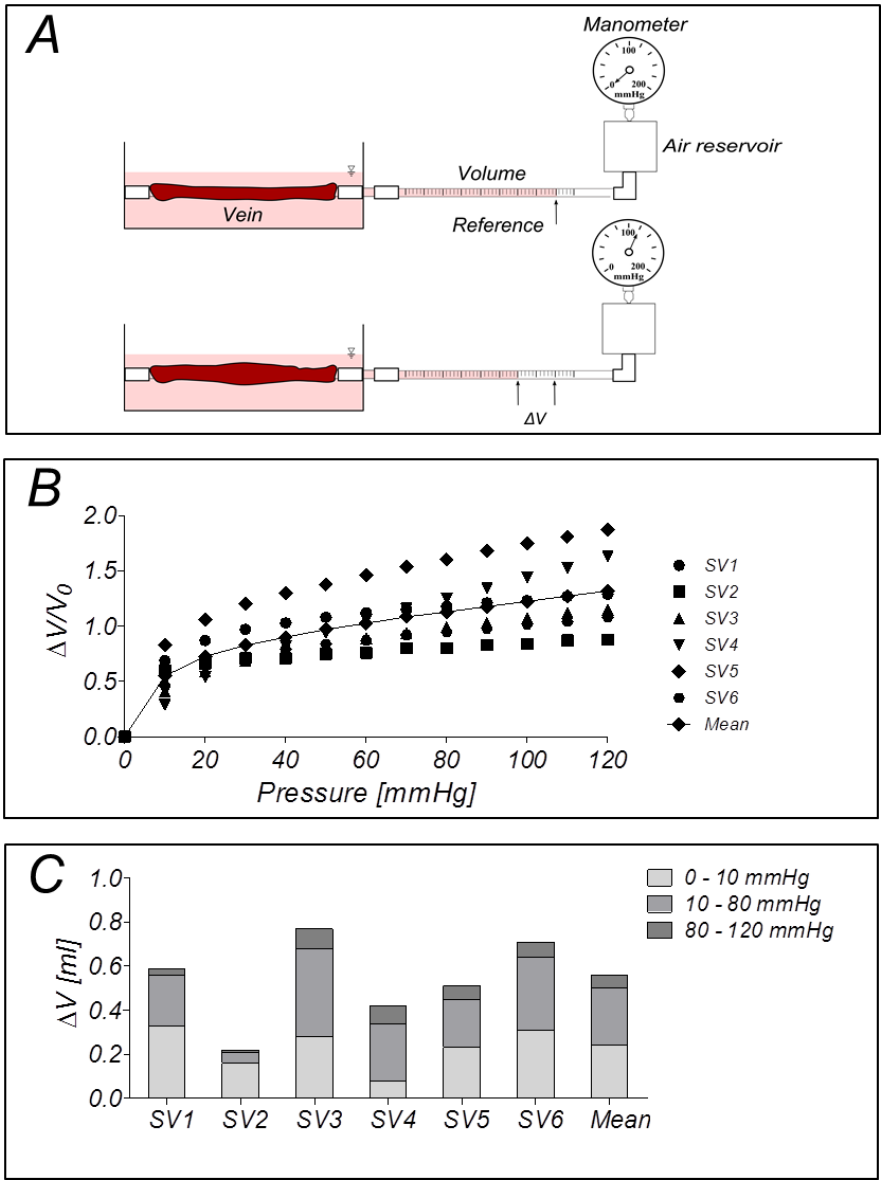

Figure 2. (A) Schematic of the custom made set-up developed for the compliance measurements. Pressure is measured with a manometer and inner volume increments (ΔV) are measured using a graduated pipette. (B) Pressure versus volume relationship for 6 human SVs. An initial rapid volume rise at physiological venous pressure range (0 - 10 mmHg) is followed by a decreasing slope at physiological arterial pressure (80 - 120 mmHg). (C) Representative histogram of the inflation volume necessary to generate pressure ranges (0 – 10 mmHg, 10 – 80 mmHg, and 80 – 120 mmHg) in the vein lumen.

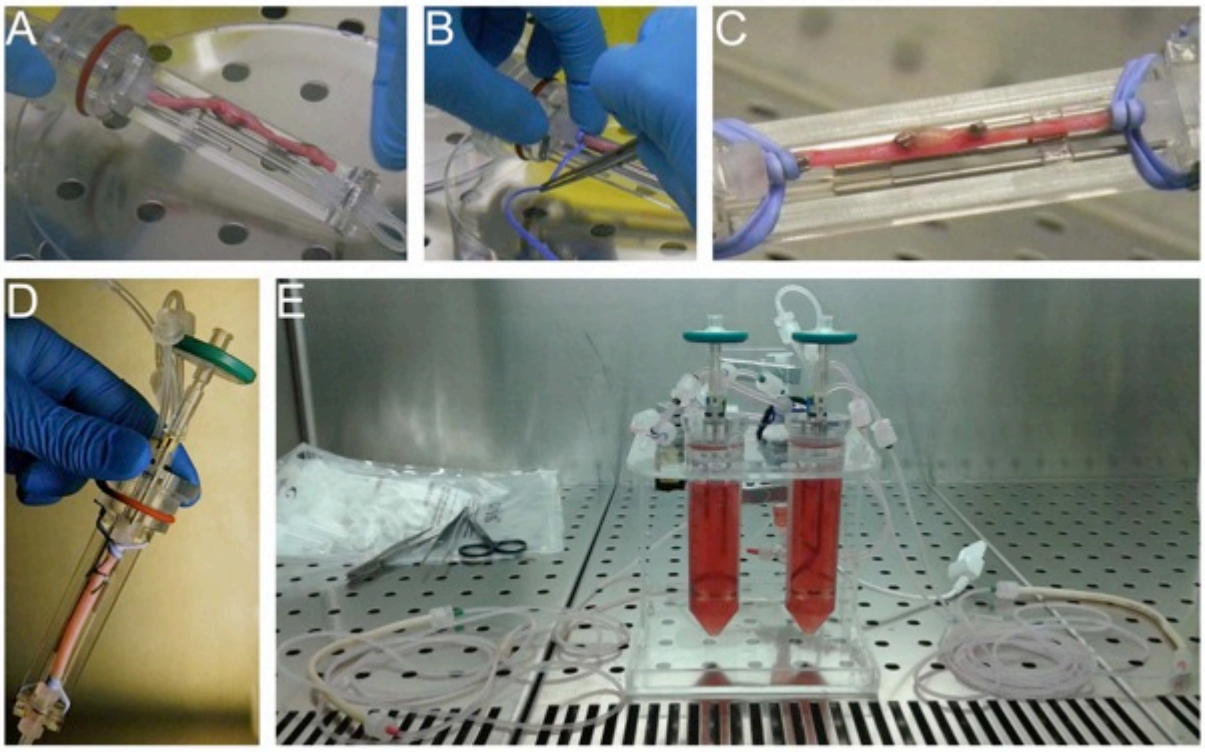

Figure 3. Prototype of the EVCS during the assembling phase. (A) The SV sample is mounted in the housing, and (B) secured via vessel loops. (C) After vessel fixing, (D) the HEPA filter is mounted onto its port, and then the vessel housing is inserted within the reservoir and connected through a silicone O-ring coupling resulting in a compact culture chamber. (E) The culture chamber is then connected to the hydraulic circuit and the entire system is filled with culture medium.

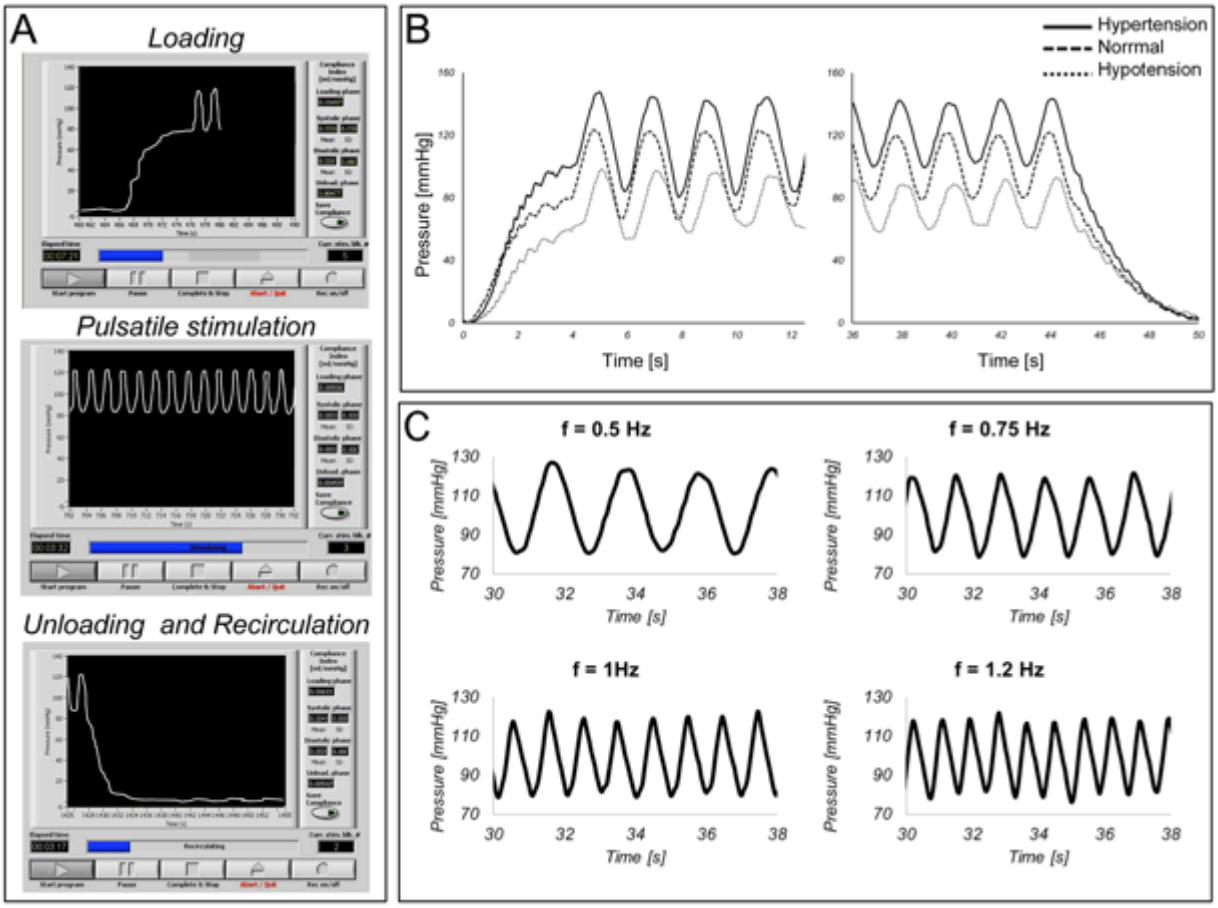

Figure 4. (A) Printout of the resulting pressure tracing during the four steps of CABG-PS cycle. (B) Pressure tracings of CABG-PS cycle of a SV segment obtained changing the pressure within the ranges: 60 – 90 mmHg (Hypotension), 80 – 120 mmHg (Normal), and 100 – 140 mmHg (Hypertension) at a pre-defined pulse frequency of 0.5 Hz. (C) Fragments of CAGB-PS cycle of a SV segment obtained changing the pulse frequency (0.5 Hz, 0.75 Hz, 1 Hz and 1.2 Hz) and applying a Normal pressure range to the vessel.

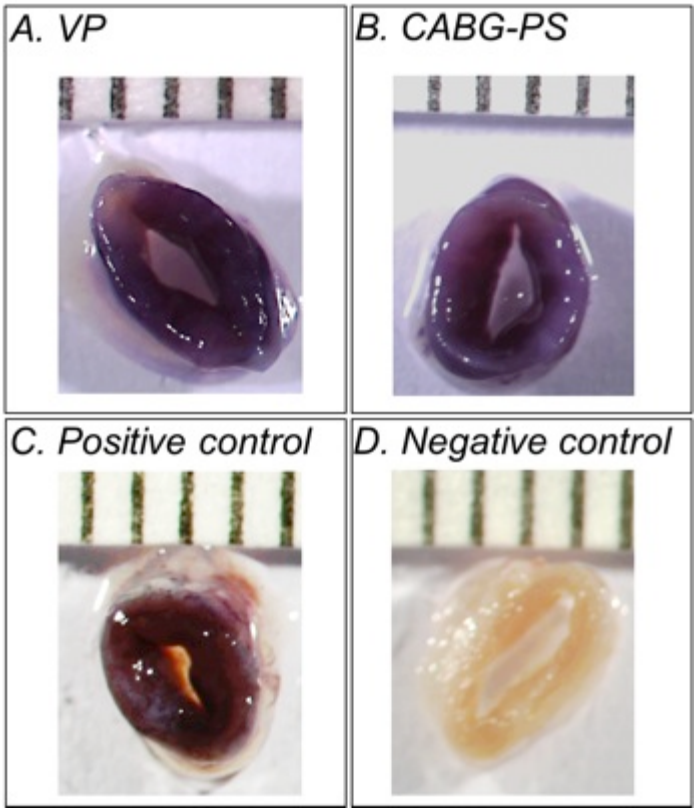

Figure 5. Images of SV rings stained with MTT after 7-days culture in the EVCS under VP (A), and CABG-PS (B) conditions. Fresh tissue and formaldehyde-fixed tissue were used as positive (C) and negative (D) control, respectively. The images in the figure are representative of the behaviour observed for 3 VP and 3 CABG-PS samples and related controls.

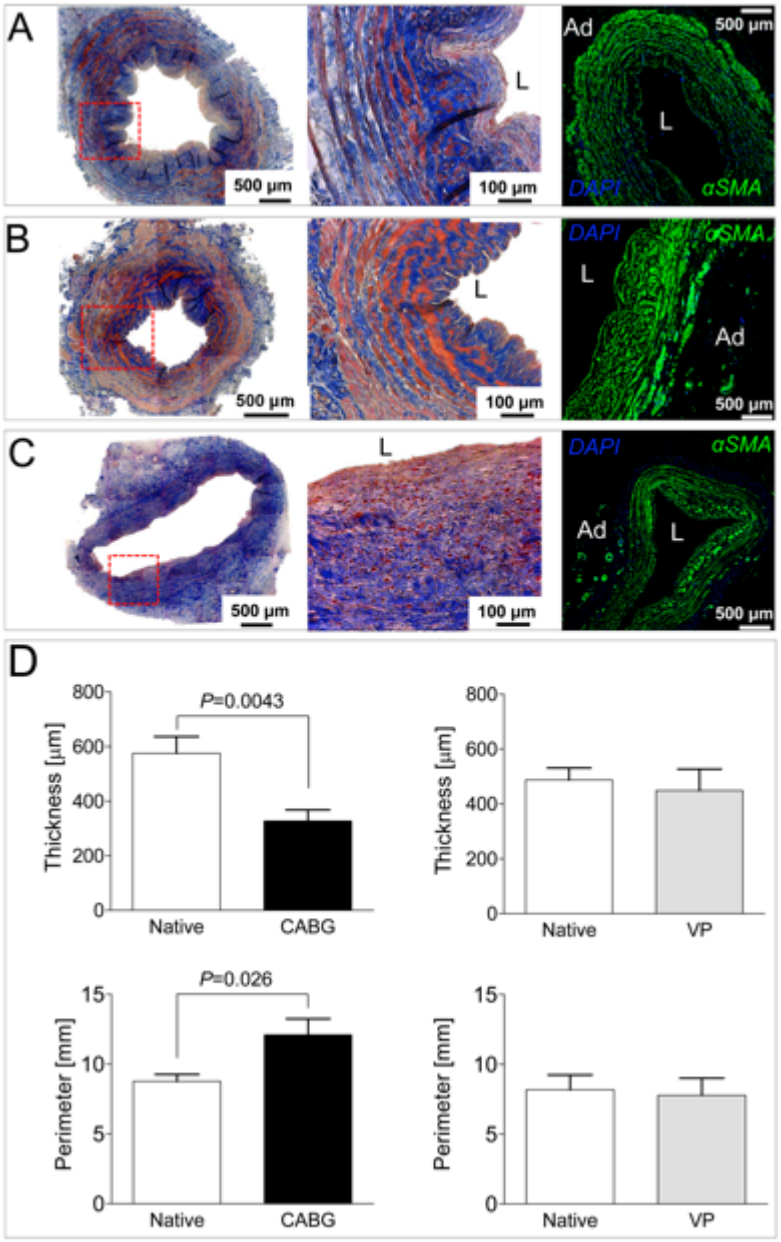

Figure 6. SV tissue sections stained with Masson's trichrome and α-SMA/DAPI after 7-days culture in the EVCS. The images are representative of the behaviour observed for 3 VP and 3 CABG-PS samples and related controls. A major rearrangement of SMCs was observed in CABG-PS vs. VP SVs. In each panel, from left to right, low and high power views of Masson's trichrome stained section and low power views of α-SMA stained sections are shown in native tissue (A), VP-cultured (B), and CABG-PS (C) SV samples, respectively. In all images, L indicates the lumen, while Ad the adventitia layer. (D) Thickness and inner perimeter measurements were performed on SV sections stained with Masson's trichrome staining. Thickness and luminal perimeter data were compared by a non-parametric Mann Whitney test; the *p* value above the bar graph indicates the significance level in these comparisons. The graphs without *p* value indication indicate absence of statistical significance.

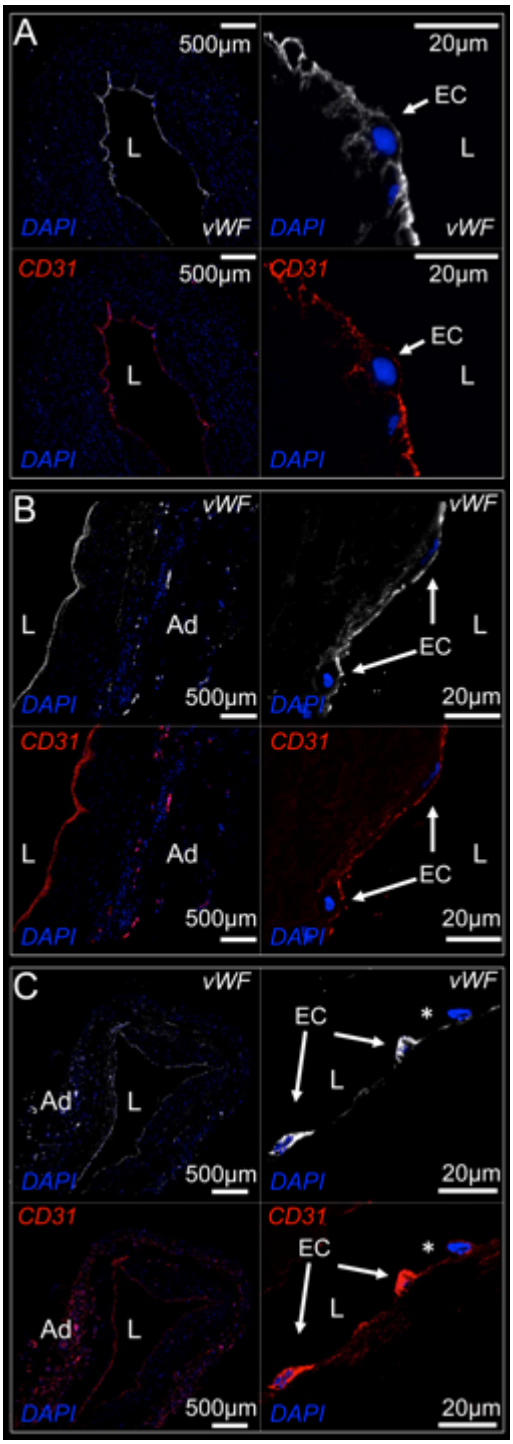

Figure 7. Low and high power views of representative IF images in native (A), VP-cultured (B) and CABG-PS (C) SV samples tissue sections stained with vWF and CD31 specific antibodies. In all panels, L indicates the vessel lumen and Ad the adventitia layer. Arrows in the panel show $vWF^{+}/CD31^{+}$ ECs lining the SV lumen. Note the lower immunostaining in the CABG-like (C) vs. VP (B)/native (A) vessels and evident endothelial cell rupture as indicated by partial detachment of the cell from the basal lamina (*).