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Cell- and Tissue-based mechanosensation in programming and progression of coronary vein graft disease

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“Never say never because limits, like fears, are often just illusions.”

Michael Jordan

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Chapter I: *General Introduction*

"Cardiovascular disease are the number 1 cause of death globally, taking an estimated 17.9 million lives each year"
(World Health Organization, 2020)

Cardiovascular diseases (CVDs) are a group of disorders involving heart and blood vessels, which includes coronary heart disease, cerebrovascular diseases, rheumatic heart disease and other conditions. CVDs are the leading cause of death worldwide, and it is supposed to dominate future trends in global mortality. Four out of five CVDs are due to heart attacks and stroke and one third of these deaths occurs prematurely in people under 70 years of age. The onset of CVDs could be prevented by adopting healthy lifestyle behaviours, such as healthy diet, physical activity, limited use of alcohol and cessation of tobacco use. The effects of behavioural risk factors may show up in individuals as raised blood pressure, high levels of blood glucose and lipids, and overweight and obesity. These factors could result in an increased risk of developing a heart attack, stroke, heart failure and other complications.

Coronary Heart Disease.

Coronary heart disease (CHD), also known as coronary artery disease, is a narrowing of the blood vessels that supply blood and oxygen to the heart. This disease is caused by the build-up of atheromatous plaque in the inner lining of large- and medium-calibre arteries. Plaques consist in an accumulation of cholesterol and other lipid compositions, covered by fibrous cap. On the basis of animal experiments and observations in human specimens, most contemporary schemes of atherogenesis posit an initial qualitative change in the monolayer of endothelial cells that line the inner arterial surface. The normal endothelium does not in general support the binding of white blood cells. However, when subjected to atherogenic stimuli (dyslipidaemia, hypertension or pro-inflammatory mediators), arterial endothelial cells begin to express, on their surface, selective adhesion molecules that bind to various classes of leukocytes [1]. Interestingly, the foci of increased adhesion molecule expression overlap with sites in the arterial tree prone to develop atheroma, in particular at branch points where the endothelial cells experience disturbed flow [2]. Once adherent to endothelium, leukocytes penetrate into the intima, differentiate into tissue macrophages and participate in a

local and perpetual inflammatory response [3]. Inflammatory mediators can augment expression of macrophage scavenger receptors leading to uptake of modified lipoprotein particles and formation of lipid-laden macrophages, called foam cells. As this inflammatory process continues, the activated leukocytes and vascular cells secrete cytokines and growth factors able to promote the migration and proliferation of vascular smooth muscle cells (SMCs) in the intima [4]. These cells have a crucial role in the remodelling of the atherosclerotic plaque: they produce extracellular matrix (ECM) molecules, including interstitial collagen and elastin, and form a fibrous cap that covers the plaque [5]. This cap typically overlies a collection of macrophage-derived foam cells, some of which die, for example due to apoptosis, and release lipids that accumulate extracellularly. The inefficient clearance of dead cells, a process known as efferocytosis, can promote the accumulation of cellular debris and extracellular lipids, forming a lipid-rich pool called the "necrotic" core of the plaque. Atherosclerotic plaques are responsible for clinical manifestations that can be chronic, limiting the blood supply to the heart and leading to tissue ischemia, or acute, resulting from a sudden rupture of the plaque and formation of a thrombus or blood clot. Despite the "aberrant" recruitment of medial SMCs promotes plaque formation, these cells, in advanced state of the

disease, have a beneficial role in preventing the rupture of the cap. Generally, plaques that undergo to the rupture, have thin, collagen-poor fibrous caps with few smooth muscle cells but abundant macrophages [6]. The inflammatory cells may hasten plaque disruption by elaborating collagenolytic enzymes that can degrade collagen, and by generating mediators that provoke the death of SMCs, the source of arterial collagen. Plaque macrophages also produce the pro-coagulant tissue factor that renders the lipid core thrombogenic, providing a powerful substrate for the activation of the coagulation cascade and to induce thrombus formation [6]. Although blood flow continues over the lesion, microemboli of plaque material and thrombus may be washed away, leading to distal embolization of the myocardium [7]. Therefore, different clinical manifestations could occur, depending on the specific blood vessel involved: clot can block the blood vessel that feeds the heart causing a heart attack; otherwise, it could affect a blood vessel that feeds the brain provoking a stroke. The interruption of blood supply to the arms or legs could cause difficulty in walking and, eventually, also gangrene [8].

Treatment of Coronary Heart Disease.

There is no definitive cure for atherosclerosis, but treatments that can slow the worsening of the disease. The major treatment goal is to prevent significant narrowing of the arteries so that symptoms never develop and vital organs are never damaged. With this aim, the frontline primary prevention strategy is to follow a healthy lifestyle; secondary pharmacologic treatments may then follow. To slow down the progression of the disease and consequently thrombus formation, it is important to maintain the normal function and continuity of the endothelium of the arteries. This can be achieved with drugs that reduce cholesterol levels and blood pressure and, at the same time, exert direct effects on the endothelium [9]. The guidelines on the dyslipidemia treatment provide the use of statin (HMG-CoA reductase inhibitor) in combination with ezetimibe and, in high-risk patients, the treatment with a proprotein convertase subtilisin/kexin type 9 (PCSK-9) inhibitor [10]. However, its long-term efficiency and safety still needs to be proven and costs may limit its practical use. Medications are prescribed to reduce the risk of death by reducing the risk of heart attack, stroke, and heart failure. For example, antianginal medications (such as beta-blockers, nitroglycerin and

calcium channel blockers) reduce the amount of oxygen that the heart requires and/or increase the blood flow through the arteries, resolving the symptoms of angina [11]. These medications are indicated for patients with coronary narrowing that do not limit blood flow, but are equally important for those patients undergoing coronary revascularization with angioplasty, stenting or bypass surgery. Indeed, the optimal therapeutic strategy depends largely on the severity of disease. In patients with coronary atherosclerosis that limits blood flow in the coronary arteries, percutaneous coronary intervention (PCI) can be proposed. In patients with multiple areas of coronary artery narrowing or blockage, coronary bypass grafting (CABG) remains the only choice to restore sufficient perfusion of the downstream myocardial tissue [12].

Coronary artery bypass.

Coronary artery bypass graft surgery is among the most frequently performed surgical procedure for revascularization in patients with multivessel coronary heart disease [13]. The surgical technique of the bypass is performed under general anaesthesia and with the support of extracorporeal circulation, representing thus an invasive procedure. Although it has been used for more

than 40 years, no detailed guidelines on the choice of coronary bypass grafting conduits have been published to date. In CABG surgery, the preferred vascular conduits are autologous arteries that are harvested from the patient itself during the intervention. Autologous saphenous vein is also widely used as a conduit to bypass atherosclerotic lesions in coronary arteries. The effectiveness of CABG in relieving symptoms and prolonging life is directly related to graft patency. Because arterial and venous grafts have different patency rates and modes of failure, conduit selection is important in determining the long-term efficacy of CABG. The arterial conduits used for myocardial revascularization are the internal mammary artery (IMA) and left internal mammary artery (LIMA), which are the ideal conduits for their ease of use. Indeed, the advantage of the saphenous vein (SV) derives from its higher length and the ease of harvesting from the patient legs. Despite SV segments are liable to stenosis within relatively short time after implantation, their employment as bypass conduits continues due to the use of multiple grafting procedures, technical ease of harvesting/manipulation and increasing need of redo surgery [14].

Complications of saphenous vein grafts: Vein Graft Disease.

Within one year of CABG surgery, 10% to 15% of venous grafts occlude, and almost half of venous graft conduits fails at 10 years [15]. SV grafts normally fail at short- or at mid/long-term. The first can occur prematurely within the first week after grafting, due to the formation of a thrombus that obstructs the lumen of the vessel. This event is the consequence of the endothelial denudation of the vessel conduit that exposes the subendothelial layer to platelets with a consequent activation of the coagulation cascade. Mid/long-term graft stenosis is instead caused by modification of the graft biology, which is exposed to an altered flow with a high luminal shear stress, different oxygen content and high/pulsatile pressure. While the first causes of SV graft failure can be circumvented in most of the cases by anti-coagulation therapy, the biological mechanisms leading to SV graft stenosis cannot be controlled, if not indirectly, by maintaining a low blood pressure and low cholesterol level.

Indeed, the biological mechanisms involved in late bypass occlusion are parts of a complex process, which determines several events: inflammatory responses due to invasion of monocytes; the remodelling and the production of new extracellular matrix; activation,

migration and proliferation of SMCs, particularly in the sub endothelial layer of the graft with progressive intimal thickening and stenosis of the conduit. This pathological condition is known as Vein Graft Disease (VGD), and it is the principal cause of recurrent cardiac ischemia in patients. This requires stent implantation and, in most severe cases, re-intervention.

Several strategies aim at improving vein graft patency. Preoperative measures to avoid graft failure include adequate physiological and anatomic characterization of coronary lesions in the cardiac catheterization laboratory. During surgery, instead, different procedures may be adopted to reduce early graft failure: avoidance of distention, no-touch technique, and minimizing trauma to the graft handling during harvesting and implantation. However, none of these options have a significant effects on insurgence of late graft failure [16]. A large number of pharmacological interventions have been investigated for the prevention of VGD. Since hypercholesterolemia is associated with graft failure, aggressive lipid-lowering therapy with statins has shown significant success in preventing neointimal hyperplasia in CABG surgery [17]. Antithrombotic therapy has the potential to reduce thrombotic bypass graft occlusion and may also prevent either distal embolization, either associated ischemic myocardial injury [18]. Evidences has demonstrated that

aspirin reduces thrombosis-induced early graft failure via an antiplatelet action, but it seems to have minimal effect on long- term patency and it does not inhibit the development of neointimal hyperplasia [19]. In this context, the results of large randomized trials investigating the employment of aspirin and of lipid-lowering agents (e.g. statins) to prevent graft have shown these drugs not only extend early and late graft patency, but also have beneficial effects on native coronary atherosclerosis [20]. Unfortunately, a substantial proportion of CABG patients (>50%) are relatively resistant to aspirin and may therefore be more prone to VGD [21]. Subsequent studies that combined an anticoagulant with aspirin to prevent VGD and post- operative ischemic events resulted inconclusive or negative [22].

The involvement of various cellular pathways in vein graft disease has been addressed by gene therapy approaches. The underlying idea was to exploit the transient availability of the vessel out of the patient's body and proceed with the treatment with gene transferring agents (plasmids, viral vectors) able to reduce the impact of vein graft disease after vein implantation [23]. For example, these studies have addressed the role of metalloproteinases and their inhibitors. TIMP-1 or -2 gene transfer resulted in the reduction of neointimal lesions of rat carotid arteries after balloon injury [24]. It has been

demonstrated also that, in the mouse model of vein graft failure induced by grafting vena cava into carotid interposition, TIMP-2 gene transfer significantly reduced the diameter of vein graft *in vivo* [25]. This involves the inhibition of MMP activities, which are essential for smooth muscle cells to remodel the extracellular matrix and migrate in response to injury [26]. Several recent studies have finally identified microRNAs that are differently regulated during VGD. For example, miR-21 levels were elevated following engraftment and genetic ablation of miR-21 in mice dramatically reduced neointima formation [27]. Up to date, the only randomized controlled trial available involving gene modulation in CABG surgery is the PREVENT-IV study [28]. This trial included 3014 patients and studied the role of edifoligide, an inhibitor of transcription factor E2F responsible for upregulating several genes believed to play a key role in the initiation of neointima hyperplasia. The primary efficacy endpoint of the study was angiographic VGF at 12 to 18 months after CABG surgery. Unfortunately, edifoligide was no more effective than placebo in preventing VGF.

Hemodynamic forces contribute to vein graft failure.

A crucial component contributing to vein graft failure is the change in the pressure load and cyclic strain consequent to arterialization of the saphenous vein. In fact, under normal conditions, the saphenous vein is subject to a quasi-steady flow patterns that are associated to very low shear stresses (0.1-0.6 Pa) and constant pressure loads (5-10 mmHg). By contrast, after CABG, veins experience a fast pulsatile flow, which is supposed to cause an adaptive remodelling in the vessel wall, leading to progressive bypass occlusion [29]. Vascular tissues are subjected to several mechanical forces: shear stress, acting principally on endothelial cells and influencing their orientation in the direction of flow, luminal pressure, wall strain, mainly in the circumferential direction, and longitudinal tension. Clearly, all these stimuli may have important effects on the structure of the venous conduits and may determine profound alterations in their mechanical characteristics either at the time of their surgical preparation, either following implantation in coronary position. The action of mechanical forces on blood vessels has been for long time uniquely associated to the consequences of blood flow sensed at the free surface of endothelial cells paving the lumen [30]. Indeed, 'physiologic' (laminar) shear stress has

a generalized vasoprotective role via, e.g., release of nitric oxide by endothelial cells (ECs) or factors (e.g. prostacyclin, PDGF-BB and TGF-β1) controlling the vascular tone and maintaining a quiescent smooth muscle cellular phenotype [31]. By contrast, transition from laminar to turbulent (oscillatory) shear stress disturbs vascular homeostasis, and leads to changes in vascular tone, permeability and, ultimately, atherosclerotic plaque development or neointima accumulation [32]. The action of mechanical force in blood vessels is not, however, limited to perception of the flow by endothelial cells. In fact, cells present in other vascular districts, such as the smooth muscle cells (SMCs) in the media in arteries and veins, are able to sense the tissue mechanics through the ability to respond to elongation and compression forces propagated in the vascular wall through the extracellular matrix [33]. Given that variations in physical characteristics of the surrounding extracellular matrix (ECM) can lead SMCs to reversibly transit from quiescent (contractile) to secretory phenotypes [34] and EC cells are subject to direct flow-controlled transcriptional regulation [35], the maladaptive remodelling of blood vessels is likely the result from the combination of different forces acting all in concert.

Mechanical stimulation is an integral component of the cardiovascular developmental process. Hemodynamic

forces have an important role in development of primitive vessels, which undergo remodelling in the presence of the flow forming a branched, hierarchically organized network of large and small-caliber vessels delivering blood to the embryo [36]. Paradoxically, at the earliest developmental stages, the mechanical component of the blood flow could be even more important than the oxygen and nutrients supply for the tissues. In fact, several studies show that blood flow is necessary for vessel and cardiac morphogenesis independent of the trophic function [37]. In addition to controlling morphogenesis and homeostasis of the vascular system, mechanical factors can also accelerate vascular dysfunction and pathology consequent to metabolic, genetic or inflammatory-related conditions. For example, endothelial cells are highly responsive to modifications in flow patterns, and this maintains the fluid shear stress within a desired range [38]. Variations in shear stress are sensed by mechano-receptors (e.g. integrins) expressed by endothelial cells, and downstream signalling cascades are activated to induce rapid changes in cytoskeleton structure and triggering specific gene-expression programs as well [39]. The consequence is the synthesis/release of vasoactive mediators reducing shear stress, of ECM remodelling enzymes promoting vascular wall repair, and of growth factors controlling SMCs survival and proliferation [40]. This

is a compensatory process needed to maintain an anti-proliferative, anti-thrombotic and anti-inflammatory phenotype of the endothelium. A hypothesis for intracellular transduction of flow-related mechanical stimulation involves the function of the primary cilia. Primary cilia are cellular protrusions composed of microtubules connected to cytoskeleton. Endothelial cells have abundant cilia in regions subjected to low shear stress or disturbed blood flow, while they are absent in regions with high laminar shear stress [41]. While shear stress induces endothelial cell differentiation, there is evidence that the cyclic strain, generated by the pulsatile nature of blow flow, is responsible for maintaining smooth muscle cells within the wall in an active, contractile rather than in a secretory/proliferating status [42]. Moreover, cyclic strain may play a similar role in ensuring the quiescence of resident progenitor cells. In particular, SMCs sense this oscillatory pressure by cyclic compression and strain forces, depending on their geometrical position inside the vessel. The orientation of SMCs is not fixed but adaptable in response to the magnitude of strain [43]. Adaptation to an elevated pulsatile flow promotes also extracellular matrix-regulated SMC migration and proliferation. A prerequisite for SMC migration is the degradation of ECM through secretion of metalloproteases (MMPs) from SMCs [44]. Hemodynamic variations are known to regulate MMPs

expression and activation [45]. In a murine model of blood flow cessation, interruption of the flow in carotid arteries caused MMP-9 upregulation and arterial enlargements, and this was reverted using a nonselective MMP inhibitor [46]. Variations in the composition of ECM also change the response of SMCs to mechanical strain, suggesting that specific patterns of matrix-integrin engagement orchestrate downstream intracellular signalling pathways [47]. This results in a phenotypic switching of SMC, from the contractile state to the synthetic/proliferative. This process is a hallmark of intimal hyperplasia in saphenous vein grafts [48].

Altered cell mechanosensing may regulate the pathological programming of vascular cells.

All cells and tissues in multicellular organisms are continuously subjected to mechanical stresses. These forces have various origins, from pressure forces linked to gravity, to forces related to motion (e.g. muscle contraction, blood flow). It is now accepted that these applied forces are able to modify cellular behaviour by affecting transcriptional cell machinery and driving either the cell fate specification either differentiation [49]. Under

physiological conditions, the responses of the cells to biochemical signals, such as growth factors and small molecules, may be then the result of a cooperation with 'physical' signalling, instructed by the mechanical characteristics of the tissue, such as the elasticity or the higher or lower amplitude of strain forces, transmitted to the cytoskeleton by the surrounding extracellular matrix.

Mechanotransduction is a general modality of intracellular signal transduction, not only related to motion, but also to position information that is transmitted through acquisition of specific geometric features and/or traction/compression forces transmitted from neighbouring cells [50]. Reciprocally, forces generated inside the cell by the tension produced by the actomyosin cytoskeleton are transmitted through adhesion sites to surrounding structures. Thus, the cytoskeleton rapidly senses and adapts to changes in the mechanical properties of the microenvironment. Among the major cellular elements that are involved in mechanosensing, there are the sites of cell-ECM and cell-cell adhesion. At a molecular level, cell-ECM adhesion is mediated by integrins, which are the main substrate adhesion receptors and provide a direct physical link between the ECM and cytoskeletal adaptors, thereby connecting the ECM with the actin cytoskeleton. The relationship between filamentous actin (F-actin) and the ECM is reciprocal: integrins promote bundling of actin

filaments to generate tension within a cell and, at the same time, the activity of actin regulatory molecules, the rate of actin polymerization/spatial organization affects integrin function, and thereby the adhesive state of cell [51]. The structure and dynamics of the actomyosin cytoskeleton are regulated by the Rho family of GTPases. Inhibition of Rho and the downstream kinases ROCK and MLCK causes a generalized depolymerisation of the F-actin cell tensioning apparatus that converts cells into a state of low tensile forces [52]. This establishes a bi-univocal relationship between the degree of cell spreading and the tensioning of the cytoskeleton, with a higher actomyosin traction forces in spread cells *vs*. rounded cells attached to ECM through looser connections [53]. The importance of substrate compliance in stem cell growth and pluripotency has been recently demonstrated by establishing a link between the cytoskeleton tensioning and the activity of the YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) transcriptional coactivator duo with a consequent relationship between gene expression and mechanical and cytoskeletal signalling [54].

YAP/TAZ are transcriptional coactivators that shuttle between the cytoplasm and the nucleus where they associate with several promoter-specific transcription factors. TEA domain family member (TEAD) transcription

factors emerged as the main partners of YAP and TAZ on DNA, although RUNX2, T-box5 (TBX5) and p73 have also been reported to interact with YAP and TAZ to regulate gene expression [55]. These transcriptional factors have key roles in cell proliferation, survival, differentiation, tissue regeneration and organ size determination. The best characterized regulators of YAP/TAZ are the components of the Hippo pathway. The Hippo pathway becomes activated when MST kinase phosphorylates LATS proteins, which then phosphorylate YAP and TAZ on multiple sites. Phosphorylated YAP and TAZ are excluded from the nucleus and accumulate in the cytoplasm, where they are degraded by the proteasome. When the Hippo pathway is inactivated, YAP and TAZ are dephosphorylated, accumulate in the nucleus and regulate gene transcription together with DNA-binding transcription factors such as TEAD [56]. YAP/TAZ nuclear translocation is also controlled by the activity of the Rho/ROCK kinase. In particular, YAP/TAZ activity requires stress fibres and cytoskeletal tension induced by ECM stiffness and cell spreading and it results inactive when Rho is inhibited. Dupont S. et al. [57] have been demonstrated that the inhibition of Rho and of actin cytoskeleton also inhibited YAP/TAZ transcriptional activity. Conversely, triggering F-actin polymerization and stress fibres formation by overexpression of activated diaphanous protein promoted YAP/TAZ activity.

Extracellular forces promote cell-ECM adhesions via integrins and the development of intracellular contractile F-actin structures containing myosin molecules. This event is regulated by a bidirectional signalling between Rho-ROCK, integrins and myosin activity. In fact, F-actin may sequester the YAP/TAZ inhibitor factor (LATS1-LATS2) or promote its post-translational modification and this, in turn, prevents the interaction with the transcription factor. When cells are in contact with soft substrates or are confined onto small areas, LATS1 and LATS2 are released or activated by the remodelling of actin cytoskeleton [58]. This is supported by the evidence that Rho inhibition activates LATS, contributing to complete nuclear exclusion of YAP and TAZ [59]. In summary, the mechanical forces induce a rearrangement in the cytoskeletal of the cell, which results into a force-specific changes in gene expression through the activity of YAP/TAZ transcription factors with consequences for cell differentiation and, potentially pathologic commitment.

Vessel-resident and vessel-extrinsic players in the Vein Graft Disease.

Intimal hyperplasia in VGD could be widely considered as a maladaptive repair process initiated by a combination of metabolic, inflammatory and mechanical cues caused by

vein graft manipulation and implantation into a non-physiologic environment. Indeed, the shift of the flow dynamics toward the pulsatile coronary pressure pattern and the exposure to arterial oxygen content may be sensed by vein-resident and vessel-extrinsic cells as a primary vascular damage signal.

Among vessel-extrinsic cells involved in VGD, circulating progenitors seem to participate to neointima accumulation through an active homing process related to the post-grafting vascular inflammation. Based on the evidences that bone marrow progenitor cells can differentiate into different vascular lineages, such as endothelial cells and SMCs [60], animal models of vein transplant vasculopathy have shown that bone marrow cells may give rise to a substantial percentage of VSMCs that contribute to arterial remodelling [61]. For example, Boehm M. et al have found that p27Kip1, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors, directly regulates the proliferation of bone marrow-derived cells that migrate into damaged blood vessels and reconstitute vascular lesions [62]. These findings are also in agreement with those of Xu et al., who showed that, by transplanting vein conduits in mice engrafted with TIE2-LacZ bone marrow cells, the endothelial cells layer of arterialized vein grafts originated from BM-derived

circulating monocytes but not from the remaining endothelial [63].

Endothelial cells are highly responsive to modifications in flow patterns and they proliferate and migrate to "reline" the endothelial layer damaged due to surgical manipulation of the graft. However, the newly formed endothelium is functionally compromised and increases the risk for early thrombosis, promoting platelet adhesion and inflammation [64]. This process of re-endothelialization in the early post-operative period is crucial for the vessel patency. In the subsequent 4 to 12 weeks, a complete matrix remodelling of the vessel wall accompanied by an increased wall stiffening occurs in the graft. Neointima formation starts with SMCs activation and recruiting vessels-resident/extrinsic progenitors. Indeed, SMCs, shortly after vein implantation into arterial position, loose their typical contractile phenotype, become secretory cells and invade the intima layer, leading to a progressive reduction of the vascular lumen [65]. Within the native vein, in their physiological environment, SMCs predominantly exist in a quiescent phenotype and regulate contraction and blood pressure. Unlike either skeletal or cardiac myocytes that are terminally differentiated, SMC retain remarkable plasticity and can undergo reversible phenotypic changes in response to modifications in local environmental cues. After different stimuli, including hypoxia and vein

arterialization, indeed, SMCs lose their contractile functions, show an increased rate of proliferation and migration and enhanced ECM and inflammatory cytokines production [65]. This contributes to the development of the intimal hyperplastic lesion in the vessel wall and progressive stenosis of the graft.

SMCs are not the only players in VGD. In fact, adventitial fibroblast cells (AFs) may play an important role for neointima formation. In porcine models of balloon injury, AFs are converted into myofibroblasts and are involved in the wall repair process through abundant ECM deposition, responsible for adventitial thickening. The adventitia is an important structural component of the vessel, consisting of a loose ECM network containing fibroblasts, blood and lymphatic vessels, nerves, progenitor and immune cells. Adventitial cells are able to sense and directly response to a stimuli through reciprocal communication with other adventitial cells or with cell of the neighbouring vascular layers. In response to hormonal, inflammatory, and environmental stresses, resident adventitial cells are often the first vascular wall cells to exhibit evidence of "activation" by increase in cell proliferation, upregulation of ECM proteins, as well as by the secretion of chemokines, cytokines and angiogenic factors able to directly affect resident vascular wall cell phenotype and regulate *vasa vasorum* expansion [66]. Given the function of the

adventitia as a homeostatic element for the integrity and the functioning of the vessels, it has been hypothesised that activation of these cells due to vascular insults may have an impact on the progression of VGD. Experimental evidences demonstrate that the adventitial layer, in both the developing and adult blood vessels, serves as a niche for stem and progenitor cells, including endothelial progenitor cells, mesenchymal progenitor cells, smooth muscle and pericyte progenitors, mesenchymal stem cells and mesoangioblasts [67]. The presence of resident progenitor cells in the adventitia of various vessels derives from the embryonic origins of pluripotent cells with mesoangioblast characteristics, which maintain a high level of mesenchymal and vascular differentiation capacity in cells located in association with the vascular wall [68]. This supports the hypothesis that distinct progenitor cell subtypes reside, even in the adult life, in the vessels and may play a role in various pathogenic events, thus serving for postnatal vasculogenesis, tumour vascularization and local immune responses [67]. For example, it was described that a population of adventitial progenitors, expressing Sca-1+, cKit, CD34 and Flk-1 markers, participated to lesion formation and progression in mouse models of vein graft disease by migrating from the adventitia to the media and the intima layers [69]. This result is consistent with the finding that about 60% of SMCs in the

atherosclerotic lesions of vein grafts were derived from the vessel wall, with the remaining 40% originating from the circulating monocytes [70]. The existence of progenitor stem cells in the adventitia of human saphenous vein has been recently demonstrated [71]. These cells, the so-called Saphenous Vein Progenitors (SVPs), express Desmin, Vimentin, NG2 and PDGFRβ together with mesenchymal (CD44, CD90, CD73, CD29) and stemness antigens (Oct-4 and Sox-2) and are located in perivascular zone of *vasa vasorum*. SVPs showed to have proangiogenic capacity. Transplantation into mouse ischemic limbs proved to be superior to a comparable dose of circulating proangiogenic cells in supporting neo-vascularization. The benefit was attributed to enhancement of reparative angiogenesis by a yet incompletely understood interaction between SVPs and endothelial cells [72]. The ability of SVPs to contribute to ischemic tissues recovery has been demonstrated also in a mouse model of myocardial ischemia. Transplanted cells improved myocardial recovery mainly by promoting reparative angiogenesis with a biphasic modality, consisting in early increase in capillaries and arterioles density and late potentiation of arteriole formation [73].

Taken together, these evidences show that human SVPs are potent inducers of reparative vascularization and cardiac healing through an integrated mechanism that

involves reciprocal interactions between donor cells and the ischemic environment. It is not yet known whether SVPs are also pathologic cells whose *in situ* activation in response to SV insults determined by surgical manipulation and implantation into coronary position, contributes to the progression of vein graft disease.

Scope of the thesis.

The objective of the work contained in the present thesis was to understand the cellular and molecular basis of intima hyperplasia associated to VGD, using a yet un-attempted multidisciplinary approach focused on mechanobiology.

To achieve this goal an *ex vivo* approach was carried out to perform biomechanical conditioning of human saphenous vein segments. This work took advantage from the design of an *ex vivo* vein culture system able to replicate the altered pressure pattern experienced by saphenous vein after CABG (80-120 mmHg) and the physiologic venous-like perfusion (3 ml/min, 5 mmHg) [74].

Chapter II describes the results of an investigation performed with this bioreactor indicating the relevance of a still unrelated matricellular component in VGD, while Chapter III illustrates in more details the effect of *in vitro* mechanical stimulation of human SV advential progenitors with the validation of the YAP/TAZ dependent pathway as a pathologic effector.

The translational outcome of this project is to prevent the onset of intima hyperplasia in SV grafts by treating vessels with drugs impacting on these two aspects of mechanical responsivity of the vessel-resident cells.

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Chapter II: Coronary artery mechanics induces human SV remodelling

Coronary artery mechanics induces human saphenous vein remodelling via recruitment of adventitial myofibroblast-like cells mediated by Thrombospondin-1

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Introduction.

Coronary artery bypass grafting is used in cardiac surgery for more than 50 years to combat the consequences of coronary artery disease [1-3], a pathology with a wide incidence in Western world (> 1000 cases/ 106 adult people) and rapidly increasing in emerging countries – e.g. China [4]. The most represented coronary-compatible vessels used in CABG are the internal mammary artery (IMA), the radial artery (RA) and the SV. The patency of arterial grafts is relatively well preserved, while the SV conduits are subject to intima hyperplasia determining progressive graft occlusion. In a high percentage of cases, this requires re-hospitalization with graft stenting and, ultimately, re-intervention [1, 2]. More in details, early vein graft failure due to acute thrombosis occurs in as many as 18% of cases. Intermediate graft failure (up to 2 years after surgery), and late graft failure (> 2 years after surgery), occurs in 20% to 50% of cases at 5 years. Finally, by 10 years after surgery, 40% of grafts result completely blocked and a further 30% have a compromised flow. Even if early remodelling of the vein is predictive for later graft patency, the aetiology of long-term failure is still poorly understood [5]. Initially, both the surgical procedure and exposure to high flow and pressure compromise the endothelial layer,

which induce SMCs proliferation [6, 7], changes in matrix composition, and thickening/stiffening of the vessel wall. This limits the vein's adaptability to the arterial circulation and ultimately leads to clinically apparent stenosis [8]. Animal models revealed that occlusion of autologous SV grafts is a consequence of neointima formation, driven by proliferation and migration of SMCs and possibly of adventitial progenitors into the intima [9], inducing extracellular matrix (ECM) deposition, and formation of a thick neointima [10].

The role of mechanical forces in the progression of graft failure has been recognized, although the nature of cell-based mechanosensing in the vascular tissue remains unclear due the difficulty of decoupling distinct components *in vivo* [11, 12]. In fact, the existing studies have not yielded conclusive results on the effect of mechanical stimulation on SMC growth and phenotype, with results depending on direction, frequency, duration and modality of the stimulus, but also on the origin and initial phenotype of the SMCs. For example, cyclic strain of human SV-derived SMCs resulted in increased DNA synthesis and cell number with a decrease in smooth muscle a-actin (aSMA) [13-15], while cells of arterial origin showed opposite effects [16, 17]. *Ex vivo* vessel culture systems (EVCSs) and bioreactors to stimulate cells mechanically offer the unique possibility to investigate the

effects of isolated or combined mechanical stimuli under well-controlled and reproducible biomechanical and/or metabolic conditions in human large vessels. In this framework, the aim of the present investigation was to characterize the effect of coronary mechanical conditions on molecular programming of vein graft disease using an integrated tissue/cell biomechanical approach.

Material and Methods.

Extended Methods are provided in the Methods section in the online only Data Supplement.

1. Ethics

The experimental investigation on human-derived tissues and cells was approved by the local ethical Committee at Centro Cardiologico Monzino, IRCCS. Patients were required to sign an informed consent. The use of human material was done in compliance with the Declaration of Helsinki. The main patient characteristics are shown in Table S1. Arteriovenous bypass procedures in pigs were performed in female Landrace or Large White/Landrace crossbred pigs weighing 20 to 30 kg. All animals received humane care in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 and the Guide for the Care and Use of Laboratory Animals published by the US

National Institutes of Health (NIH Publication No. 85- 23, revised 1996). For surgery, anaesthesia was induced with a single dose of intramuscular ketamine into the neck (0.1 mg/Kg ketamine: Ketaset Injection Fort Dodge Animal Health Ltd, Southampton, UK). After endotracheal intubation, anaesthesia was maintained using 2-3% halothane and oxygen, the animals ventilating spontaneously throughout. Animals were euthanized with 100 mg/Kg intracardiac injection of pentobarbitone in a single dose (Euthatal; 200 mg/mL pentobarbital sodium, J.M. Loveridge Plc, Southampton, UK).

2. Tissue/cells mechanical stimulations

Mechanical stimulations of SV grafts was performed using a custom-made bioreactor [18] tailored to reproduce the coronary mechanics. Cell straining was performed with Flex-Cell system. Mechanical stimulation times ranged from 7 to 14 days for SV grafts and from 1 to 3 days for cells.

3. Tissue/cells analyses

After mechanical stimulation, tissues, cells and culture supernatants were prepared and appropriately processed for histological, immunohistochemistry, immunofluorescence, protein/secretome and RNA analyses, as already published [19], and described in the extended online methods.

4. In vitro cell culture

Isolation of cells for in vitro experiments was performed as previously described [20, 21], using immunomagnetic and/or plastic adherence selection. Migration experiments were performed using Transwell-based assays followed by quantification of Crystal-Violet cell staining.

5. Molecular analyses

Tissue/cells RNA and protein content was analysed by Q-RT-PCR and Western blotting performed with protocols already published [19], while the secretome analysis was conducted using a MALDI-TOF methods as already described [22].

6. Data representation and statistical analyses

In all graphs throughout the manuscript, data were plotted as mean ± standard error using GraphPad Prism 7. A P value < 0.05 was considered significant. The type of statistical test employed for data comparison is specified in figure legends. As a general rule, comparisons between two independent samples were performed by unpaired/paired t-test (two-tails), while for comparisons between 3 or more groups we adopted one-way ANOVA with post-hoc comparisons tests. The specification of the number of independent samples included in the analyses and the

type of statistical tests used to compare data are specified in the legends to figures. Further details about data processing are provided in the online methods section.

Results.

1. CABG-like hemodynamics induces consistent remodeling of human SV wall associated to SMCs phenotypic switching

Our previous contributions showed that application of an 80-120 mmHg pulsatile pressure regimen determined a change in the SV structure at 7 days, consisting of a significant thinning of the vessel wall, and elevation in cell death by apoptosis and enhanced proliferation [18] [19]. These changes were less evident in SVs exposed to venous flow (VP), characterized by a constant low pressure (5 mmHg). Confirming these findings, morphometric analyses of SVs treated with coronary flow showed a significant thinning of the medial layer (**Fig. 1 A, 1 B**). In order to demonstrate the specific effects of mechanical forces on intima thickening, we compared results obtained in the presence of venous or coronary flow/pressure patterns with a conventional vein 'rings' culturing model [23]. Figure S1 and S2 show results of this experiment, which demonstrate that in the presence of coronary flow-pressure pattern

intima thickening does not occur, at least at the considered time points.

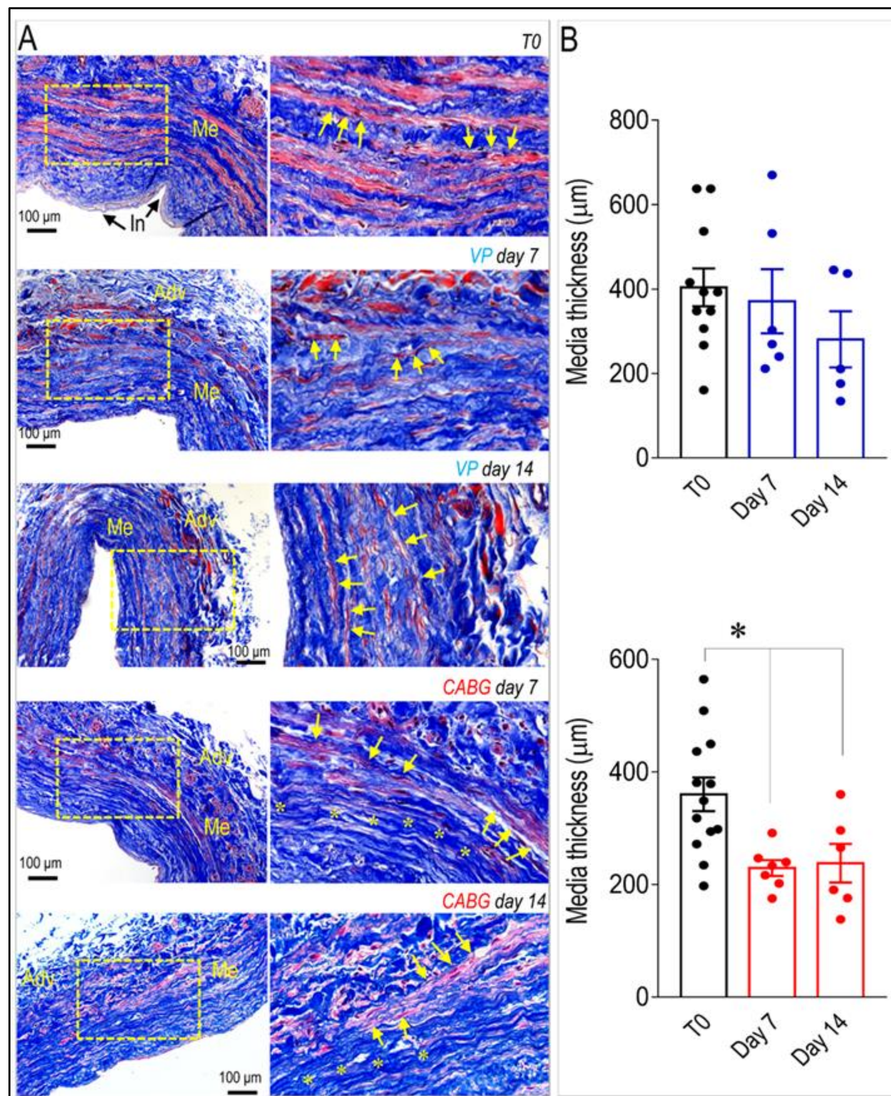


Figure 1. (A) Low and high magnifications (zones enclosed in the yellow areas) of transversal sections of native SVs (T0) and SVs exposed to venous perfusion (VP) or coronary flow (CABG) for 7 and 14 days, stained with Masson's trichrome. While in VP samples, the circumferentially arranged SMCs bundles (characterized by the red staining, yellow arrows) are present throughout the culturing period, in SVs exposed to CABG mechanics, part of these bundles

disappeared (areas stained in blue, asterisks) leaving zones rich in collagen and deprived of cells. This suggests that circumferential SMCs bundles are a specific target of coronary flow mechanics. (B) Quantification of media thickness, confirmed a major effect of the CABG stimulation on SV wall remodeling. * indicate $P < 0.05$ by one-way ANOVA with Newman-Keuls post-hoc. Bar graphs represent mean and SE. Me = Media; Adv = Adventitia: In = Intima.

Since ex vivo cultured SVs exhibited a similarly elevated cellular apoptosis (**Fig. 2 A, 2 B**), we investigated whether application of a coronary-like flow pattern affected the phenotype of the surviving cells. To this aim, we performed IF staining with antibodies recognizing SMCs contractile markers aSMA (**Fig. 3 A**)/Calponin (**Fig. 3 B**), and the synthetic SMCs marker Vimentin (**Fig. 3 C**). Cells quantification (**Fig. 3 D**) demonstrated a clear reduction in the percentage of aSMA+ and Calp+ cells in SV conduits stimulated with coronary flow/pressure pattern, and a consistent increase in cells with synthetic characteristics, suggesting a SMCs phenotypic transition.

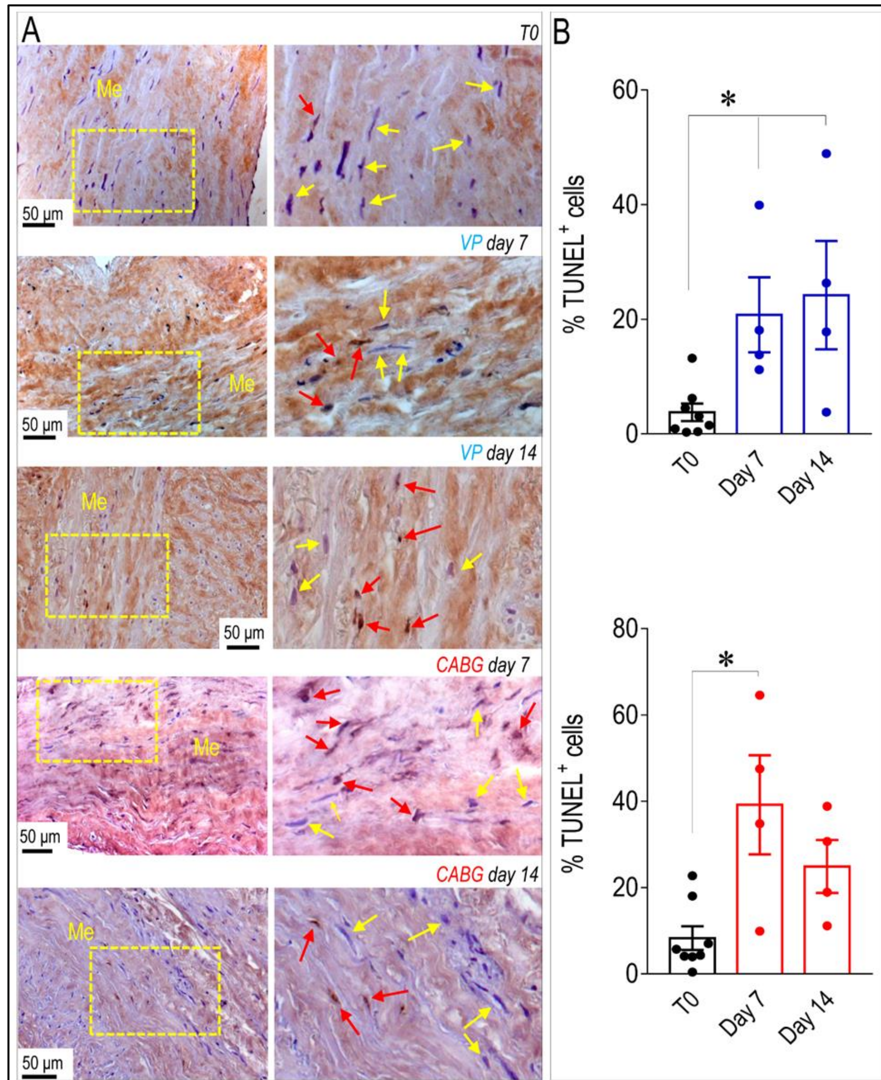


Figure 2. (A) Low and high magnifications (zones enclosed in the yellow areas) of transversal sections of native SVs (T0) and SVs exposed to venous perfusion (VP) or coronary flow (CABG) for 7 and 14 days stained with TUNEL assay for detection of apoptotic cells. Yellow arrows indicate TUNEL- cells while red arrows indicate TUNEL+ apoptotic cells. (B) Apoptosis quantification in the SV media at the two time points. * indicate $P < 0.05$ by one-way ANOVA with Newman-Keuls post-hoc. Bar graphs represent mean and SE. Me = Media.

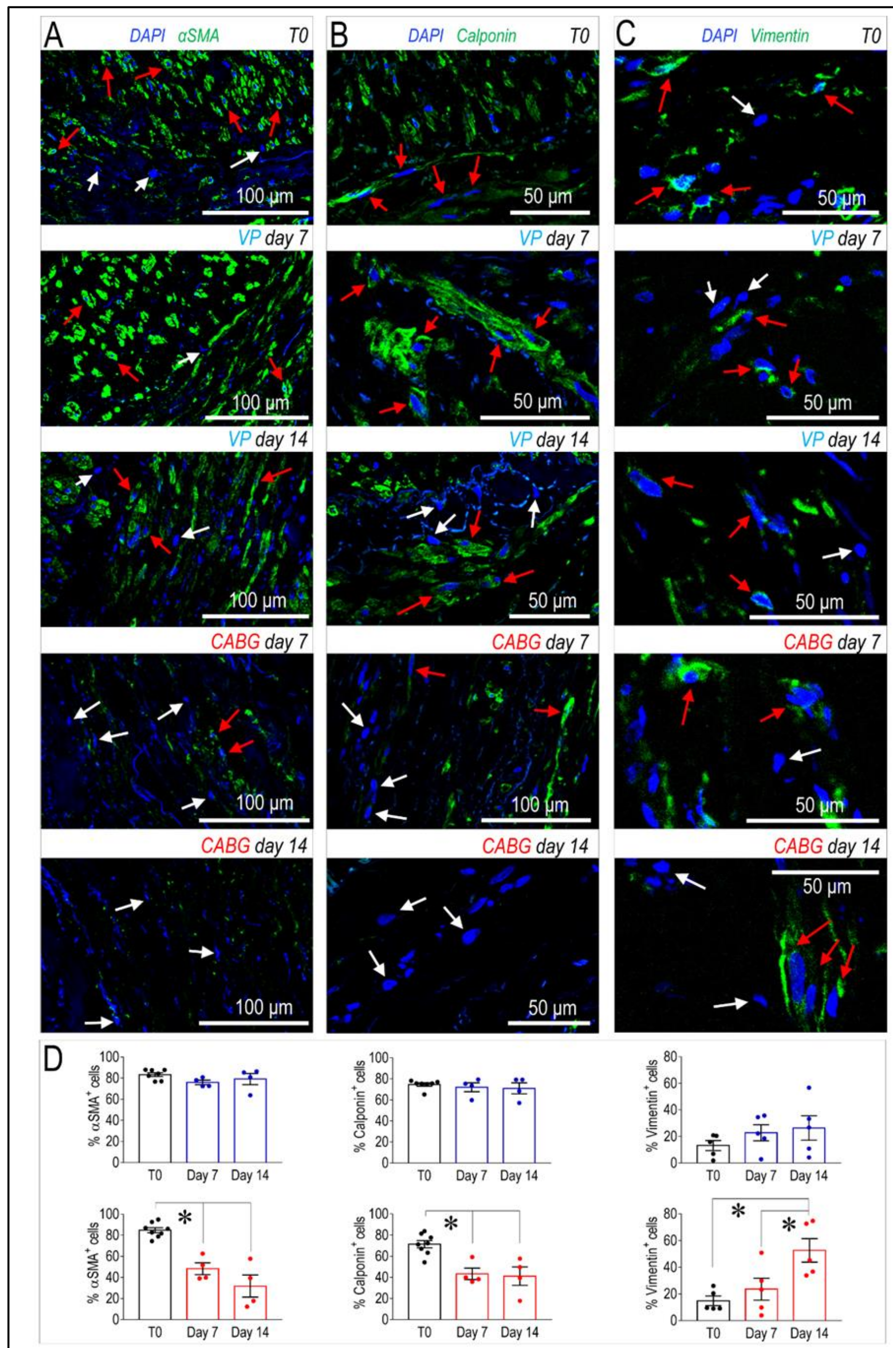


Figure 3. (A-C) Confocal microscopy images of transversal sections of native SVs (T0) and SVs exposed to venous perfusion (VP) or coronary flow (CABG) for 7 and 14 days stained with antibodies recognizing contractile (aSMA, Calponin) and secretory (Vimentin) SMCs markers. Red arrows indicate cells

expressing the indicated markers, while the cells indicated by white arrows are marker- cells. (D) Quantification of the marker+ cells expressed as a percentage of the total nuclei count present in the SV medial layer. Data are presented per treatment groups with blue bars indicating VP and red bars indicating CABG. * indicate $P < 0.05$ by one-way ANOVA with Newman-Keuls post-hoc comparison. Bar graphs represent mean and SE.

2. Coronary mechanics induces recruitment and proliferation of CD44+ cells in SV medial layer

Recruitment of cells from the vascular adventitia has been identified as a key early event in VGD setting in animal vein arterialization models [24]. Furthermore, microscopic observations performed in explanted CABGs or SV grafts exposed ex vivo to trans-wall hypoxia gradients showed an enhanced growth of adventitia vessels [25]. Since CD44 marker expression has been associated to myofibroblast programming of stromal cells in fibrotic diseases [26, 27], we investigated the expression of this marker after culturing SV grafts. In order to assess if cells expressing CD44 also expressed SMCs markers, we performed co-staining with anti-aSMA and -SM22a specific antibodies in control and ex vivo cultured SVs. As shown in **Fig. 4 A, 4 B** very few CD44+ cells were present in the media of the vessels before the beginning of the culture. However, these cells increased time-dependently, in particular in CABG samples, where they exhibited a clear co-staining with SM22a. Quantification of cells confirmed that CD44+ and

SM22a+ cell percentages increased under either VP or CABG stimulation at day 7, and continued to rise in CABG-stimulated vessels at day 14 (**Fig. 4 C**). The presence of CD44+/SM22a+ cells in CABG-treated SVs exhibited, finally, a substantial and constant increase (**Fig. 4 C**).

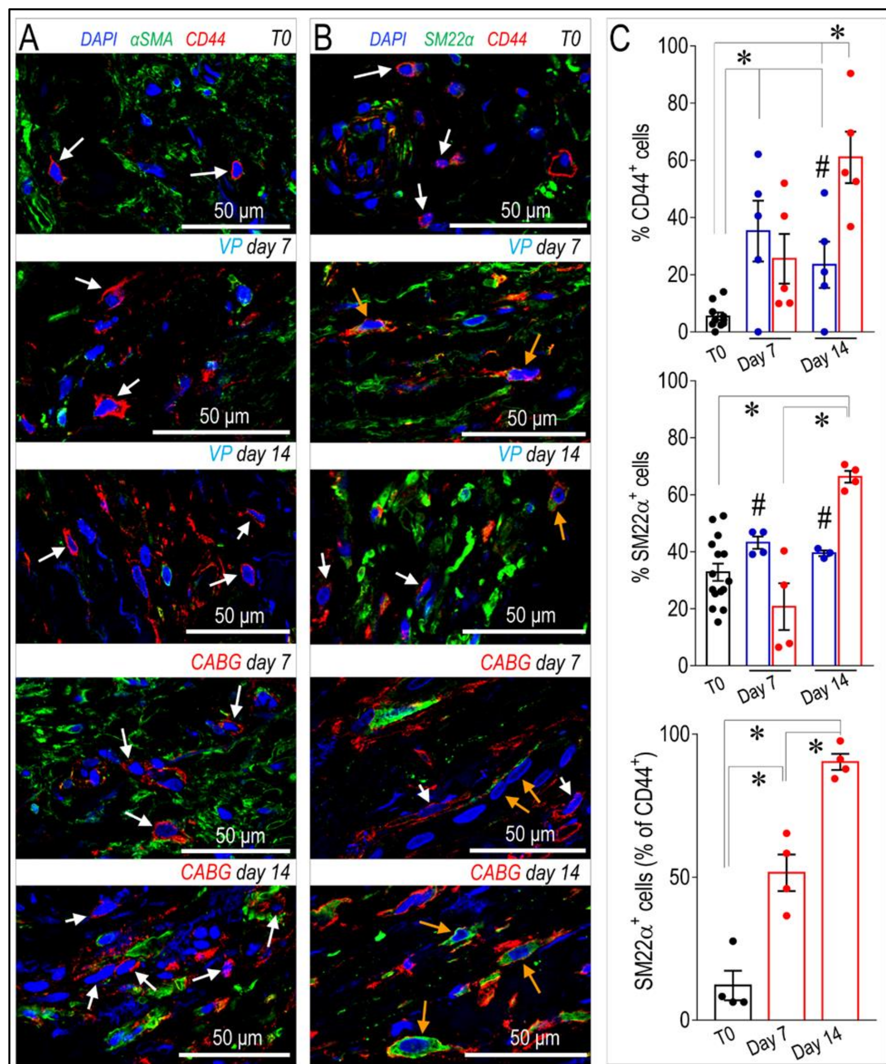


Figure 4. (A-B) Low and high magnifications of confocal images of SV tissue sections stained with CD44 with aSMA, (A) or SM22a (B) antibodies. While

expression of aSMA was independent of that of CD44 (white arrows) a staining overlapping of CD44+ and SM22a+ (orange arrows) was observed especially in CABG samples at day 14 of stimulation. (C) Quantification of single marker+ cells or SM22a+/CD44+ cells in the medial layer of SV conduits exposed to venous perfusion or coronary flow revealed a sharp increase in double positive cells in the CABG conditions. * indicate $P < 0.05$ by one-way ANOVA with Newman-Keuls comparison tests; # indicate $P < 0.05$ by unpaired Student's tests at the corresponding time points between the two treatments. Bar graphs represent mean and SE.

Since the majority of CD44+ and SM22a+ cells at the beginning of the culture were confined in the adventitia in close association with the vasa vasorum (Figure S3), this suggests that coronary flow/pressure pattern activates adventitial cells expressing myofibroblasts/immature SMCs markers. In keeping with this conclusion, increase in CD44+/SM22a+ cells in CABG-stimulated grafts was associated to an elevated proliferation level (**Fig. 5 A, 5 C**), which was more pronounced initially in the adventitia (in particular in the vasa vasorum region), and later in the media, as detected by expression of PCNA marker.

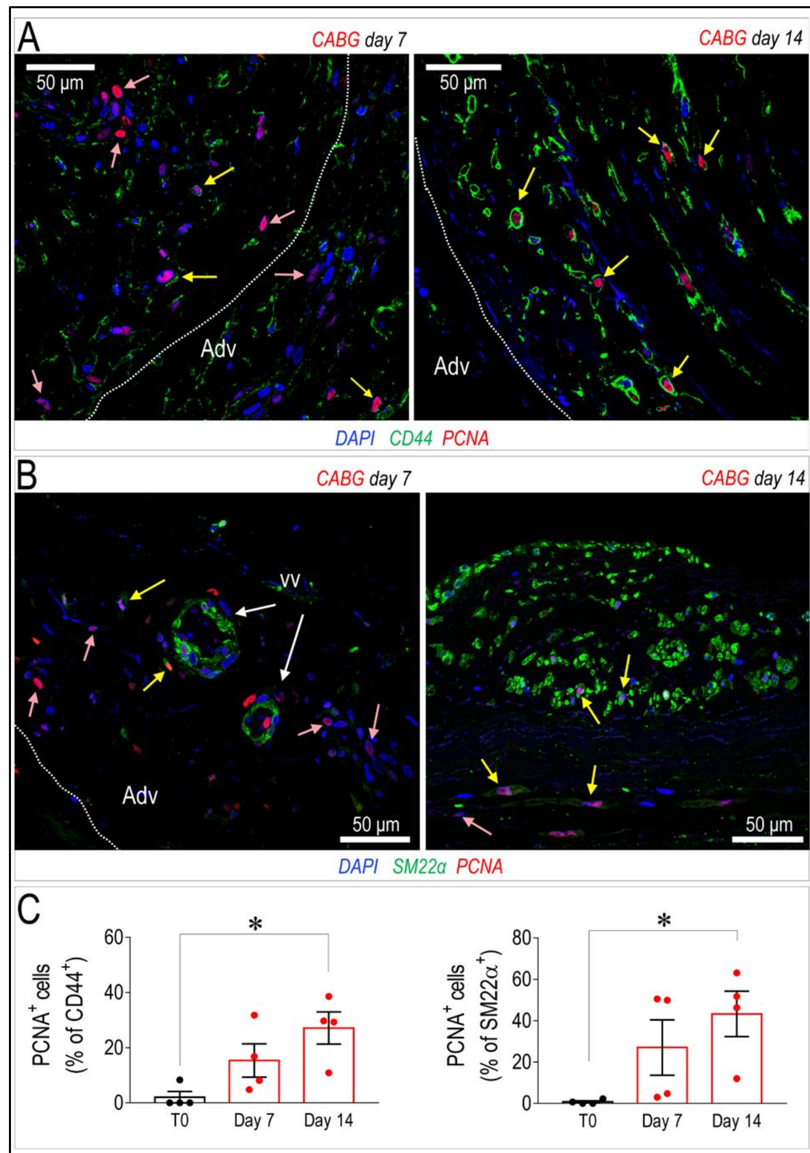


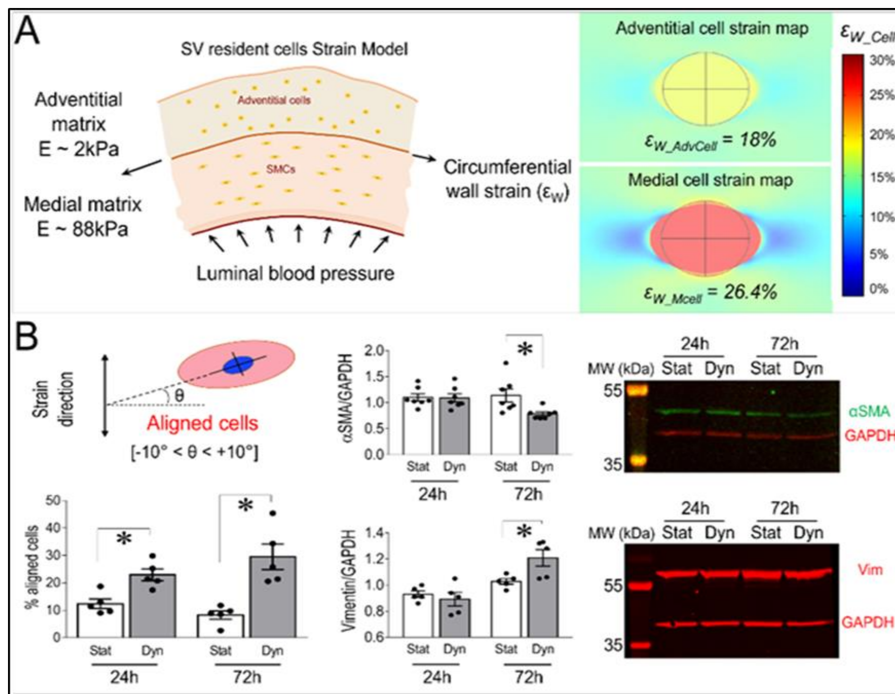
Figure 5. (A-B) Immunofluorescence staining of CABG-treated SV conduits sections with PCNA marker in conjunction with CD44 (A) or SM22a (B). PCNA+ cells co-expressing (yellow arrows) or not co-expressing (rose arrows) the mesenchymal and the SMC markers were localized in the adventitia (Adv) at 7 days also in association with the vasa vasorum (VV) Dotted lines indicate the position of the external elastic lamina. (C) Quantification of CD44+/PCNA+ and SM22a+/PCNA+ cells in the medial layer. * indicate $P < 0.05$ by one-way ANOVA with Dunnett's post hoc comparison test. Bar graphs represent mean and SE of observations.

3. Thrombospondin-1 is a mechanically regulated factor in the SV wall associated to SMCs switching from contractile to synthetic phenotype

Mechanical forces exerted by the counter-pulsed coronary flow on vein wall expose vein-resident cells to a high level of strain, whose distribution is modified compared to the natural venous perfusion [12]. We then modelled in silico the level of cell deformation associated to the circumferential strain of the two major SV layers occurring in the presence of coronary flow mechanics. We were interested in this strain component considering the orientation of the circumferentially arranged SMCs bundles, which appeared the most affected structures in the media of CABG-stimulated SV conduits (Fig. 1 and 3). As shown in **Fig. 6 A**, the model predicted a more pronounced strain in the stiffer media (~ 26% elongation), and a lower strain value (~ 18% elongation) in the softer adventitia. Based on this evaluation, we decided to investigate the effects of uniaxial cell deformation on SMCs isolated from human SVs (Figure S4) using an in vitro cyclic cell strain setting. **Fig. S 5 and 6B** show the results of 24 and 72 h SMCs mechanical stimulation, which determined a significant reorientation of the cells. Under these conditions, these cells downregulated the contractile phenotype

marker aSMA, and upregulated the levels of Vimentin (**Fig. 6 B**). They also exhibited a substantial rearrangement of the contractile cytoskeleton organization, which acquired an arrangement consistent with the synthetic phenotype [21] (Figure S6). In order to find a relationship between the phenotypic switch occurring in SMCs and the observed activation and presence of CD44+ cells, we performed a secretome analysis of proteins released in the culture medium by mechanically strained SMCs using a mass spectrometry-based approach. This indicated the matricellular protein Thrombospondin-1 (TSP-1)[28] as a factor released at high levels both at 24 and 72 h of mechanical stimulation (Table S2). Mass-spec data were validated in independent biological replicates using IF and ELISA tests, which confirmed TSP-1 release from SMCs (**Fig. 6 C**). Interestingly, Western analysis of the protein extracts revealed a decrease of the intracellular content of TSP-1 in mechanically stimulated SMCs (**Fig. 6 C**). Altogether, these data indicate that mechanical strain induces release of TSP-1 from intracellular stores and a concomitant upregulation at transcriptional level, thus identifying TSP-1 as mechano-responsive gene. Finally, we performed immunohistochemistry and Western blotting analyses on tissue sections and protein extracts of samples treated with VP or CABG flow. This clearly showed a specific elevation of TSP-1 in the intima and media compartments of CABG-

stimulated vs. VP and control SVs (**Fig. 6 D, 6 E**). Immunofluorescence staining with anti-TSP-1 and -SM22a antibodies indicated the presence of TSP-1+ cells with or without co-expression of the early SMCs marker in CABG-treated samples (Figure S8).



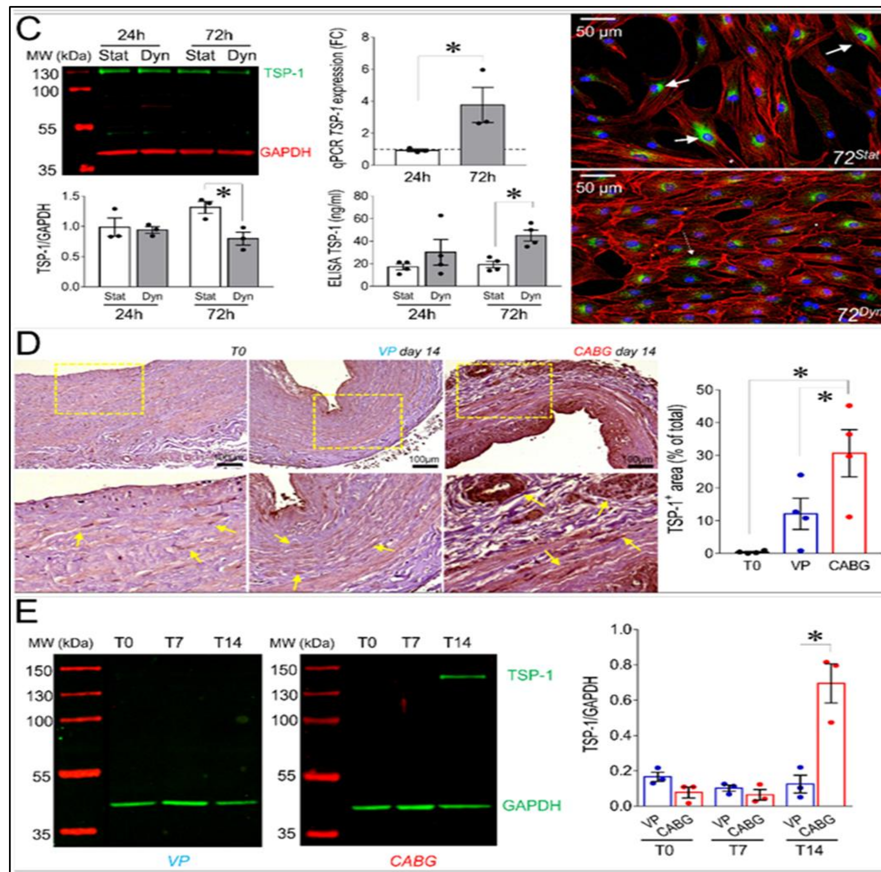


Figure 6. (A) On the left side of the panel, a schematic of the structural components, the stiffness characteristics (expressed as Young's modulus, E (kPa) and the circumferential wall strain (εW) employed to compute cell stretching experienced by cells in the adventitial and in the medial layer when coronary blood pressure is applied. On the right side of the panel it is represented the predicted cell deformation maps in the medial (εW_Mcell) and adventitial (εW_AdvCell) layers, as a result of the circumferential wall strain. (B) Results of 10% cyclic stretching on orientation of SV-SMCs (see scheme showing the procedure adopted to calculate cellular orientation above the bar graph on the left); modulation of αSMA and Vimentin by Western blot analysis (right). (C) Western blotting and IF (left side and right side, respectively) of TSP-1 into mechanically stimulated cells; ELISA tests of TSP-1 release in the medium. TSP-1 gene expression analysis by Q-RT-PCR (central part of the panel). (D) TSP-1 immunohistochemistry in VP and CABG-stimulated SV samples. Statistical analysis indicated a significant difference in signal intensity at day 14 in CABG (red bars) vs. VP (blue bars) and T0 (white bars) samples. (E) Western blotting analysis of whole protein extracts from SV samples. As shown in the quantification graph, CABG-treated samples at day 14 (red bars) underwent a dramatic upregulation of TSP-1 compared with VP samples (blue bars) and earlier CABG time points, or controls. * indicate $P < 0.05$ by, (B, C)

paired t-test, (D) one-way ANOVA with Newman-Keuls multiple comparison post-hoc test and unpaired t-test (E). Bar graphs represent mean and SE of observations.

4. TSP-1 induces migration of SV adventitial progenitor cells

The human SV adventitia contains progenitor cells with pericyte characteristics, the so-called saphenous vein progenitors (SVPs) [20]. These cells are characterized by CD44 as well as other fibroblast/stromal cells markers (Figure S9)[26], and may represent a potential source of cells participating to SV graft pathologic evolution by differentiating into myofibroblasts and SMCs [12]. In order to correlate the mechanical-dependent TSP-1 regulation in the SV wall and activation/recruitment of CD44+ cells, we performed migration assays in Transwells (**Fig. 7 A**). Results showed that TSP-1 elevated the migration of the cells compared to serum only. As established in literature, TSP-1 exerts its cellular functions through specific receptors [28]. We therefore investigated the expression of TSP-1 receptors in SVPs using specific antibodies for CD36 and CD47, and this highlighted a high CD47 expression level (**Fig. 7 B**). To substantiate the role of TSP-1 on SVP migration, we treated cells with a blocking CD47 antibody in migration assays against the protein (**Fig. 7 C**) or the stretched SMCs conditioned medium (**Fig. 7 D**); in both cases, treatment with the antibody inhibited SVPs migration.

5. Integration of mechanosensing- and humoral-driven pathways in pleiotropic SVPs responses in saphenous vein graft failure

Taken together, the previous data suggested that CD44+/SM22a+ cells growing in SV conduits exposed to coronary mechanics are actively recruited from the adventitia where they initially reside; conversion from contractile to synthetic SMCs due to circumferential strain component attracts these cells in the medial layer through secretion of TSP-1 and CD47-mediated chemotaxis. To get further insights in this process, we reasoned that transitioning from the adventitia to the media could expose cells to a combination of stimuli resulting from TSP-1 and classical vascular pro-remodeling factors, such as TGF-β [29]. On the other hand, we already showed that arterial-mimicking pressure elevates the expression of TGF-β in the SV, and literature reported a crucial function of TSP-1 for TGF-β pathologic activation of cells with mesenchymal and smooth muscle cells phenotypes [30, 31]. In order to substantiate this hypothesis, we stimulated SVPs with TGF-β (10 ng/mL) and TSP-1 (50 ng/mL), alone or in combination, followed by analysis of SM22a, Collagen1, TGF-βR, and cell proliferation. Results showed an increase in the expression level of the SMC/myofibroblast differentiation markers (**Fig.**

7 E-S 10) and proliferation (**Fig. 7 F**-S 11) at both times in cells treated with the factors combinations.

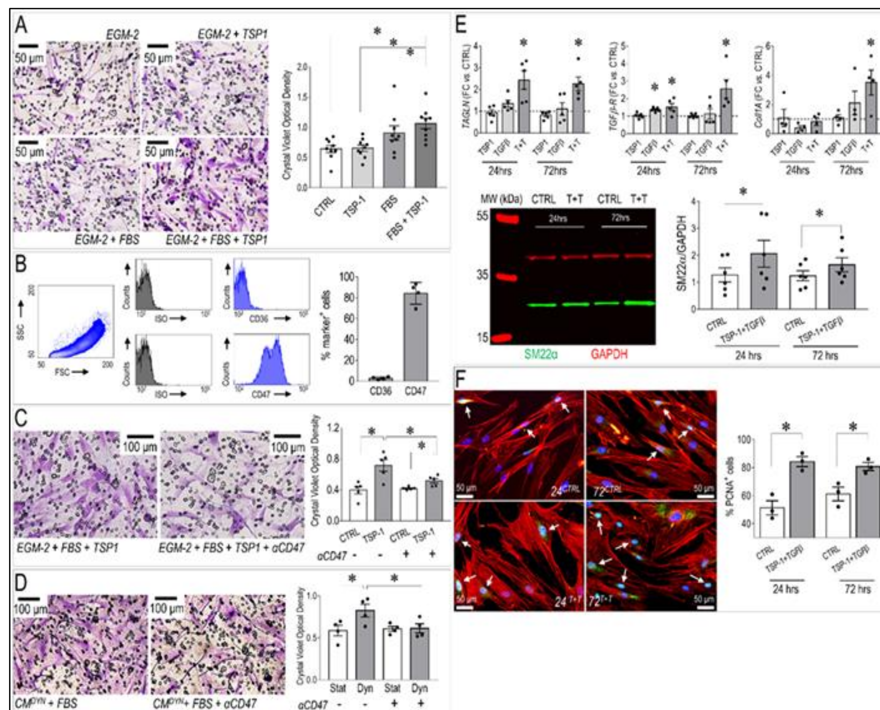
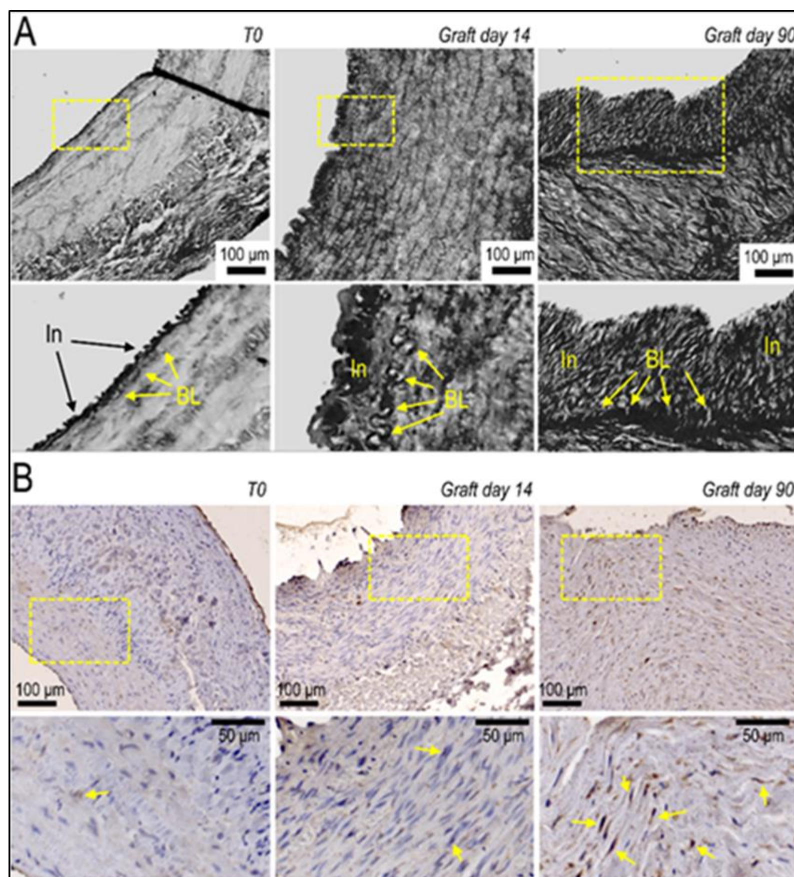


Figure 7. (A) SVPs transwell migration assay; micrographs show the cells that migrated through the membrane pores stained with Crystal Violet (purple color). Bar graph indicates quantification of the Crystal Violet optical density (see methods). * indicate $P < 0.05$ by one-way ANOVA (repeated measures) with Newman-Keuls multiple comparison post hoc test. (B) FACS analysis of the main TSP-1 receptors in human SVPs (n = 4). (C-D) Inhibition of SVPs migration against recombinant TSP-1 (C) and strained SMCs conditioned medium (D) by CD47 blocking antibody. * in both panels indicate $P < 0.05$ by paired student's t-test. (E) Effect of TSP-1/TGF-β treatment on SVPs phenotype. Q-RT-PCR analysis of TAGLN (SM22a), TGFβR, Col1A genes expression (upper). Data are represented as fold change (FC = 2-ΔΔCT) vs. untreated cells cultured for the same amount of time (dotted line). * indicate $P < 0.05$ by paired t-test performed on ΔCT values. Western blotting analysis of SM22a protein expression (lower). * indicates $P < 0.05$ by paired t-test. (F) Effect of TSP-1/TGF-β treatment on SVPs proliferation (PCNA immunofluorescence, green). * indicate $P < 0.05$ by paired t-test. Bar graphs represent mean and SE of observations.

For a final confirmation of TSP-1 expression in failing of arterialized veins, we performed immunostaining with TSP-1 antibodies in sections of SV grafts transplanted into carotids in pigs [32]. As shown in **Fig. 8 A, 8 C**, SV grafts underwent a significant thickening of the intima layer, which peaked at 90 days post-transplantation. Concurrently, an increased number of cells expressing TSP-1(**Fig. 8 B, 8 C**) and of SM22a+/CD44+ cells (**Fig. 8 D**) was observed in the wall of the implanted SVs.



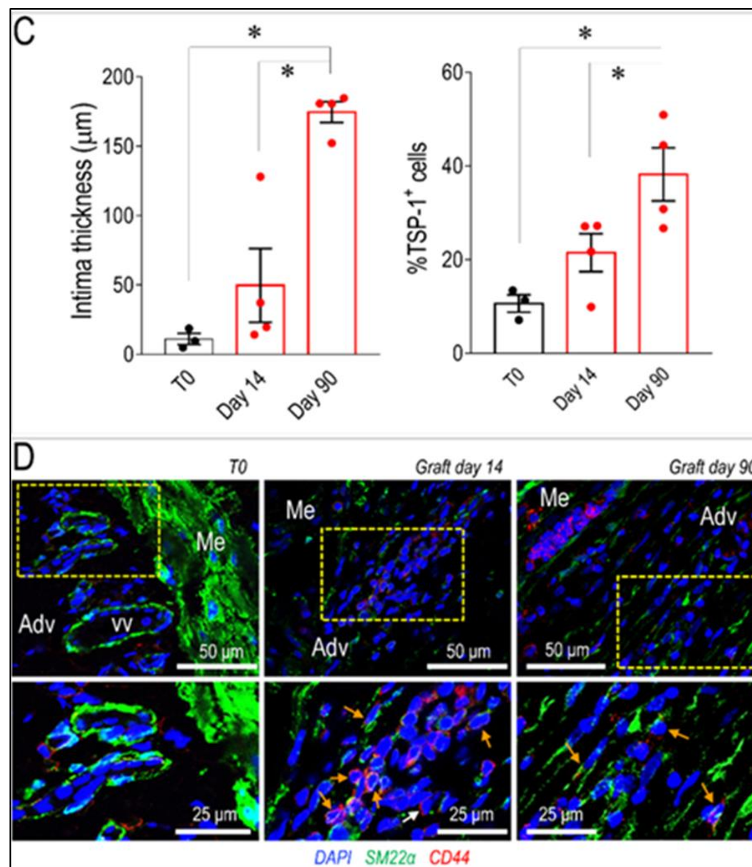


Figure 8. (A) Low and high magnifications (zones enclosed in the yellow areas) of transversal sections of porcine SV grafts before, and after 14 and 90 days in vivo arterialization. Yellow arrows indicate the intima basal lamina (BL). (B) Low and high magnifications (zones enclosed in the yellow areas) of section stained with anti TSP-1 antibody. Yellow arrows indicate TSP-1+ cells. (C) Quantification of intima thickening and of TSP-1+ cells. (D) Staining with antibodies directed against SM22a and CD44 of control (T0) and in vivo arterialized (day 14; day 90) SV in pigs. In the high magnifications on the bottom (correspondent to the zones enclosed in the yellow areas), orange arrows indicate cells expressing the two markers. * indicate $P < 0.05$ by one-way ANOVA with Newman-Keuls post-hoc test. Bar graphs represent mean and SE of observations.

Discussion.

We and others have hypothesized an important role of hemodynamic forces in molecular programming of the SV

graft disease [12]. On the other hand, the lack of platforms mimicking the peculiar flow/pressure pattern existing in the coronary circulation has prevented the identification of molecular pathways connected to alterations of SV mechanics directly in human samples. In the present investigation we filled this gap using a platform customized to maintain vessel viability and reproducing the hemodynamic conditions of the coronary circulation [18]. We found a major vessel remodeling effect consisting in, *i)* a substantial rearrangement of the intima and the media layers structure, *ii)* a major change in cell composition resulting from an early induction of apoptotic death in a significant portion of the SMCs, accompanied by a transition from a contractile to a synthetic phenotype of the surviving SMCs associated with TSP-1 release in the media, and *iii)* proliferation of a vessel-resident cell subset arising from the SV adventitia, characterized by expression of CD44 and SM22a, leading to a fibrotic-like response associated to TSP-1 release.

1. Coronary mechanics induce SMCs phenotypic transition toward a synthetic phenotype and growth of a myofibroblast-like cell population in the SV media.

The first questions that we aimed to resolve was to define the structural rearrangement of the vessel wall subjected to CABG conditions and the cellular dynamics occurring in consequence of the coronary stimulation *vs.* the venous flow/pressure pattern.

As shown in Figures 1, S1 and S2, under CABG conditions, the SV wall underwent a consistent thinning with reduction of the overall media thickness. Interestingly, the coronary-like stimulation was not associated to intima growth, while VP-stimulated vessels (analogous to the conventionally accepted SV culture method) exhibited significant intima thickening at day 14. This result, although contra-intuitive, is in line with observations performed in SV CABGs explanted *post mortem* showing narrowing of the vein wall and absence of intima hyperplasia at early times after implantation[33]. It further supports the hypothesis, already made in two other previous publications from our groups [18, 34], that the SVs contain a certain degree of damage before the culture that depends on surgical manipulation (e.g. interruption of collateral vessels, partial removal of the adventitia). In support of this hypothesis is, finally, the finding that TUNEL staining showed a similar level of apoptotic cells, especially at 7 days of culture, irrespective of the flow pattern (Figure 2).

IF data indicated that coronary mechanics induced synthetic SMCs phenotype, with a decrease in aSMA and

Calponin and a parallel increase in Vimentin in the media layer (Figure 3). This is consistent with observations performed in animal models, in which medial SMCs lose contractile markers in favor of a synthetic phenotype[35, 36]. It is interesting to note that while disappearance of cells with contractile markers peaked at day 7, a consistent increase in Vimentin^{+} cells occurred at day 14 of CABG stimulation. Thus, the shift in SMCs phenotype in the media was likely an effect of the adaptation of the cells surviving the early apoptotic peak to the new mechanical conditions. This conclusion is in line with reported differences in biological responses of synthetic SMCs to mechanical strain compared to their contractile counterparts[37]. The different response of these cells in the CABG mechanical condition may be finally part of the TSP-1-mediated pathway, given the reported protective effects from apoptosis of TSP-1/CD47 interactions in SMCs[38].

Our immunofluorescence analyses of the SV wall revealed that in CABG condition, the vascular wall was repopulated by cells expressing the mesenchymal marker CD44 and early SMC marker SM22a. Due to marker overlapping between these strictly associated cell types, and the absence of methods to perform lineage-restricted cell tracking in the human tissue, it is at present not possible to conclude definitively whether these cells derive from direct

activation of pre-existing CD44^{+}/SM22a^{+} SMCs or of progenitors with pericyte characteristics associated to *vasa vasorum*[39]. CD44 is, in fact, the ligand for hyaluronan, and it is involved in cell-cell/cell-matrix interactions in SMCs, myofibroblasts and inflammatory cells. This glycoprotein has been associated with SMCs differentiation[40], and high levels of CD44 have been associated with cell migration, injury-induced remodeling and fibrosis[41, 42]. SM22a/Transgelin is a F-Actin associated protein expressed in smooth muscle cells and fibroblasts, whose control has been recently associated to cell mechano-transduction[43]. Interestingly, in unstimulated samples SM22a^{+}/CD44^{+} cells were found almost uniquely in the adventitia in the *vasa vasorum* region, from where they appeared to invade the media at the later time point, especially in CABG condition (Figure S3, Figure 5). This finding is suggestive of a two-step adventitial cells recruitment process consisting of, at first, activation in the adventitia and, thereafter, active migration/proliferation in the media. Although these results are in contrast with findings obtained by passive culturing of SV vein rings (where there is no intervention of mechanical forces, Figure S1-S2)[23], they confirm findings in real CABGs explanted *post mortem*, where no intima hyperplasia was observed up to one month after implantation[33] and previous results obtained in our

laboratory showing a structural rearrangement of *vasa vasorum* in response to hypoxia or mechanical injuries[19, 34]. Furthermore, they are in line with studies showing the contribution of adventitial progenitors to vein graft failure in animal models of vein arterialization[24].

2. A matricellular pathway mediated by Thrombospondin-1 accounts for activation of resident myofibroblast-like cells in SVs exposed to coronary mechanics

Cell sensitivity to mechanical cues is becoming more and more relevant for the progression of chronic fibrotic diseases as a fundamental part of damage repair process[44, 45]. Our *in silico* modelling of the cell strain in the two main layers of the SV wall predicted a particularly elevated level of mechanical stress for SMCs in the media (particularly those arranged in the circumferentially arranged bundles) (Figure 6). It was therefore crucial to expose these cells directly to cyclic mechanical stress *in vitro* and assess existence of signals secreted by these cells potentially involved in activation of adventitial resident cells. Given that *in vivo* mechanical stress sensed by these cells has a major uniaxial component [12], we performed a stimulation protocol along a unique direction with a 10% deformation level. Although this protocol led cells to

experience a nominal deformation level lower than the maximal predicted by the model in the SV wall, we reasoned that mechanical forces in the real tissues is sensed by cells with a complex dynamics involving partial absorption by surrounding matrix due to its viscoelastic properties, while in the 2D condition cellular deformation occurs through direct transmission of elastic forces to cytoskeleton by the focal adhesion complexes [46]. The choice of a mass spectrometry approach to analyze the secretome of strained SMCs was made to not restrict our search for possible paracrine factors only on growth factors and chemokines that have been classically involved in progression of VGD, but also on the potential role of secreted proteins such as ECM components or matrix remodeling enzymes [47]. On the other hand, recent investigations by global proteomic profiling have disclosed new roles for ECM/matricellular vascular composition changes in early remodeling responses after injury [48]. Our secretomic analysis revealed TSP-1 as the factor more robustly released in the conditioned medium by SMCs subjected to mechanical stress. The role of TSP-1, and in a more general view, of members of the Thrombospondin family, have been so far connected to various effects on SMCs and vascular cells, such as focal adhesion kinase function, ERK1/2, p38 and CD44 regulation, migration, proliferation and arterial remodeling [49], but never

specifically linked to VGD. This is important, especially in the view of recent evidences showing the susceptibility of TSP-1 expression/function to mechanosensitive control in formation of aortic aneurysm [50] or in disturbed flow-dependent arterial stiffening [51]. Although the objective of the present study was not to establish links between the response to mechanical cues and TSP-1 secretion by SV-SMCs, or to unveil the identity of mechanotransduction-dependent machineries controlling *TSP-1* gene expression at transcriptional level, it was remarkable to observe a sustained release of this factor specifically in the medial layer of CABG-stimulated human vessels (Figure 6, Figure S8), or in *in vivo* arterialized SV grafts (Figure 8). Together, these evidences support the hypothesis that release of TSP-1 in the vascular wall by SMCs is a damage response caused by SMCs mechanical stress linked to the altered vessel flow dynamics, and potentially activating/recruiting adventitial cells with a myofibroblast phenotype.

Inspired by the vision of a mechano-paracrine mechanism involving TSP-1 in SV remodeling process, we finally established a mechanism for activation of adventitial cells in SV graft pathophysiologic process. Even if these cells have been extensively characterized for their vascular regeneration potential[20], the expression of several markers in common with mesenchymal cells such as CD105, CD90 and CD44 (Figure S9) suggests a specific

vascular pathophysiologic role [39]. In our cell migration setting, we observed a chemotactic effect of TSP-1 on SVPs. This effect was specific and involved CD47, one of the common TSP-1 receptor, expressed at high levels in these cells (Figure 7). Furthermore, compared to the treatment with the single factors, the combined treatment with TSP-1 and TGF-β increased SVPs maturation toward a SMC/myofibroblast phenotype, as shown by results of *TAGLN1*, *TGFBR1* and *Col1A* genes expression, SM22 protein expression and proliferation (Figure 7, Figure S10-S11). Taken together, these results support a multiple role of TSP-1 in activating myofibroblast-like progenitors migration from adventitial *vasa vasorum* and in modulating the latent TGF-β signaling, to promote maturation and proliferation of these cells in the media [52]. This hypothesis is in line with previous observations performed in mesenchymal progenitors[53] and SMCs [49], and establishes a new function of TSP-1 in early mechanical-dependent modification of matricellular composition in human SV grafts. The potential of our mechano-dependent graft pathology model was finally tested in the SV into carotid interposition in pigs, a widely accepted, robust and reproducible large animal system to assess vein arterialization. Data obtained in this system (Figure 8) confirmed the overexpression of TSP-1 and in the vascular wall of transplanted SVs and a similar dynamics of

SM22α/CD44^{+} in the adventitial region, and this correlated with neointima accumulation.

In conclusion, our study unravels for the first time a molecular mechanism linking mechanical injury occurring in coronary SV grafts with programming of intima hyperplasia by a mechano-paracrine effect. While this evidence demonstrates the relevance of cell-based mechanosensing in fibrotic diseases of the cardiovascular system, it calls for more focused studies addressing the potential reduction of intima thickening, e.g. by treatments with peptides inhibiting the TSP-1 function to resolve the timely issue of venous CABGs occlusion [54].

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Chapter III: Implication of YAP/TEAD pathway in mechanical strained SVPs

Implication of YAP/TEAD transcriptional circuitry in mechanical strain-dependent fibrotic activation of human saphenous vein progenitors

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In preparation

Introduction.

Coronary artery bypass grafting (CABG) is a standard surgical procedure to re-vascularize the chronically ischemic myocardium since ‘60s [1]. Despite the undoubted clinical benefits, patients receiving saphenous vein (SV) bypass undergo mid- and long-term complications due to progressive reduction of bypass patency around 50% at 10 years after implantation [2, 3]. The choice of arterial conduits, such as the inner mammary or the radial arteries, have significantly alleviated the impact of coronary artery surgery on patients’ outcome [4, 5]. On the other hand, the use of SV is unavoidable, especially in cases of multi-vessel coronary artery disease, which required multiple conduits to be grafted [6]. Numerous *in vivo* studies have addressed the pathophysiology of vein graft disease in order to *i*) understand the contribution of different cell types to intima hyperplasia (IH) [7, 8], *ii*) assess the phenotypic changes occurring in vein resident cells during arterialization process [9], *iii*) test intervention strategies with gene transfer or gene modulation approaches [10-12] and, lately, *iv*) to identify novel arterialization-specific molecular pathways [13].

SV implantation in coronary position determines remodeling of the vessel due to accumulation of smooth

muscle cells and production of new extracellular matrix in the intima layer (intima hyperplasia, IH). Indeed, IH is primarily a consequence of uncontrolled SMCs proliferation, induced by inflammatory response, vein de-endothelialization and modified luminal flow [14]. The cell response to the altered wall strain has also recently emerged as a primary player in vein arterialization. In fact, wall-resident cells respond to the switch from a rather constant, low level venous pressure to a cyclic high level mechanical load associated to arterial-like conditions. As an example, endothelial cells (ECs) and, more importantly, SMCs respond to cyclic strain with apoptosis [15], modified proliferation [16], enhanced or reduced migratory activity [17], as well as with a change in redox state and cytokine synthesis [18]. In addition, the early structural adaptation of the vessel to arterial flow, as detected by CT-scan or 3D-Echo-derived imaging data [19, 20], may suggest the implication of altered hemodynamics in vein grafts failure.

In the present contribution, we provide novel insights on the cellular and molecular bases of intima hyperplasia associated to VGD, using an approach focused on mechanobiology. For this aim, we used an *in vitro* bioreactor allowing to investigate the effect of cyclic deformation on phenotype and functions of human saphenous vein derived-adventitial progenitors (SVPs).

Material and Methods.

1. Ethics

Patients enrolled as SV donors were recruited at the Cardiac Surgery and Vascular Surgery Divisions at Centro Cardiologico Monzino, IRCCS. Tissue was collected after securing informed consent, under protocols approved by the ethical Committee of Centro Cardiologico Monzino, in accordance with the principles outlined in the Declaration of Helsinki for use of human tissue. Arteriovenous bypass surgery in pigs were performed in female Landrace or Large White/Landrace crossbred pigs weighing 20 to 30 kg. All animals received humane care in accordance with the Home Office Animals (Scientific Procedures) Act of 1986 and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85- 23, revised 1996). Anaesthesia was induced with a single dose of intramuscular ketamine into the neck (0.1 mg/Kg ketamine) and, after endotracheal intubation, it was maintained using 2-3% halothane and oxygen, the animals ventilating spontaneously throughout. A single dose of 100 mg/Kg intracardiac injection of pentobarbitone was used to euthanize animales (Euthatal; 200 mg/mL pentobarbital sodium).

2. Isolation and culture of SV-derived cells

SVs of patients subjected to unilateral saphenectomy were collected for isolation of SVPs, following the protocol described previously [21]. In brief, the vein was mechanically minced and digested for 4 hours at 37°C with 3.7 mg/mL Liberase 2. Remaining aggregates were removed through filtration with 70µm and 40µm cell strainer. $CD34^{POS}/CD31^{NEG}$ cells were isolated by magnetic bead-assisted cell sorting. Cells were grown in a humidified atmosphere (95% air, 5%CO2) at 37°C in Endothelial Growth medium (EGM-2) supplemented with 2% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S).

3. *In vitro* cell straining

To investigate the effect of isolated mechanical strain on cultured cells, SVPs were subjected to cyclic strain using the FlexCell Tension Plus FX-5000T system. Before cell seeding, six-well Bioflex plates were surface-coated with human fibronectin (10µg/ml) in PBS after covalent crosslinking with a crosslinking reagent (sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate; Sulfo-SANPAH) at 0.2 mg/ml in Hepes 50 mM (pH 8.5), photo-activated by exposure to UV-light (365 nm). Cells were subjected to uniaxial cyclic deformation protocol (0-10% deformation, 1 Hz frequency), for 24 and 72 hours, while static controls were provided by

seeding an equal amount of cells, under the same atmospheric conditions, but without mechanical stimulation.

4. Tissue mechanical stimulations

Human SV segments (~5 cm in length) were stimulated for 14 days using a custom-made bioreactor [22] tailored to reproduce the coronary mechanics (CABG-like stimulation, luminal pressure: 80 - 120 mmHg; pulse frequency: 1 Hz; mean flow rate: ~150 ml/min) or venous conditions as controls (VP, continuous luminal pressure: 5 mmHg; flow rate: 5 ml/min). Fixed stimulated SV were embedded in paraffin and cut at 5 µm using a rotary microtome for histological analysis.

5. RNA sequencing

Total RNA was extracted from cell lines using TRIzol, quantified with NanoDrop-1000 spectrophotometer. Superscript III was used for reverse transcription. Quantitative real-time PCR analysis were performed with Power SYBR Green PCR Master Mix in an ABI 7900 Fast thermal cycler to detect *CTGF*, *CYR61*, *ANKRD1*, *YAP* and *COL1A* gene amplification products (listed in Table 1). Expression levels were calculated relative to *GAPDH* mRNA, used as an internal standard control. The fold change of the genes in stimulated cells vs. control cells was

calculated as 2 $^{-\Delta\Delta CT}$ and the statistical analysis was done on the ΔCT values.

Table 1. PCR oligo sequences of primers employed in Q-RT-PCR analysis

GENE	***SEQUENCE***
hCTGF	Forward: 5'-AGGAGTGGGTGTGTGACGA-3' Reverse: 5'-CCAGGCAGTTGGCTCTAATC-3'
hCYR61	Forward: 5'-CCTTGTGGACAGCCAGTGTA-3' Reverse: 5'-ACTTGGGCCGGTATTTCTTC-3'
hANKRD1	Forward: 5'-AGTAGAGGAACTGGTCACTGG-3' Reverse: 5'-TGGGCTAGAAGTGTCTTCAGAT-3'
hYAP	Forward: 5'-CACCTGTATCCATCTCATCCAC-3' Reverse: 5'-ACGACCAATAGCTCAGATCCT-3'
hCOL1A	Forward: 5'-GGACACAGAGGTTTCAGTGG-3' Reverse: 5'-CCAGTAGCACCATCATTTCC-3'
hGAPDH	Forward: 5'-AATCCCATCACCATCTTCCAG-3' Reverse: 5'-AAATGAGCCCCAGCCTTC-3'

RNA sequencing experiment was performed and paired-end sequencing was chosen (Read1:R1, Read2:R2), where short reads (101 base pair in length) are obtained from ends of DNA fragments for high-throughput sequencing. Prior to further analysis, a quality check using FASTQC bioinformatics tool was performed on sequencing data. The obtained reads were aligned on human reference genome (assembly hg38) using as annotation the RNAs obtained from GeneCode v.24 with the bioinformatics tool

STAR. The transcripts were identified using HTSeq-count. We obtained the differential expression probability for each one of the features using DESeq2. Genes with adjusted P-Value less equal 0.05 and absolute Fold Change (FC) more equal 1.5 was considered differentially expressed.

6. Immunofluorescence/Immunohistochemistry

After fixation with 4% paraformaldehyde, cells were permeabilized for 1 hour with PBS containing 3% (w/v) BSA and 1% (v/v) Triton X-100, followed by incubation with primary antibodies anti- YAP (Santa Cruz, 500 ng/ml) and anti- Vinculin (Invitrogen, 5 ng/ml) at 4°C overnight. Cells were then incubated for 1 hour at room temperature with the appropriate secondary antibodies, Phalloidin-TRITC and DAPI nuclear dye. Digital images were obtained using an ApoTome fluorescence microscope (Carl Zeiss). For immunohistochemistry on tissue sections, after heat-induced epitope unmasking (citrate buffer, pH 6, 10 minutes) and quenching with hydrogen peroxide (0.6%, 20 minutes), nonspecific binding was blocked with 3% (w/v) BSA for 45 minutes and sections were incubated overnight with a primary antibody against YAP (Santa Cruz, 500 ng/ml). Subsequently, sections were incubated with a secondary antibody (rabbit anti-mouse IgG HRP, Invitrogen) for 1 hour, after which the reaction was

developed with diaminobenzidine and nuclei were counterstained with haematoxylin.

7. Migration

Migration assay was performed as described previously [23]. In brief, SVPs (15,000) were mechanically stimulated for 72 hrs and then seeded in the upper part of the insert in a 24 well PET transwell (8 µm pore membrane) in a FBS gradient. After 24 hours, migrating cells were fixed with 4% paraformaldehyde, permeabilized with methanol and stained Crystal Violet. Images were acquired with Axiovert 200M (Zeiss). For Crystal Violet quantification, the staining was solubilized with 2% (v/v) SDS and optical density (550 nm) was measured using Infinite M200 PRO reader (Tecan). To inhibit YAP-dependent migration, transwell assay was performed in presence of YAP inhibitors (Forskolin, Sigma-Aldrich, 100 µM, or Verteporfin, SelleckChem, 10 µM).

8. Western Blot and Immunoprecipitation

Western blotting analyses were performed following protocols already published [23]. In brief, stimulated cells were lysed in a buffer containing 10mM Tris-Cl, pH 7.4, 150mM NaCl, 5mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate and 1% (v/v) protease and phosphatase inhibitor mixture. Whole cell lysates were sonicated, centrifuged for 15

minutes at 14 000g; cell supernatants were then collected and proteins were quantified by BCA protein assay kit. Cell lysates (30 µg per lane) were diluted in Laemli sample buffer, heated at 95°C for 5 minutes, run onto a 4-12% gradient SDS-polyacrylamide gel, and transferred to nitrocellulose membrane. The blots were blocked with Tris Buffered-saline containing 5% (w/v) BSA for 1 hour. Overnight incubation at 4°C with primary antibodies (listed in Table 2) was performed to examine individual protein expression. Membranes were finally incubated with appropriate secondary antibodies for 20 minutes. Images were taken by LI-COR Odyssey and band densities were quantified using ImageJ software.

Table 2. List of the antibodies employed for immunoblotting.

Name	***Producer***	***Host***	***Dilution***
Anti- YAP	Santa-Cruz	Mouse	100 ng/mL
Anti- P-YAP	Cell Signalling	Rabbit	Dilutes as indicated
Anti- LATS	Santa-Cruz	Goat	200 ng/mL
Anti- P-LATS	Cell Signalling	Rabbit	Dilutes as indicated
Anti- TEAD 1-4	Cell Signalling	Rabbit	Dilutes as indicated
Anti- 14 3 3	Cell Signalling	Rabbit	Dilutes as indicated
Anti- GAPDH	Santa-Cruz	Mouse	80 ng/mL

For immunoprecipitation, lysates (100 μg) were incubated overnight at 4°C with antibody against YAP (Santa Cruz, 2 μg) and protein G- agarose beads. The beads were then washed three times with the lysis buffer, and the immune complex was eluted in Laemli sample buffer. Lysates and immunoprecipitates were subjected to SDS-PAGE electrophoresis, followed by immunoblotting. Proteins were visualized using western blot chemiluminescence reagent.

9. Data representation and statistical analyses

Data are presented as mean ± standard error using GraphPad Prism 7. A P value < 0.05 was considered significant. The type of statistical test employed for data comparison is specified in figure legends.

Results.

1. Mechanical stimulation induces a cytoskeletal and morphological rearrangement of human SVPs associated to increased migratory ability

The results exposed in the previous chapter suggested that mechanical strain provides a chemotactic stimulus for adventitial progenitors to migrate from the adventitia to the media, where they may activate a fibrotic-like process by interacting with preexisting SMCs and/or differentiating

into secretory SMCs. Given that these cells could be also direct targets of mechanical stress, we evaluated their mechanosensitivity using the FlexCell system, by employing a protocol of uniaxial straining (10% nominal value) for 24 and 72 hrs. Results showed a clear realignment of the cells at the 72 hrs time point with a complete cytoskeletal and morphological rearrangement (**Fig. 1 A**). Cell morphology was evaluated by calculating cell spread area and cell shape index [24]. Results of these analyses showed clear differences between strained *vs.* control cells (**Fig. 1 B**). In particular, the strain caused an increase of cell shape index at 24 hours, indicating a trend of the cells to become round when subjected to the strain, possibly due to strain-induced cytoskeleton reorganization. By contrast, at 72 hours, cells become more spread, suggesting that after an initial depolymerisation, the F-actin fibres underwent a complete re-orientation causing an increased spreading level (**Fig. 1 B**). Taken together, these results clearly indicate a biphasic response of SVPs to mechanical strain that consists in an early cytoskeleton rearrangement phase, followed by a more spread phenotype due to a later adaptation to strain. In keeping with the hypothesis that cyclic elongation affects cell migration [25], results of cell migration in Transwells showed a significant increase in motility of SVPs stimulated for 72hrs with the mechanical stimulus.

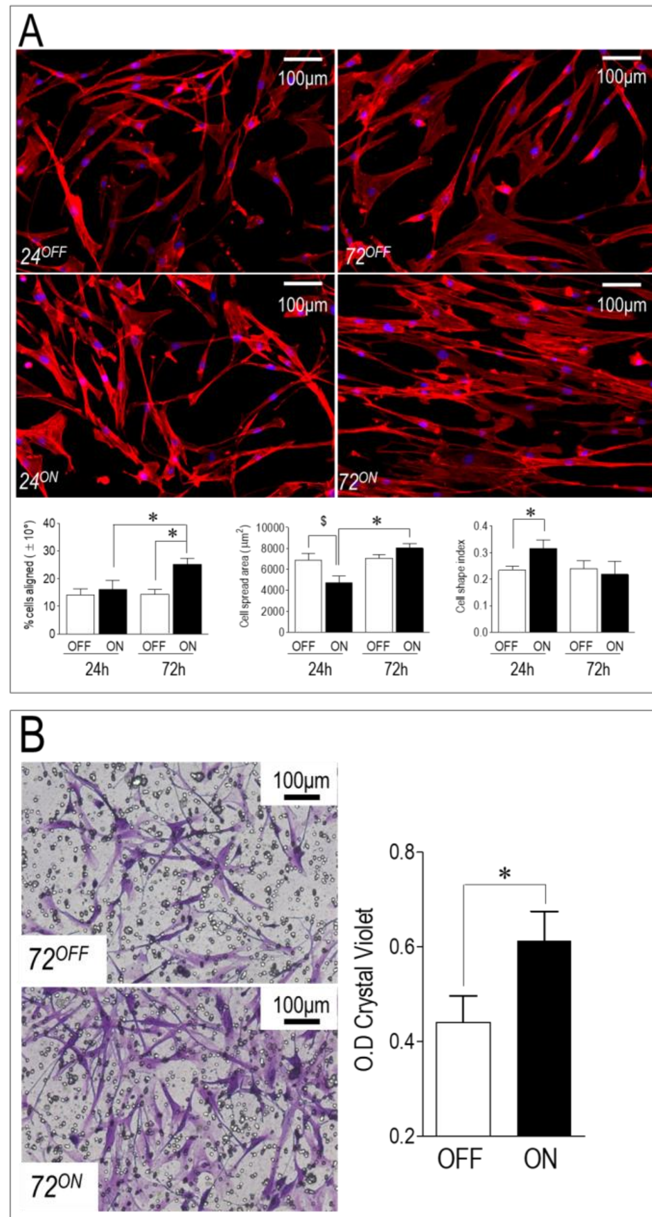

Figure 1. (A). Cytoskeleton staining of stretched SVPs revealed a clear trend of these cells to align perpendicularly to the direction of the strain. The graphs illustrates the percentage of the cells with a nuclei angle between 0±10°C that were considered aligned. Mechanical stimulation affected also SVPs morphology, as showed by cell shape index and cell spread area. (B) The migration rate of SVPs in presence of 10% serum increased after 72 hours of mechanical stimulation. OFF = unstimulated cells; ON = stretched cells. In red Phalloidin, in blue DAPI. * indicate $P < 0.05$ by paired t-test student. Bar graphs represent mean and SE of observations.

2. RNA sequencing analysis identifies a significant mechanosensing-dependent gene expression signature in the stretched advential progenitors

In order to find potential molecular players activated by the cyclic mechanical stimulus in SVPs, we performed a RNA-sequencing analysis of control and strained SVPs at 24 and 72 hrs of stimulation in 5 independent SVPs lines. Gene sets were built taking into consideration genes that were up/downregulated (±1.5 folds) significantly in pairwise comparisons in all conditions (time and straining). These data were then at first analysed with an unsupervised gene expression analysis tool to identify the experimental condition mostly affecting the transcriptome of the cells. We performed four different comparisons: cells stimulated for 24 hours *vs*. controls (24 OFF *vs.* 24 ON), cells stimulated for 72 hours *vs.* controls (72 OFF *vs*. 72 ON), cells cultured in static condition for 24 and 72 hours (24 OFF *vs*. 72 OFF) and cells stimulated for 24 and 72 hours (24 ON *vs.* 72 ON). Analysis was conducted on differentially regulated genes with an adjusted P-value ≤ 0.05 and log2 Fold Change > 1.5. By this, we observed 103 genes with differential expression in 24 OFF *vs.* 24 ON, 245 genes in 24 OFF *vs*. 72 OFF and 74 genes in 24 ON *vs*. 72 ON. (**Fig. 2**). Gene ontology analysis in these three conditions revealed an

enrichment of up/down modulated pathways involved in the normal cellular functions, such as cell cycle, proteins and lipids synthesis, metabolism (**Fig. 2**).

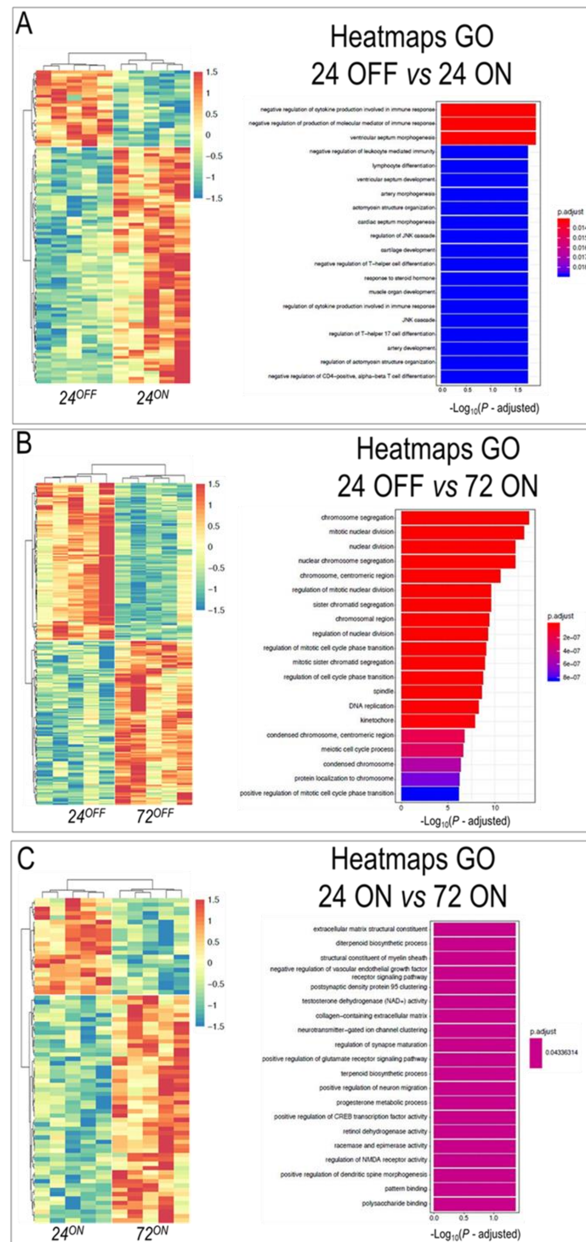

Figure 2. RNA sequencing analysis identified four different comparisons: cells stimulated for 24 hours vs controls (24 OFF vs. 24 ON)(A), cells stimulated for 72

hours vs controls (72 OFF vs. 72 ON), cells cultured in static condition for 24 and 72 hours (24 OFF vs. 72 OFF) (B) and cells stimulated for 24 and 72 hours (24 ON vs. 72 ON) (C). Very low number of genes resulted differentially expressed in 24 OFF vs. 24 ON, 24 OFF vs. 72 OFF and 24 ON vs. 72 ON conditions and gene ontology analysis revealed an enrichment of pathways normally involved in cell functioning.

The comparison performed at 72 hrs revealed a potent mechanical-dependent response of the cells in term of gene expression pathways with the identification of 819 differently expressed (**Fig. 3 A**) and a top 20 gene ontology terms associated to mechano-dependent enriched genes were upregulated in cells strained for 72 hours. Among these, an enrichment of pathways involved in extracellular matrix remodelling and cell differentiation suggested the potential crucial role that prolonged altered mechanics could have in the pro-pathological activation of advential progenitors in vein graft failure. To further contextualize results into a cellular mechanosensing-related process, the gene set was interrogated for up/downmodulation of genes related to the Hippo pathway. Cells are in fact able to transfer mechanical signals into the nucleus to exert gene expression regulation through the activation/repression of the YAP/TAZ transcriptional complex [26] (see introduction). Results of this gene enrichment analysis rendered numerous genes belonging to the Hippo pathway to be significantly upregulated by mechanical strain, with particular reference to genes involved in profibrotic cell activation and canonical

YAP/TAZ transcriptional targets (e.g., *CTGF, CYR61, ANKRD1*). Finally, by a bioinformatics search performed to assess all the possible target genes of the YAP/TAZ complex, we interrogated the dataset of genes differentially expressed at 72 hours in mechanically stimulated vs. statically cultured cells for genes containing known consensus TEAD binding sequences. TEADs are in fact the DNA binding protein functioning as bridging factors in YAP/TAZ-dependent transactivation [27]. Results of this analysis showed an enrichment of genes and relative pathways related to extracellular matrix remodelling (**Fig. 3 C**). Together with the results of cell morphology and migration, these results show that cyclic straining induces remarkable changes in cell structure and migratory activity in combination with activation of mechanically regulated-gene expression pathway related to induction of fibrosis.

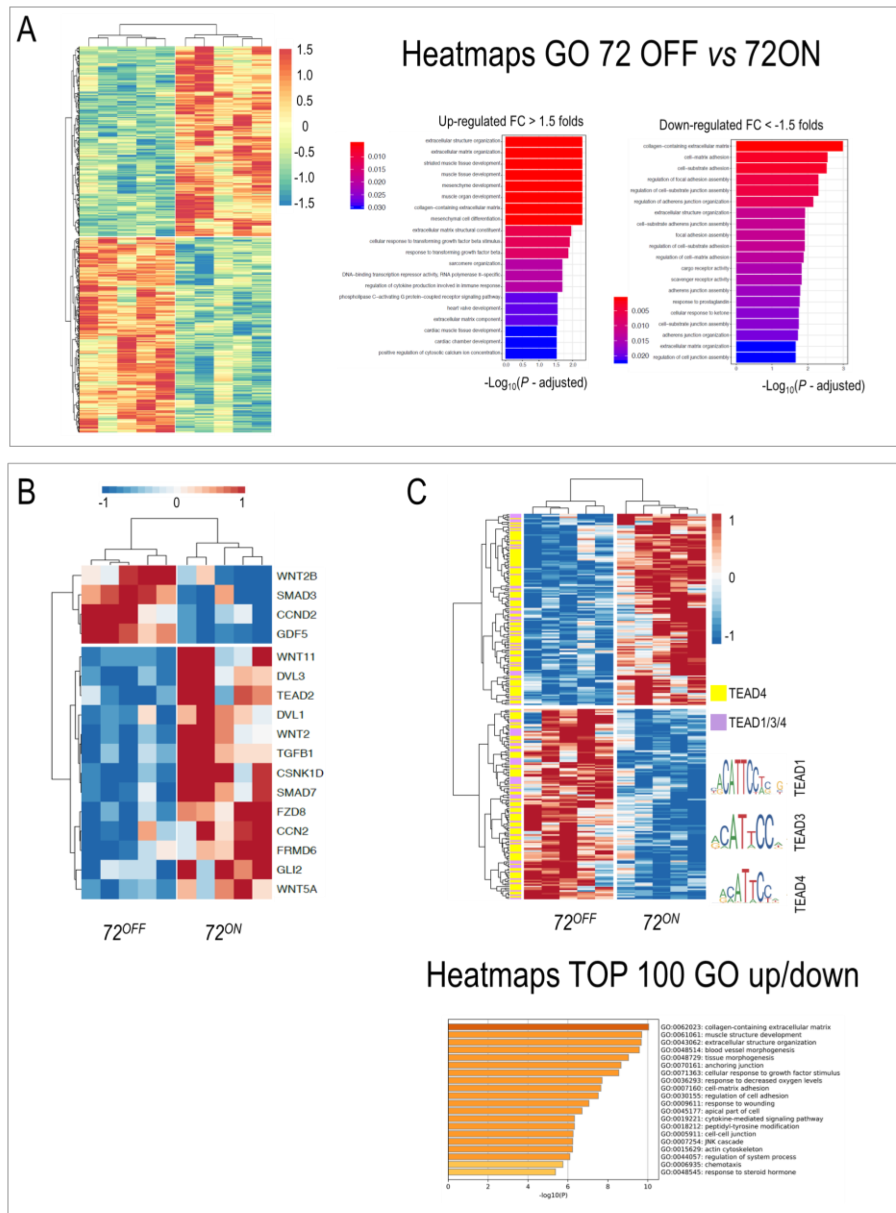

Figure 3. (A). RNA sequencing analysis identified a mechano-dependent signature in cells stimulated for 72 hrs vs control. On the right side, top 20 up/downregulated pathways in the same strain condition. (B-C) Analysis of transcripts differentially up/downmodulated in strained vs. control cells showed a significant enrichment of the HIPPO/YAP/TEAD transcriptional circuitries in cyclically strained cells for 72 hours. In particular, we found an upregulation of genes involved in profibrotic cell activation and canonical YAP/TAZ transcriptional targets.

3. YAP transcriptional activity is mechanically regulated in strained human adventitial progenitors and in arterialized SVs in vivo

In order to mechanistically correlate the altered mechanical stress to YAP function, we decided to further explore the function of this transcription factor in SVPs subjected to mechanical strain.
Firstly, we assessed the localization of the transcription factor with immunofluorescence analysis. Images and quantifications showed a significant increase in nuclear positioning of the transcription factor in strained cells (**Fig. 4 A**). In addition, since YAP activity can be negatively regulated by the HIPPO pathway through the phosphorylation of the upstream Serine/Threonine kinase cascade, via LATS/14-3-3 proteins [28], we performed a Western blot analyses to assess YAP^{Ser127} phosphorylation. Results showed a consistent decreased of the phosphorylation in mechanically-treated SVPs (**Fig. 4 B**), but this was not due to a decrease in the activity of the HIPPO pathway as shown by the lack of reduction in Phospho-LATS. Based on these results we hypothesized that increase of YAP nuclear translocation is a result of mechanical stress determined by tensioning of the cytoskeleton and independent of the activity of the HIPPO signaling [29].

In order to confirm activation of a YAP/TAZ/TEAD signature in mechanically stimulated SVPs, we controlled the formation of a transcriptional complex including YAP and TEAD by co-immunoprecipitation (**Fig. 4 C**). Results showed an increase in the amount of TEAD immunoprecipitated with YAP under dynamic conditions at 72hrs. Finally, since dataset interrogation for YAP-specific target genes showed an increased amount of genes up/down-modulated by mechanical stress we analyzed directly the expression of YAP canonical targets by real-time PCR. This showed a specific increase of the expression of CTGF, CY61 and ANKRD1(**Fig. 4 D**). Taken together these data highlight a strain-controlled YAP transcriptional activity in advential progenitor cells.

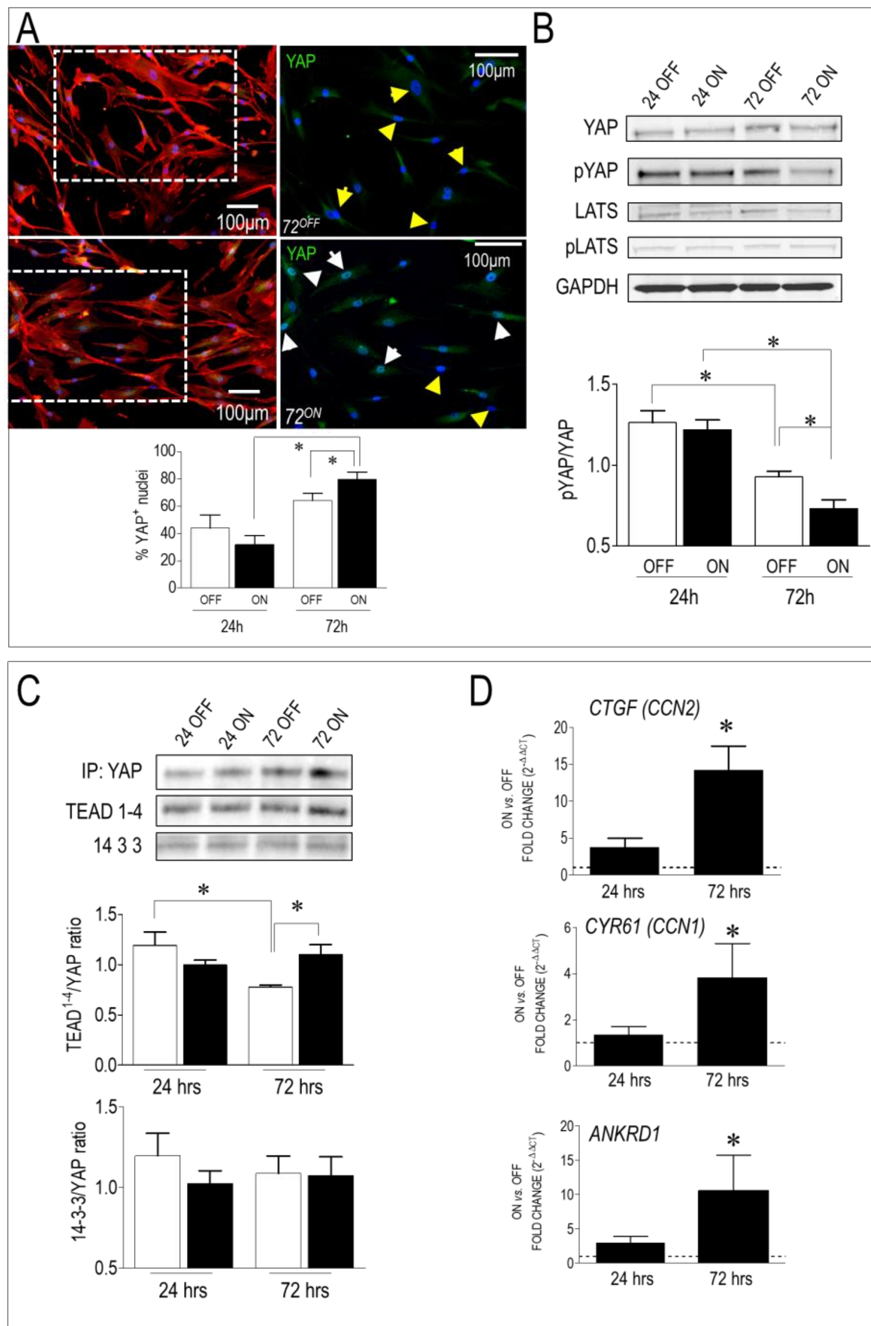

Figure 4. Cyclic strain caused a significant increase of YAP nuclear localization after 72 hrs. A consistent reallocation of YAP from cytoplasm to the nucleus was observed in strain conditions after 72hrs (A). Western Blot analysis revealed a decrease of the ratio between phosphorylated/total YAP at the same

mechanical time point (B). Immunoprecipitation analysis showed a preferential association of nuclear YAP with TEAD 1-4 (C). YAP target genes were upregulated in mechanically stimulated vs. static cultures SVPs at 72 hrs (D). OFF = unstimulated cells; ON = stretched cells. In greed YAP, in red Phalloidin, in blue DAPI.* indicate $P < 0.05$ by paired t-test student. Bar graphs represent mean and SE of observations.

In our previous contribution [23], we showed that elevated levels of wall strain with a potentially major uniaxial component is generated by the coronary hemodynamics in the medial layer of the SVs. To assess whether cells populating the medial layer of the SV exposed to coronary flow/pressure patterns show activation of the YAP-dependent pathway, we performed immunohistochemistry. Images showed the presence of cells with nuclear-localized YAP only in samples exposed to coronary flow (**Fig. 5 A**). By contrast, no YAP nuclear staining was present in cells of SVs exposed to physiologic venous perfusion. We obtained similar findings by analyzing the presence of YAP in cells of the pig saphenous vein arterialized by carotid interposition for 90 days (**Fig. 5 B**) [30]. These two evidences show the implication of YAP signaling in VGD pathological context.

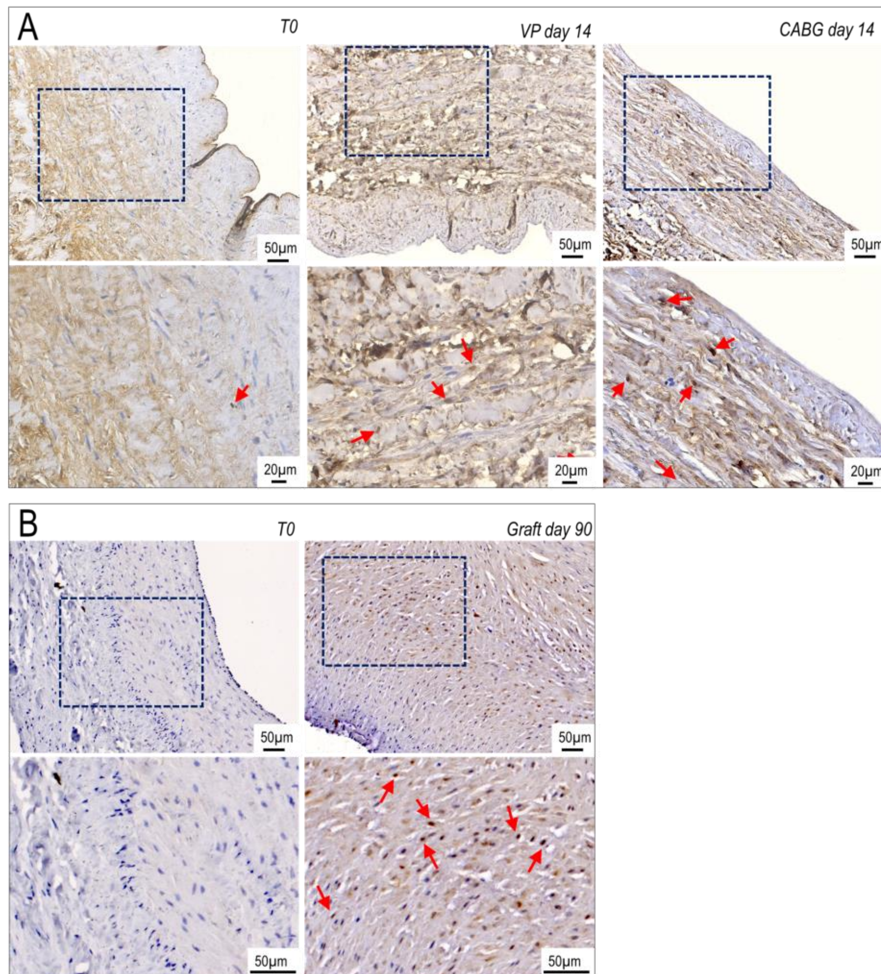

Figure 5. Low and high magnifications (zones enclosed in blue areas) of transversal sections of SV conduits stimulated with venous or CABG (A) and porcine SV grafts before and after 90 days in vivo arterialization (B). In high magnifications, red arrows indicate cells with YAP nuclear localization.

4. Forskolin induces F-actin cytoskeleton depolymerization associated to YAP phosphorylation and reduced migration in SVPs

In order to demonstrate that YAP nuclear localization is associated with cytoskeleton tensioning we performed experiments in the presence of Forskolin (FRSK), an activator of cAMP known to interfere with the signaling promoting polymerization of the actomyosin cytoskeleton, by inducing the activation of protein kinase A and Rho GTPase [31]. Treatment with FRSK (100 µM for 6 hours) determined a completely reversible relocation of YAP from the nucleus to the cytoplasm, as verified by immunofluorescence and Western blotting (**Fig. 6 A, 6 B**). Interestingly, after inhibitor washout, YAP protein returned into the nucleus. The same biphasic response of SVPs to the inhibitor was confirmed by the expression of YAP canonical target genes verified by Q-RT-PCR (**Fig. 6 C**). Cytoskeleton defects due to FRSK treatment induced also a reduction in SVPs migration in Transwell assays (**Fig. 6 D**).

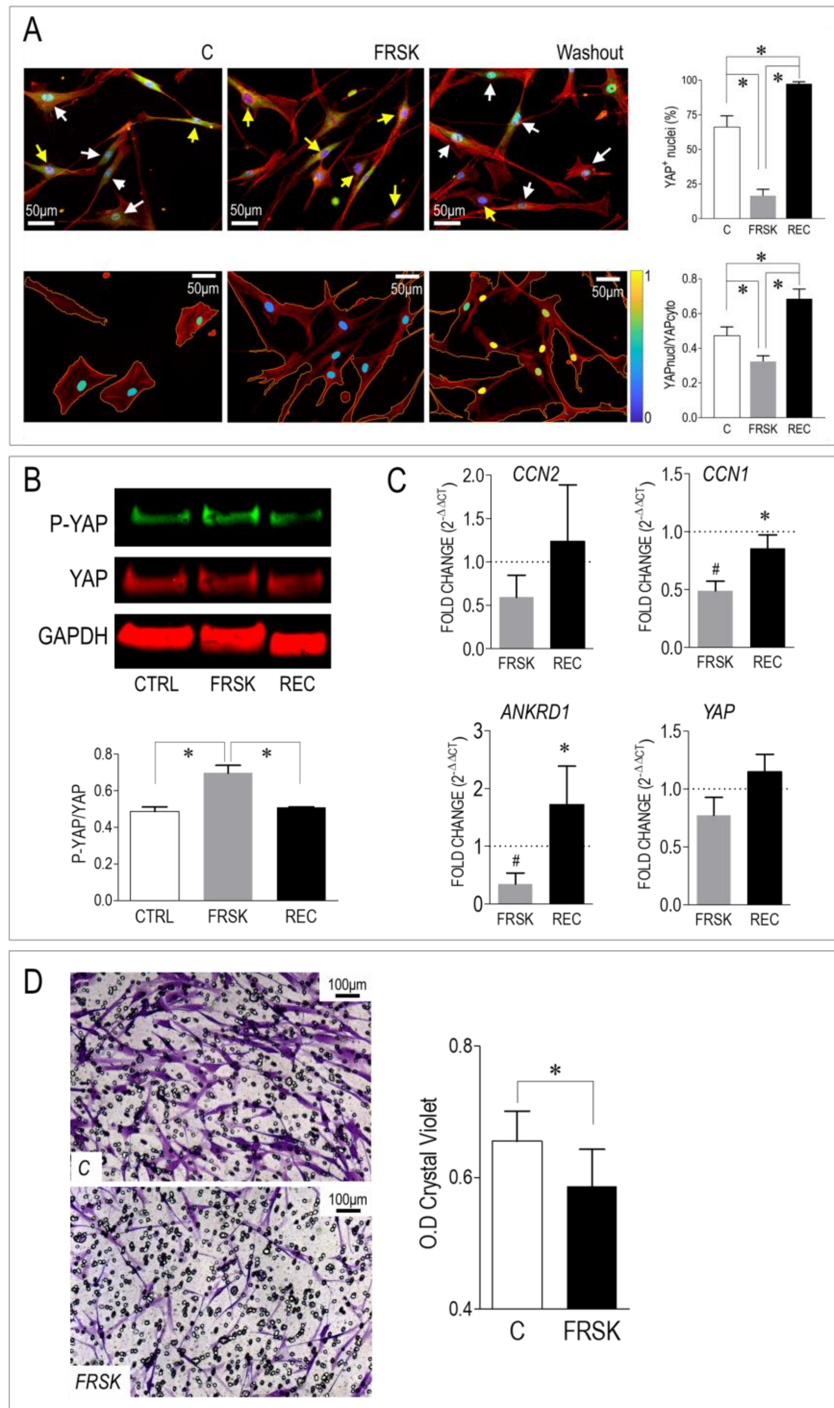

Figure 6. Actomyosin polymerization inhibition using Forskolin determined the shuttling of YAP protein from nucleus to cytoplasm, as verified by

immunofluorescence and Western blot analysis (A-B). With FRSK washout, YAP protein returned into SVPs nuclei. This reversibility of YAP nuclear translocation was also notable in the expression levels of YAP canonical target genes (C). The effect of FRSK on cytoskeleton reflected on the reduced SVP migratory ability after treatment (D). In green YAP, in red Phalloidin, in blue DAPI. * indicate $P < 0.05$ by paired t-test student. Bar graphs represent mean and SE of observations.

5. Verteporfin suppresses the YAP-TEAD complex and inhibits focal adhesions formation and cell migration

Since Forskolin has an indirect effect on YAP function interfering with F-actin polymerization, we decided to inhibit directly YAP transcriptional activity using Verteporfin (VTP), a drug known to interfere in the crosstalk between the YAP/TAZ complex and their TEAD1-4 transcriptional coactivators [32]. Unlike the effect of FRSK on YAP phosphorylation, 6 hours of incubation with VTP (10 µM) did not affect YAP nuclear localization, as shown by immunofluorescence (**Fig. 7 A**); however, it induced a downregulation of YAP targets, even in the presence of cytoskeleton tensioning (**Fig. 7 A**). It was interesting to note that VTP also inhibited the transcription of the *YAP* gene itself. In keeping with this hypothesis, the expression of YAP protein was in part reduced in cells treated with the drug. Hence, treatment with VTP is able to block the mechanical-dependent transcription of YAP target genes without affecting the cell cytoskeleton and reduces the expression

of the transcription factor further reinforcing inhibition of pro-pathologic SVPs activation. VTP treatment, analogously to FRSK, inhibited SVP migration (**Fig. 7 B**). One possible explanation for a reduction in cell motility after the treatment was downregulation of specific components of the cytoskeleton machinery. Since cell mechanosensing strictly depends on focal adhesion (FA) assembly to translate mechanical cues from integrin-ECM to the cytoskeleton and YAP is involved in the FAs formation [33], we investigated the ability of VTP to prevent focal adhesion formation by assessing Vinculin distribution. Results showed a significant decrease in the number of FAs in SVPs treated in presence of cytoskeletal tensioning (**Fig. 7 C**). These data suggest that YAP/TEAD complex actively participate to the FA formation and VTP is able to interfere with this process affecting SVP migration.

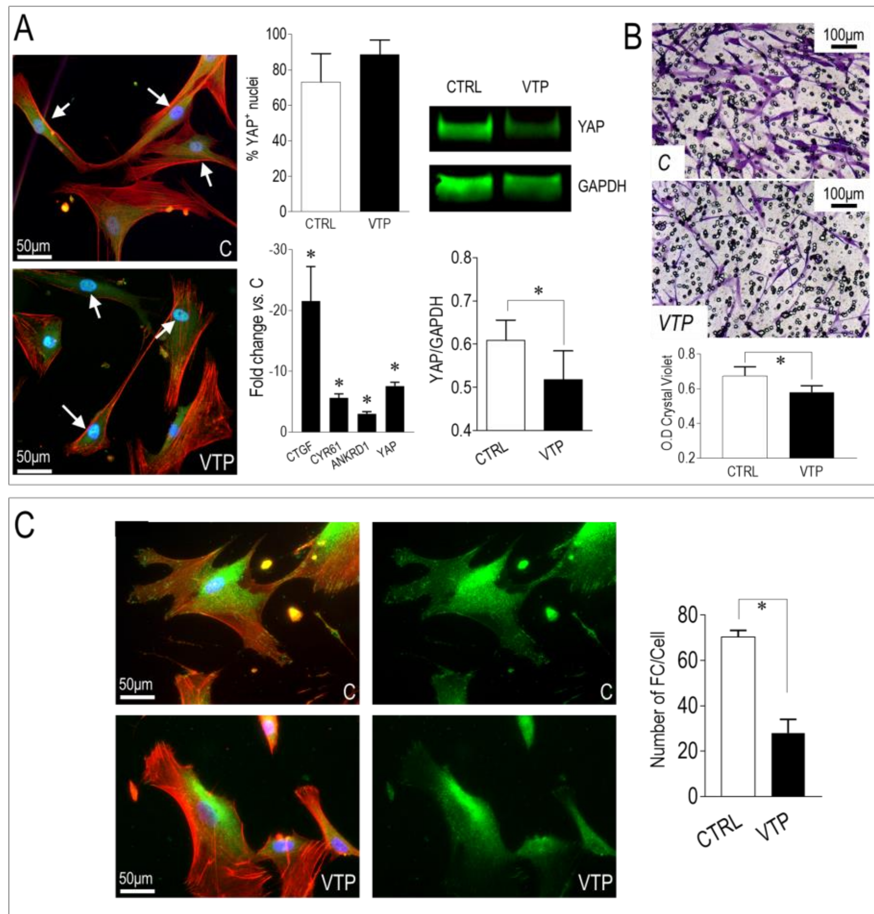

Figure 7. (A) VTP treatment determined YAP protein degradation, without affecting YAP nuclear translocation with the consequent downregulation of YAP target genes. (B) Like FRSK, VTP treatment affected the ability of SVPs to migrate in presence of serum. (C) YAP inhibition interfered also with focal adhesions formation, as showed by Vinculin staining. In green YAP or Vinculin, in red Phalloidin, in blue DAPI.* indicate $P < 0.05$ by paired t-test student. Bar graphs represent mean and SE of observations.

6. Myofibroblast-like activation of Human SVPs is mediated by YAP/ TGF-β signaling

In our previous contribution [23], we observed a cooperation between mechanical strain and humoral

factors (TGF-β/TSP-1) in recruiting and activating advential progenitors from the adventia to the medial layer in SVs segments exposed to coronary flow. To investigate whether this potential convergence between mechanosensing and TGF-β/TSP-1 pathway was mediated by YAP transcriptional activity, we cultured SVPs in the presence of TGF-β and TSP-1, alone or in combination, followed by analysis of the YAP target genes and fibrosis markers. Data showed that the two factors, only in combination, increased the level of nuclear-localized YAP compared to controls (**Fig. 8 A**), upregulated YAP-dependent target genes (*CCN2*, *ANKRD1*) (**Fig. 8 B**) and these events were completely inhibited by VTP treatment. One of the key mechanisms of myofibroblast activation is the overproduction of extracellular matrix components, such as collagen [34]. Based on this evidence, we assessed the ability of SVPs to synthesize collagen in conditioned medium after treatment. Data showed an upregulation of collagen 1A transcripts when SVPs were cultured in presence of TGF-β/TSP-1 (**Fig. 8 C**) and consequent increased release of collagen content (**Fig. 8 D**). Verteporfin restored the quantity of soluble collagen to the basal levels, even in the presence of TGF-β/TSP-1 (**Fig. 8 C, 8 D**). These data suggest that YAP acts as a converging factor between the known paracrine activation of vessel fibrosis determined by TGF-

b/TSP1 and mechanical signaling deriving from the altered flow/pressure patterns.

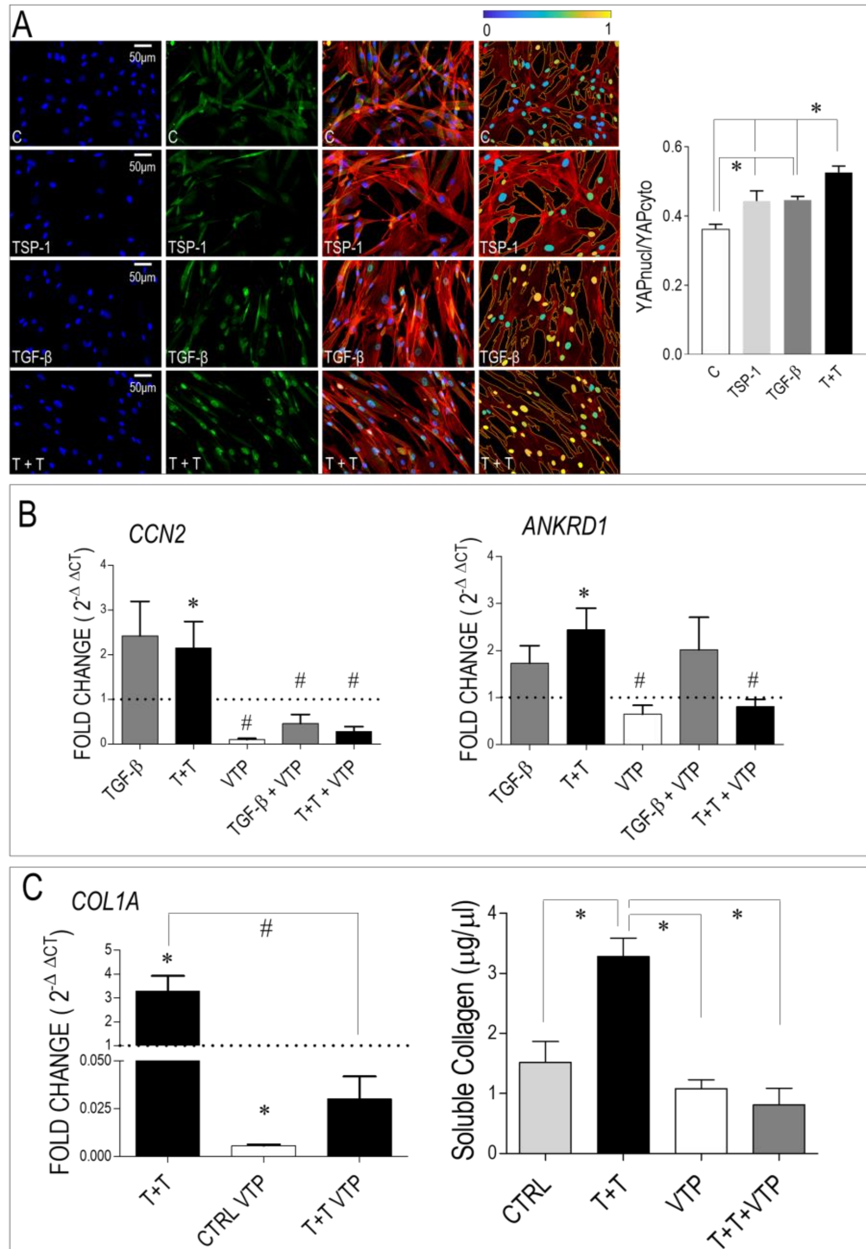

Figure 8. YAP nuclear localization was found increased in SVPs treated with TSP1 and TGF-β together (A). This was confirmed also by the upregulation of YAP targets (CCN2 and ANKRD1) (B). VTP, even in the presence of TSP1 and TGF-β,

was able to interrupt the YAP-dependent pro-fibrotic activation of SVPs, as showed by collagen quantification. In green YAP, in red Phalloidin, in blue DAPI.* indicate $P < 0.05$ *vs* CTRL, # indicate $P < 0.05$ *vs no* VTP by paired t-test student. Bar graphs represent mean and SE of observations.

Discussion.

In the present contribution, we established, for the first time, the direct susceptibility of human advential progenitor cells to mechanical stimulation mimicking the coronary cyclic wall strain. This might confirm the potential role of hemodynamic forces in molecular programming of the SV graft and can produce an outstanding opportunity for the devise of novel translational protocols to reduce the consequences of Intimal hyperplasia.

1. YAP signaling predisposes pathologic evolution of SVPs by altered mechano-perception

For long time, activation of vessel-resident SMCs has been considered the unique cause of IH. However, recent investigations have proposed that cells associated to the so called *vasa vasorum*, may retain an undifferentiated phenotype and may thus be recruited by local activation to proliferate, differentiate and contribute to IH [35, 36][8]. In a previous contribution, we found that these cells were

recruited in the medial layer via a mechano-dependent signalling, determined by a strain-dependent SMC phenotypic switching. Based on this evidence, we decided to assess SVPs response to mechanical strain. The reason for choosing *in vitro* uniaxial stimulation was based on the likely direction of mechanical forces acting in the human SV after implantation in coronary position. In fact, the pulsatile wall motion in the arterialized SV is acting in a tangential direction, thus exposing cells to an essentially unidirectional deformation that may cause major rearrangements of the cytoskeleton in a perpendicular direction to the main straining force. SVPs stimulated for 72 hours were mainly aligned along the orthogonal direction to the strain field (Figure 1 A). This shows that vein progenitors respond to mechanical cues by adjusting the tension and overall organization of their actin-cytoskeleton. Also changes in cell morphology suggest an active cell structure rearrangement process in consequence to mechanical stimulation in human SVPs (Figure 1 A). Since cell migration is driven by the continuous reorganization and turnover of actin cytoskeleton [37], we assessed the ability of SVPs to migrate in presence of 10% serum after 72 hours of mechanical stimulation. As shown in Fig. 1 B, stretched SVPs migrated more compared to the static controls, likely due to the active role of mechanical stimuli in the formation of

contractile stress fibres and focal adhesion complexes promoting cell migration through lamellipodial protrusions [38].
To investigate in more detail the possible interaction of the SVPs mechano-sensing machinery with gene regulation pathways, we performed an RNA sequencing analysis on
control *vs.* strained SVPs derived from 5 different donors. While the comparison of the transcriptomes in cells stimulated for shorter times did not highlight regulation of relevant pathways, cells stimulated for 72 hours showed a robust change in gene signatures (Figure 3 A) with an enrichment of several pathways involved in extracellular matrix remodelling and organization. These findings, are in line with evidences showing that cells respond to the cyclic strain through the regulation of ECM protein synthesis and MMP activity ([39],[40]), thus explaining the complete vessel wall remodelling observed in SVs after implantation in coronary position that affects graft patency [41]. On the other hand, ECM network is actively subject to continuous remodelling due to mechanical stress and strain that induce changes in fibre orientations and their connectivity [42].
Since SVPs demonstrated a direct susceptibility to mechanical strain with cytoskeletal and morphological changes (Figure 1 A), we interrogated RNA sequencing database to assess the possible implication of

HIPPO/YAP/TEAD mechanosensing pathway. Data showed an enrichment of genes involved in this pathway in SVPs stimulated for 72 hours compared to static cells (Figure 3 B) and of genes potentially regulated by the YAP/TAZ/TEAD complex (Figure 3 C).

Based on these evidences, we decided to investigate, at molecular level, the effect of mechanical strain on YAP transcriptional activity. YAP/TAZ are mechanically-activated transcription factors able to transduce in the nucleus signalling deriving from sensing structural and mechanical features of the microenvironment [43]. These activities are relevant in various cellular processes from developmental biology to molecular setting of several diseases, such as cancer [44], but have a less consolidated role in cardiovascular disease. The results of our immunofluorescence staining showed a significant reallocation of YAP from cytoplasm to the nucleus in dynamic condition after 72 hours of stimulus (Figure 4 A). Phosphorylation by components of the HIPPO signalling machinery has been identified as a canonical mechanism for YAP/TAZ regulation. In particular, phosphorylated LATS1/2 phosphorylate serine residues in YAP/TAZ leading to their cytoplasmic retention and degradation. Otherwise, when YAP/TAZ are active in the nucleus, the HIPPO signalling is inactivated and dephosphorylated [45]. Western blot analysis showed a decrease of YAP^{Ser127}

phosphorylation in SVP stimulated for 72 hours (Figure 4 B), supporting the data showed in Fig. 4 A. However, HIPPO signalling was not involved in YAP nuclear translocation. Indeed, mechanical strain did not affect LATS phosphorylation in strained SVPs (Figure 4 B). Several evidences demonstrated the same HIPPO pathway-independent YAP regulation [29, 43]. In fact, mechanical cues are able to regulate YAP activity through focal adhesions and F-actin cytoskeleton. Contraction of stress fibres, connected focal adhesions to nuclear lamina, promotes nucleus flattening and therefore increases YAP/TAZ import [46]. This could explain the same phenomena that occurred in SVPs after cyclic strain stimulation.

Nuclear YAP mainly interacts with transcription factors of the TEA domain family members (TEAD 1-4) to regulate gene transcription [47]. For this reason, we performed YAP co-immunoprecipitation. We observed a preferential association of YAP with TEAD 1-4 in SVPs mechanically stimulated (Figure 4 C) and this was confirmed also by the increase in the expression levels of YAP/TEAD target genes (Figure 4 D).

Further confirmation to YAP-mediated mechanosing involvement in vein graft failure was obtained in *ex vivo* and *in vivo* models. In particular, in human SV segments subjected to coronary flow for 14 days, cells in the medial

layer showed an increase YAP nuclear localization (Figure. 5 A), as well as in porcine SV grafts after 90 days *in vivo* arterialization (Figure 5 B), suggesting that YAP function is also relevant in VGD pathophysiology. Further investigations to better characterize nuclear YAP^{+} cells in the media of both human and porcine SVs are necessary to deeper explore its functions.

To demonstrate that morphological changes in the cells after mechanical stimulation are directly connected to nuclear localization of the YAP protein, we used a specific inhibitor of the YAP upstream signalling pathway that interferes with the polymerization of the actomyosin cytoskeleton. Forskolin activates cAMP/PKA signaling pathway affecting F-actin rearrangement [48]. Interfering with the intracellular transmission of mechanical forces induced YAP to translocate from the nucleus to cytoplasm (Figure 6 A), with consequent increased levels of Serine 127 phosphorylation (Figure 6 B) and a downregulation of YAP canonical targets (Figure 6 C). To inhibit directly YAP transcriptional activity, we used also Verteporfin, an already clinically tested drug able to disrupt the interaction between YAP and TEAD complex [32]. Treatment with VTP had the same transcriptional effects of Forskolin without affecting cytoskeleton integrity (Figure 7). In addition, unlike Forskolin, Verteporfin induced a reduction of YAP gene transcription and a related decrease in its protein

level. This suggests a possible therapeutic use of this inhibitor as an attenuator of nuclear transduction of mechanical cues inside the nucleus of SVPs and their derivatives in the SV wall exposed to non-physioloical hemodynamic conditions. Interestingly, Verteporfin reduced also the SVPs ability to migrate in presence of serum (Figure 7 B), even if it did not induce cytoskeletal changes inside the cells. For this reasons, we investigated the effect of the inhibitor on focal adhesion formation. Immunofluorescence revealed that SVPs treated with Verteporfin showed a reduced number of FAs, suggesting that inhibiting YAP also reduces invasion of the vein graft by adventitial cells.

2. Convergence of YAP/TGF-β pathways in the fibrotic differentiation of advential SV progenitors

We have recently found that experimentally exposing human SVs to coronary-like flow induced the release of matricellular protein Thrombospondin-1 (TSP-1) by resident smooth muscle cells due to cell strain dependent effects. Since TGF-β is a well-known factor involved in vein graft disease and was found upregulated in CABG-stimulated veins [49], we cultured SVPs in presence of TGF-β and TSP-1, alone or in combination, and investigated a possible

crosstalk of the mechanosensing-related pathway converging onto YAP and the TGF-b-depedent pro-fibrotic signaling. Data showed that YAP inhibition by VTP was able to revert completely the SVPs pro-fibrotic phenotype (Figure 8 A) and reduced the soluble collagen release (Figure 8 B, 8 C) induced by TGF-b. These results suggest that targeting these two pathways *in vivo* may retard the progression of the SV bypass remodeling caused by pro-fibrotic activation of adventitial cells.

In conclusion, this study unravels, for the first time, a novel mechanical-dependent effect in an important population of the human saphenous vein deputed to preserve the vessel integrity. In particular, we discovered a cooperation between the matricellular changes in the SV wall exposed to coronary mechanics, and pro-fibrotic signaling mediated by convergent activation of Hippo/TGF-β pathways in failure of the human aorto-coronary bypass. These results have a strong translational applicability to reduce the burden of venous bypass failure in patients with chronic coronary artery disease.

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Chapter IV: Summary, Conclusions and Future Prospective

Summary.

Coronary artery bypass grafting (CABG) has been used in vascular surgery procedures for more than 50 years to combat the consequences of myocardial ischemia in patients with coronary artery disease, a primary ageing-related cardiovascular disease in Western countries. The two most represented coronary-compatible autologous vessels to be used in CABG are the inner mammary artery and the saphenous vein (SV). Despite the advantages of SV deriving from its length and the ease of harvesting from patient legs, the implantation in coronary position determines remodelling of the vessel due to accumulation of smooth muscle cells and production of new extracellular matrix in the intima layer, a process called intima hyperplasia (IH). In 50-60% of cases, IH leads to a progressive bypass occlusion with consequent graft failure on average at 10 years from the surgery. This pathological condition is known as Vein Graft Disease (VGD). A crucial component contributing to this disease might be the change in hemodynamic load consequent to arterialization of the saphenous vein. An altered flow with high pulsatile pressure and different oxygen content may determine alterations in the mechanical characteristics of the graft and the activation of vein-resident cells. This

hypothesis is supported by the view of the vascular wall as a reservoir of cells with progenitor characteristics, which may act as mechanical damage sensors. The specific rationale of the PhD project was to investigate the response of saphenous vein adventitial progenitors (SVPs) to mechanical stimulation mimicking the cyclic wall strain experienced by SV in coronary position. We have pursued this objective using an interdisciplinary approach in order to reproduce arterialization both in cultured human veins (Chapter II) and *in vitro* (Chapter III). Exposure to pulsatile flow determined a remodelling process of the vascular wall involving reduction in media thickness and a phenotypic conversion of smooth muscle cells. Proliferating cells expressing mesenchymal (CD44) and early SMC (SM22α) markers were recruited from the SV adventitia in CABG-stimulated vessels. A consistent release of Thrombospondin-1 (TSP-1), a matricellular protein involved in TGF-β-dependent signalling was observed both *ex vivo* and *in vitro*. TSP-1 showed a direct chemotactic effect on SV adventitia resident progenitors (SVPs) and this effect was inhibited by blocking TSP-1 receptor CD47. The involvement of TSP-1 in adventitial progenitor cells differentiation and graft intima hyperplasia was finally contextualized in the TGF-β-dependent pathway, and validated in a saphenous vein into carotid interposition pig model (Chapter II). In chapter III, SVPs mechanosensitivity was assessed by

analysis of nuclear alignment and cell shape index/spread areas at both time points. The susceptibility of SVPs to uniaxial strain was revealed by a trend of the cells to orientate in orthogonal direction to the strain field and morphological changes. Mechanically stimulated cells for 72 hrs showed a significant increase in their motility as verified by migration assays in the presence of medium supplemented with 10% serum. A gene enrichment analysis of transcripts up/downmodulated by mechanical stress revealed an involvement of the HIPPO/YAP/TEAD transcriptional circuitry in cells stimulated with the cyclic strain. In keeping with this hypothesis, mechanical stimulation increased significantly the nuclear translocation of the YAP protein and its transcriptional activity. In order to mechanistically correlate the effect of mechanical stress to YAP function, we inhibited the intracellular transmission of mechanical forces by treating cells with a drug interfering with the polymerization of the actomyosin cytoskeleton (Forskolin, FRSK) and another molecule preventing association of YAP/TAZ complex with DNA binding protein TEADs (Verteporfin, VTP). Treatment with FRSK determined a completely reversible relocation of YAP from the nucleus to the cytoplasm, and this decreased expression of canonical target genes. Treatment with VTP caused a similar downregulation of YAP targets in the presence of cytoskeleton tensioning. The two inhibitors also inhibited

SVPs motility in migration assays. Interestingly, YAP was found with an intranuclear localization also in cells of human SVs exposed to coronary flow mechanics *ex vivo* and in porcine SVs arterialized by carotid interposition *in vivo*. Finally, convergence of the Hippo and TGF-β/TSP-1 pathways was confirmed by culturing SVPs in the presence of TGF-β and TSP-1, alone or in combination, followed by analysis of the YAP target genes and fibrosis markers expression.

Conclusions.

The biological data achieved from these two studies confirm an important contribution of the arterial-like wall strain in SV structural and biochemical changes, in activation of vessel resident cells and in the expression of molecular signals involved in the pathogenesis of IH. Indeed, our results provide the evidence of a matricellular mechanism involved in the human vein arterialization process controlled by alterations in tissue mechanics. In particular, we demonstrated a cooperation of Thrombospondin 1, a known component of the TGF-β pro-fibrotic pathways, with a mechano-dependent YAP intracellular pathways in the fibrotic differentiation of the adventitial SV progenitors. These results could open the

way to novel potential strategies to block VGD progression based on targeting cell mechanosensing-related effectors.

Future perspectives.

Although there are numerous successful experimental treatments for vein graft disease in animal models, no accepted strategy for management of neointimal hyperplasia has been translated into clinical use. The present project reaches important results that elucidate the role of coronary artery-like stimulation on vein graft pathogenesis. The achievements of these two studies are essential not only for better clarifying the role of hemodynamic forces in SVs, but also for their translational aspects, from basic research to the clinical use. Indeed, the discovery of molecular targets regulated by mechanical strain in the SV will produce an outstanding opportunity for the devise of novel translational protocols to reduce the consequences of IH. Possible strategies will include, for example, the use of peptide inhibitors to interfere with upregulation of Trombospondin-1. Another approach will be the use of drugs selectively targeting specific signalling pathways activated by arterial-like wall strain in SV cells. This will allow to perform saphenous vein preconditioning procedures using ad-hoc designed *ex vivo* vein culture

systems with specific cocktail of factors and drugs in order to inhibit signals crucial for the development of IH before the vein implantation.

Publications

Peer-reviewed Publications.

1. Harnessing Mechanosensation in Next Generation Cardiovascular Tissue Engineering.
   G Garoffolo, S Ferrari, S Rizzi, M Barbuto, G Bernava and M Pesce. *Biomolecules*. 2020

2. Coronary artery mechanics induces human saphenous vein remodelling via recruitment of adventitial myofibroblast-like cells mediated by Thrombospondin-1.
   G Garoffolo, M S Ruiter, M Piola, M Brioschi, A C Thomas, M Agrifoglio, G Polvani, L Coppadoro, S Zoli, C Saccu, G Spinetti, C Banfi, G B.Fiore, P Madeddu, M Soncini and M Pesce. *Theranostics*. 2020.

3. Mechanotransduction in the Cardiovascular System: From Developmental Origins to Homeostasis and Pathology.
   G Garoffolo and M Pesce. *Cells*. 2019.

4. Activation of human aortic valve interstitial cells by local stiffness involves YAP-dependent transcriptional signaling.

R Santoro, D Scaini, LU Severino, F Amadeo, S Ferrari, G Bernava, **G Garoffolo**, M Agrifoglio, L Casalis, M Pesce. *Biomaterials*. 2018.

5. Cell based mechanosensing in vascular patho-biology: more than a simple go-with the flow.
   G Garoffolo, R Madonna, R de Caterina, M Pesce. *Vascular Pharmacology*. 2018