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The Effects of Celastrol on Endothelial Cells Survival and Proliferation

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The Effects of Celastrol on Endothelial Cells Survival and Proliferation

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Résumé

Introduction: La chirurgie de pontage coronarien requiert, dans la grande majorité des cas, l'utilisation de l'artère mammaire interne en combinaison avec un ou des greffons provenant de la grande veine saphène. Malgré le taux de perméabilité inférieur aux artères, la veine saphène reste un choix populaire de conduit en raison de son accessibilité et de sa longueur. De ce fait, le greffon veineux devient la cible de multiples approches et le sujet de nombreuses études visant à optimiser sa perméabilité. Celles-ci incluent le raffinement des techniques de prélèvement, les solutions de préservations, les agents pharmacologiques ainsi que la thérapie génique. Il est davantage intéressant de combiner les approches afin de joindre leurs bénéfices, comme, par exemple, ajouter un agent pharmacologique à une solution de préservation. Un agent potentiel serait le Celastrol, connu pour être un inhibiteur du HSP90 et possède des propriétés anti-oxydantes et anti-inflammatoires.

Méthodologie: Des cellules endothéliales humaines provenant de la veine ombilicale (HUVEC) sont pré-conditionnées à de multiples concentrations de Celastrol (10^{-10}M, 10^{-8}M and 10^{-6}M) pendant une heure avant d'être soumises aux conditions de stress. Pour reproduire les conditions per-opératoires de prélèvement, les cellules endothéliales ont été préservées dans du salin (NS) héparinisé. Pour mimer le stress secondaire à l'ischémie/reperfusion, les cellules ont aussi été soumises à diverses concentrations de H_2O_2. Une analyse de la viabilité cellulaire fut conduite par le test de LIVE/DEAD. La capacité de ré-endothélialisation est étudiée grâce à l'épreuve de scratch test. Les voies intracellulaires de survie telles que le *RISK pathway* (Akt, ERK1/2), le *Heat shock response* (HSP70) et la réponse anti-oxydante (via l'activité de HO-1) ont été examinées par immunoblot.

Résultats: Les résultats démontrent que la préservation des cellules endothéliales dans du NS héparinisé est associée à une augmentation de la mortalité comparativement au milieu de culture (20.4% vs 1.9%, p=0.004). Toutefois, un traitement au Celastrol n'affecte pas significativement la survie des cellules endothéliales dans le NS héparinisé. Le stress oxydatif induit aussi une augmentation de la mortalité, et ce à dose-dépendante. Suivant un court stress

oxydatif (H_2O_2 4 mM), un pré-traitement au Celastrol 10^{-10}M est associé à une meilleure viabilité comparativement au véhicule (76.6% vs 66.1%, p=0.005). Lorsque soumises à un stress oxydatif prolongé (H_2O_2 0.5 mM pendant 24h), les HUVEC pré-traitées au Celastrol à 10^{-8}M et 10^{-10}M démontrent une amélioration significative de la viabilité, 93.7% vs 76.9% (p=0.001) et 93.6% vs 76.9% (p=0.002) respectivement. Quant à la ré-endothélialisation, un traitement au Celastrol 10^{-6}M est associé à une fermeture plus rapide et complète comparativement au véhicule. Un court traitement au Celastrol active précocement les kinases de la voie de RISK (Akt et ERK). Le traitement induit aussi l'expression de HSP70 et HO-1 qui reste soutenue jusqu'à 48 heures post-traitement.

Conclusion:

Le Celastrol active plusieurs voies de protection intracellulaire tels que le RISK pathway, le Heat Shock Response et la réponse antioxydante via l'activité de HO-1. En corrélation avec cette réponse, il améliore la survie des cellules endothéliales dans un milieu oxydatif. Le Celastrol promeut aussi une ré-endothélialisation plus complète et rapide. Cette étude met en valeur les bénéfices potentiels du Celastrol sur les cellules endothéliales. Afin d'optimiser la protection du greffon, le Celastrol pourrait donc être considéré comme agent adjuvant à une solution de préservation.

Mots-clés : endothélium, celastrol, pré-traitement pharmacologique, fonction endothéliale, greffon veineux, défaillance du greffon, chirurgie cardiaque, solution de préservation

Abstract

Introduction: Coronary artery bypass grafts are most commonly performed using saphenous vein grafts to complement the internal thoracic artery. The saphenous vein will remain popular despite its lower patency rate because it is easily accessible and lengthy enough to perform multiple bypasses. Therefore, several approaches have been studied, with the common goal of finding the optimal conditions that reduce graft failure. They include novel harvest techniques, new preservation preparations, innovative genetic therapies and experimental drugs. We believe a pharmacological pre-conditioning with an anti-oxidative and anti-inflammatory drug during the crucial time of harvest may spark beneficial survival response from the endothelial cells. One particular compound is Celastrol, an HSP90 inhibitor, which displays those antioxidant and anti-inflammatory properties.

Methods: Human umbilical vein endothelial cells (HUVEC) were pretreated with various concentrations of Celastrol (10^{-10}M, 10^{-8}M and 10^{-6}M). In order to reproduce oxidative stress found in ischemia/reperfusion, cells were exposed to hydrogen peroxide for a short and extended period (1h and 24h). To mimic storage condition encountered in clinical settings, cells were also exposed in heparinized normal saline. The viability was assessed by LIVE/DEAD assay. As for migrative and proliferative properties, scratch tests were performed. Finally, various protective intracellular pathways were evaluated by Western blot.

Results: This study shows that pre-treatment with Celastrol promotes survival in HUVEC submitted to oxidative stress. Notable improvement in cellular viability was detected as early as 1 hour after oxidative stress (H_2O_2 4 mM), 76.6% vs 66.1% (p=0.005). Significant survival benefits are also reported after prolonged oxidative stress (H_2O_2 0.5 mM for 24 hours); viability was 93.7% vs 76.9% (p=0.001) for Cel 10^{-} 8 M and 96.6% vs 76.9% (p=0.002) for Celastrol 10^{-10}M when compared to the vehicle. Celastrol, however, did not significantly affect viability of HUVEC stored in heparinized normal saline. Celastrol at 10^{-6} M promotes faster and more complete wound closure compared to the vehicle or to lower dosages. Celastrol triggers early activation of the RISK pathway, inducing activation of both Akt and ERK1/2 within the first 15 minutes of

treatment. Celastrol also induces the expression of HSP70 and HO-1, effectors of the Heat Shock Response and the anti-oxidative response respectively.

Conclusion: Pre-treatment by Celastrol provides survival benefits in endothelial cells under oxidative stress. It also stimulates endothelial cell proliferation and migration, promoting faster and more complete re-endothelialisation. Celastrol can potentially be used as an additive to storage solutions to limit endothelial injury and promote graft protection.

Keywords: endothelium, endothelium dysfunction, vein graft failure, vein graft, coronary artery bypass grafting, Celastrol, pharmacological pre-treatment, preservation solution

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Abbreviations

ACS: Acute Coronary Syndrome

AHA: American Heart Association

ADP: Adenosine diphosphate

Ang 1/Ang2: Angiopoietin1/angiopoietin 2

AP-1: Activator protein 1

ASA: acetylsalicylic acid or aspirin

ATP: Adenosine triphosphate

AWB: Autologous whole blood

Bcl-2: B-cell lymphoma 2 protein

BAX: Bcl-2-associated X protein

BIMA: Bilateral Internal Mammary Arteries

CABG: Coronary Artery Bypass Graft

CAD: Coronary Artery Disease

Cel: Celastrol

CO: Carbon Monoxide

COX: Prostaglandin H-synthase

CVD: Cardiovascular Disease

DAPT: Dual-Antiplatelet Therapy

DR: Death Receptor

EBM: Endothelial bovine medium

EC: Endothelial cells

EGM: Endothelial Growth medium

ESC: European Society of Cardiology

eNOS: endothelial nitric oxide synthase

ERK: extracellular signal-regulated kinases

FAD: flavin adenine dinucleotide

FGF: Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor Receptor

FMN: flavin mononucleotide

GADPH: glyceradehyde 3-phosphate dehydrogenase

GAG: Glycosaminoglycan

GEA: Gastroepiploic artery

GLUT-1: Glucose Transporter 1

HBSS: Hank's buffered saline solution

HIF-1a: Hypoxia inducible factor 1a

HRE: Hypoxia Response Element

HSP: Heat Shock Protein

HSR: Heat Shock Response

HUVEC: Human umbilical vein endothelial cells

IBD: Inflammatory bowel disease

IGF-1: Insulin-like Growth Factor

IH: intimal hyperplasia

IL: Interleukin

IMA: internal mammary artery

LAD: Left anterior descending artery

LDL: Low-density Lipoprotein

LIMA: Left internal Mammary Artery

LPS: Lipopolysaccharide

LSGS: Low Serum Growth Supplement

MAPK: mitogen-activated protein kinase

MI: Myocardial Infarct

MMP: Matrix Metalloproteinase

mRNA: messenger RNA

NFkB: Nuclear Factor Kappa B

NO: Nitric Oxide

NS: Normal saline

Nrf2: Nuclear factor erythroid related factor 2

NTT: No Touch technique

PAF: Platelet-activating Factor

PAR: Polyarthritis Rheumatoid

PCI: Percutaneous Coronary Intervention

PDGF: Platelet derived Growth Factor

PGI_2: Prostacyclin or Prostaglandin I2

PLC: Phospholipase C

PMN: Polymorphonuclear cell

PRV: Pressure-valve release

PSGL-1: P-selectin glycoprotein ligand-1

RA: Radial artery

RITA: Right internal Mammary Artery

ROS: Reactive Oxygen Species

SiRNA: Small interfering RNA

SLE: Systemic Lupus Erythematosus

SMC: Smooth muscle cell

StRE: Stress-responsive elements

SV: Saphenous vein

SVG: Saphenous vein graft

TF: Transcription factor

TFPI: Tissue factor pathway inhibitor

TIE2: Tyrosine-protein kinase receptor

TIMP: Tissue inhibitor of metalloproteinase

TNF: Tumor necrosis factor

t-PA: Tissue plasminogen activator

TRAIL: TNF-related apoptosis-inducing ligand

TxA_2: Thromboxane A 2

VEGF: Vascular endothelial Growth Factor

VEGFR: Vascular endothelial Growth Factor receptor

VLDL: Very-low-density Lipoprotein

VGF: Vein raft failure

vWf: von Willebrand factor

WPB: Weibel-Palade bodies

Health is the greatest gift; contentment is the greatest wealth.

-Buddha

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Chapter 1 – Introduction

1.1 Coronary artery bypass grafting and choices of grafts

The coronary artery bypass grafting (CABG) is the most frequently performed cardiac surgery, not surprisingly as coronary artery disease remains the number one cause of death worldwide, according to the World Health Organization.[1] CABG has shown to be effective at relieving anginal symptoms, improving functional status and quality of life measures.[2-5] Despite increasing numbers of percutaneous coronary interventions (PCI), the undeniable survival benefits of CABG cement its status as the preferred treatment for patients with high cardiovascular risk such as diabetic patients, those with complex and extensive coronary disease, or those with ventricular dysfunction.[6-14]

1.1.1 Arterial Grafts

Generally, CABG requires the use of both arterial and venous grafts harvested from the patient during the same procedure. The available choices of arterial grafts are the internal mammary artery (IMA), radial artery (RA) and gastroepiploic artery (GEA), listed in order of frequency of use. IMA shows the best patency rate of 85-95% at 10 years [15]. RA has a patency rate of 88% at 5 years, whereas GEA, 85% at 5 years and 66% at 10 years [16, 17]. IMA are known to be the gold standard graft and thus, is used in over 95% of CABG cases [3, 9, 18].

IMA has intrinsic characteristics that confer its superior patency rate. First, it has low collagen content, which grants its elastic properties. Compared to the RA, the IMA responds better to nitric oxide (NO), shows less reactivity to vasoconstrictive agents (i.e., KCl, norepinephrine, phenylephrine) and have a thinner muscular component, making it resistant to vasospasms [19-21]. The IMA endothelium few fenestrations, limiting cellular migration and lipoproteins entry into the subendothelial space. It exhibits antithrombotic molecules such as heparin sulfate and higher NO production. All those features are protective against the development of

atherosclerosis [22, 23]. Furthermore, IMA avoids ischemia as it is typically harvested and anastomosed *in situ*.

One major drawback of arterial grafts is their limited availability. The left IMA is unquestionably used to revascularize the most significant vessel, predominantly the left anterior descending artery (LAD). In multivessel disease, there is still debate about the choice of second graft. The use of both IMA is supported by the theoretical advantage of offering gold standard quality grafts to two coronary territories. However, the largest clinical study comparing bilateral internal mammary artery (BIMA) revascularization to single mammary artery revascularization showed an increased rate of sternal wound infection and modest to no clinical benefits [24, 25]. While the RA was compared to the right internal mammary artery (RIMA) with similar clinical results, its popularity remains limited because of its propensity to spasm, especially during the perioperative period [19, 26]. Additional skepticism stems from aesthetic motives, risk of nerve injury in the arm, the morbidity associated with upper limb infection, hematoma or ischemia [27]. The GEA is rarely used. Its harvest requires a bigger incision, with entry into the abdominal cavity and secondary increased risk of contamination. It also requires technical expertise not widely trained and mastered [28]. As for prosthetic grafts, its use is limited in CABG due to its poor long-term patency rates and thus, considered as a last resort option [29].

1.1.2 Venous grafts

Since 1967, when Favarolo first successfully reconstructed a conduit with a segment of a saphenous vein to revascularize the right coronary artery, saphenous vein grafts (SVG) quickly became the ideal complement to the left IMA for the revascularization in multivessel disease [30]. It provides a longer segment to be used for multiple grafting. Its harvest is comparatively less complex and time-consuming, potentially shortening operative time and is particularly advantageous in cases of emergency [31]. When concerns about competitive flow arise, SVG also becomes an interesting alternative. While arterial grafts are living grafts reacting to the reduction of flow, SVG acts as a large conduit with minimal regulation and resistance [32]. Those are reasons why venous grafts, despite their lower performance, remain the most frequently used

conduit in combination with the IMA [33]. The biggest caveat to SVG is its patency rates, reported to be 85-90% at 1 year, 80% at 5 years and 50-60% at 10 years, which measures poorly to its arterial counterparts [15, 32]. Inherent elements of SVG contribute to the poor patency rate. It has a media composed mostly of concentric collagen and multiple layers of longitudinal and circumferentially oriented smooth muscle cells (SMC). This characteristic confers mechanical stiffness, particularly at non-physiological pressures (≥ 50mmHg), a factor implicated in triggering hyperplasia.[34] SVG is also susceptible to accelerated atherosclerosis due to its higher collagen content, abundant fibroblast growth factor receptors, increased intercellular junction permeability, rapid lipid uptake, lower redox responses as well as lower NO sensitivity and production [23, 35-37]. As the use of SVG will likely prevail, extensive research has focused on ways to target vein graft failure (VGF). A detailed description of VGF physiopathology and a review of those advancements are discussed in the next sections.

1.2 Endothelial Physiology

The endothelium constitutes a single layer of endothelial cells lining the whole vasculature. It is a large interface responsible for the nutrients and gas exchange between the intravascular space and the surrounding tissue. It is central in preserving vascular homeostasis, playing an essential role in moderating the vascular tone, initiating and regulating the inflammatory response as well as maintaining the thrombotic/antithrombotic balance. Endothelial dysfunction results from the imbalance of those functions and plays a critical role in cardiovascular diseases [38].

1.2.1 Vasoactive regulation

The regulation of vascular tone is dependent on the presence and integrity of the endothelium. Endothelial cells (EC) constitutively produce NO which is the primary regulator of the vascular basal tone. NO diffuses and reaches the smooth muscle layer where it activates the soluble guanylate cyclase to produce cGMP, activating downstream pathways resulting in SMC relaxation [38, 39]. Beside vasodilatation, NO also has anti-inflammatory properties by inhibiting platelet

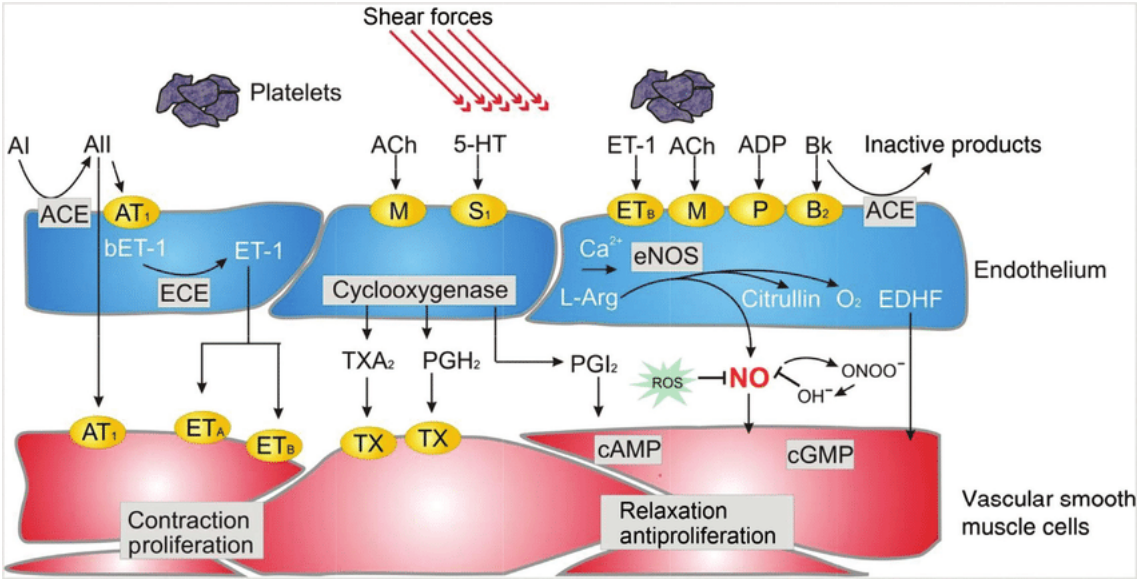
aggregation, cytokines productions as well as reducing endothelial permeability, leukocyte adhesion and migration [40].

NO production is stimulated by various triggers such as acetylcholine, bradykinin, histamine, arachidonic acid derivatives, β-adrenoceptor agonists and serotonin, among others [40]. NO is synthesized by the nitric oxide synthase (NOS) enzyme family, using NADPH, O_2 and L-arginine as substrates and flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) tetrahydrobiopterin, heme, Ca^{2+}/calmodulin acting as cofactors. There are 3 types of NOS, named after the cell type from which they were first isolated and cloned: eNOS (endothelial), nNOS (neuronal) and iNOS (inducible form) [41]. eNOS is the predominant endothelial isoform.[40] It maintains a basal production of NO [42]. Oxidized low-density-lipoprotein (LDL) reduces expression of eNOS, whereas increased transcription follows the presence of vascular endothelial growth factor (VEGF), insulin, fibroblast growth factor (FGF) or hypoxia [39]. iNOS is normally not expressed, as it is an inducible form of NOS. Pro-inflammatory cytokines (IL-1, TNF-α and IFγ) or oxidative environment stimulate transcription of iNOS through the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) [43].

Shear stress provokes a rapid enzymatic activation and transcription of eNOS. Aside from the resulting vasodilatory response, shear stress stimulates the release of antithrombotic agents and factors that reduces leukocyte migration as well as SMC proliferation. A steady laminar flow provides protection against atherosclerosis by inducing the release of both NO and prostacyclin (PGI_2) [44]. PGI_2 is produced by cyclooxygenases and induces SMC vasorelaxation via activation of adenylate cyclase and production of cyclic adenosine monophosphate (cAMP). PGI_2 also plays a role in the resting vascular tone and increases in response to thrombin, histamine, serotonin and arachidonic acid derivatives [45]. Additional to the release of vasodilatory factors, shear stress provokes EC conformal changes. They adopt an elongated form along the direction of blood flow to alleviate mechanical stress [39].

Endothelium-dependent vasoconstriction is mediated by endothelin (ET). ET is continuously synthesized *de novo,* modulated by shear stress, hypoxia, thrombin and angiotensin II among others [46]. EC releases pre-endothelin in the non-luminal extracellular space where it is

converted to functional ET by endothelin-converting enzymes (ECE). There are 3 isoforms of ET: ET-1, ET-2 and ET-3. Additional to its vasoconstrictive property, ET-1 also has a mitogenic effect on SMC. ET exert their effects upon binding with endothelin receptors ET_A and ET_B. ET_A has an affinity for ET-1 and ET-2 and induces SMC contraction through phospholipase C activation. ET_B has an affinity for all ET isotypes. Activation of ET_B causes a counterregulatory response by stimulating the release of NO/PGI_2. The existence of other endothelial-derived vasoconstrictive agents is not known. Many factors, however, have vasoconstrictive effects (i.e., superoxide anion, vasoconstrictive prostaglandins PGF2 and PGH2, TxA2) [39, 46].



Endothelium-derived vasoactive substances (5-HT, serotonin; Ache, Achetycholin; ADP, adenosine phosphate; AI, angiotensin 1; AII, angiotensin II; ACE, angiotensin converting enzyme; AT_1, angiotensin type 1 receptor; B_2, bradykinin receptor; Bk; bradykinin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; bET-1, precursor of endothelin 1; ET-1, endothelin 1; ET_A: endothelin receptor type A; ET_B: endothelin receptor type B; NO, nitric oxide; PGI_2, prostaglandin I 2; PGH_2, prostaglandin H 2; ROS, reactive oxygen species; TX, thromboxane receptor; TxA_2, thromboxane A2) [47]

Figure 1. – Endothelium-derived vasoactive substances

1.2.2 Inflammatory response and leukocyte recruitment

Inflammation is a protective mechanism necessary for defense and repair of damaged or infected tissue. There are 3 components in the inflammation response: a vasodilatory process, which results in a rise of blood flow; an increase in vessel permeability and exudation of protein-rich fluids; and the recruitment and activation of leukocytes at the damaged site. The endothelium initiates and tightly coordinates these 3 responses [38].

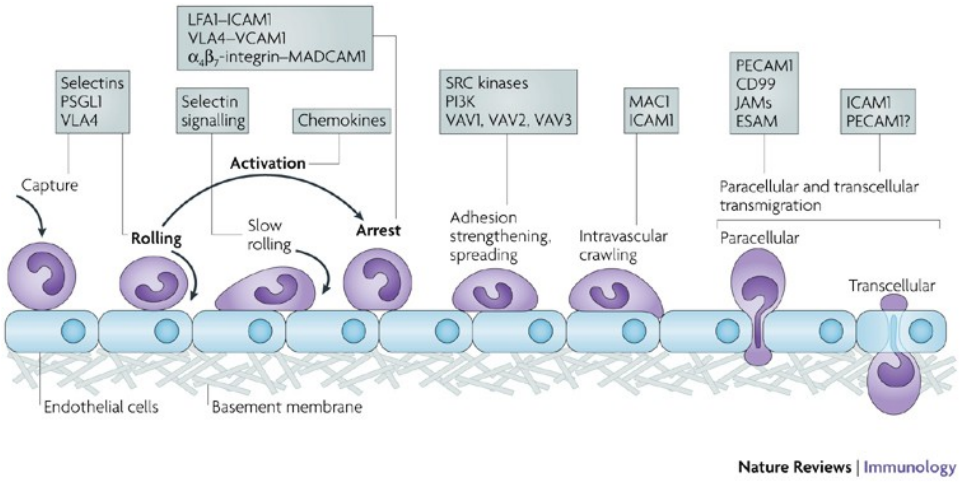
There are 2 types of EC activation. Type 1 is a rapid and transient response. It starts and lasts within minutes. It is typically mediated by ligands that bind to G-protein-coupled receptors which leads to an increase in intracellular Ca^{2+}, an example of this being the action of histamine [38]. The formation of the Ca^{2+}/calmodulin complex is required for the activation of eNOS.[48] A calcium-mediated activation of phospholipase A2 allows cleavage of membrane phosphatidylcholine into arachidonic acid, which is converted into PGI_2 by cyclooxygenases [49]. Ca^{2+} also allows contraction of actin filaments leading to the opening of intercellular gaps, resulting in an increase in vascular permeability and leakage of protein-rich plasma into the extravascular space [50].

Type 2 is a more sustained inflammatory response. It is mediated by TNF-α and IL-1, which initiate a cascade of kinases leading to activation of NF-kB and activator protein 1 (AP-1) [38]. Both transcription factors modulate the expression of proinflammatory cytokines such as TNF-α, IL-1, IL-8 and MCP-1. TNF-α and IL-1 have autocrine and paracrine effects on EC, ensuring positive feedback to the inflammatory response [39]. IL-8 and MCP-1 are chemoattractant to neutrophils and monocytes/macrophages, respectively [51, 52]. EC also secretes the platelet-activating factor (PAF), necessary for platelet aggregation as well as neutrophil adhesion [53]. A modulation of adhesion molecules expression controls the transition from neutrophilic to mononuclear infiltration. The duration of the type 2 response lasts as long as there are cytokines[38].

The migration of lymphocytes, monocytes and neutrophils from the intravascular space towards the tissue is possible owing to the presence of adhesion molecules. Selectins initiate the process

by allowing tethering of the lymphocyte to the EC. The lymphocyte is seen “rolling” to the inflammation site. There are 3 types of selectins: L-selectins, E-selectin and P-selectin [54]. L-selectin is constitutively expressed on most leukocytes and binds to glycoproteins (i.e., GlyCAM-1, MAdCAM-1, CD34) [39]. Attachment requires the induction of L-selectin ligands on the surfaces of platelets and EC. E-selectin is specific to EC. Its expression is induced by IL-1, TNF-α and LPS. It is found in both platelets and EC. P-selectin is stored in Weibel-Palade bodies (WPB), storage granules within endothelial cells, and released upon activation by cytokines. It interacts with its ligand, PSGL-1 found on leukocytes, and mediates their activation [54].

EC stimulates leukocytes by anchoring and presenting chemokines on its proteoglycan-rich glycocalyx. The stimulated leukocyte initiates a conformation change of its integrins, rendering them capable of binding to immunoglobulins on the endothelial surface [39]. Integrins are transmembrane glycoproteins that control intercellular and cell-matrix interactions. Leukocyte-EC interaction is mediated by 3 integrins: LFA-1 being the predominant integrin for lymphocyte migration, Mac1 responsible for neutrophil adhesion and VLA4 expressed on monocytes [39, 55]. EC, on the other side, expresses adhesion molecules ICAM-1, ICAM-2, VCAM-1 and e-selectin. The complementary ligand for Mac-1 is ICAM-1, minimally expressed during normal conditions, but markedly induced by cytokine activation. LFA-1 also binds to ICAM-1 and ICAM-2 whereas VLA-4 binds selectively to VCAM-1. Firm adhesion requires the binding of integrins [55]. The migration of leukocytes through the endothelium involves the interaction of Mac-1 and LFA-1 with ICAM-1. Monocytes use the interaction between VLA-4 and VCAM-1. The migration through gaps between the EC is a complex process called diapedesis and requires a cycle of adhesion and detachment [38, 39]. Anchoring of neutrophils causes a rise of intracellular calcium leading to EC contraction and disruption of junctional complexes. Concomitantly, attachment of leukocytes disrupts the VE-cadherin-catenin complex opening up cell junctions. All of this contributes to widening the intercellular space for leukocyte migration [39]. PECAM-1 is an immunoglobulin expressed on the surface of platelets, EC, monocytes and neutrophils. It is more concentrated at the intercellular junctions of EC but is evenly distributed on the neutrophil surface. The contributive role of PECAM-1 is to guide leukocyte migration [56].



Adhesion molecules and leukocyte adhesion process (ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular cell adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen-1; MAC1, macrophage-1 antigen; MADCAM1, mucosal addressin cell adhesion molecule 1; PECAM1, platelet endothelial cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; PSGL1, P-selectin glycoprotein ligand-1; SRC, selective neural cell adhesion molecule; Vav, the Vav protein family; VLA4, integrin receptor for VCAM-1;) [54]

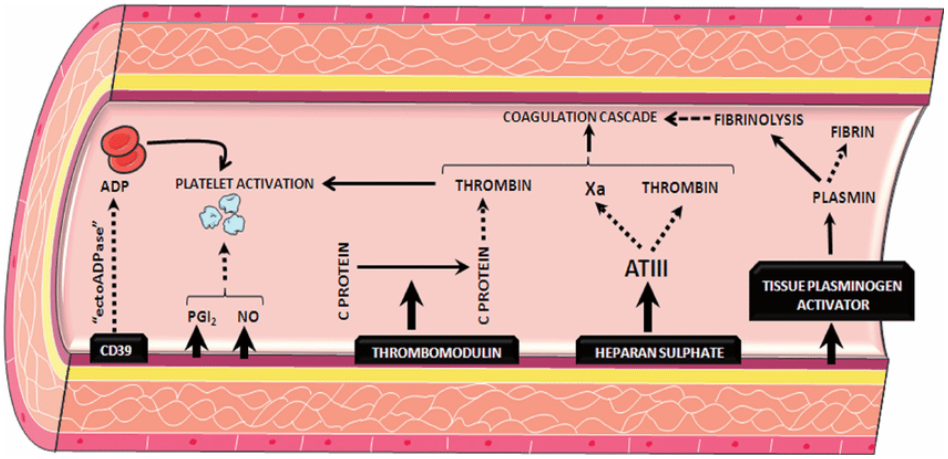
Figure 2. – Leukocyte adhesion process

1.2.3 Regulation of the coagulation cascade by the endothelium

In a normal state, the endothelium maintains the balance in favor of thrombolysis and anticoagulation. The endothelial surface displays ectonucleotidases, enzymes capable of hydrolyzing adenosine triphosphate (ATP) and adenosine diphosphate (ADP), therefore having an anti-aggregating effect [39]. The surface is also rich in heparan sulfate proteoglycans, a heparin-like molecule that binds antithrombin III and inactivates thrombin. EC expresses thrombomodulin, a membrane protein that regulates the activity of the protein C/S complex. Protein C has anticoagulant properties through the inactivation of factors Va and VIIIa. Furthermore, the production of PGI_2 and NO contribute to the inhibition of platelet activation and aggregation [38]. Additionally, EC releases low concentrations of tissue factor pathway inhibitor (TFPI), the primary inhibitor of the coagulation cascade through inactivation of factor Xa and tissue factor-factor VIIa complex [57]. EC also produces tissue plasminogen activator (t-PA) and urokinase, fibrinolytic agents. t-PA converts plasminogen into plasmin, allowing

degradation of the thrombus. EC counteracts t-PA with the production of plasminogen activator inhibitor (PAI-1), thereby tightly regulating the balance of fibrinolysis [58].

Following vessel injury or stimulation by cytokines, the equilibrium is tipped in favor of a prothrombotic state. Damages on the endothelial surface trigger the platelet activation. Following activation by cytokines, LPS or thrombin, ECs synthesize and express tissue factor on their surface. Tissue factor is the starting key of the extrinsic pathway. The end product of the coagulation cascade is the formation of thrombin (factor IIa). Thrombin converts fibrinogen to fibrin, which allows the creation of the clot matrix [58, 59]. Thrombin prompts the production of the platelet-activating factor (PAF) and encourages further cytokines release. PAF is a potent platelet activator and promotes adhesion of platelet to EC. Thrombin also stimulates the release of von Willebrand factor (vWF) and P-selectin from WPB, promoting further platelet activation and aggregation [39]. vWF stabilizes factor VIII and is required for platelet binding [60].



Antithrombotic properties of the normal endothelial (ADP, adenosine diphosphate; ATIII, antithrombin III; NO, nitric oxide; PGI_2, prostaglandin I 2; Xa, activated factor X) [61]

Figure 3. – Antithrombotic properties of the normal endothelial

1.2.4 Angiogenesis and proliferation

Angiogenesis is the formation of new capillaries. Arteriogenesis refers to the growth and maturation of a vessel [62]. EC proliferation is generally limited to the fetal period and the wound

healing process. It is particularly important for the development of solid tumors and is a major target in cancer therapy [63]. Triggers of angiogenesis can be inflammation, tissue growth, genetic mutations or hypoxia.

Failure of the inflammatory response to resolve induces T cells and phagocytes to produce and release angiogenic factors to increase blood flow to the affected site. In this situation, the angiogenic process is achieved by the action of the vascular endothelial growth factor (VEGF), which binds to its receptor (VEGFR), fibroblast growth factor (b-FGF) which binds to FGFR-1 and angiopoietin-1 and angiopoietin-2 which bind to tyrosine kinase receptor 2 (TIE2). Three pathways are typically activated: ERK1/2, PI3K/Akt and the Ca2+-PLCγ complex. The effect is survival, growth and migration of EC [38].

Hypoxia induces the hypoxia inducible factor (HIF-1), which activates the transcription of genes implicated in glycolysis (i.e.; glycolytic enzymes, GLUT-1), erythropoiesis (i.e., erythropoietin, transferrin) and angiogenesis (i.e. VEGF) [62]. HIF-1 is considered the main pathway behind the adaptative response to hypoxic stress. The complete maturation of the vessels involves the formation of a new basal membrane and recruitment of pericytes and SMC. Platelet derived growth factor (PDGF) is a SMC chemoattractant [39]. As for Angiopoietin 1, it is responsible for the remodeling and maturation of the vessel [62].

1.3 From harvest to vein graft failure

1.3.1 Early injury, ischemia and endothelial dysfunction

Routine vein graft preparation requires considerable manipulation and stretching, leading to endothelial injury and loss [64]. The use of marking pen for orientation has proven to be toxic and most preservation solutions cause endothelial dysfunction [65, 66]. Additionally, the use of a pressure-syringe for irrigation and distention exerts supraphysiologic pressures, up to 300mmHg, that induce structural and functional damage to the endothelium and SMC [67, 68]. This explains why samples of harvested SVG are often found to be dilated, flaccid and decreased in reactivity to vasoactive substances [69]. Loss of endothelium exposes the subendothelial

matrix and tissue factor and activates the platelets and coagulation cascade [32]. Although an overwhelming pro-coagulant response to injury can lead to an acute graft occlusion, most events are driven by surgical techniques (i.e., restrictive anastomoses, kinking, graft orientation and arrangements) and underlying coronary disease characteristics (i.e., small target vessels, poor distal runoff, diffuse CAD, heavily calcified target) [32, 70-72]. Early graft occlusions occur in 5-12% of cases [15, 35, 73-75].

Endothelial injury and dysfunction are characterized by impaired production of anticoagulant and vasodilatory factors such as NO, prostaglandins and tissue plasminogen activator (t-PA). At the same time, activated platelets continue secreting prothrombotic and vasoactive factors such as TxA2, PAF, ADP and ATP [67]. This imbalance of vasoactive substances promotes vasoconstriction, thrombosis and inflammatory response. Interestingly, in a study on canine saphenous grafts, venous rings denuded of endothelium maintained a vasoactive activity through prostaglandins produced by the SMC [76]. It suggests that PGI2 may play a more important role in vaso-activity of veins than arteries. Vein graft samples demonstrate upregulated expression of pro-inflammatory mediators (NF-kB, TNFα), cytokines (IL-1) and adhesion molecules (ICAM) [69]. Concomitantly, platelets express adhesion molecules on their surface (P-selectin), vWF and CD40 ligand. It leads to neutrophil recruitment and adhesion [77, 78]. Activation of local polymorphonuclear cells (PMN) causes the release of oxygen-derived free radicals, proteases and cytokines (TNF, IL-1, IL-6 and IL-8) and perpetuate inflammation [67]. The subendothelial space becomes edematous and infiltrated with leukocytes [79, 80]. Increasing levels of reactive oxygen species (ROS) from the inflammation and vascular injury further exacerbate graft dysfunction [81].

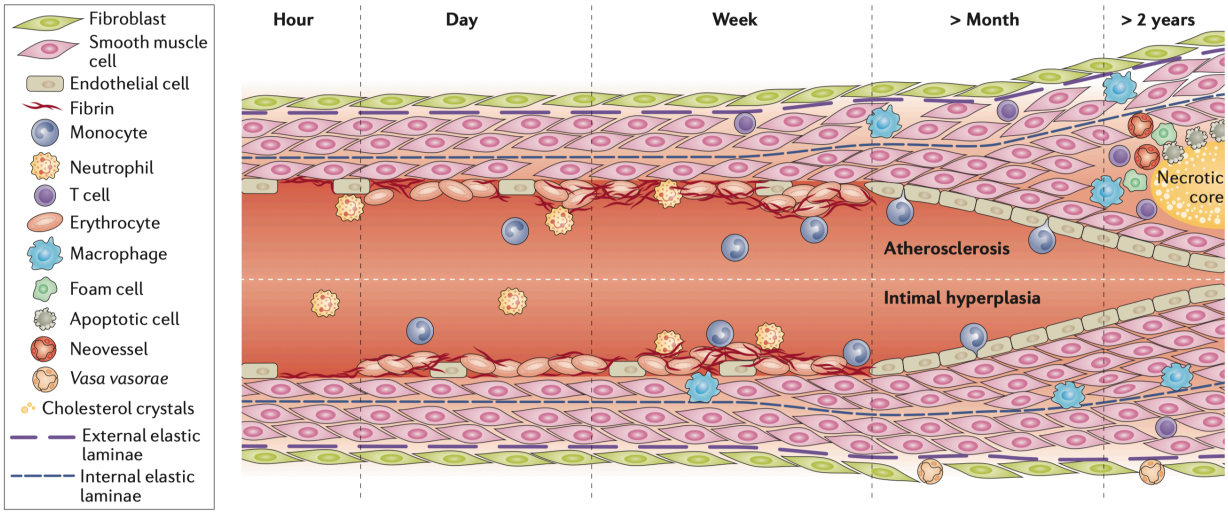
Moreover, vein grafts are often stripped from their adventitia and vasa vasorum, which are important for oxygen and nutrients delivery [33, 35]. Vein grafts sustain ischemia until grafting. Once grafted, the arterial pressure causes additional damage to the venous conduit. The higher pressure further damages the endothelium while flooding blood increases ROS formation, leading to ischemia-reperfusion injury [64, 67].

1.3.2 Exposure to arterial pressure and remodeling

Veins are poorly adapted to the higher arterial pressure. Saphenous vein (SV) has a much larger diameter and relatively thinner wall when compared to IMA, resulting in much greater internal wall stress.[34] Early on, SVG initiates the remodeling process, often called 'arterialization' [79]. The process results in matrix reorganization and development of a neointima in response to the arterial blood flow. Shear stress on the wall triggers the migration of SMC to the media. Activated platelets and injured endothelium secrete PDGF, b-FGF, VEGF, IGF-1 and TGF, promoting SMC proliferation [67, 70]. ROS are also powerful stimuli for SMC proliferation [80, 81]. Matrix metalloproteinases (MMP), predominantly MMP2 and MMP9, stimulate SMC migration and proliferation and participate in the matrix turnover [79, 82]. Imaging assessments of vein grafts in lower extremity bypasses found that the lumen diameter nonuniformly increases by 25% on average and wall thickness by 35% [80]. Within 2 weeks, the endothelium is mostly restored in studies on animal models. Duration for complete re-endothelialization remains unknown for humans and highly depends on the quality and the length of the graft used. Progenitor cells from the bone marrow and the adventitia contribute to the process [79]. Reestablishment of a fully functional endothelium marks the last stage of vein graft remodeling. At 30 days, the neointimal hyperplasia is mostly established [32]. Indeed, mature SVG end up displaying endothelium-dependent relaxation [80, 83]. The early remodeling response is a determinant of long-term function and patency of grafts. *Owens et al.* found that poor remodelers, grafts with inappropriate and insufficient remodeling, have a 13-fold increase in the risk of failure at 2 years compared to robust remodelers [80]. Intimal hyperplasia (IH) is in fact, a self-limited process that stabilizes and does not compromise luminal flow [35]. It is suggested that focal areas of stenosis may be due to an inappropriate hyperproliferative response superimposed on a restrictive pattern of insufficient outward remodeling [80]. Due to this ununiform response, IH is responsible for most graft failures during the first year of surgery.

1.3.3 Atherosclerosis and late graft failure

Most late graft failures result from unstable atherosclerotic plaques. Veins are prone to accelerated atherosclerosis, which differs from spontaneous atherosclerosis by being diffuse and more concentric appearance as well as having denser cellularity and varying degrees of lipid and mononuclear infiltration compared to arterial vessels [35]. The higher collagen content, a greater concentration of dermatan sulfate, a glycosaminoglycan with higher affinity for lipoproteins (LDL and VLDL), as well as high intercellular permeability all contribute to accelerating the atherosclerotic process [32, 36]. Plaques within SVG have a large necrotic core, are rich in inflammatory and foam cells, and are poorly calcified with a thinner cap. Those plaques are more susceptible to rupture [31]. Virtually all SVG show atherosclerotic plaques formation at 1 year [23, 83]. Development of atherosclerosis in SVG is associated with the same risk factors as native coronary arteries, highlighting the importance of maintaining an adequate lifestyle and following an optimized pharmacotherapy following surgical revascularization [84].



Development of vein graft failure [79]

Figure 4. – Development of vein graft failure

1.4 Strategies of VGF prevention

The severity of vein graft disease correlates with worse survival, higher MI and more frequent repeat revascularization [85, 86]. The early remodeling response is determinant and predictive of the long-term evolution of graft disease. The early recovery of a physiologically functional endothelium is pivotal [71]. Consequently, approaches focusing on minimizing initial injury and promoting the prompt restoration of endothelial function will likely have an impact on later development of VGF. Exploring therapies for vein graft failures has been an extensive area of research and still, to this day, stems from continuous innovative ideas. The strategies can broadly be grouped as either improvement in techniques of harvest and revascularization, optimized preservation solutions, pharmacotherapy or gene therapy.

Technical approaches	Preservation solutions	Pharmacotherapy	Others
• Optimal revascularisation strategy • External stenting • *No Touch* Technique • Endoscopic harvesting • Pressure controlled valve/distention	• Heparinised NS • Autologous whole blood • Buffered solutions *(see Table 2)*	• Statins • Anti-platelet therapy • Fibrinolytics and coagulotherapy • Direct-delivery intended compounds	• Glues • Carbon monoxide • Gene therapy *(see Table 3)*

Table 1. Strategies for prevention of vein graft failure

1.4.1 Technical approaches

1.4.1.1 Revascularization strategy

Surgeons play an essential role in the fate of their grafts. Conscientious harvest techniques are as important as planning the revascularization procedure. Choosing the proper conduits as well as the appropriate target is the basis of a successful surgical revascularization [87]. The target site should take into consideration the location, presence and severity of the disease. Non-

restrictive anastomoses on an appropriately sized vessels (≥2mm) lower the risk of graft failure [88]. A larger runoff area is associated with higher outflow potential and is associated with a lower risk of VGF [89]. Careful measurement of the graft for proper length, to avoid kinking or tension, is also necessary.

1.4.1.2 Pressure-controlled vein distention

A pressure-syringe is used for flushing and distending the vein to overcome vasospasm during harvest. Pressure can reach up to 400mmHg and can cause extensive endothelial denudation. [67, 68, 90] Vein distention is found to trigger various markers of inflammation [90]. In a study looking at veins samples from 21 patients, a pressure-controlled bulldog clamp was used to maintain intraluminal pressure under 80 mmHg. The protected vein segments showed less intimal loss and damage than the unprotected segments where mean pressure was measured up to 260mmHg [91]. *Li et al.* looked at porcine and human saphenous veins harvested with a pressure-release valve (PRV), which limits pressure to 140mmHg. Distention with the PRV reduced de-endothelialization and intimal thickening and preserved endothelial-dependent relaxation compared to regular distended veins [92]. While the use of a PVR is not widely adopted, there is a consensus that cautious flushing and minimal distention of grafts is warranted.

1.4.1.3 Endoscopic harvest

Endoscopic harvest is subject to extensive debate. While the technique requires longer harvest time, specialized material and expertise, faster wound healing and better aesthetic results support its use [93]. The technique is associated with less leg wound complications, particularly in patients affected by diabetes and obesity [94, 95]. However, reported clinical outcomes are mixed. A meta-analysis of 11 studies comparing endoscopic to the standard open technique, which includes over 18 000 patients, found that graft patency was superior with the open SVG harvest technique [open 82.3% vs. endoscopic 75.1%; OR 0.61 (95% CI, 0.43-0.87), p=0.01]. MI

incidence and mortality were not significantly different.[96] Reduced patency rate may be related to the significant traction-induced trauma. Histologic findings report stretched grafts devoid of adventitia as well as endothelial injury and loss [97-99]. The VICO clinical trial showed that the open approach demonstrated marginally better endothelial integrity compared to the endoscopic approach. However, the differences in clinical outcomes with regards to major adverse cardiac events did not reach significance. The study also supports that high-level experience with the endoscopic technique is needed for optimal results [98, 100]. Operator-dependent factors, types of devices and patient selection biases make the comparison of both techniques often difficult. Therefore, endoscopic harvest should be performed only in experienced hands and in selected patients.

1.4.1.4 No-Touch technique

The No-Touch technique (NTT) consists of harvesting the vein with the surrounding tissue and avoids vein distention or trauma. It allows preservation of the adventitia and vasa vasorum, rendering a better resistance to ischemia and vasospasms to the graft. Limiting manipulation also minimizes endothelial injury. NTT grafts showed a lower expression of KLF4, an indicator of SMC proliferation and differentiation [101]. Multiple studies have reported superior patency rates with the NTT. *Souza et al.* reported long-term graft patency that was significantly better for the NTT [conventional 76% vs. NTT 90%, p=0.01] at 8 years follow-up [102]. *Kim et al.* reported angiographic results from 368 patients, 103 of which benefitted from the NTT. NTT veins showed a better patency rate at 1 year [NTT 97.4% vs. conventional 92.4%; p=0.024], although it did not affect clinical outcomes [103]. Harvest of the surrounding tissue potentially leads to increased risk of saphenous nerve injury and leg infection. However, the PATENT SVG trial did not report a higher incidence of leg swelling and discomfort, nor infectious complications at 1 year [101].

1.4.1.4 External stenting

External stents are metallic, tissue or biodegradable sheaths wrapped around vein grafts at the time of surgery. Besides providing structural support to the vein, it provides an environment for neo-adventitial development and microvasculature growth. Regeneration of a new vasa vasorum within a week following implantation may allow the graft to respond adequately to wall stress. It is shown that inflammatory cells tend to infiltrate the space between the stent and the graft, promoting outward migration of SMC [104]. The VEST trials reported performances of external stents on operative patients. Stented grafts did not show changes in flow dynamics [105]. The studies have consistently demonstrated reduced intimal hyperplasia and lumen irregularities and conduit ectasia. Yet, VGF rates are comparable between stented and non-stented grafts at short (1 year) and long-term follow-up (up to 5 years) [106-108]. Evidences show that fixations with sutures are preferable to metallic clip [106]. Most studies on external stents involve a limited number of patients. External stents use remains mostly experimental at this point.

1.4.2 Preservation solutions

Preservation solutions are used to conserve and protect vascular conduits from desiccation until grafting. They can also serve as a vessel for drug delivery. They can be categorized into heparinized saline, autologous whole blood and buffered solutions. In a survey conducted among cardiovascular surgeons in top-ranked hospitals around the United States, 40% uses a pH-buffered solution, 28% uses saline and 27% uses autologous blood. The solution is heparinized in 89% of the cases and kept at room temperature in 74% of the time [109]. This variable practice is partly due to the lack of clinical data from randomized clinical trials. Most evidences come from in vitro experiments, animal studies and posthoc analysis.

1.4.2.1 Saline solutions and its harmful effects

Normal saline (NS) is an isotonic crystalloid, universally used for volume resuscitation. Its osmolality is 308mOsm/L. It contains 9g of NaCl per liter of water (Na concentration of 154mEq

and 154mEq for Cl). The pH is acid and varies between 4.5 to 7 [110]. NS repeatedly shows to be a deleterious solution for veins grafts. Storage in saline significantly impairs endothelium-dependent vasodilatory function [111, 112]. NS has an acidic pH. It is not buffered and thus, prone to acidification by ambient carbon dioxide. *Wise et al.* suggested that endothelial function is dependent on pH, with dysfunction occurring when pH is below 6 [112]. *Harskamp et al.* looked retrospectively at the 3000 patients from the PREVENT IV study (45% had SVG preserved in NS, 32% in autologous whole blood (AWB) and 17% in buffered saline). The VGF rate at the one-year angiographic follow-up was lower in the buffered saline group compared to NS (OR 0.59 [95% CI, 0.45-0.78; P<0.001]) and AWB [OR 0.62 95% CI, 0.46-0.83; P=0.001]. The composite endpoints of all-cause mortality, MI and revascularization were also lower in the buffered saline group compared to NS and blood (HR 0.81 [95% 0.64-1.02; p=0.08]) [113].

1.4.2.2 Autologous whole blood (AWB)

AWB is retrieved from the patient during the procedure, and heparin is added. The theoretical principles supporting the use of AWB are the capacity to deliver oxygen and the profile of a medium closest to physiologic conditions. Interestingly, however AWB is found to have a lower partial pressure of oxygen (PO_2) than NS [114]. This is because blood has, through the Bohr effect, an oxygen affinity dependent on pH. During the preservation period, the acidotic blood (pH 7.35) induces cellular anaerobic metabolism, which increases the consumption of oxygen and reduces its extracellular release and thereby the PO_2. This hypoxic environment induces expression of HIF-1α [114]. This transcription factor has been implicated in angiogenesis, apoptosis, cellular proliferation, survival and glucose metabolism [115]. Preservation in AWB was also found to induce higher expression of the antioxidant enzyme glutathione peroxidase, providing better protection against oxidative stress [114]. On a canine model, femoral veins were stored in heparinized saline or heparinized AWB for 1 hour. The morphological analysis showed superior preservation of endothelial cells and re-endothelialization of the grafts following AWB storage [116]. *Wilbring et al.* reported that SVG kept in AWB for 30 minutes retain greater contractility and endothelial functions. They also conserve higher cellular energy compounds.[117] On the

other hand, both AWB and NS were found to reduce vascular reactivity [118]. Clinical outcomes can be found in a sub-study of the PREVENT IV trial. VGF was reported to be 32.2% with AWB storage, compared to 44.4% in NS and 16.8% in buffered solutions [113]. Taking into consideration all the studies, AWB was either equal or superior to NS, but still inferior to buffered solution use [119, 120].

1.4.2.3 Buffered Saline solutions

Buffered solutions contain a weak acid and its conjugate base, which keep pH constant at different experimental and clinical conditions. They include a vast array of formulations whose components vary according to their clinical application. More evidences show their superiority for vein grafts preservation compared to NS and autologous blood.

1.4.2.4 Ringer's solution

Ringer's solution is an isotonic buffered solution. Its ionic concentration is closer to physiologic plasma than NS. It contains Na (130mM), Cl (109mM), K (4mM), Ca (1.5mM) and sodium bicarbonate as the buffer. When the solution contains sodium lactate, it is called Ringer's lactate, a crystalloid used clinically as a fluid replacement. The osmolarity is 273 mOsm/L and pH 6.5 [121]. *Ronbos et al.* studied SVG segments treated with a solution of Ringer's Lactate and an added vasodilator, either papaverine or trinitrate/verapamil, and found the combination of verapamil and trinitrate to preserve the endothelium optimally [122]. No study was found to compared Ringer's solution to other preservation media.

1.4.2.5 PlasmaLyte

PlasmaLyte reproduces plasmatic concentrations of Na, K, Cl, K, Mg with slight variations depending on the manufacturer. The solution is devoid of calcium as is thus compatible with blood components. Some formulations contain metabolizable bases that replace bicarbonate such as acetate, gluconate or lactate [123]. *Sanchez et al.* described a preserved vasorelaxation

response in canine SVG kept in PlasmaLyte. Similar results were found on human veins when stored at 37° C, but not at room temperature [124]. In a porcine model, an optimized vein preparation using pressure-regulated distention, non-toxic vein marker and Plasmalyte limited disruption of the endothelial layer, impairment of vasoactive responses and cellular metabolic dysfunctions when compared to the traditional method of vein preparation (standardized distention, use of toxic ink marker and heparinized saline solution) [125]. PlasmaLyte appeared as effective as other buffered media (University of Wisconsin, Celsior® and GALA) in preserving cellular functional integrity but is far more cost-effective [112].

1.4.2.6 The St-Thomas solution

The St-Thomas solution was the first cardioplegic solution, developed in the 1970s. It is the still most used cardioplegic solution among cardiac surgeons. It is composed of NaCl 110mM, $NaHCO_3$ (10 mM), KCl (16 mM), $MgCl_2$ (16mM) and $CaCl_2$ (1.2mM) with a pH of 7.8 [126]. The rapid onset and efficacious cardiac arrest support its popular use to this day. The one caveat is that repeated dosages need to be administered every 15 to 20 minutes to maintain cardioplegia, which makes it inconvenient if a long cardiac arrest is needed. Only one study reported the greatest constrictor response after storage in St-Thomas solution, compared to other buffered solutions, while relaxation responses were similar [118].

1.4.2.7 Organ preservation solutions

The **University of Wisconsin solution** (UW) was initially developed for liver grafts preservation in the 1980s before expanding its use to kidneys, pancreas and heart preservation. It is composed of Na (25mM), K (125 mM), $MgSO_4$ (5 mM), KH_2PO_4 (25mM), a colloid carrier HES (hydroxyethyl starch) (50g/L), penicillin 200,000U/L, insulin 40 IU/L, dexamethasone (16mg/L), glutathione (GSH) as oxygen radical scavenger, allopurinol 1mM and adenosine 5mM, raffinose (30 mM) and lactobionate (100mM). The pH is 7.4 [127-129]. *Wise et al.* reported improved contractile responses and preserved endothelial-dependent relaxation in human SVG preserved with UWS

compared to NS and AWB [112]. Following cold UWS storage for 2 to 3 hours, grafts demonstrated recuperation of both its contractility and vasorelaxation capacity. Vasoreactivity was well preserved even after 24 hours of conservation, declining steadily between 24 and 96 hours of conservation time [130]. UWS was superior in preserving vasoactive properties when compared to a Histidine-Tryptophan-Ketoglutarate (HTK) solution (see below), particularly during long periods of storage [130].

Celsior® was initially developed as a cardiac preservation solution in the early 1990s. It contains glutathione (3 mM), lactobionate (80 mM) and potassium (15 mM) [131]. The ionic components are Na (100 mM), Cl (28 mM), Ca (0.25 mM) and Mg (13 mM). It has an osmolality of 320 mOsm/L and a pH of 7.3 [132]. *Wise et al.* reported improved contractile responses and preserved endothelial-dependent relaxation in human SVG preserved with Celsior® compared to NS and AWB [112].

HTK preparations are intended for donor organs' perfusion and flushing. Its constitution relies on the principle of inactivating organ function by the withdrawal of extracellular sodium and calcium while offering extreme buffering of the extracellular space. It contains mannitol as an osmotic, histidine (198 mM) as a buffer, tryptophan (2 mM) as a membrane stabilizer and ketoglutarate (1 mM) as an energy substrate. HTK has a higher buffer capacity than UW. Because it does not contain HES, it is not as viscous as UW and can be used to flush organs [132]. HTK solutions contain relatively similar concentrations of histidine, tryptophan and ketoglutarate with differences met in the added components. TiProtec® and Custodiol® are marketed HTK formulations currently used in cardiac surgery.

TiProtec® is a vascular storage solution that has been developed for cold storage. It contains Na 16 mM, K 93 mM, Cl 103 mM, Ca 0.05 mM, Mg 6 mM. Glucose (10 mM) and Sucrose (37 mM). It has glycine and alanine to prevent hypoxic injury and an iron chelator, deferoxamine, for protection against cold-induced iron-dependent cell injury. Its pH is 7 and osmolality is 305 mOsm/L [132, 133]. *Wilbring et al.* reported superior vasoactive functions in SVG segments preserved in TiProtec® compared to NS [111].

Custodiol® is a cardioplegic solution. It contains Na (15 mM), Ca (0.02 mM), K (9mM), Cl (50 mM), Mg (4 mM) and mannitol (30 mM). The pH is 7.0 and osmolality 310 mOsm/L. It is an attractive solution for minimally invasive cardiac surgery and complex cases. A single dose provides an extended period of myocardial arrest and protection. However, it has not shown superior clinical outcomes compared to conventional cardioplegia [134]. *Aavik et al.* stored venous allografts in different cold buffered solutions for extended periods of time, up to 35 days, before looking at the generated histologic changes. Custodiol® induced the least damages on the structure of the graft and the intima [135].

DuraGraft®

It is a pH and ion-balanced solution designed and marketed for vascular graft preservation. It contains antioxidants (glutathione, L-ascorbic acid), L-arginine, which allows the synthesis of NO as well as glucose and high-energy phosphates to support anaerobic metabolism. DuraGraft® is also known as GALA, which stands for glutathione, ascorbic acid and L-arginine [136]. After a storage time of 60 to 300 minutes, human vein grafts showed greater preservation of endothelial viability, calcium mobilization and NO generation compared to NS, AWB and Hank's buffered saline solution (HBSS) [137]. Other data suggest maintenance of the functional integrity of SVG in GALA [112]. There are currently 2 ongoing clinical trials studying outcomes of patients receiving isolated CABG with the use of DuraGraft® as a preservation solution for SVG segments [138, 139].

		Use	Ionic composition (mmol/L)	pH	Osmolarity (mOsm/L)	Additional compounds
NS		Fluid resuscitation	Na 154 Cl 154	4.5-7.0	308	Heparin
AWB		Oxygen and nutrients delivery	*Patient's plasmatic ionic concentrations*	7.35-7.40	275-295	Heparin
Buffered solutions						
Ringer's Solution		Fluid resuscitation	Na (130) CL (109) K (4) Ca (15)	6.5	273	Sodium bicarbonate as buffer When lactate is present, solution is named Ringer's Lactate
Plasmalyte			Plasmatic concentration of Na, K, Cl, Mg *No calcium	7.4	280-310	-
UWS		Organ preservation	Na (30) K (120) $MgSO_4$ (5) $HaPO_4$ HPO_4	7.4	320	HES, glutathione, adenosine, allopurinol, raffinose, lactobionate
Celsior			Na (100) Cl (28) Ca (0.025) Mg (13)	7.3	320	Glutathione, lactobionate
HTK	**TiProtec**		Na (16) Cl (103) K (93) Ca (0.05) Mg (6)	7.0	305	Histidine, tryptophan, ketoglutarate, Glucose, Sucrose, Glycine, alanine, deferoxamine
	Custodiol	Cardioplegia	Na (15) K (9) Ca (0.02) Mg (4)	7.0	310	Histidine, tryptophan, ketoglutarate, Mannitol
St-Thomas solution			Na (110) $NaHCO_3$ (10) KCl (16) $MgCl_2$ (16) $CaCl_2$ (1.2)	7.8	303	-
DuraGraft		Vascular Graft Preservation	pH and ion-balanced†		Similar to plasma†	Glutathione, ascorbic acid, L-arginin

†According to manufacturer's website (https://www.somahlution.com/products-duragraft/)

Table 2. Preservation solution composition

1.4.3 Gene Therapy

The growing interest in genetic therapy started in the early 1990s. Gene therapies combine approaches that influence and control the content or expression of genetic material. It is done by delivering an active transcriptional unit to a cell by a vector. Vectors are either viral or non-viral. Non-viral vectors encompass naked plasmid DNA, oligodeoxynucleotides, small interfering RNA (siRNA) and polymeric nanoparticles. While they provide relative safety as they are devoid of viral genetic material and induce less host immune response, their transducing efficiency is inferior to viral vectors [140, 141]. Viral vectors include retrovirus, lentivirus, adenovirus and adeno-associated virus [141]. Viruses have long evolved and adapted to become potent vectors. However, they can cause direct cytotoxicity and trigger a stronger immune response.

In choosing the ideal vector, specific clinical circumstances of application are important to consider. In the context of CABG, the particular operative setting opens a window during which gene therapy can be performed. Once harvested, the SVG is preserved in a solution for a period of 15 to 45 minutes before being grafted. The chosen vector should be efficient enough to deliver the transgene into the graft during that brief period. As an example, the PREVENT trial protocol detailed an *ex vivo* administration of medication through a controlled-pressure delivery system for 10 minutes before grafting [142]. Additionally, the vector should be easy to store and handle. It also needs to be safe to endothelial cells and SMC, generate little to no inflammatory response and confers a desirably lasting effect on the targeted gene [142]. An *ex vivo* therapy allows local treatment of the target tissue and minimizes systemic exposure. Adenoviral vectors have emerged as the agent most suitable under these criteria, specifically adenovirus type 5 [143-145].

The transgene may be of human origin or exogenous (e.g. viral or bacterial). It can be a new transcriptional unit or a sequence that affect the expression of a native gene. Expression of the transgene depends on cell transcription and translation activity. Generally, transgenes are transient unless they cross the nuclear membrane and integrate themselves into the genome or adopt an episomal form, a stable cytoplasmic form of DNA which can provide long-term expression [144].

On the other hand, inhibition of a gene can be done through the use of oligonucleotides. They are short antisense sequences, 15 to 20 bases in length, and specific to a target mRNA. Binding of oligonucleotides to mRNA results in enzymatic degradation of mRNA, preventing translation of RNA into proteins. Transcription factors decoys are oligonucleotides that mimic binding sites of the transcription factor. One major advantage of oligonucleotides is their small size, which makes them easier to deliver to cells [144]. E2F decoy oligonucleotides were used to prevent E2F-mediated transcription of pro-proliferative genes. E2F is a family of transcription factors that plays a role in cellular proliferation and differentiation. It is also a regulator of cell death [146]. Despite promising phase I and phase II trials, the clinical application of the decoy in phase III trials failed to demonstrate improvement in rates of vein graft failure as well as major cardiovascular outcomes in peripheral and cardiac revascularization [142, 147-149].

Understanding of the pathophysiology of a disease is essential in choosing a therapeutic target. VGF mechanisms detailed earlier help us recognize 3 stages: early thrombosis, IH and atherosclerosis. Early graft failure is mainly influenced by technical errors and underlying vascular pathology. It also occurs too soon for a transduced gene to take effect. Development of atherosclerosis, on the other hand, is a multifactorial process, thus making it a challenging target for gene therapy [150]. Therefore, the majority of strategies for the prevention of vein graft failure focus on tempering SMC migration and proliferation to slow the progression of intimal hyperplasia (IH) [143]. Table 3 summarizes a comprehensive list of gene therapies/targets explored and experimented from the literature.

By attenuating growth factors such as TGF-b or Egr-1, SMC proliferation is limited and so is IH. On the other hand, inducing endothelial growth factors (VEGF, PDGF) promotes faster re-endothelialization, earlier recovery of vascular protective functions, as well as limiting neointimal growth. Higher production of NO through the induced expression of NOS (iNOS, eNOS and nNOS) has an anti-aggregatory effect on platelet, limits leukocyte migration and adhesion and promotes vasorelaxation. Limiting inflammation, either by inhibiting adhesion molecules (E-selectins, VCAM, ICAM), inhibiting monocyte chemoattractant chemokine (MCP-1) or suppressing the expression of pro-inflammatory transcription factor NF-kB have all shown to reduce IH. As matrix degradation and remodeling by MMP plays a crucial role in SMC migration and proliferation,

adjusting the balance of MMPs and TIMPs activity in favors of a reduced matrix remodeling was associated with less IH. Other transcription factors involved in cellular replication were also targeted. The complex PTEN/PI3K (phosphatase and tensin homolog/ phosphatidylinositol 3-kinase) is a tumor suppressor gene involved in the regulation of the cell cycle [151]. They were found to be an important regulator of vascular SMC migration and proliferation [152, 153]. Inhibition of G$\beta\gamma$ protein, a G-protein associated with SMC proliferation, was also found to reduce IH in dogs. Suppression of PCNA (proliferating cell nuclear antigen) and cyclin-2 kinase, which are critical for progression into mitosis, showed a decreased in the neointimal formation and medial hypertrophy. E2F is a group of genes encoding a family of transcription factors involved in the cell cycle and synthesis of DNA. E2F decoy has been extensively studied in vivo both in animals and humans. The PREVENT studies marked the only large-scale clinical trial of the use of gene therapy to address graft failure [142, 147-149]. Although clinical benefits have yet been observed, we know that gene therapy in the context of vein grafts manipulations is a feasible and safe approach.

	Gene		Vector/expression	Gene function	Model	Action
NO production	NOS	eNOS[154]	Inducible	NO production	Goat	↓IH
		eNOS[155]	Inducible Plasmid encapsulated in virus of Japan-liposomes		Canine femoral vein	↓IH
		eNOS[156]	Adenovirus Inducible		Human SVG	Increase NO release
		iNOS [157]	Increased expression through cDNA		Rabbit	↓IH
		iNOS[158]	Adenovirus Inducible		Porcine	↓IH
		nNOS[159]	Adenovirus Inducible		Rabbit	↓Adhesion molecule expression ↓Inflammatory cell infiltration ↓IH ↓Vascular superoxide production
Vasodilatory	PGI2/COX-1	PGI2/COX-1[160]	Adenovirus Inducible	COX-1: production PGI2 PGI2: vasorelaxation, inhibition of platelets and reduction in monocytes attachment	Rabbit	↑Blood flow and lumen size
Inflammation	MCP-1	MCP-1[161]	Adenovirus Inhibitory	Monocyte chemoattractant protein when coupled with CCR-2	Canine	↓IH
		MCP-1[162]	Electroporation of plasmids Inhibitory		Murine	↓IH ↓SMC proliferation
		MCP-1[161]	Adenovirus Inhibitory		Canine	↓IH
	CCR-2	CCR-2[163]	siRNA	Forms an active complex with MCP-1	hypercholesterolemic Leiden mice	↓SMC migration and proliferation graft thickening
	NF-kB	NF-kB[164]	Oligonucleotide Inhibitory	Transcription factor responsible for mediating inflammation	Rabbit	↓IH
		NF-kB[165]			Canine	↓IH
	Elastase	Elastase [166]	Inhibitory Hemagglutinating virus of Japan	Leukocyte infiltration and serine elastase activity lead to SMC proliferation.	Jugular vein graft interposition in rabbits	Reducing early inflammation response Decrease in neointimal elastin deposition: more resistant to atherosclerosis
Adhesion molecules		Adhesion molecules (E-selectins, VCAM-1, ICAM-1)[167, 168]	siRNA inhibitory	Monocytes and endothelial cells interactions and recruitment	HUVEC on a developed perfusion model	Induce SMC apoptosis
Coagulation thrombolysis	PAI-1	PAI-1[169]	Adenoviral Inhibitory	Inhibition of plasmin generation	Murine	↓Wound healing ↓IH
	Thrombomodulin	Thrombomodulin/ endothelial cell protein C receptor (EPCR)	Adenovirus Inducible	Upon binding to thrombomodulin, thrombin incapable of cleaving fibrinogen or thrombin receptors and acquires the ability to	Rabbit	IH not affected. Inhibition of local thrombin activity.

				activate protein C which, in turn, degrades factor Va and VIIIa.		
	t-PA	t-PA[170]	Adenovirus Inducible	Converts plasminogen into plasmin which allows thrombolysis	Porcine	↑Graft blood flow ↓Flow-restricting thrombi
Matrix modulator	TIMP	TIMP-1[171]	Adenovirus Inducible	Regulate breakdown of the local matrix by inhibiting MMP activity. Migration of SMC requires active breakdown of local matrix surrounding the cell.		↓ vein graft thickening
		TIMP-1[172]			Human SVG	↓IH ↓SMC migration
		TIMP-2[173]			Murine	↓ MMP activity ↓ vein diameter and remodeling
		TIMP-2[174]			Human SVG	↓IH
		TIMP-3[175]			Human SVG Pig SVG	↓IH
	MMP	MMP-2 and MMP-9[176]	SiRNA Inhibitory	Degradation of matrix facilitating SMC migration and proliferation	Cultured human SMC	↓ invasive capability of SMC in Matrigel
Tumor suppressor gene and cell cycle mediators	Rb	Rb[177]	Adenovirus Inducible	Retinoblastoma protein is a tumor suppressor gene	Rabbit	↓IH
	PCNA	PCNA Cdc2[178]	Oligonucleotides Inhibitory	PCNA and Cdc2 are critical in modulating cell cycle leading to progression to mitosis	Rabbit	↓ IH and medial hypertrophy ↓ susceptibility to develop atherosclerosis
	PTEN/P13K	PTEN/PI3K[153]	Adenovirus inhibitory	Tumor suppressor gene : regulators of vascular SMC proliferation, migration, and cell death	Canine	↓IH
		PTEN/PI3K[152]	Adenovirus inducible		Rabbit	↓IH
	E2F	E2F[179]	Oligonucleotide inhibitory	E2F induces expression of critical cell cycle genes (PCNA, cdc2, c-myc) leading to progression to mitosis	Rat	↓ IH
		E2F[180]			Rabbit	↓ IH ↓ atherosclerosis
Growth factor / proliferation	Fibromodulin	Fibromodulin [181]	Adenovirus Inducible	TGF-b antagonist activity	Human vein grafts	↓IH
	Activin A	Activin A [182]	Adenovirus Inhibitory	Family of TGF-b	Rat	↓IH
	TBF	TGFb[183]	Oligonucleotides Inhibitory	Profibrotic and pro-proliferative effect on SMC	Rat	↓IH ↓collagen synthesis
	VEGF	VEGF-D[184]	Adenovirus Inducible	vasculo-protective; associated with reduced restenosis; limit IH progression through enhancement of endothelial recovery	Rabbit	↓IH
	PDEGFR	PD-ECGF[185]	Plasmid vector Inducible	inhibits SMC migration and proliferation and proliferation	Rat	↓IH
		PDEGFR-β [186]	Plasmid Inducible		Rabbit	↓IH
		miR-221/miR-222[187]	miRNA Inhibitory	proliferative, pro-migratory and anti-apoptotic effects on SMCs	Rat	↓IH
	HSV-1	herpes virus (HSV-1)[188]	Mutated HSV-1	Mutant HSV-1 selectively targets proliferative cells		↓SMC proliferation and IH
	Egr-1	Egr-1 [189]	Decoy Inhibitory	Early growth response gene	rabbit	↓SMC proliferation ↓IH
	Gβγ protein	Gβγ protein[190]	Adenovirus Inhibitory	G protein associated with SMC proliferation	canine	↓IH
	CREB[191]	CREB[191]	Oligonucleotides Inhibitory	Transcription factor associated with activation of IH	murine	↓IH

Table 3. Gene Therapies for VGF protection

1.4.4 Current pharmacological approach to prevent vein graft failure

Current pharmacological treatments addressing VGF can be divided into 2 categories, those that are administered systemically over a long period and those administered locally and directly on the vein graft. Currently, only systemic-administered medications are clinically used. They include antiplatelet therapy and statins. Those drugs have long cemented their role in the prevention of graft failure with parallel benefits in reducing cardiovascular mortality and morbidity. On the other hand, preventing VGF by direct drug delivery is still under development. None have reached clinical application.

1.4.4.1 Antiplatelet therapy

Platelet activation and thrombin formation are the starting events leading to VGF. Injured grafts or vessels are particularly at risk for platelet aggregation and stenosis. Clinical trials showed a reduction in the rate of graft occlusion when patients are started early on aspirin in the postoperative period [192-195]. Starting aspirin later, between 3 to 5 days after surgery, did not prove to be efficient at improving graft patency at 1-year follow-up [195, 196]. This emphasizes the fact that the pathologic process leading to VGF starts early and so should the administration of an antiplatelet drug.

Aspirin inhibits COX-1 and COX-2, enzymes that catalyze the conversion of arachidonic acid to prostaglandin H2, the precursor of TXA2 [197]. TXA2 induces platelet aggregation and vasoconstriction, which largely contributes to the vessel thrombosis [198]. The ACC/AHA guidelines suggest starting aspirin (ASA) 100-325mg daily as soon as 6 hours after a CABG procedure, based on results of the studies mentioned above [199]. Life-long treatment with ASA is recommended for all patients following revascularization procedures in both the European Society of Cardiology (ESC) guidelines and the American Heart Association (AHA) [199, 200].

Dual antiplatelet therapy (DAPT) consists of adding another antiplatelet therapy to aspirin (ASA), usually a $P2Y_{12}$ receptor inhibitor (i.e., clopidogrel, ticagrelor, prasugrel, ticlopidine). *Ex vivo* experimental models show that their combination enhanced their antithrombotic effect compared to their individual efficacy [201]. Among patients presenting with non-ST-elevation acute coronary syndromes in the CURE trial there were lower death and cardiovascular complications with the combined therapy at 12 months. However, it should be considered that the addition of a P2Y12 receptor inhibitor also comes with an increased risk of bleeding [199, 202]. DAPT strategy is therefore recommended in patients considered at risk for thrombosis, such as those presenting with acute coronary syndrome (ACS) or who had a recent PCI [203].

1.4.4.2 Antithrombotic therapy

Using an anti-thrombotic therapy seems intuitive in the context of the early thrombotic risk. A late meta-analysis by *Fremes et al.* reported results from 6 small trials that showed favorable graft patency with the use of anticoagulation therapy additional to ASA.[204] By contrast, in the most recent and larger Post-CABG trial, angiographic assessments performed between 4 and 5 years after CABG surgery in 1352 patients did not show significant improvement in terms of graft disease progression among those treated with warfarin compared to the placebo group [205].

A small trial looking at the use of fibrinolytic reported that the added administration of t-PA during the early operative period on top of the conventional treatment (ASA and clopidogrel) did not change the degree of stenosis at the 3-month angiographic follow-up [206].

Systemic anticoagulation by heparin or bivalirudin is required during the revascularization procedure to prevent clot formation during flow-limiting steps (i.e. stasis due to occlusion of graft for anastomotic purposes or hemostasis within the resting heart) and within the extracorporeal circuit, but not to prevent graft failure [207].

In conclusion, antithrombotic therapy showed limited success in preventing graft disease post-CABG. Therefore, the use of antithrombotic therapy post CABG is not supported by current guidelines [199].

1.4.4.3 Statins

Statins are HMG-CoA reductase inhibitors, an enzyme playing a central role in endogenous cholesterol production [208]. Its discovery dates back in the early 70s when research has already documented the function of HMG-CoA reductase. Akira Endo, a Japanese biochemist suggested the idea that an inhibitor of HMG-CoA reductase must exist in microorganisms to produce mevalonate, an organic compound produced by HMG-CoA from Acetyl-CoA and acts as a precursor of many substances required for building their cytoskeleton [208, 209]. The first molecule identified was mevastatin, produced by *Penicillium citrinum*, but it never made it to the market due to its numerous adverse effects [209]. It was in 1975 that the first commercialized compound, lovastatin, was released. Since then, statins have become one of the most prevalent drugs used worldwide.

Statins are well known to be effective at reducing LDL [210]. The current AHA guidelines recommend statins as secondary prevention in all patients with diagnosed CVD [211]. They also recommend as primary prevention of cardiovascular disease in a population with elevated LDL, those with documented CVD despite their LDL levels and those considered to be at intermediate or higher risk of developing CVD [212]. It is supported by the fact that statin use reduces all-cause mortality and cardiovascular mortality as well as cardiovascular complications in these populations [213, 214]. In cardiac surgery, all patients are required to continue their statin therapy indefinitely [215]. The POST-CABG trial showed that aggressive LDL-lowering treatment significantly prevented progression of atherosclerosis and reduced the incidence of repeat revascularization compared to conservative treatment [205]. A sub-study of POST-CABG found that intensive treatment had a protective effect on the incidence of SVG stenosis, independent of the LDL-lowering effect [216]. A more recent trial by *Kulik et al.* came to the same conclusion; despite lower lipid levels with the higher dosage of atorvastatin, both intensive and conservative treatments similarly prevented vein graft disease [217]. Perioperative treatment with rosuvastatin is found to significantly reduce C-reactive protein, an inflammatory marker [218]. This later supports the now established knowledge of statins' pleiotropic effects. Beyond its lipid-

lowering capacity, statins benefits range from induction of eNOS expression, prevention vasospasm and acute thrombosis through inhibition of ET1 activity, inhibition of platelet aggregation, attenuation of SMC proliferation with suppression of IH, reduction of chemokine receptor and inflammatory gene expression to increase in endogenous antioxidants and improved redox defenses [208]. It is suggested that those effects stem from the implication of the Rho/ROCKs pathway [208, 219].

1.4.5 Direct delivery-intended compounds

The methods of direct administration of drugs to a vascular graft can be broadly categorized into periadventitial delivery or direct exposure during the preservation period. Periadventitial delivery is possible thanks to the development of biocompatible material. Developed since the early 2000s, they include the use of hydrogels, polymer wraps and nanoparticles. Hydrogels are the most commonly used. Its properties are adjustable depending on the concentration, the structure and the molecular weight of the polymer [220]. Some compounds that have been coupled to such gels are paclitaxel and nitric oxide-releasing agent [221, 222]. Significant disadvantages of hydrogel usage is the early diffusion of drugs into the surrounding tissues, generating high drug concentration and increasing the risk of toxicity. Hydrogel degradation is dependent on the environment, and thus the net release effect is poorly controllable. Wraps are polymer film loaded with drug and are deemed more durable. Nanoparticles helps improved delivery efficacy while reducing potential inflammatory process generated by polymer films or gels [220]. Finally, drugs can also be mixed into preserving solutions.

Rapamycin, also known as Sirolimus, was initially an antifungal but is now used in various clinical settings such as in cancer therapy, tuberous sclerosis complex or lupus, among others. Rapamycin limits proliferation by targeting the mTOR signaling pathway which is implicated in cell growth and metabolism [223]. Rapamycin is currently used as a coating drug for intracoronary stents. Rapamycin in nanoparticle form has been tested on rabbit jugular vein interposition and found to reduce intimal hyperplasia [224]. Rapamycin infused-wraps were studied in a model of arterial injury on rats and founds also to reduce IH [225].

Resolvins are a family of mediators derived from omega-3 fatty acids that promote resolution of the inflammatory response. They reduce the expression of adhesion molecules (i.e., ICAM-1, VCAM-1) and inflammatory cytokines (i.e., TNFα, IL-1b, MCP-1, IL-6). A pre-treatment with resolvins decreases activation of SMC isolated from human SVG. They seem to have a cytostatic effect on SMC rather than a cytotoxic one [226]. Perivascular delivery to a model of an injured artery in rats showed similar findings in addition to reducing oxidative stress [227].

Suramin is an anti-parasitic drug and acts as an antagonist of PDGF receptor. Locally applied suramin gel reduces neointimal thickness in mouse veins submitted to balloon-induced mechanical injury. Suramin is reported to limit migration and proliferation of SMC [228]. Suramin was also reported to reduce proteoglycan synthesis from human SMC. Modified proteoglycans allow retention of lipoproteins and following the development of atherosclerosis [229].

NO is recognized as a marker of endothelial health. It has counteractive effects on the development of vein graft failure. NO-releasing nanoparticles have been used on a model of arterial injury in rats' carotid and found to decrease inflammation and IH [230]. *Kown et al.* documented a higher production of NO by rabbit vein grafts treated with L-arginine polymer compared to treatment with a buffered saline solution. Production of NO was dose-dependent to the concentration of L-arginine. Neointimal progression on histologic assessment at 4 weeks also slowed down [231].

Drugs can be mixed in preserving solutions. The advantage of this method is the lower if not absent risk of systemic toxicity as there is no direct administration of the drug to the patient. One disadvantage remains the short and variable period of storage. Hence, the compound needs to have a rapid onset and lasting action. In this application, a few drugs have been tested. Leoligin is an extracted compound from Edelweiss (*Leontopodium alpinum Cass*.), a plant found in mountainous regions and used in folk medicine to treat diarrhea, fever and abdominal cramps. The drug has been studied and associated with a wide variety of effects such as lipid-lowering, antioxidant, antihypertensive, anti-inflammatory and antithrombotic. *Reisinger et al.* demonstrated leoligin-associated inhibition of IH in human SVG cultures [232]. Resveratrol is a polyphenol present in wine and provides cardiovascular benefits through vasodilatory,

antioxidant and inflammatory effects. *Kaplan et al.* demonstrated a reduction in expression of ICAM-1 and VCAM-1 associated with a decrease in neutrophil adhesion on SVG stored in AWB with Resveratrol compared to storage in AWB alone [233]. Vascular reactivity experiments on human SVG and ITA also revealed improvement in endothelium-dependent relaxation [234]. *Ronbos et al.* studied SVG segments treated with a solution of Ringer's Lactate with a vasodilator, either papaverine or trinitrate/verapamil. They found the combination of verapamil and trinitrate to preserve the endothelium optimally [122]. The use of antiproliferative agents such as cytochalasin D, paclitaxel or rapamycin on porcine SVG did not show reduction in neointima development or wall thickness after 12 weeks [235]. Oxidative stress is implicated in the development of IH. One team assessed the use of intraoperative treatment with polyethylene glycol infused with superoxide dismutase (PEG-SOD) on rabbit vein grafts and found reduced IH at 28 days [236]. Carbon monoxide (CO) has also been reported to reduce IH. *Nakao et al.* used a model of an arterialized graft by transplanting an inferior vena cava as an interposition graft in a rat abdominal aorta. Grafts were preserved in a carbon-monoxide-saturated Lactate Ringer. The study showed that EC death is significantly reduced with CO treatment. The proposed underlying mechanism is CO ability to inhibit ICAM-1 expression [237].

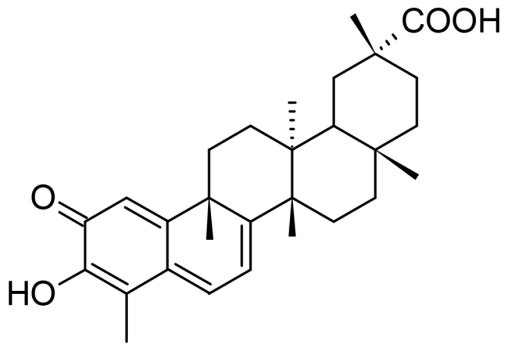
1.4.5.1 Combining pharmacotherapy and optimized preservation solutions

One important message to retain from the prior studies is that local therapy at a focal time is a feasible strategy. Contrary to surgical techniques, preparing preservation solutions are relatively simple and reproducible. It is not operator-dependent and does not require expertise nor specialized equipment. The preservation period is a valuable timeframe when adding pharmacologic treatments is possible. Such a strategy allows to act during a determinant time when the graft is most susceptible to injury. The challenge is finding the right compound. Suitable drugs need to be compatible with the preservation solution and deemed safe for endothelial cells. It must be able to activate vital protective responses early and effectively. The drug should also be capable of activating long-term cellular pathways for lasting benefits. An anti-

inflammatory profile, a redox potential or regenerative endothelial properties are some of the characteristics we should look at.

1.5 Celastrol

Tripterygium wilfordii (TW), also referred to as “Thunder of God Vine”, is a poisonous plant whose roots contain several therapeutic compounds such as terpenoids, alkaloids and steroids. One of the most abundant and active components is Celastrol. It is commonly used in Chinese medicine as an anti-inflammatory treatment against immunologic diseases such as rheumatoid arthritis. Celastrol belongs to the triterpene quinine methides family, which are compounds made up of five cyclic rings. Celastrol has seen a recent surge of scientific interest in the fields of cancer, inflammatory disease and anti-obesity research [238]. It was also investigated in the fields of neurodegenerative diseases and cardiovascular protection [239-241]. Celastrol is a known HSP90 inhibitor, which is a central mechanism leading to its known effects [242]. HSP90 is present in all cells and interact with hundreds of different proteins. The end-result of HSP90 inhibition on those proteins is dependent on the cell type and its baseline status [243].



Chemical structure of Celastrol [244]

Figure 5. – Chemical structure of Celastrol

1.5.1 Anti-inflammatory properties

Celastrol exerts its anti-inflammatory and immunomodulatory effects through inhibition of NF-kB. NF-kB is a transcription factor that forms a complex with its inhibitory protein Ik-Ba in the cytosol. Activation of various membranous receptors allows activation of IKK, which phosphorylates Ik-Ba, dissociating it from NF-kB. Once released, NF-kB translocates to the nucleus, where it modulates the transcription of genes involved in the inflammatory response [245]. Celastrol blocks the activity of IKK, thus preventing phosphorylation of IkBa [246]. Additionally, Celastrol is found to inhibit several transcription factors (TF) involved in the inflammatory response (*see Figure 6*). Consequently, Celastrol manages to suppress the production of various pro-inflammatory cytokines (i.e., TNF, IL-2, IL-6, IL-8, IL-1β, IFN-γ) [241]. Celastrol has also shown to inhibit the expression of adhesion molecules such as E-selectin, VCAM and ICAM-1 on activated endothelial cells [247]. Many autoimmune diseases such as polyarthritis rheumatoid (PAR)are the result of the excessive production of pro-inflammatory cytokine and thus, can be a potential target for the drug. Many small clinical trials have tested the use of Celastrol in PAR patients. A meta-analysis of 14 of these studies revealed that Celastrol reduces PAR symptoms, but is not superior compared to the current treatment [248]. Its application has also been studied in many other inflammatory diseases such as asthma, inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE) and psoriasis. A few small clinical trials explored treatments of TW extract in patients with Crohn's disease. They reported reduced inflammatory markers and activity index comparable to standard treatment with azathioprine or mesalazin. TW extract reached similar efficacy as disease-modifying anti-rheumatic drugs (DMARDs). However, it shows a high prevalence of adverse effects, mostly of gastrointestinal origin, such as diarrhea, nausea abdominal pain, dyspepsia. Adverse effects can reach an upsetting 50% of treated patients in clinical studies. [249] It should be noted that TW extract can contain up to 70 different active components, which does not translate to specific effects due to Celastrol [248]. Also, the major limitations of those studies are their small and heterogeneous study population, the lack of long-term follow-up as well as objective radiological assessments. The poor tolerability of systemic administration of Celastrol remains a significant constraint to designing larger trials. One area of interest is studying the chemical structure of the compound

and its related structure/activity relationships to redesign similarly inspired molecules with lower toxicity, higher tolerability and perhaps, even better efficiency [238, 239].

1.5.2 Molecular targets for anti-cancer therapies

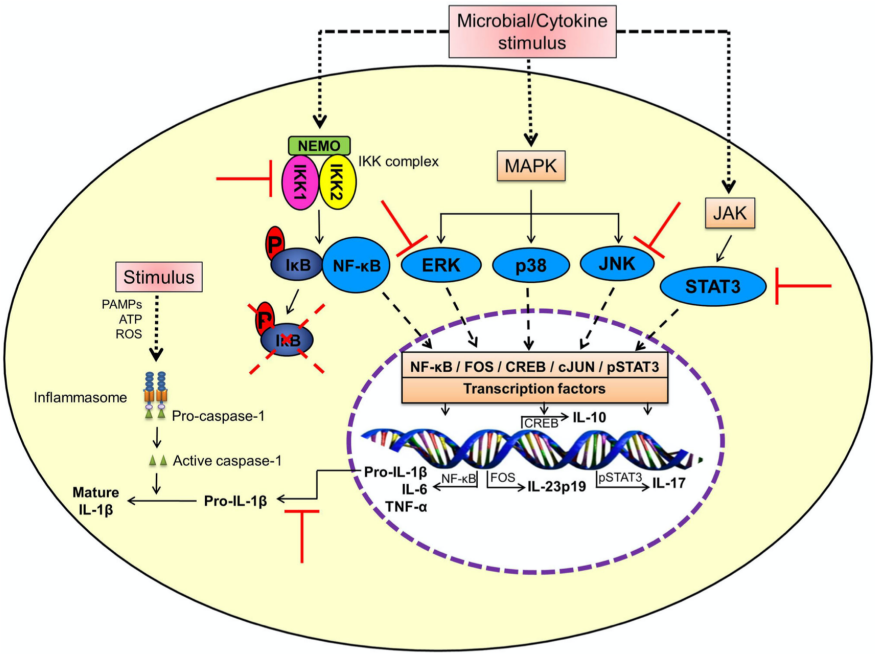
Cancer is a complex process implicating multiple genetic alterations to the cell, which confer its dysregulated proliferation and invasion properties. Celastrol acts as an anti-neoplastic agent by tempering various molecular pathways involved in neoplastic cell survival, proliferation and metastasis. It has been studied in multiple cancerous cell lines such as myelogenous leukemia, multiple myeloma, cervical cancer, hepatocellular cancer, prostate cancer, melanoma, breast cancer, ovarian cancer, osteosarcoma, nasopharyngeal carcinoma and others [238].

One of the ways Celastrol interferes with cancer progression is through inducing apoptosis. The intrinsic apoptotic pathway, or mitochondrial pathway, is activated following intracellular damage and oncogenic stress. It involves activation of caspase-3 and caspase-9 by *cytochrome c*. The resistance to apoptosis characterizes and promotes the survival of cancer cells [250]. Celastrol was found to be able to activate of caspase-3, caspase-7, caspase-8 and caspase-9 [251]. It also up-regulates the expression of pro-apoptotic BAX and down-regulates the anti-apoptotic Bcl-2 gene [252]. On the other hand, the extrinsic apoptotic pathway is activated by death receptors in response to external stimuli [250]. Celastrol enhances protein expression of death receptors (i.e. DR4 and DR5) which deliver downstream apoptotic signals [253, 254]. Celastrol was also impeding other pro-survival pathways such as the proteasome complex, the PI3K/Akt/mTOR, PI3K/AKT/NF-kB or PI3K/Akt/JNK pathways in a wide variety of tumor cell types [251, 255-257]. The suppression of autophagy, cellular catabolic response to starvation or stress, has been linked to cancer growth. Celastrol induces autophagy through the action of HIF-1α, a TF playing a central role in the cellular response against hypoxic stress [258]. Under inflammatory conditions, Celastrol promotes translocation of Nur77, an apoptosis-inducible nuclear receptor, from the nucleus to the mitochondria rendering them sensitive to autophagy [259].

Celastrol gains anti-proliferative properties through the ability to induce cell cycle arrest. It can decrease cyclin D1 and cyclin E levels, proteins required for progression of the cell cycle [260]. It also inhibits activation of STAT3, a cytokine-activated TF associated with oncogenesis. This causes cell arrest at the sub-G1 phase [260]. NF-kB is upregulated in various cancers. It promotes the expression of pro-inflammatory cytokines and growth factors that contribute to cancer development. Thus, the inhibition of NF-kB previously discussed also prevents cancer growth [261].

Celastrol also exerts anti-metastatic properties. It decreases VEGFR-1 and VEGFR-2 density, reducing endothelial sensitivity to VEGF and thereby limiting endothelial proliferation and angiogenesis [262]. The suppression of angiogenesis impedes the tumor's growth and survival. Celastrol hinders cell migration through suppression of MMP-9 expression [263]. It is also seen to downregulate CIP2A protein, an oncoprotein able to promote cancer cell proliferation, resistance to apoptosis tumor invasion capacity [264].

Finally, Celastrol potentiates the effect of some chemotherapeutic agents, synergistically enhancing apoptotic effects of bortezomib, thalidomide, vinblastine or paclitaxel, just to name a few. It can also enhance radiation-induced damages and suppression of cancerous cell proliferation [265]. The numerous pathways linked to Celastrol illustrates its complex yet incomplete understanding of the mechanism in suppressing neoplasia.



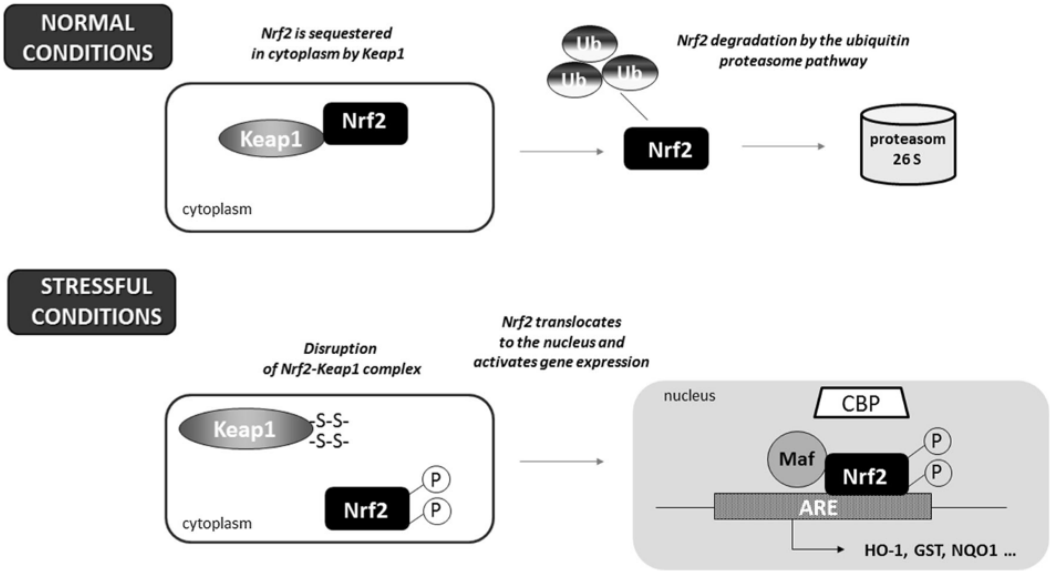
Targeted pathways inhibited by Celastrol (red ⊥). (ERK, extracellular signal-regulated kinase; IKK, IkB kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NEMO, NF-kB essential modulator; NF-kB, nuclear factor-kappa B; P, phosphorylated; PAMP, pathogen-associated molecular patterns; STAT3, signal transducer and activator of transcription 3). [241]

Figure 6. – Celastrol anti-inflammatory response

1.5.3 Antioxidative properties

Celastrol increases Nuclear factor erythroid-related factor 2 (Nrf2) activity in human umbilical vein endothelial cells (HUVEC) [266]. A Celastrol-induced production of endogenous ROS is the proposed mechanism behind Nrf2 activation. Studies have found that treatment with Celastrol led to increased ROS levels in human keratinocytes and rat cardiomyoblasts [266, 267]. The regulated production of endogenous ROS allows the modulation of various intracellular signaling pathways, including the MAPK, the PI3K-Akt, the NF-kB or the Keap1-Nrf2-ARE signaling pathway [268]. The Keap1-Nrf2-ARE pathway is implicated in the protective response against oxidative stress. In normal condition, Nrf2 is a transcription factor sequestered by Keap1, a protein

anchored to the cytoskeleton, and remains in the cytoplasm. Coupling with Keap1 enhances the ubiquitination and proteolysis of Nrf2. Oxidative stress conditions promote the dissociation of Nrf2 from Keap1. Nrf2 can then translocate to the nucleus and allow transcription of the antioxidant response elements (ARE), namely heme oxygenase-1 (HO-1), glutathione (S) transferase (GST) and NADP(H) quinine oxidoreductase (NQO-1) (*See Figure 7*) [257, 269].



Activation of Nrf2. (ARE, antioxidant response elements; CBP, cAMP-response-element-binding protein; Keap1, Kelch-like ECH-associated protein; Nrf2, Nuclear factor erythroid-related factor 2; Ub, ubiquination; p, phosphorylated) [269]

Figure 7. – Activation of Nrf2

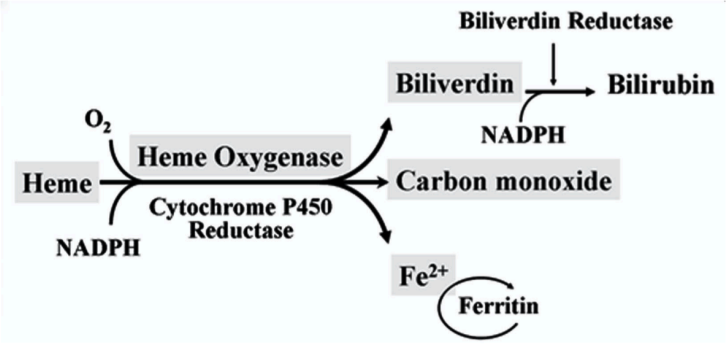
Heme oxygenases (HO) are enzymes responsible for the initial rate-limiting step of heme breakdown [270]. Heme is an organic molecule composed of four pyrrole rings and a central iron ion (Fe2+). Heme biosynthesis is complex and requires the precursor 5-aminolevalinic acid (ALA), O_2 and Fe^{2+}. Heme is present in all cells and important for the proper functioning of many enzymes such as catalases, cyclooxygenases and nitric oxide synthases. It is also essential for the formation of hemoglobin, myoglobin and cytochrome complexes [271, 272]. In addition, heme participates in many biological processes such as erythropoiesis, cell growth and differentiation[271].

Excess heme is damaging to cells and tissue. Heme catalyzes the formation of ROS and is pro-inflammatory [272]. Exposure of EC to heme promotes the expression of adhesion molecules (i.e., ICAM-1, VCAM-1 and e-selectin) [273]. There are mechanisms responsible for heme neutralization. Hemopexin is an intravascular protein binding free heme for transport or clearance and has been shown to protect against cytotoxicity *in vitro* [274]. Ultimately, heme is degraded by HO [275].

HO exist in 3 isoforms: HO-1, HO-2 and HO-3. HO-3 is a pseudogene derived from HO-2 and has negligible activity [276]. HO-2 is constitutively expressed in all cells and confers some level heme degradation, although it is not induced by external stressors and thus, is not responsible for activating the protective mechanisms [277]. Although HO-2 does not respond to stress-induced transcriptional activation, both HO-2 and HO-1 catalyze the same biochemical reaction, which is the degradation of heme into 3 components: CO, biliverdin and free iron [278].

HO-1 is highly expressed in the spleen and tissues implicated in red blood cell degradation. In most tissues, however, HO-1 is expressed at low levels in normal conditions and is induced following various forms of insults (i.e., inflammation, lipopolysaccharides, hydrogen peroxide, ischemia, hypoxia, hyperoxia, hyperthermia or radiation) [270, 277, 278]. HO-1 is also known as HSP32, due to its molecular weight, 32 kDa. Although HO does not share amino acid homology with heat shock proteins (HSP) nor does it have chaperone activity, it belongs to a family of so-called stress proteins and responds to hyperthermia [278]. HO-1 is mostly localized in the endoplasmic reticulum, more specifically within caveolae [278, 279]. It is also found, to a lesser extent, around mitochondria and within the nuclear region. Transcriptional regulation of the HO-1 encoding gene, Hmox-1, is achieved by targeting the stress-responsive elements (StRE) in the promoter region. StRE are targeted by Bach1, Nrf2, NF-kB and AP-1 [280]. Bach1 has a negative effect on the transcription of HO-1 by binding *maf* proteins and competing with NrF-2 binding. Bach1 effect dominates and is responsible for the low levels of HO-1 in normal conditions [281]. MAPK and PI3/Akt also participate in HMOX regulation [279, 280, 282, 283]. Phosphorylation of HO-1 by Akt led to changes in its activity *in vitro* suggesting the implication of PI3/Akt pathways in both transcriptional and post-transcriptional regulation of HO-1[283].

HO-1 exerts its antioxidant effect by eliminating the pro-inflammatory and pro-oxidative heme and by releasing antioxidative by-products. (*see Figure 8*) [284]. Free heme contains a Fe^{2+} atom than can react with hydrogen peroxide (H_2O_2) to produce hydroxyl radicals. The degradation of heme releases Fe^{2+} which is then sequestered by ferritin, relieving its pro-oxidative effect [285]. Ferritin is a cytoprotective and antioxidant agent for endothelial cells [286]. Biliverdin is cytoprotective against oxidative stress induced by H_2O_2 in cell culture media of EC, VSMC and cardiomyocytes. As for CO, it has vasodilatory, immunomodulatory and cytoprotective effects [280, 287, 288]. CO is also linked to stimulation of cellular proliferation. All bilirubin, ferritin and CO have antioxidant properties [277]. Many polyphenols are classified as antioxidants or anti-inflammatory owing to their capacity to induce HO-1 (i.e., curcumin, resveratrol, carnosol) [283, 289]. HO-1 polymorphisms associated with reduced expression and/or activity are found to correlate with increased cardiovascular risks [290]. *Taha et al.* demonstrated that the most active promoter profile was associated with cytoprotective, pro-angiogenic and anti-inflammatory effects on human EC [291]. Some therapeutic agents induce HO-1 such as rapamycin, paclitaxel, nitric oxide, aspirin and some statins. All these drugs have one common denominator; they are all current therapy for cardiovascular diseases.



HO-1 enzymatic activity [284]

Figure 8. – HO-1 enzymatic activity

While the antioxidant effect conducted by the Nrf2/HO-1 pathway can take hours to initiate, Celastrol may be able to rapidly respond to oxidative stress by inhibiting NADPH oxidase (NOX) isoforms [292]. NOX are enzyme complex in contact with the extracellular space. It catalyzes the production of superoxide free radicals by transferring an electron to oxygen from NADPH (nicotinide adenine dinucleotide phosphate), which results in the production of O_2^- [293]. Celastrol impedes the activity of NOX1, NOX2, NOX4 and NOX5 on human neutrophils within minutes of exposure [292]. Endothelial NOX2 is reported to contribute to angiotensin 2-induced endothelial dysfunction [294].

1.5.4 Other fields of application

Celastrol made headlines in 2015 after research found that it may help with the treatment of obesity. Celastrol suppresses food intake and leads to significant weight loss in obese mice through an increase in leptin sensitivity [295]. Celastrol also limited renal injury in diabetic rats through the NF-kB pathway [296]. In the field of neurodegenerative diseases, Celastrol was credited for slowing disease progression. Celastrol dampened neuronal loss and depletion of dopamine levels in a model of Parkinson's disease in drosophila. In a rat model of Alzheimer's disease, it reduces amyloid β production and deposition. It delays disease onset and slow neuronal loss in mice affected by amyotrophic lateral sclerosis [265].

1.5.5 Cardiovascular effects of Celastrol

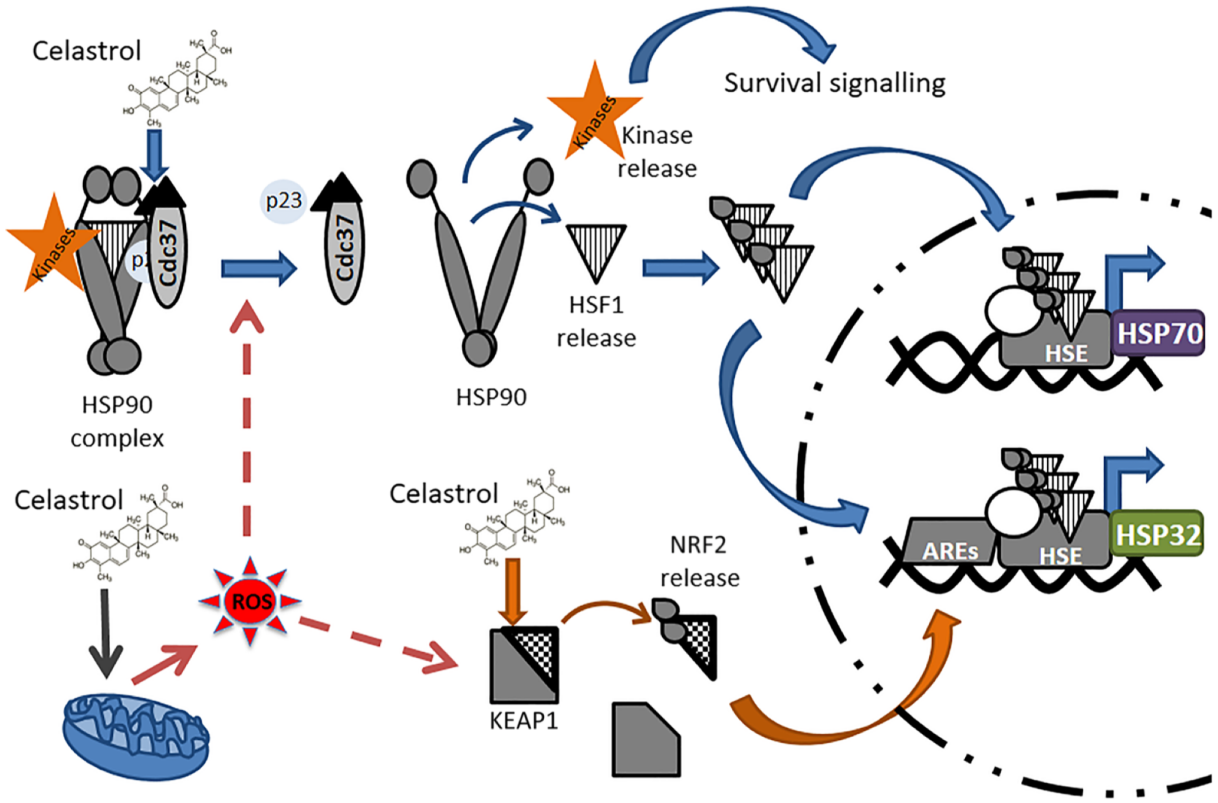
In an experimental model of atherosclerosis, systemic administration of Celastrol in nano-micelles was associated with a decrease in the size of atherosclerotic plaques as well as a reduction in the inflammatory immune cell populations within them [297]. Celastrol lowers blood pressure in a model of hypertension in rats through the inhibition of Ang II-induced SMC activation. It was reported that treatment with Celastrol decreases vascular and cardiac remodeling [298].

Our group previously showed that Celastrol conferred a better survival against hypoxic stress in rat cardiomyocytes *in vitro*. In a rat model of myocardial infarct, Celastrol limited infarct size and ventricular remodeling 14 days after the ischemic event [267]. More recent findings link the survival benefits of Celastrol with its HSP90-inhibitory activity. Following ischemia-reperfusion, Celastrol improved rat cardiomyocytes' survival which was associated with an increase in cardiac functional indexes, reduction in infarct size and tissue injury in *ex vivo* heart perfusion experiments. Celastrol inhibitory effect on HSP90 led to increased cardioprotective mechanism through induced expression of HSP70 and HSP32 (Figure 9) as well as activation of the RISK pathway (through phosphorylation of Akt and ERK1/2). Interestingly, chemical modifications of Celastrol let to analogous molecules which also exhibit HSP 90 inhibition but were not as effective in inducing the Heat Shock elements (HSE) and the ARE [239].

One might question how Celastrol has such different effects on different types of cells. Like previously mentioned, Celastrol is used as an anti-tumor drug due to its antiproliferative, anti-angiogenic and apoptotic effects. While HSP90 inhibition leads to improved survival on cardiomyocytes, it also leads to arrest in proliferation in leukemia cells [299] or promotes glioblastoma cell death [300] and pancreatic cancer cells [301]. Extended discussion about differences in neoplastic cells' intracellular mechanisms compared to normal cells goes beyond the scope of this study, but they likely explain for the diverging effects of Celastrol reported in the literature. It is also worth to mention that the concentrations and the periods of treatment with Celastrol differ in studies which can also lead to different effects.

So far, it is believed that Celastrol can exert cytoprotective effect on healthy cardiomyocytes. Our group findings support Celastrol as the most potent HSP90 inhibitor for cardio-protection yet. On the same line of thought, this study aims to uncover if similar mechanisms exist on endothelial cells and if those could lead to similar response to oxidative stress and survival. We believe that ROS play a major role in driving the early response and is responsible of much of the endothelial injury and death. Using Celastrol as a local treatment for vascular grafts would avoid systemic administration, thus preventing higher dosage and systemic toxicity. It would then be important to determine if the treatment can generate the desire effects within the limited time frame of

graft preparation. This project wants to determine if there are any beneficial effects of Celastrol on human endothelial cells (HUVEC) in vitro.



Celastrol inhibits HSP90 causing the release of Heat Shock Factor 1 (HSF1). Celastrol also induces the Nrf2 activity by endogenous production of ROS. Both nuclear factors, Nrf2 and HSF1, allows transcription of HSP70, effector of the Heat Shock response, and HSP32, protective against oxidative stress (ARE, antioxidant response element; HSE, heat shock elements, HSE; HSP90, heat shock protein 90; HSP32, heat shock protein 32 or heme-oxygenase-1, Keap1, Kelch-like ECH-associated protein; Nrf2; Nuclear factor erythroid-related factor 2). [302]

Figure 9. – Celastrol inhibitory activity on HSP90 and downstream effect on expression of HSP70 and HSP32

Chapter 2 – Hypothesis and Objectives

2.1 Hypothesis

Celastrol pre-conditioning can improve endothelial cell survival during oxidative stress and storage in normal saline, a common graft storage solution.

Celastrol pre-treatment can promote faster re-endothelialization.

Celastrol is capable of triggering survival pathways such as the RISK pathway and the Heat Shock Response.

Celastrol activation of HO-1 is contributive to the cellular acquired resistance against oxidative stress.

2.2 Objectives

In vitro assessment of the effects of Celastrol on human umbilical vein endothelial cells:

1. Following short oxidative stress (reproducing early period after reperfusion)
2. Following long-term oxidative stress (reproducing the delay response with prolonged reperfusion injury)
3. Following storage under heparinized normal saline (reproducing clinical condition of graft preservation).
4. On their proliferation and migration potential: indicative of their potential to re-endothelialized

Assessment of the pathways implicated in the actions of Celastrol:

1. RISK pathway: Akt and ERK1/2
2. HO-1 expression
3. Expression of HSP70 from the heat shock response

Chapter 3 – Materials and Methods

3.1 Celastrol

Celastrol (Cayman Chemical, Ann Arbor, MI) is a commercially available compound. Stock solutions were prepared in dimethyl sulfoxide (DMSO, 99.9%) (Sigma-Aldrich, Canada Oakville, ON) at a concentration of 10^{-3}mM and stored at -20^{o}C.

3.2 Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from the *American Type Culture Collection (ATCC)*. Every surface for treatment or culture purposes are coated with sterile gelatin A 1% in phosphate buffered saline (PBS) for 2 hours before seeding. HUVEC are cultured in M200 medium (Life technologies. #cat: M200-500) containing FBS 5% (Life technologies. #cat: 12483-020), 1% Low serum growth supplement (Life technologies. #cat: S-003-10) and 1% penicillin/streptomycin (Life technologies. # 15140-122). For the sake of simplification, we would call that solution EGM 5%, or endothelial growth medium (EGM). Cells are incubated at 37^{o}C in a 5% CO_2 humidified incubator. Medium is replaced every 2 to 3 days. HUVEC are passaged once they reach at least 90% confluence on culture plates, usually every 5 days. 500 000 cells are seeded in 25cm^2 flask and confluence is reached with around 2 million cells. Cells in their 4th to 8th passage were used in this study.

3.3 Stress and treatment preparations.

For the experimental conditions, 96-well plate (Eppendorf, Mississauga, ON) gets pre-coated with Gelatin A 1%/PBS. 5000 cells are seeded per wells in EGM 5% and incubated for 24 hours before the experimental conditions.

All treatments require the dilution of Celastrol aliquots (10^{-3}M) in M200 (Life Technologies. #cat: M200-500) supplemented by 1% FBS (Life technologies. # cat: 12483-020) (EBM 1%). Dosages

between 10^{-10}M and 10^{-6}M were prepared. Every treatment lasts one hour. Following the treatment, cells are carefully washed 3 times with EBM 1%.

Oxidative stress is replicated by diluting hydrogen peroxide 30% (*Fisher Scientific*) in EBM 1%. Concentrations between 0.3mM and 0.5mM are prepared for the prolonged stress condition (24 hours) and between 1 mM and 6 mM for the short stress (1 hour).

Heparin 25U/mL is added to normal saline (*Sandoz Canada*) in order to reproduce the preservation solution used in the operating room. Duration of storage in heparinized saline was 3 hours.

3.4 Viability testing

Following any prior stress condition or treatment, HUVEC are washed with EBM 1% 3 times. Viability testing is done by using the LIVE/DEAD kit (*Life Technologies*) according to the manufacturer's recommendations. The LIVE/DEAD assay discriminates live cells stained green-fluorescent by calcein-AM which indicates intracellular esterase activity from dead cells, stained red-fluorescent by ethidium homodimer-1, which indicates loss of plasma membrane integrity (see Figure 13). The total number of cells is determined by nuclei staining by Hoechst 33342 (*Life technologies, 1:10 000*). Images were captured using the *Operetta system*, a high content screening system, and analyzed using the *Harmony High-Content Imaging and Analysis* software ver. 4.1 (*Perkin Elmer*, Waltham, MA). Living cells emit a green fluorescent dye, whereas dead cells emit a red fluorescent dye. The total number of cells are determined by Hoechst 33342 staining and is used to correlate with the sum of dead and alive cells recorded. Viability is reported as the percentage of living cells over the total of cells, dead and alive.

3.5 Scratch tests

HUVEC are seeded on gelatin A 1%/PBS pre-coated 96-wells for scratch (Essen, #4379); 15 000 cells per wells. They are incubated in EGM 5% for 24 hours at 37°C. Cells are then exposed to the

treatment preparations previously described. After 1 hour of stimulation, wells are washed 3 times with EBM 1% before adding 100uL of EBM 1% per well. A scratch is standardly made with the *WoundMaker* (*Essen*, cat: #4493) according to the manufacturer protocol. The 96-wells plate is then incubated in the *IncuCyte Zoom* machine (*Essen*) at 37°C, where picture is taken at 10x every hour. Wound closure analysis is accomplished using the *IncuCyte Zoom Scratch Wound* software.

3.5 Protein Analysis

For Western blot analysis, p-Akt (*Cell signaling technology* # cat: 9271, rabbit, 1:2000), Akt (*Cell signalling technology* # cat : 9272, rabbit, 1:4000), p-ERK1/2 (*Cell signaling technology* # cat: 9102, mouse, 1:2000), ERK1/2 (*Cell signaling technology* # cat: 9106, mice, 1:4000), HSP70 (*Enzo life science* # cat: ADI-SPA-810, mouse, 1:1000), and HO-1 (*Enzo Life science* # cat: ADI-SPA-950, rabbit, 1:2000) primary antibodies were used. Goat anti-mouse HRP (*Santa Cruz biotechnology* #cat: sc-2005, 1:3000) and goat anti-rabbit HRP (*Santa Cruz biotechnology* #cat: sc-2004, 1:3000) were used at secondary antibodies.

HUVEC are seeded in pre-coated 6-cm plates (*Sarstedt*, Montreal, Quebec) at a density of 400,000 cells/plate. After seeding, HUVEC are kept at least 24 hours before the stimulation conditions. Following the treatment, plates are washed 3 times with EBM1 % before all trace of medium is aspirated. The dry plates are then kept at -80°C until extraction.

Protein extraction is performed by incubating plates in lysis buffer (preparation: 50mM Hepes, 1% NP-40, 4mM EDTA, 1mM Na_3VO_4, 10mM NaF, 1mM phenylmethanesulfonyl fluoride and 1mM Na pyrophosphate in 10 mL of distilled water) for 30 minutes on ice. Lysed cells are collected and centrifuged in a refrigerated (4°C) microcentrifuge for 10 minutes at 13 000 RPM at 4°C. The supernatant is then transferred in Eppendorf tubes, where protein dosage is performed by Bradford protein assay. Protein extracts are prepared by adding a loading buffer (*Bioland Scientific 6X Laemmli SDS sample buffer*) for a 5:1 ratio and 5 uL of b-mercaptoethanol. Samples were then heated at 95°C for 5 minutes before being stored at -20 °C.

8% and 10% Separating gels (preparation: 30% acryl-bisacrylamide mix, 1.5M Tris pH 8.8, 10% SDS, 10% ammonium persulfate and TEMED were mixed in 10 mL of distilled water) were used. Stacking gels are prepared using 30% acryl-bisacrylamide mix, 1.5M Tris pH 6.8, 10% SDS, 10% ammonium persulfate and TEMED mixed in 10 mL of distilled water.

Protein migration is performed over 90 to 120 minutes at 120V in running buffer (SDS, Tris, glycine). Proteins are transfered onto polyvinylidene (PVDF) membrane is performed overnight at 30V in a refrigerated chamber (4°C). Membranes are then blocked using 5% milk (no fat) in PBS. Membranes are incubated with primary antibodies (mixed in 5% milk-PBS) overnight at 4°C on a shaker. Membranes are washed with PBS containing 0.1% Tween20 before incubation with secondary antibodies for 1 hour on a shaker. Concentrations of antibodies follow manufacturer's recommendations. Membranes are washed again in PBS- 0.1% Tween20 before incubation with ECL 1X solution (following the proportion solution A and B provided by the manufacturer) for 1-2 minutes.

Following exposure in a darkroom, quantitative analysis was performed with *NIH Image J software version 1.52 (*https://imagej.nih.gov/ij/).

3.6 Statistical analysis

All experiments were performed at least three times, using triplicate samples for each data point. Data is presented as means and standard error of the mean. Exploratory analyses (N less than 5) on viability and protein expression were performed using one-way ANOVA followed by the two-tailed t-test performed on Microsoft® Excel® for Mac version 16.37.

For viability and cell number analyses, one-way ANOVA followed by a Dunnett's multiple comparisons test or a two-way ANOVA followed by Boniferroni's multiple comparisons test was performed.

For scratch test, results were converted to an area under the curve, which was then analyzed by one-way ANOVA followed by a Dunnett's multiple comparisons test. The analysis was performed

on *GraphPad Prism* version 8.4.3. An equivalence test (TOST) was performed by 2 one-side t-test and run on XLSTAT version 2020.3.1.1006 by *Addinsoft*.

For all analyses, $P \leq 0.05$ was considered significant.

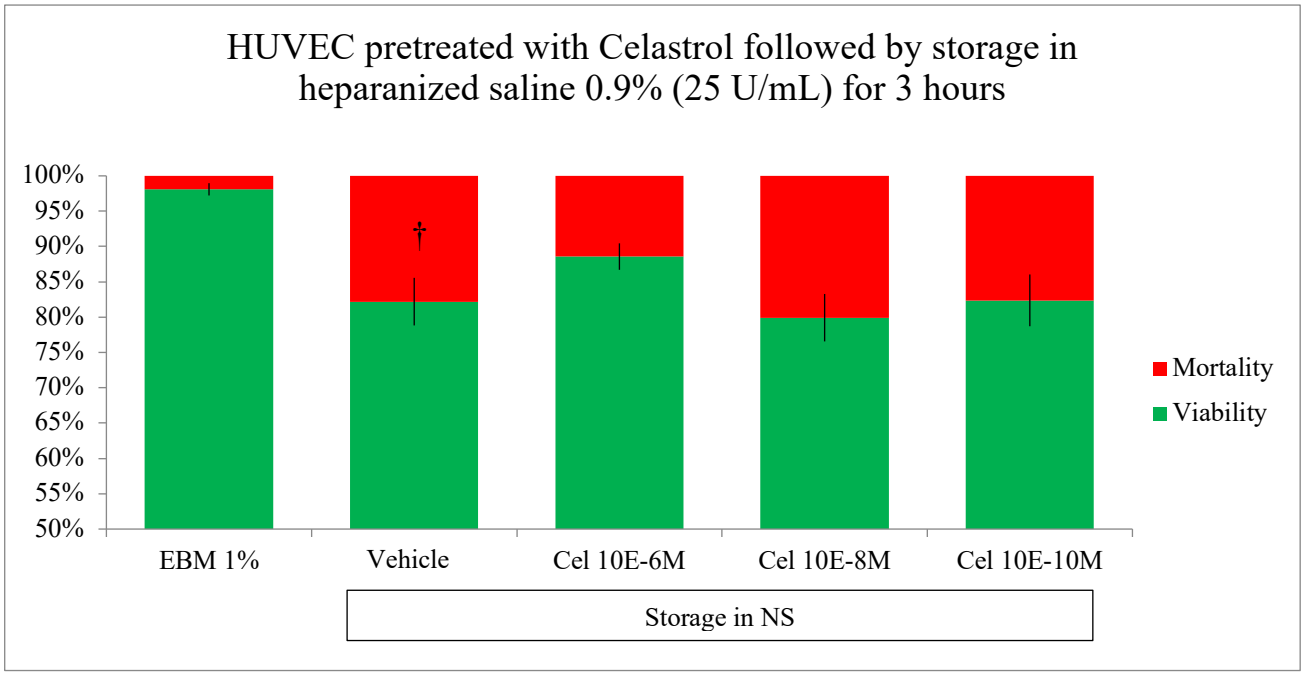
Chapter 4 – Results

4.1 Viability of Endothelial Cells following exposure to heparinized NS

Venous grafts are commonly stored in heparinized NS during the surgery. In order to reproduce the clinical environment in vitro, HUVEC are exposed to heparinized NS in order to assess the impact of the solution on their survival. The period of storage was 3 hours as shorter times did not induce enough mortality to highlight the effect of Celastrol. In clinical settings however, grafts are usually kept for much shorter periods.

Figure 10 shows the ratio of viable and dead cells after storage in NS and how a pre-treatment with Celastrol affect their viability. In the EBM 1% group, which in the negative control group, minimal mortality is expected since no stress was stimulated. Indeed, there is a reported 1.90±0.01% mortality. Storage in heparinized normal saline is shown to be significantly more cytotoxic than EBM 1%, where mortality reaches 17.8 ± 3.3 % ($p=0.009$). Pre-conditioning with Celastrol 10^{-6}M decreases the mortality rate to 11.9 ± 1.9 %, although that difference did not reach significance ($p=0.09$). The lower concentrations of Cel 10^{-8}M and 10^{-10}M revealed a mortality of 20.1% and 17.6% respectively, which were also not statistically different from the non-treatment group. These results raise the possibility of a dose-dependent response where the highest Celastrol concentration seems to reveal some level of benefit.

4.1.1 Viability after preservation in heparinized saline



Once HUVECs are treated with Celastrol at 10^{-10}M, 10^{-8}M and 10^{-6}M for 1 hour, they were stored in heparinized normal saline (NS) for 3 hours (n=3). In the EBM 1% group, HUVEC were kept in a culture medium low in glucose and devoid of growth factor and serve as the control group. This group was a negative control group. Thus, it was not exposed to NS nor to Celastrol. In the vehicle group (DMSO devoid of Celastrol), HUVEC were not treated with Celastrol and were exposed to NS for 3 hours. (HUVEC, human umbilical vein endothelial cells; EBM 1%, endothelial basal medium with FBS 1%; CTRL, control; Cel, Celastrol)

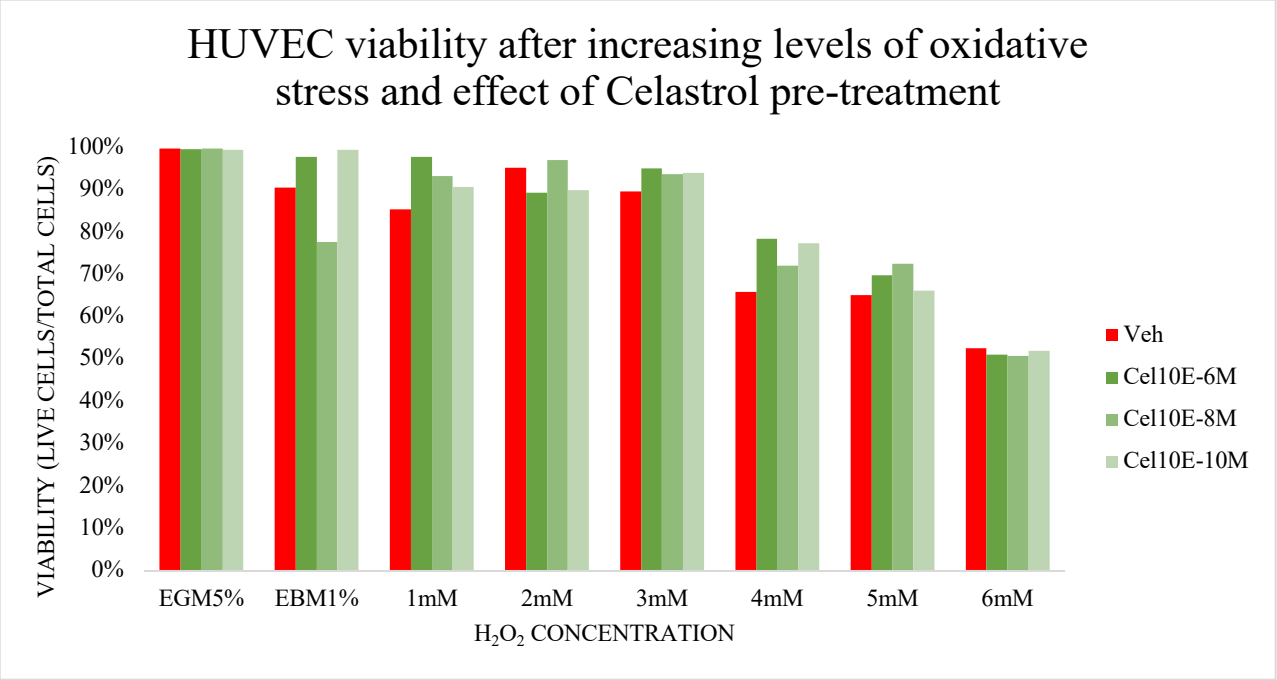
† Significant increase in mortality in HUVEC exposed to NS (vehicle) compared to EBM 1% : 1.9 vs 17.8% (p=0.009).

Figure 10. – HUVEC survival after prolonged storage in heparinized normal saline and effects of a Celastrol pre-treatment on their survival

4.2 Viability under oxidative stress

4.2.1 Response to increasing oxidative stress and determination of H_2O_2 experimental concentration for the 1-hour stress experiments

In order to reproduce the oxidative stress condition following reperfusion, HUVEC are exposed to different concentrations of H_2O_2. *Figure 11* shows the viability testing done after exposure to different concentrations of H_2O_2 for 1 hour. This preliminary step aims to determine the condition generating the most noticeable treatment response on mortality. 4 mM is the concentration where we begin to see a noticeable difference in terms of induced mortality. Therefore, we limited further testing for H_2O_2 3 mM and 4 mM. Lower concentrations lead to lower mortality and are likely going to accentuate experimental variability and inconsistency as seen for dosages 1 mM and 2 mM. Under a higher concentration of H_2O_2 (5mM and 6mM), mortality increases in a dose-dependent manner which was also associated with a decline in Celastrol's recorded effectiveness. In both the 5 mM and 6 mM groups, Celastrol's effect, if any, seemed blunted by the overwhelming mortality generated. It was observed that wells exposed to H_2O_2 5 mM and 6 mM succumbed to higher mortality than what is reported by the numbers. In fact, visually, mortality approached 100% in all wells stressed with doses higher than 5 mM. Thus, it was deemed futile to repeat conditions of 5mM and 6mM. We reported results from the concentration H_2O_2 4 mM where effect was most highlighted (*Figure 13*). Results for the concentration H_2O_2 3 mM can be consulted in the Appendix (Figure 1).

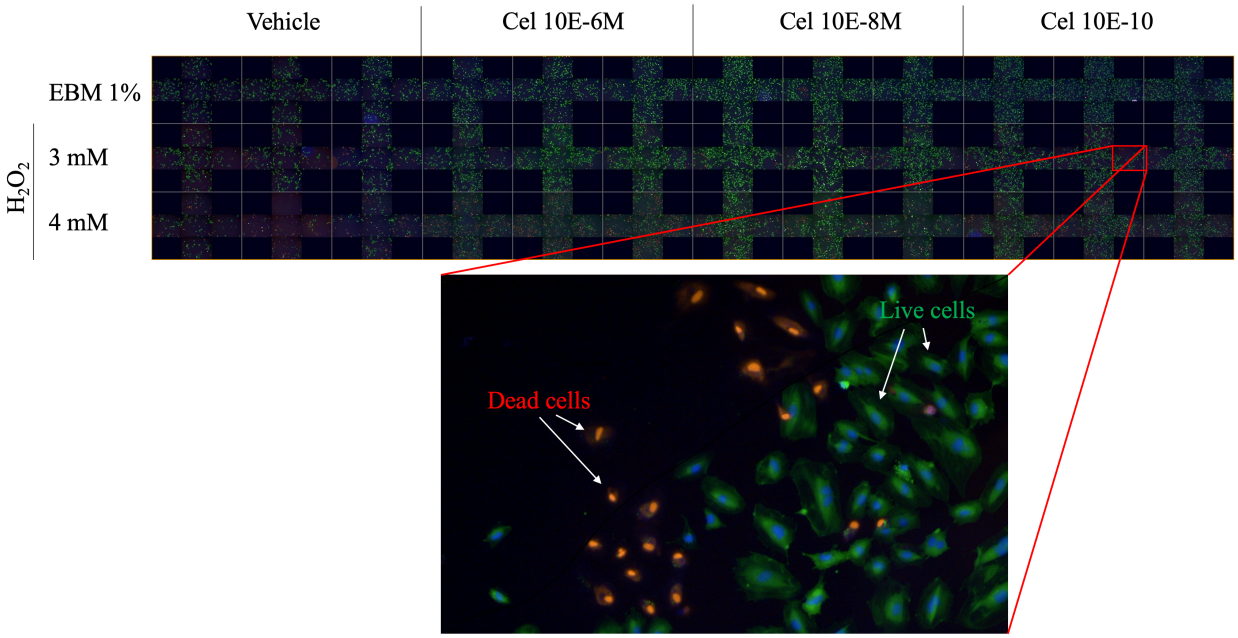


This figure shows HUVEC survival in various concentrations of H_2O_2 and variable concentrations of Celastrol treatment. Cells were exposed to 1 hour of oxidative stress at different concentrations. Both the EGM 5% and EBM 1% groups were not submitted to oxidative stress. EGM 5% is the standard medium for culture, containing 5% FBS and growth factors. EBM 1% is devoid of growth factors and contains only 1% FBS. EBM 1% is the base medium used for H_2O_2 preparations and therefore serve as the control group. Both culture media are reported here for the purpose of showing that lack of growth factors and low FBS stress HUVEC and viability measures should be analyzed in consideration of that. No error bar is shown as this test was done once (n=1). (HUVEC, human umbilical vein endothelial cells; EGM 5%, endothelial growth medium with FBS 5%; EBM 1%, endothelial basal medium with FBS 1%; CTRL, control; Cel, Celastrol; Veh, vehicle)

Figure 11. – HUVEC viability after increasing oxidative stress (1h) and response with Celastrol treatment

4.2.2 Viability following oxidative stress (short period)

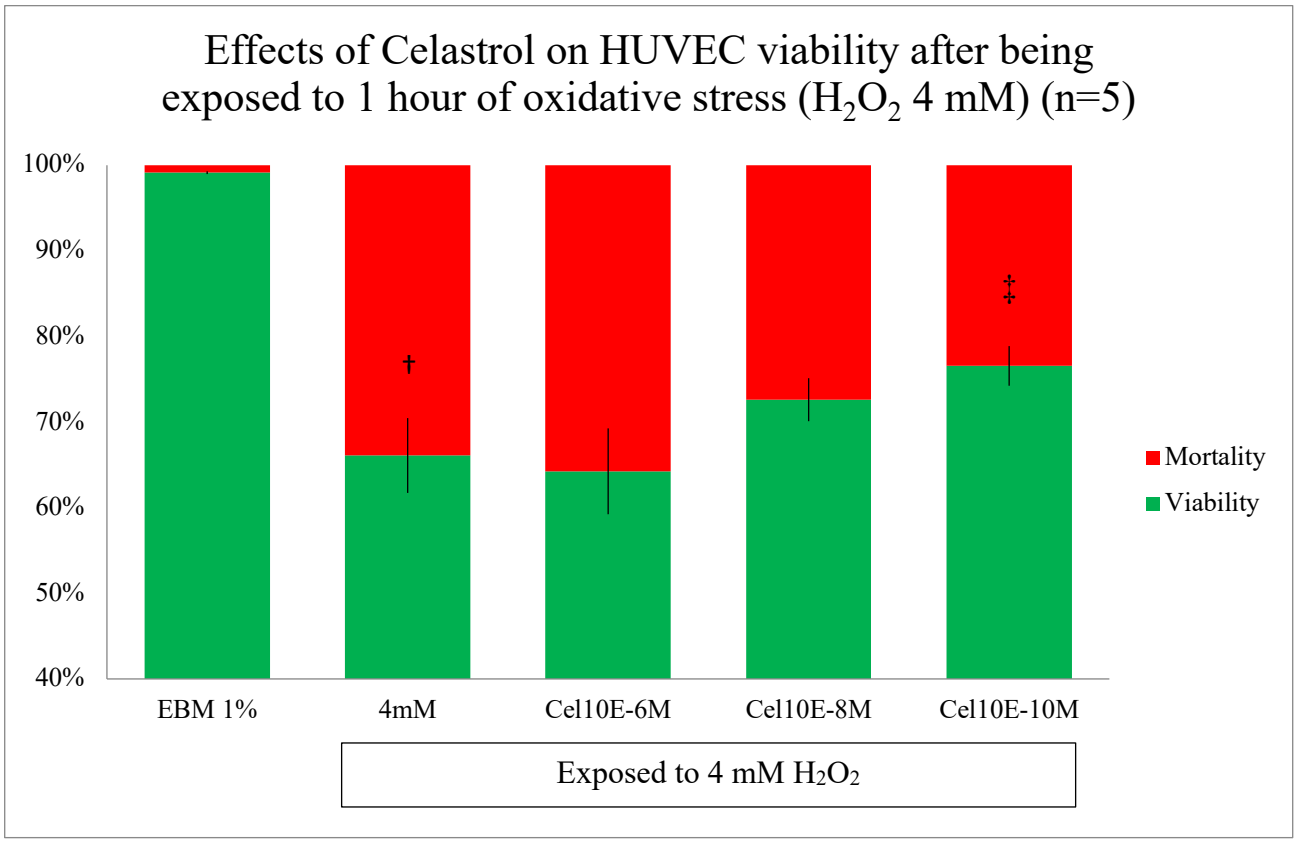
Figure 12 is a photo taken from one of the experiments described in 4.2.1. It shows that the vehicle groups experience significant death and cell loss compared to groups treated with Celastrol. The compilation of 5 experiments are reported in Figure 13.



Picture was taken from a 96-wells plate by the *Operetta system*. Each well (a rectangle) is seeded with 5000 cells. The viability assessment was done by LIVE/DEAD assay, which allows marking of living cells in fluorescent green by calcein AM and dead cells in fluorescent red by ethidium homodimer. The *Operetta system* is set to take 5 pictures in each well, covering the center and 4 cardinal areas. Every row represents the oxidative stress concentration in which the cells were submitted. The wells in row EBM 1% were not submitted to oxidative stress and, therefore serve as the control group for the stress conditions. The columns show the treatment received. The wells in the column marked as Vehicle were not treated with Celastrol and only received the vehicle, DMSO. All the Celastrol-treated wells received the treatment for 1 hour before exposure to the oxidative stress.

Upon visual inspection, oxidative stress influenced the number of living cells present in the wells. Additionally, there seems to be more living cells in wells treated with Celastrol. (EBM 1%, endothelial basal medium with FBS 1%; CTRL, control; Cel, Celastrol).

Figure 12. – LIVE/DEAD assay following short oxidative stress and treatment with Celastrol



This figure shows the compilation of 5 experiments exposing HUVEC to H_2O_2 4 mM. The EBM 1% group was not exposed to Celastrol nor H_2O_2 and served as the control group. The 4 mM group did not receive Celastrol but was exposed to 4mM H_2O_2 for 1 hour. All groups pre-treated with Celastrol for 1 hour prior to stress.

†Exposure to 1 hour of H_2O_2 4 mM significantly increase mortality compared to EBM 1%; 0.9 vs 33.9% ($p<0.0001$)

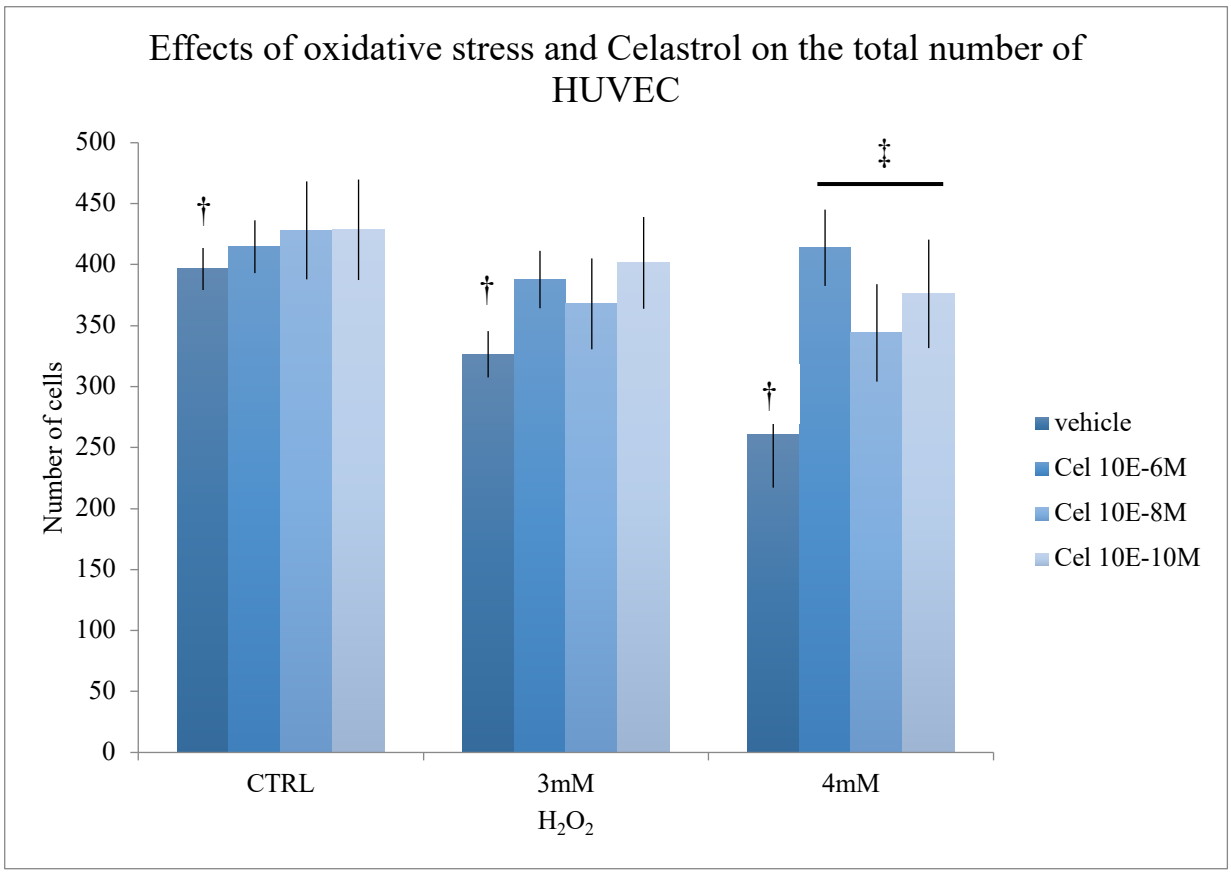
‡ HUVEC pre-treated with Cel 10^{-10}M showed higher viability compared to the stress condition, 76.6% vs. 66.1% ($p=0.035$). (HUVEC, human umbilical vein endothelial cells; EBM 1%, endothelial basal medium with FBS 1%; CTRL, control; Cel, Celastrol)

Figure 13. – Effects of Celastrol on HUVEC survival following short oxidative stress

Figure 13 shows viability in the negative control group to be 99.1%. Viability markedly drops to 66.1% in H_2O_2 4mM compared to the control group ($p<0.0001$). Pre-conditioned cells showed an improvement in viability with concentrations of Cel 10^{-8}M and 10^{-10}M, reporting 72.6% ($p=0.07$)

and 76.6% (p=0.005) viability respectively. However, Cel 10^{-10}M is the only concentration that significantly improves the survival of HUVEC. Opposite to the findings for heparinized NS, no dose-response is found in the case of a short oxidative stress. In this case, a lower concentration seems to be the most effective.

As observed in *Figure 12*, the number of cells within wells exposed to oxidative stress seems significantly reduced. It is noted that dead HUVEC tend to detach from the wells. Floating dead cells are inevitably discarded during washes and do not account in the total number of dead cells. The effect was observed in a more dramatic manner the higher the mortality. This is why a concomitant look at the total number of cells is relevant in order to draw a more complete picture of cell survival. *Figure 14* shows the total number of cells detected by *Hoescht 33342* staining, done concomitantly with the LIVE/DEAD assay. The CTRL groups did not undergo oxidative stress. In the CTRL vehicle group, 397 ± 18 cells are counted. There is a consistent trend towards an increase in the number of cells in wells treated with Celastrol compared to the vehicle groups in the same stress condition. At 4 mM H_2O_2, Celastrol 10^{-6}M is the only dose associated with a significantly higher cellular count: 414 ± 31 cells compared to 261 ± 44 for the vehicle (p=0.02).



This figure shows the total number of cells detected as per Hoescht 33342 staining. The CTRL groups were not exposed to oxidative stress. Treated groups received Celastrol for 1 hour prior to oxidative stress. The vehicle groups received DMSO only. (n=5)

† The number of cells in the wells are inversely dependant on the degree of oxidative stress. Compared to the control group, there is a decline of 17.6% (p=0.03) and 34.3% (p=0.05) for the 3mM and 4mM conditions respectively.

‡ In HUVECs exposed to H2O2 4 mM, Celastrol 10^{-6}M is associated with a significantly higher cellular count when compared to control ($p<0.05$ for all comparisons).

(HUVEC, human umbilical vein endothelial cells; CTRL, control; Cel, Celastrol)

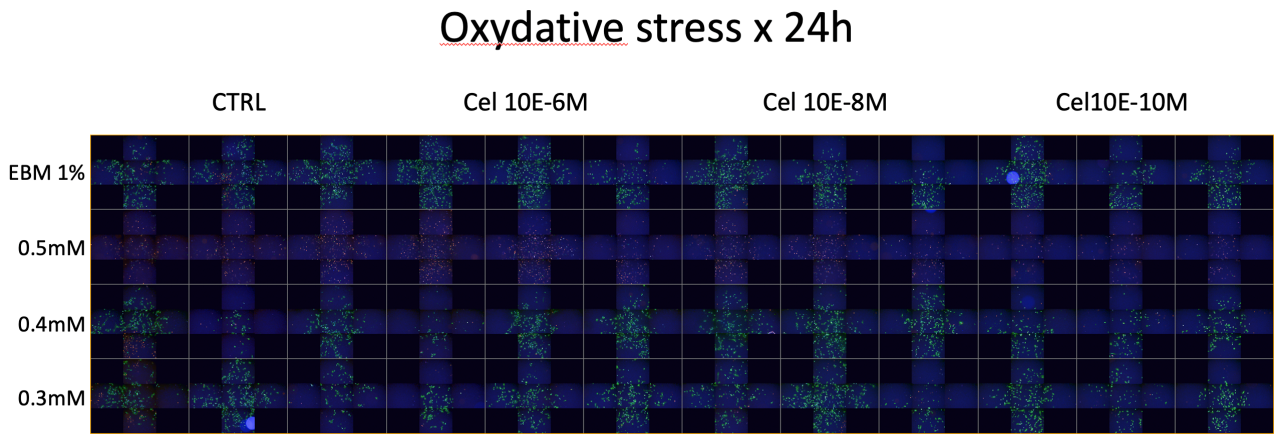
Figure 14. – Effects of oxidative stress and Celastrol on the total number of HUVEC

4.2.3 Viability following prolonged oxidative stress (24h)

Unfortunately, following anastomosis of a graft, oxidative stress likely continues beyond the one-hour mark. Specific duration of that stress is hard to evaluate in clinical settings, but we believe that the first 24 hours are crucial in dictating the later evolution of the graft. Therefore, evaluating the influence of a Celastrol treatment on HUVEC during the first 24 hours of reperfusion is our next step.

Following multiple trouble-shootings, we isolated H_2O_2 doses between 0.3 mM and 0.5 mM for long-term oxidative stress testing (24 hours). We decided to test the 3 dosages due to noticeable variability in terms of mortality; for the same dose, different lineage showed markedly different mortality rates. *Figure 15* is an example of this variability. It shows a snapshot at one of our experiments which seems to show significant cell death in all wells exposed to 0.5 mM. It was observed that it is, at least partly, related to the number of cell passages. Further experiments actually shows that 0.5mM that shoes the most consistent death rate and response, which compilation is showed in *Figure 16*. The figure distinguishes 5 mM as the condition where the most substantial benefit of Celastrol was detected.

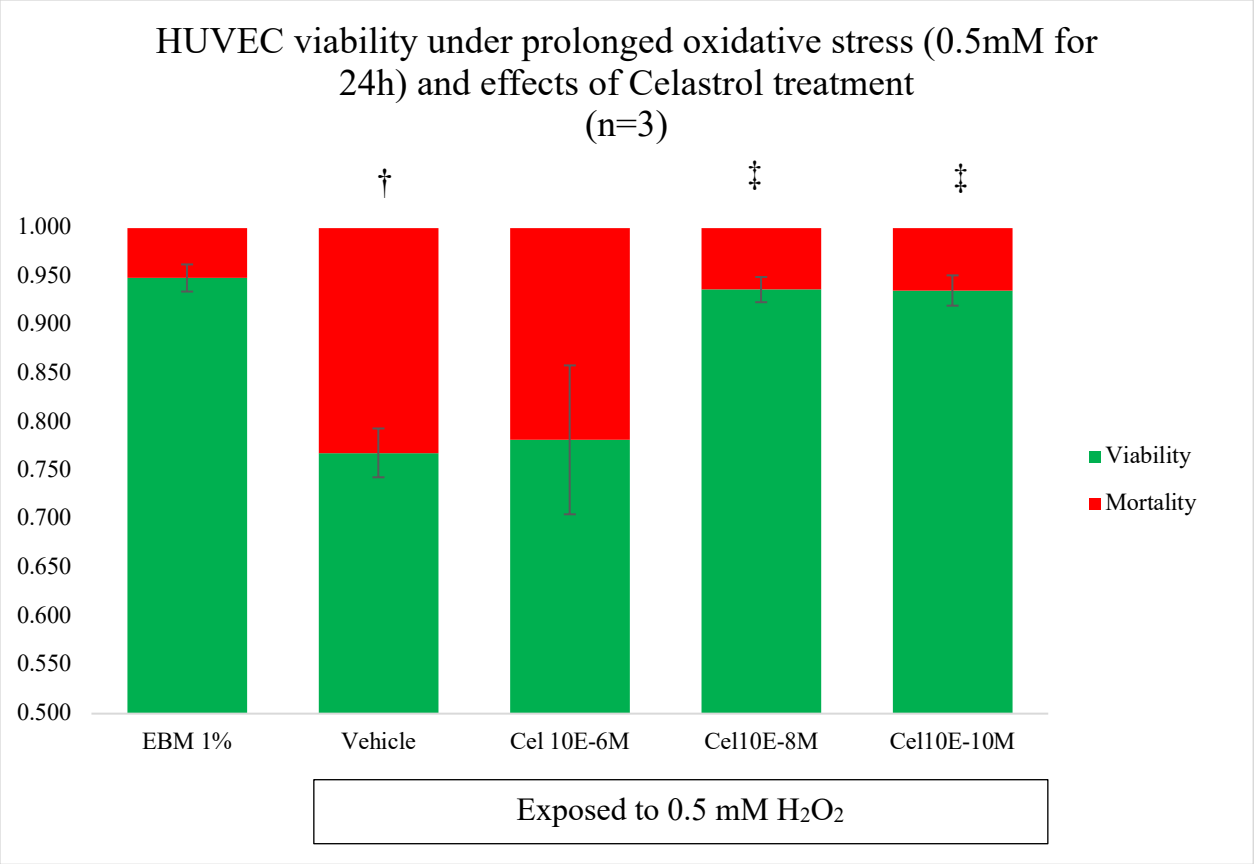
Figure 16 shows that prolonged oxidative stress (0.5mM for 24 hours) significantly reduces viability in HUVEC: 76.9% vs 94.9% (p=0.001). HUVEC pre-treated with Cel 10^{-6}M did not show a significant benefit in terms of survival compared to the stress condition, 78.2% vs 76.9% (p=0.9). However, 1 outlier experimental result contributed to the greater error interval and a decreased viability percentage in the compiled result. Excluding data from that experiment resulted in an analysis showing significant improvement of viability with the Cel 10^{-6}M treatment. (*see Figure 2 in Appendix*). Interestingly, HUVEC pre-treated with Cel 10^{-8}M and Cel 10^{-10}M showed a significantly higher viability compared to the stress condition, 93.7% vs 76.9% (p=0.001) and 96.6% vs 76.9% (p=0.002) respectively.



Picture was taken from a 96-wells plate. Each well (represented by a square) is seeded with 5000 cells. The viability assessment was done by LIVE/DEAD assay, which allows marking of living cells in fluorescent green by calcein AM and dead cells in fluorescent red by ethidium homodimer. The *Operetta system* is set to take 5 pictures in each well, covering the center and 4 cardinal areas. Every row represents the oxidative stress concentration in which the cells were submitted. EBM 1% is the base solution in which various concentrations of H_2O_2 (0.3 mM, 0.4 mM and 0.5 mM) were prepared. Therefore, the wells in row EBM 1% were not submitted to oxidative stress. The columns show the treatment received. The columns marked as CTRL were not treated with Celastrol and only received the vehicle, DMSO. All the wells received a pre-treatment with Celastrol for 1 hour before being submitted to the oxidative stress.

This is a snapshot from one of the first experiments on HUVEC. In this experiment, 0.5 mM seems to be particularly lethal to HUVEC. Upon visual examination, there seems to be arguably more living cells in wells treated with Cel 10^{-8}M. It should be noted that the same conditions in younger lineages often showed less mortality for the H_2O_2 5 mM condition and results in visually different pictures. This figure was chosen to show the most apparent dose-response to oxidative stress. (EBM 1%, endothelial basal medium with FBS 1%; CTRL, control; Cel, Celastrol)

Figure 15. – LIVE/DEAD assessment following a prolonged oxidative stress



This figure shows the compilation of 3 experiments exposing HUVEC to H_2O_2 0.5 mM for 24 hours. The *Vehicle* group was not treated to Celastrol and served as the control group. Treatment with Celastrol lasted 1 hour and was done prior to stress. The EBM 1% group was not exposed to Celastrol nor H_2O_2 and served as the control group. (HUVEC, human umbilical vein endothelial cells; EBM 1%, endothelial basal medium with 1% FBS; Cel, Celastrol)

†Prolonged oxidative stress (0.5mM for 24 hours) significantly reduces viability in HUVEC: 76.9% vs. 94.9% (p=0.001)

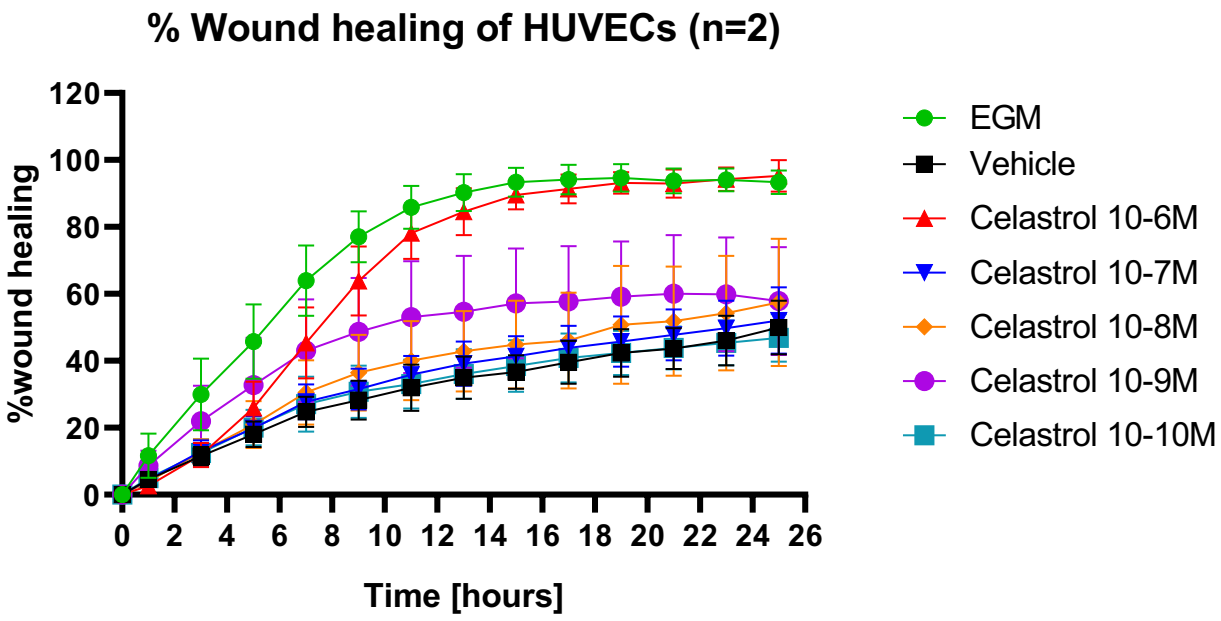
‡ HUVEC pre-treated with Cel 10^{-8}M and Cel 10^{-10}M showed higher viability compared to the stress condition, 93.7% vs. 76.9% (p=0.001) and 96.6% vs. 76.9% (p=0.002), respectively.

Figure 16. – HUVEC viability under prolonged oxidative stress (0.5 mM) and effects of Celastrol treatment

4.3 Proliferative potential of pretreated endothelial cells

Re-endothelialization is the result of proliferation and migration of EC. In order to assess the capacity of HUVEC to reendothelialize a denuded surface, scratch tests were performed.

4.3.1 Scratch Tests

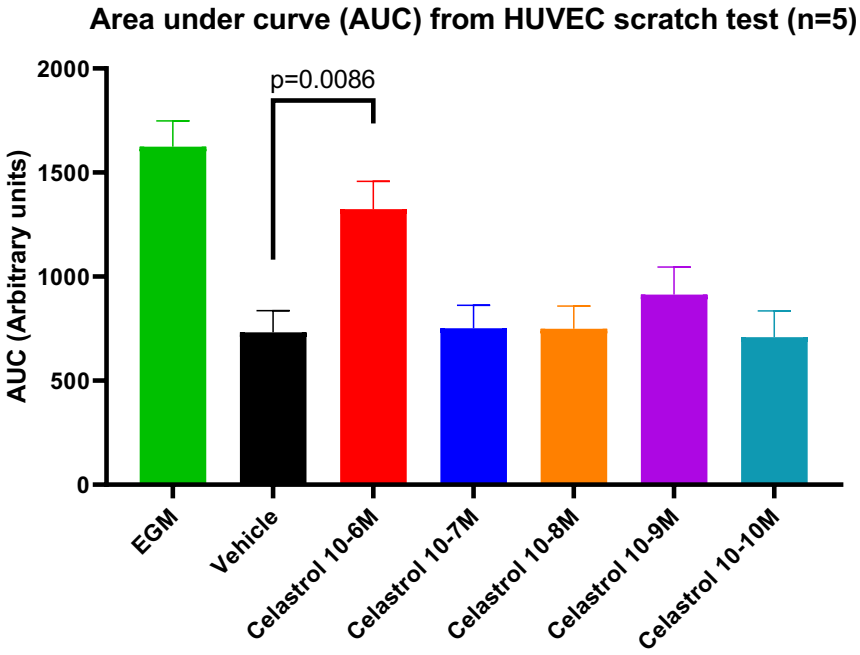


This figure shows wound closure as assessed by the scratch test. The EGM group accounts for HUVEC cultured in a growth medium. As a pro-proliferative environment, EGM serves as the positive control group. The vehicle group received the vehicle (DMSO) in a medium devoid of growth factors (EBM 1%) and serves as the negative control group. All Celastrol dilutions were also prepared in EBM 1%. The percentage of wound closure during a period of 24 hours was reported based on the area covered by HUVEC assessed every 2 hours. A complete wound closure would reach 100%. Of note, 2 different periods were recorded (24 hours and 26 hours) due to technical changes in the machine setting. (EBM, endothelial basal medium; EGM, Endothelial growth medium; HUVEC, human umbilical vein endothelial cells)

Figure 17. – Effects of Celastrol on re-endothelialization: percentage of wound healing

Figure 17 shows the percentage of wound closure by HUVECS pretreated with different concentrations of Celastrol in our first series of experiments. It depicts the most well-defined response and most representative of our final compiled results (Figure 19). The positive control group was kept in the growth culture medium (EGM), a pro-proliferative environment, and therefore expected to reach the most complete closure. 50% closure is observed between 6 and 8 hours in EGM and reached a plateau over 90% closure after 12 hours. Near-complete closure was reached in EGM groups at 24 hours. Closure for the groups treated with concentrations lower than 10^{-6} M is reported to be between 40 and 60% at 24 hours. Slower closure was expected in vehicle compared to EGM as HUVEC were kept in EBM 1%, which is devoid of growth factors.

All experiments are reported on area under the curve (AUC) in Figure 18. It shows that Cel 10^{-6} M is the only dose that significantly promotes wound closure (p=0.009) compared to the vehicle. However, equivalence test did not show equivalent closure rate between the growth medium and Cel 10^{-6} M. Lower Celastrol concentrations were not associated with differences in wound closure compared to vehicle.



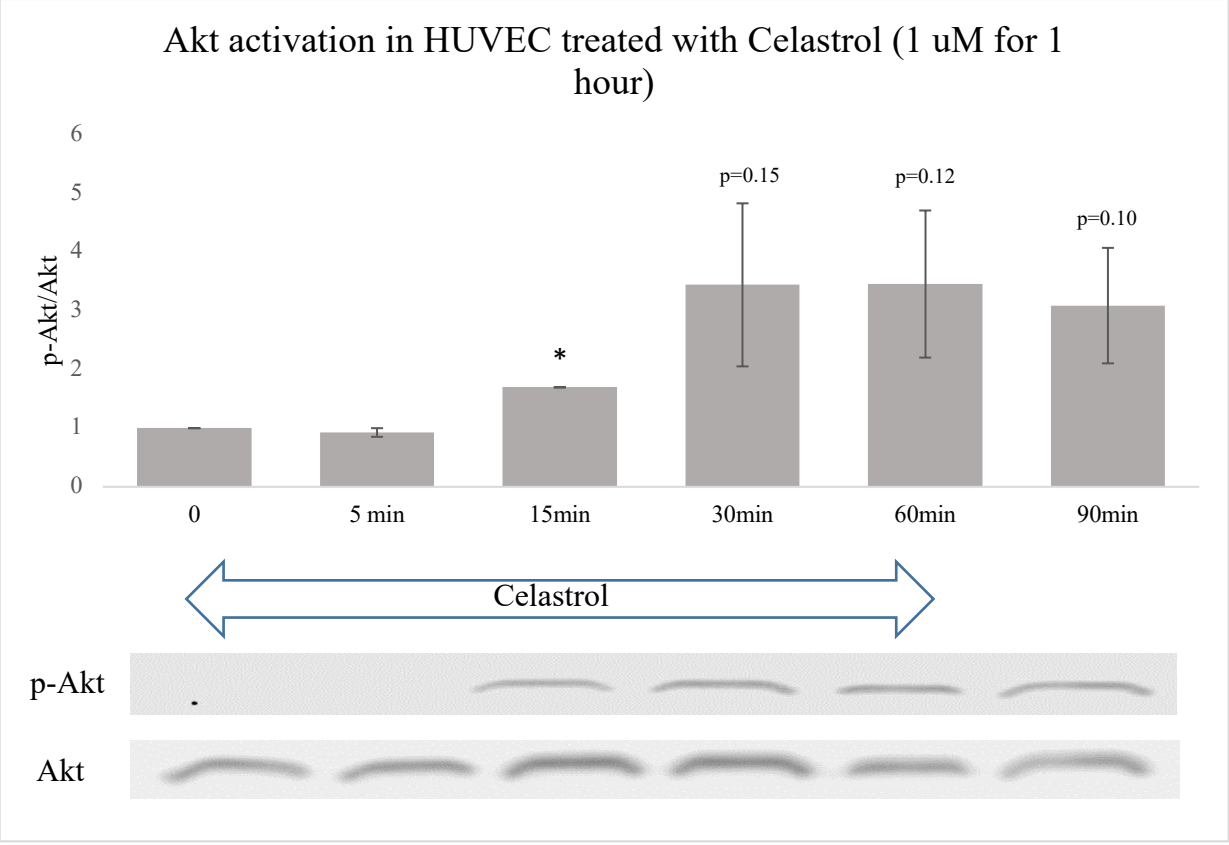
5 experiments were pooled and analysis is conducted based on the area under the curve. (AUC, area under the curve; HUVEC, human umbilical vein endothelial cells; EGM, endothelial growth medium)

Figure 18. – Effects of Celastrol on re-endothelialization

4.4 RISK pathway

In this section, we assessed the effect of Celastrol on rapid response kinases. As an HSP90 inhibitor, Celastrol is known to affect Akt activity and in the early period of the treatment. Therefore, we assessed Akt phosphorylation (activated form of Akt) in the during Celastrol treatment (1 hour) and also its response early after the end of the treatment (90 minutes).

4.4.1 Celastrol induces early Akt activation



HUVEC are treated with a dose of Celastrol 10^{-6}M for 1 hour. Time =0 is the control condition, before administration of the treatment. Following the treatment, HUVECs are allowed recuperation time in their standard growth medium

(EGM 5%) for an additional 30 minutes, which marks the 90min timepoint. (N=3) (HUVEC, human umbilical vein endothelial cells; Akt, protein kinase B; p-Akt, phosphorylated protein kinase B; min, minute)

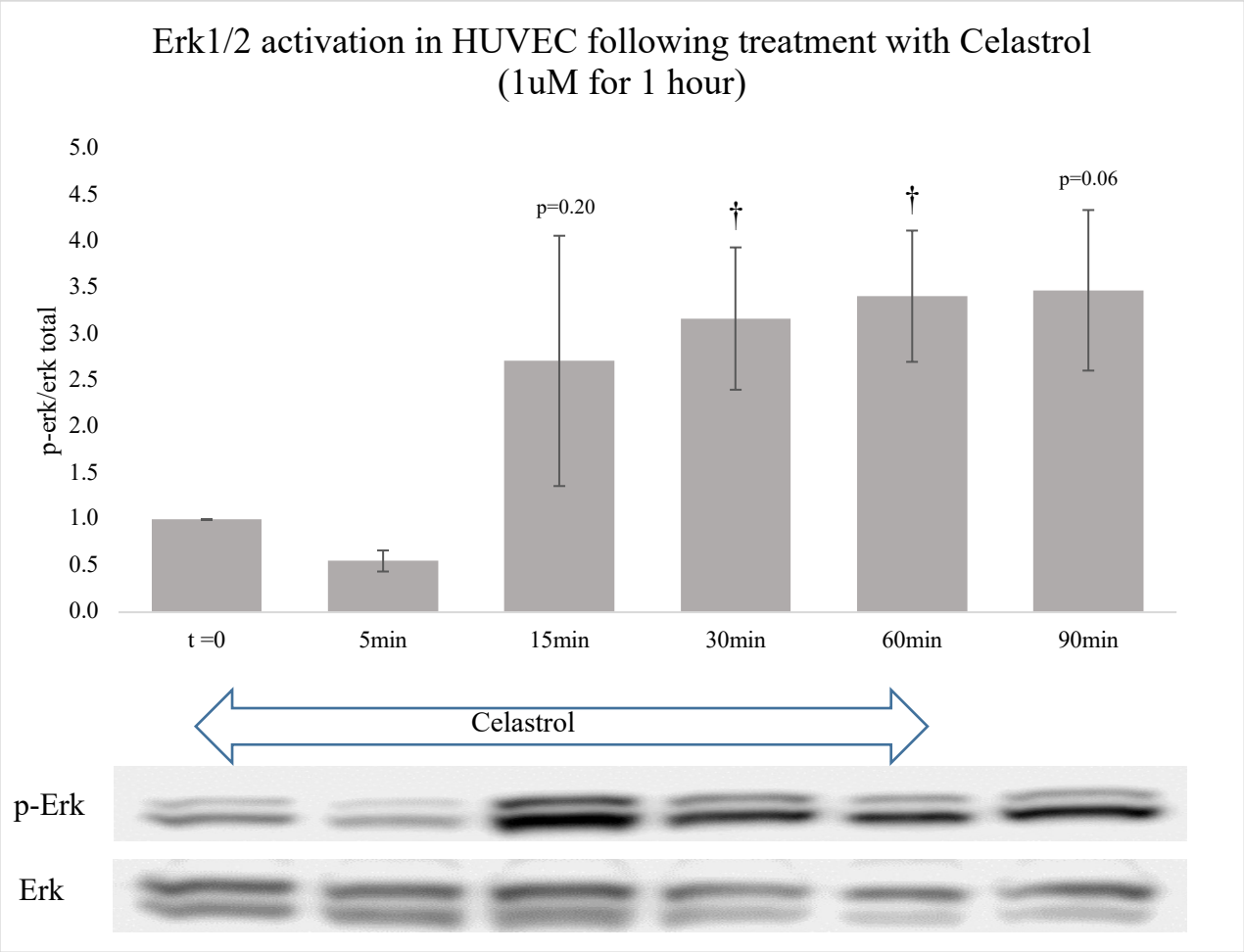
*At 15 minutes, there is a significant increase in the ratio p-Akt/Akt, 1.7-fold the control condition ($p<0.00001$).

Figure 19. – Kinetics of Akt activation following Celastrol treatment

Figure 19 shows the kinetic of Akt activation (ratio of phosphorylated Akt on total Akt). Timeline=0 marks the moment right before the start of the treatment and serves as the reference point. HUVEC are treated with Celastrol 10^{-6}M for 1 hour, which corresponds to the period between time=0 and the 60 minutes mark. At 15 minutes, there is a significant increase in the ratio p-Akt/Akt: 1.7-fold of time 0 condition ($p<0.00001$). Increased phosphorylation of Akt continues throughout the treatment and remains elevated even 30 minutes after the withdrawal of the drug. After 60 minutes, p-Akt/Akt starts to decline. Despite the noticeable increase in Akt activation between 30 and 90 minutes, as evidenced by the corresponding gels, statistical tests did not reveal significance because of the high standard deviation. It was due to one of the 3 experiments showing a much higher ratio of Akt activation (3 times the ratio found in the remaining 2 experiments). The trend, however, remains similar. (see Figure 4 in Appendix)

4.4.2 Celastrol induces early activation of ERK1/2

As an HSP90 inhibitor, Celastrol is also known to affect ERK1/2 activity and in the early period of the treatment. We assessed ERK1/2 phosphorylation (activated form of ERK1/2) in the during Celastrol treatment (1 hour) and also its response early after the end of the treatment (90 minutes).



HUVEC are treated with a concentration of Celastrol 10^{-6}M for 1 hour. Following the treatment, HUVECs are allowed recuperation time in their standard growth medium (EGM 5%) for an additional 30 minutes, which marks the 90min mark. (N=3) (HUVEC, human umbilical vein endothelial cells; Erk, extracellular signal-regulated kinase; p-Erk, phosphorylated extracellular signal-regulated kinase; min, minute)

†There is a significant increase in Erk1/2 activation at 30 and 60 minutes timepoint.

Figure 20. – Kinetics of ERK1/2 activation following Celastrol treatment

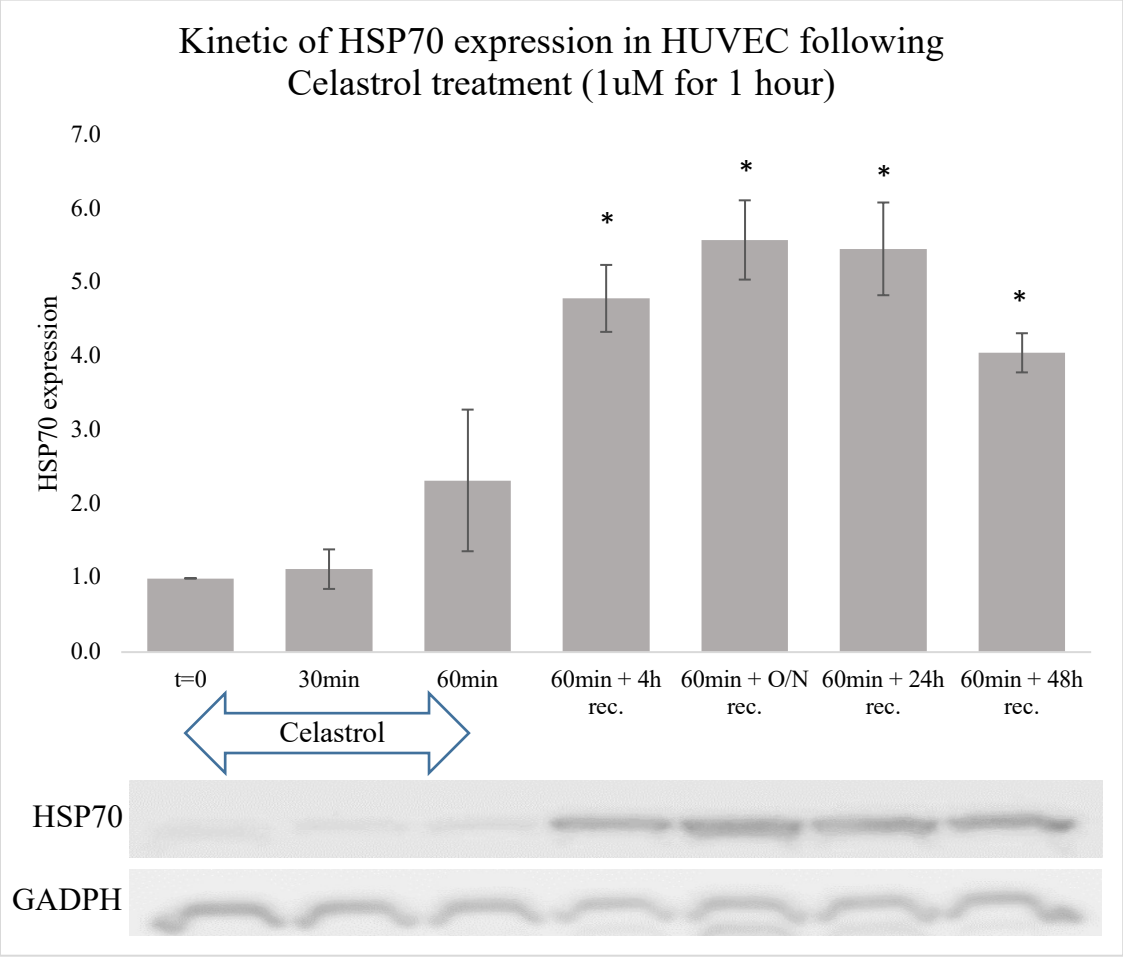
Figure 20 presents the kinetic of ERK1/2 activation during and following a 1-hour treatment of Cel 10^{-6} M. An initial drop of p-Erk is noted at 5 minutes, followed by an increase in ERK1/2 activation (p-ERK1/2) starting at 15 minutes. p-Erk/ERK remains elevated compared to baseline even after the end of the treatment, at 90 minutes. A significant increase in p-Erk/ERK is reported for the 30- and 60-minutes time points.

4.5 Activation of the Heat Shock Response

Celastrol inhibitory effect on HSP90 also influences expression of Heat Shock Response main effector protein, HSP70. The kinetic of HSP70 expression is depicted in Figure 21 following treatment with Cel 10^{-6}M. As nuclear response shows up much later then kinase activation, kinetic assessments were performed up to 48 hours following the initial treatment.

4.5.1 Celastrol induces a sustained expression of HSP70

Figure 21 shows the protein expression following treatment with Celastrol 10^{-6}M for 1 hour. The increase in HSP70 expression is seen starting at the 60-minute mark. The increased expression becomes statistically significant at the 60 min + 4h recuperation timepoint and remains so up to 48 hours after the end of the treatment. The peak expression is recorded at 60min + O/N recuperation and is 5.60-fold the baseline (t=0). The expression then declined between 24 and 48 hours but remained 4.10-fold the baseline at 48 hours.



HUVEC were treated with Celastrol 1uM for 1 hour, followed by a recuperation period in EGM 5% over 48 hours. Therefore, HUVEC was exposed to Celastrol only in the first 60 minutes. HSP70 expression was assessed at 4 hours, overnight or 16 hours, 24 hours and 48 hours during that recuperation period. (HUVEC, human umbilical vein endothelial cells; rec, recuperation; min, minute; O/N, overnight; HSP70, heat shock protein 70; GADPH, glyceraldehyde 3-phosphate dehydrogenase; t=0, 0 minute or time prior to treatment)

*There is a statistically significant increase in HSP70 expression detected after 60 minutes.

Figure 21. – Effects of Celastrol on HSP70 expression

4.6. Antioxidative pathway: induction of HO-1 expression

As reported from prior discoveries, Celastrol also induces HO-1 expression, leading to anti-oxidative response. The kinetic of HO-1 expression was followed until 48 hours after the end of the treatment and is depicted in Figure 22.

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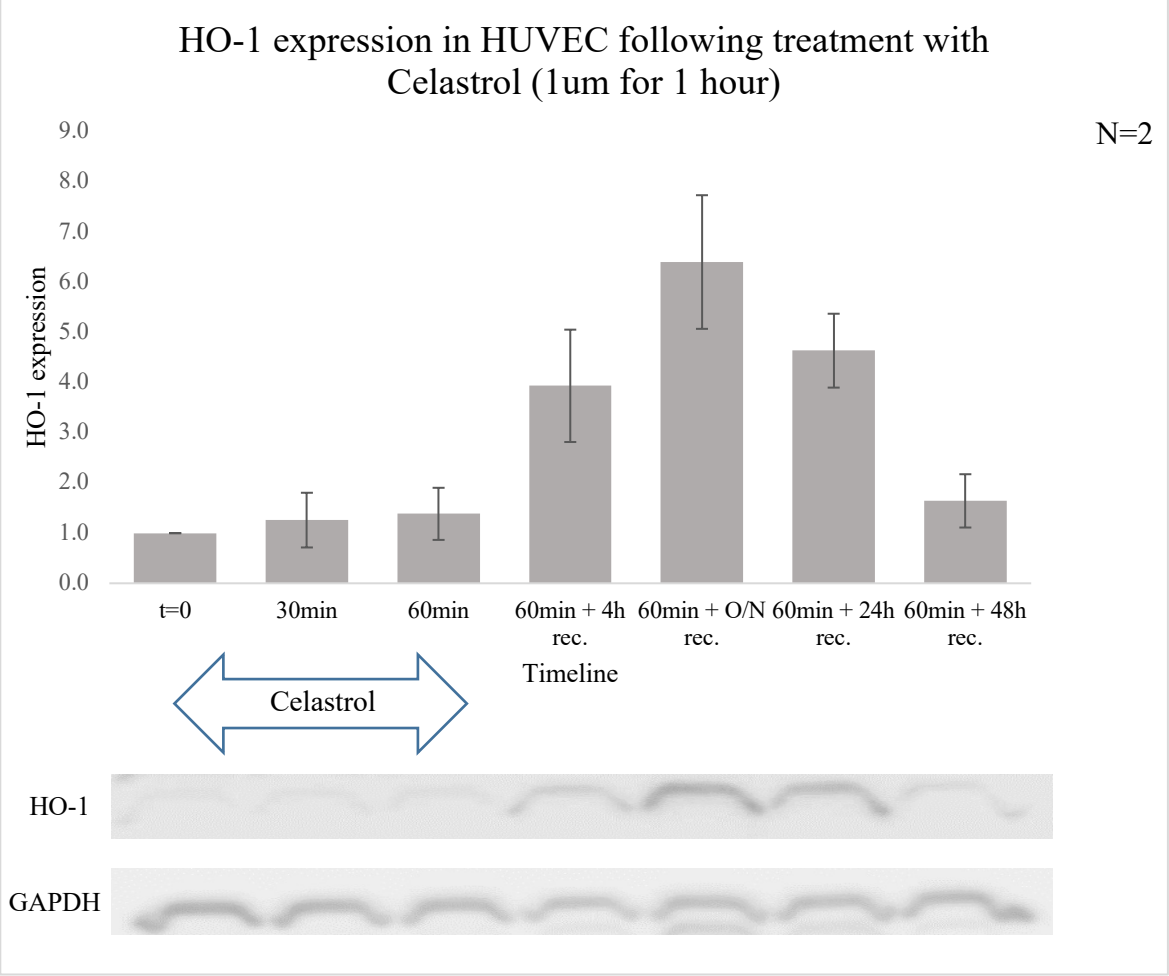
4.6.1 One short Celastrol treatment promotes sustained expression of HO-1



HUVEC were treated with Celastrol 1uM for 1 hour followed by a recuperation period in EGM 5% over a span of 48 hours. Therefore, HUVEC was exposed to Celastrol only in the first 60 minutes. HO-1 expression was assessed at 4 hours, overnight or 16 hours, 24 hours and 48 hours during that recuperation period. (HUVEC, human umbilical vein endothelial cells; rec, recuperation; min, minute; O/N, overnight; HO-1, heme oxygenase 1; GADPH, glyceraldehyde 3-phosphate dehydrogenase; t=0, 0 minute or time prior to treatment)

Figure 22. – Effects of Celastrol on HO-1 expression

Figure 22 shows a significant increase in HO-1 expression starting at 4 hours after treatment. HO-1 expression continues to rise up to 6.4-fold from baseline at 16 hours (O/N recuperation) before declining. This figure represents the compilation of 2 experiments. A third experiment was excluded for analytical purposes. Due to the skewed results of that third experiment, a statistical analysis was not performed. It should be noted, however, that the trend is similar in all 3 experiments. A compilation of the 3 experiments can be found in the Appendix (*Figure 5*).

Chapitre 5 – Discussion

The significant findings in this study are:

1) Celastrol does not confer survival benefits in endothelial cells submitted to storage in normal saline
2) Celastrol confers survival benefits in endothelial cells submitted to oxidative stress.
3) Celastrol accelerates and improves re-endothelialization
4) These observed effects are associated with Celastrol-induced activation of the *Reperfusion Injury Salvage kinase* pathway, the *Heat Shock Response*, and expression of the antioxidant enzyme, HO-1

First and foremost, determining the effective Celastrol concentrations requires taking into consideration the narrow therapeutic window of the compound [249] Celastrol treatment has been largely used in clinical and experimental settings with dosages 0.1 to 10µM *in vitro* [267, 303-306]. Similarly, *Yu et al.* determined toxic doses of Celastrol on human neutrophils to be above 10 µM [307]. Our group previously published protective effects on cardiomyoblasts with Celastrol 0.1umol/L [239, 267]. From those evidences, Celastrol at concentrations between 10^{-10}M and 10^{-6}M were used. DMSO was used as the vehicle at a concentration less than 1% v/v, which is considered to be far from its toxic level [308].

Second, we tested HUVEC viability under oxidative stress and in NS storage. These *in vitro* conditions reflect the clinical practice of using heparinized NS as the preservation solution for vein grafts and the environment rich in ROS encountered during reperfusion.

Storage in heparinized NS in clinical settings rarely exceeds 1 hour [109]. However, detection of significant mortality was seen only from the third hour of storage, which becomes the condition for the viability assessment. After 3 hours in heparinized NS, Cel 10^{-6} M marginally improved survival. That difference was not statistically significant. It is unknown if a longer storage period would reveal more appreciable differences. Investigation of more extended exposure was futile in this context because it does not represent clinical practice. In retrospect, a different

experimental setting could have been planned: starting preservation in heparinized NS for 1 hour followed by a period of recovery in the culture medium. It would allow documentation of the response in the hours or even days following a single short NS exposure. Nevertheless, these results reveal that Celastrol does not improve significantly the survival of HUVEC in NS. NS use during CABG procedure remains quite common in practice (30%) despite evidences in the literature showing that NS is possibly the worst option for graft preservation [113]. Coupled with evidences in this study, NS should no longer be used as a preservation solution for graft preservation. According to current clinical evidences, the first change that should be implemented in the operating room to optimize graft patency is the use buffered solutions. In current practice however, only 40% of cardiothoracic surgeons in the United States are using pH-buffered solutions [109]. While accessibility and cost favor NS use, surgeons' personal preference or familiarity remain important factors that influence their choice. Until a rigorous randomized clinical trial is conducted, practice will likely remain divided in the use of preservation solutions. Additional testing is needed to find if additional of Celastrol into a buffered solution can further improve graft patency.

For viability testing post-oxidative stress, a significant survival benefit was observed for the treatment Cel 10^{-10}M following a 1-hour oxidative stress. Survival benefits were also reported for concentrations of Cel 10^{-10}M and Cel 10^{-8}M following a 24-hours oxidative stress.

EBM 1% was used as the negative control group because it is the base medium used for preparations of treatment and stress conditions. HUVEC are cultured in EGM 5%, which contains EBM and additional growth factors as well as 5% FBS. EGM 5% is avoided in the stress and treatment conditions because the added growth factors may trigger competing or similar cellular responses and may hinder the studied endpoints. Using EBM 1% limits contaminating effects of the growth factors. However, the longer the HUVEC are incubated in a low-FBS medium devoid of growth factors, the more susceptible they are to die. The reported mortality was 0.9% for 1 hour compared to 5.1% for 24 hours (*see Figure 14 and 17*). Comparatively, mortality in the standard culture medium, EGM 5%, is consistently less than 1% (*see Figure 12*). In longer incubation (24 hours), EBM 1% contribution to the mortality percentage should be considered.

One caveat in the viability studies is the capacity of some dead HUVEC to detach from the wells. The floating cells are lost after the washes. In fact, drug-induced apoptosis of HUVEC was found to be associated with progressive detachment of dead cells [309]. The effect is more pronounced the higher the induced mortality. Consequently, the absolute mortality rate may be underreported. This is why the number of cells stained with Hoescht 33342 was also reported to highlight this effect and also to complement our viability analysis. It should be mentioned that Hoescht 33342 stains both living and dead cells. However, more mortality will likely correlate with fewer cells remaining in the wells. In the results, the number of cells is inversely proportional to the degree of oxidative stress (Figure 15). Interestingly, more cells were counted in the Celastrol-treated wells compared to the vehicle. It should be mentioned that the total number of cells stained by Hoescht-33342 compared to the sum of LIVE and DEAD cells correlated. For the short oxidative stress condition (1 hour), the higher number of cells is unlikely to be the result of cellular proliferation as the time between the stress and analysis is too short. Consequently, analysis of Hoescht 33342 staining will not be relevant to complement viability analysis in longer experimental conditions such as the 24-hour exposure to oxidative stress. For longer periods, Hoescht 33342 would stain both surviving cells and those resulting from proliferation. Therefore, Hoescht staining was not considered in the viability analysis for the 24-hour oxidative stress conditions.

Considering all analysis, the main finding remains that a short pre-treatment with Celastrol confers survival benefits in HUVEC exposed to oxidative stress. These benefits are seen as early as 1-hour and are still present after prolonged stress (24h). The optimal dosage of Celastrol required for optimal viability is between 10^{-10}M and 10^{-8}M. A higher concentration (Cel 10^{-6}M) did not result in better survival in the oxidative environment compared to that observed in NS. We believed that part of the survival response is orchestrated by Nrf2 through the presence of ROS. In NS, Celastrol would be the main cause of endogenous ROS production. In an established oxidative environment, a higher Celastrol concentration may induce a higher oxidative which becomes cytotoxic.

As the third step, scratch tests were performed. Since some mechanical damage and loss on the endothelium is inevitable during harvest, we focused on the potential of reendothelialization.

Because complete coverage and recuperation of a functional endothelium is crucial in recovering graft homeostasis, the earlier the graft is reendothelialized, the earlier it will gain proper homeostais and limit ongoing damages. Scratch tests aim to reproduce the denuded subendothelial surface following a mechanical injury during graft preparation. To assess the capacity of HUVEC to reendothelialize, which is a process involving both cell proliferation and migration, scratch test analysis is chosen over tests exclusively accessing proliferation (i.e., BrDu). Scratch tests are a simple and reliable method to assess endothelial cell migration and wound healing capacity.[310] It is a particularly interesting and relevant model because it reproduces the mechanical denudation encountered during harvest. However, it cannot replicate the additional mechanical stretch caused by manipulation or pressure caused by flushing the grafts. The scratch test does allow a standard and reproduceable mechanical denudation. Results show that the proliferation rate is highest within the first 12 hours after which a plateau is reached. EGM 5% shows near-complete wound closure at 24 hours, 96.0%. Cel 10^{-6} M has an average closure of 91.2% compared to 50.6% for the vehicle at 24 hours. Lower dosages report final wound closure between 46 and 70%. Comparing AUC shows Cel 10^{-6} M to significantly improve wound closure ($p=0.009$). However, the equivalence test did not confirm similar closure rate between Cel 10^{-6} M and EGM 5%.

Stemming from these results, the optimal dosage from both survival and proliferative standpoint may be somewhere between Cel 10^{-8} M and Cel 10^{-6} M. Interestingly, *Pang et al.* reported contradictory findings to ours. Celastrol treatment, in their case, resulted in reduced viability in HUVEC and inhibition of VEGF-induced migration and capillary structure formation.[305, 311] Their results correlate with other studies finding similar inhibitory effects on angiogenesis potential and supporting Celastrol's activity against cancer growth and development [303, 312, 313]. We believe dosage and period of treatment to be critical in inducing the desired effects from Celastrol. *Pang et al.* submitted their endothelial cells to prolonged periods of treatment. For example, HUVEC were incubated in Celastrol 1 µmol/L, our highest treatment dosage, for 8 to 10 hours in their wound-healing assay, which is far longer than our short treatment. The use of Celastrol at twice our highest concentration, 2 µmol/L, inhibited micro-vessels sprouting. Protein analysis was performed following 4 hours of Celastrol treatment at concentrations of 0,

0.5, 1, 2 and 5 µmol/L. While p-Akt was increased with Celastrol concentrations below 1 µmol/L, concentrations ≥ 1 µmol/L were associated with reduced phosphorylation of Akt [305]. Expectedly, higher doses and longer incubation with Celastrol will increase the risk of cytotoxicity to the cells, which explain its harmful effects on cancer cells and cytoprotective effects seen with lower and shorter treatments like ours.

As the fourth step, we assessed intracellular pathways potentially involved in Celastrol treatment. As a HSP90 inhibitor, a few of its client proteins could be affected. They include stress-induced kinases such as Akt and ERK1/2. Celastrol was found to modulate Akt activity in different cell lines (i.e., cardiomyocytes, endothelial cells, cancer cells among others), which can be either inducible or suppressive to the PI3K/Akt pathway [256, 258, 267, 314]. Looking at Akt activation kinetics during Celastrol treatment at 10^{-6} M (*Figure 20*), there is an early rise of Akt phosphorylation between 15 and 30 minutes, which peaked at 30 minutes before decreasing after 60 minutes. Phosphorylated Akt remained high compared to basal activation (4.9 folds of the control at 90 minutes). This early Akt activation may be accountable for the early survival outcomes seen from treated HUVEC.

Akt, also known as protein kinase B, is a kinase linked to downstream effectors involved in cell growth, proliferation, metabolic activity and resistance to apoptosis [315]. Shear stress, ROS, GF (i.e., VEGF-α, insulin, insulin-like growth factors, erythropoietin, PDGF and βFGF) are some of the upstream regulators of Akt [316]. Activation of Akt requires its translocation to the plasma membrane where PI3K, also known as phosphoinositide-dependent kinase 1 (PDK1), phosphorylates its threonine residue (Thr308) [317]. Phosphorylated Akt (p-Akt) detaches itself from the membrane and migrates into the cytosol and nucleus where it can exert its enzymatic activity. p-Akt can phosphorylate eNOS, promoting angiogenesis and vasorelaxation, but for most, the downstream effectors of p-Akt are involved in survival response, proliferation, metabolic homeostasis and cell growth (*see Figure 6 in Appendix*) [315]. The ability of p-Akt to optimize cell survival comes from its capacity to block apoptosis. It does so by phosphorylating caspase-9, thereby inhibiting the cytochrome-c/Apaf-1/caspase-9 apoptotic pathway. It also phosphorylates BAD, which forms a complex with Bcl-2 and Bcl-X, and, by doing so, releases Bcl-2 and Bcl-X to promote their anti-apoptotic effects [316]. p-Akt inhibits tuberous sclerosis 1

protein (TSC1) by phosphorylating it, which leads to the activation of the *mammalian target of rapamycin complex 1* (mTORC1) whose substrates are involved in cell proliferation. Akt can also inactivate GSK3β, increasing Cyclin D1 and promoting G1/S cycle progression [258]. In short, the Akt activation seen in our results can be linked to the improvement in survival and proliferation observed in HUVEC.

Another family of kinases affected by Celastrol is ERK1/2. *Li et al.* reported Celastrol-induced activation of ERK1/2, resulting in improved viability, diminished inflammatory response and enhanced antioxidant enzymatic activity in HUVEC submitted to angiotensin II stimulation for 24 hours [294]. In our study, there is a notable ERK1/2 activation at 30 minutes of treatment with Celastrol 10^{-6}M (*Figure 21*). ERK1/2 phosphorylation starts as early as p-Akt but, contrary to Akt, continues to rise after 60 minutes.

The activation of ERK1/2, a member of the mitogen-activated protein kinase (MAPK) pathway, has been implicated in EC's survival response and proliferation [318, 319]. MAPK has 4 distinct members: extracellular signal-regulated kinase (ERK1/2), p38 proteins, c-Jun N-terminal kinases (JNKs) and ERK5. These MAPK are activated by MAPK kinases (known as MEK): MEK1/2, MKK3/6, MKK4/7 and MEK5, respectively. ERK1/2 ensures cell cycle progression through the inactivation of Cdc2 inhibitory kinase (MYT1) [318]. Cdc2, or cyclin-dependent kinase 1, is necessary for cell cycle progression.[320] ERK1/2 also activates activator protein 1 (AP-1), a transcription factor with a wide range of nuclear targets involving cell growth, differentiation and apoptosis. ERK1/2 also increases expression of several of the pro-survival Bcl-2 proteins family, notably MCL-1 (induced myeloid leukemia cell differentiation protein), an apoptosis inhibitor [318]. Comparatively, p38 and JNK activation can either induce apoptosis or promote survival depending on the cell type, the intensity and duration of the stimulus as well as the crosstalk between both pathways [321].

The simultaneous activation of pro-survival kinases, Akt and ERK1/2, upon external stressor characterizes the activation of the Reperfusion Injury Salvage Kinases Pathway or RISK pathway. The RISK pathway is a group of kinases extensively studied in the mechanism of cardio-protection, specifically in the context of pre and post-conditioning [322]. RISK is activated by various factors

such as insulin, urocortin, atorvastatin, opioid receptor agonists among others. Its activation is associated with the decrease in infarct size and reduced markers of injury (i.e., troponin) following an acute ischemic event. Importantly, it is known that a short-term activation is protective. In contrast, chronic activation would be considered harmful due to the growth-inducing effect and is associated with cardiac hypertrophy [322]. Many of the kinases' downstream effectors converge to the mitochondria, where they inhibit the mitochondrial permeability transition pores (mPTP) opening, therefore preventing apoptosis [322, 323]. In essence, the ability to modulate these pro-survival kinases provides an approach to limit ischemic-reperfusion-injury-induced cell death and explains Celastrol survival benefits on HUVEC in oxidative stress.

It is noteworthy to mention that Celastrol has opposing effects in studies on cancer therapy or inflammatory diseases. Celastrol inhibits Akt pathway leading to the suppression of cellular proliferation and invasion of many neoplastic cell lines (i.e., osteosarcoma, cholangiocarcinoma, colorectal, prostate cancer cells, breast cancer) [251, 265, 305, 314]. How Celastrol promotes survival in hypoxic cardiomyocytes or stressed endothelial cells yet induces tumor cell apoptosis can be explained by a few elements. As mentioned earlier, it is hypothesized that timing and duration of treatment play a central role in guiding the cellular response to external stimuli. Chronic activation of kinases gives rise to an overwhelming proliferation response, which can lead to cardiac hypertrophy or significant vascular remodeling or atherosclerosis [315, 324-326]. This unopposed stimulation may also contribute to tumorous cell survival and proliferation. Dysregulation of Akt is associated with loss of the cell cycle control and desensitization to the apoptotic stimuli and is commonly identified in tumor cells [327]. Celastrol treatment can suppress this prominent and constant kinase activation as an HSP90 inhibitor [302]. There are 3 types of HSP90 inhibitors which are classified depending on which HSP90 cofactor they target. Celastrol targets cofactor Cdc37. Occupancy of HSP90 pocket by Celastrol alters the stabilization of the complex HSP90-Cdc37 and its client proteins, among which are Akt, ERK, Cdk4, Raf and EGFR [242, 328, 329]. Destabilized client proteins become targets of ubiquitination and proteasome degradation [329]. Hence, although Celastrol induces the phosphorylation of Akt acutely, prolonged treatment can deplete Akt stores by the sustained inhibition of HSP90 and destabilization of HSP90-Cdc37-client proteins, Akt and ERK. Findings by *Han et al.* on a

hepatocarcinoma cell line, HepG2, supports this theory. Celastrol treatment did induces Akt phosphorylation at 6 hours, but a 24-hour treatment induced a significant depletion of total Akt protein and a consequent reduction in p-Akt [258]. In untreated cells, more than 30% of Akt is found in a complex with HSP90-Cdc37 [329]. Following HSP90 inhibitor drug treatment for 24 hours, Akt half-life is seen declining from 36 to 12 hours and protein expression dropping by 80%. The decline is seen as early as after 6 hours of treatment and is time and concentration dependent. Similarly, prolonged Celastrol treatment is found to also inhibit ERK1/2 in tumoral cells [329]. Two weeks of Celastrol administration inhibited ERK1/2 phosphorylation and tumor growth in human colorectal cancer cells [330]. Three weeks of Celastrol treatment in a mice model of hepatocellular carcinoma was shown to have antiproliferative effect by repressing both ERK and Akt phosphorylation [331]. In addition to the effects of the treatment duration and concentration, the intracellular mechanisms found in neoplastic cells are drastically different from normal cells. It is possible that the cytoprotective effect on healthy endothelial cells may not be reproduceable on neoplastic cells with very different cellular response.

In this study, endothelial cells exhibited activation of pro-survival kinases after a short treatment of Celastrol. This short period of conditioning is sufficient to induce survival benefits while avoiding kinases depletion by continuous HSP90 inhibition or chronic kinase activation. Ultimately, that narrow window of opportunity during graft preparation, earlier identified as a potential limitation in the pre-treatment efficacy, becomes the perfect setting for a one-time burst treatment and Celastrol becomes the ideal candidate.

A single 1-hour exposure to Celastrol was sufficient to produce survival benefits detected even after 24 hours of oxidative stress and strong enough to elicit near-complete reendothelialization within a day of the treatment. The beneficial effects are persisting well after the end of treatment hint at the probable involvement of downstream effectors from a transcriptional level.

Through HSP90 inhibition, Celastrol also induces the HSR [332]. HSF-1, or heat shock factor 1, is a transcription factor that is complexed with HSP90 and inhibited by this later in normal conditions. When stress ensues (i.e., hypoxia, oxidative stress, heat), unfolded proteins compete with HSP90 complex and provoke the release of HSF-1 which translocates to the nucleus and binds to the heat

shock response elements (HSRE). These elements lead to the transcription of heat shock proteins (HSP). They are a family of chaperones highly expressed in response to stress. Their role in limiting cell damage facilitates cell recovery and survival [333]. HSP70 is one of the most widely studied HSP. Its chaperone activity includes folding proteins, dissembling protein complexes, preventing aggregation of misfolded proteins, refolding misfolded proteins to clearing aberrant proteins and proteins aggregates. It plays a critical role in protecting cells from pathological conditions induced by misfolded or aberrant proteins [334]. *Figure 22* shows kinetic of activation of HSP70 in HUVEC following a 1-hour treatment with Celastrol. An increased in HSP70 expression is seen at 60 minutes and rises steadily until it peaks at 24 hours. 60 minutes marks the end of the Celastrol treatment and initiation of the oxidative stress in our experiments. At that point, the cells have already initiated HSP70 expression and thus protective mechanism was already in place when external stress was introduced. A survival benefit was, in fact, already seen after a 1-hour exposure to H_2O_2. At 24 hours, HSP70 expression is highest. This is the time point when survival benefits were documented after a 24-hour oxidative stress. HSP70 expression declined gradually between 24 and 48 hours, although it remains highly expressed even after 48 hours (4.4 folds of that at t=0). In summary, Celastrol can trigger the Heat Shock Response before the actual stress and that precedent response is associated with improved survival.

In contrast, the increase in the HO-1 expression was detected much later, at the 4-hour mark (*Figure 22*). Therefore, it is unlikely that HO-1 participated in the protective effects seen after 1-hour oxidative stress (*Figure 14*). However, HO-1 may have contributed to the adaptive response observed at 24-hour oxidative stress (*Figure 17*). HO-1, as previously mentioned, is an enzyme catalyzing the degradation of heme from which 3 by-products resulted. The removal of toxic heme and the antioxidant effects from CO, bilirubin and ferritin all contribute to relieving the oxidative burden on endothelial cells [284, 285]. In fact, reduced expression of HO-1 was observed in patients with CAD [335]. There was also a correlation between genotypes associated with lower capacity of HO-1 induction and severity of CAD [290]. Physiologic cyclic strain stimulates HO-1 expression and activation on human aortic endothelial cells and HUVEC. Similar to our findings, the increase in HO-1 expression was detected 4 hours intro the stimulated cyclic strain and is

highest at 24 hours. The HO-1 induction through the mobilization of NrF-2 resulted in improved viability in those EC [336].

Celastrol-induced overexpression of both HSP70 and HO-1confers the lasting survival advantage seen after a prolonged oxidative stress. The expression of both protective proteins takes 24 hours to reach its peak, which correlates with the time of our viability assessment. The proposed mechanism behind Celastrol effect and its interaction with HSP70 and HO-1 is well illustrated in Figure 9.

Limitations of the study

One limitation on the viability studies is the partial cellular detachment of dead HUVEC. This limitation might have led to an underestimated mortality on the grafts and a blunted effect of Celastrol during analysis. However, analysis of Hoescht staining led to conclusions in sync with the LIVE/DEAD assays. This only means that Celastrol can induce an even more impressive survival benefit likely humbled by our results. Because the LIVE/DEAD assay was limited by the number of cells in the wells, and some might have been lost through washes, we might have considered using flow cytometry as an additional mean to confirm our findings. However, our results were consistent and convincing enough for to drive present conclusions.

One major limitation the study is the *in vitro* nature of the experiments. *In vitro* experiments allow to isolate specific conditions, but certainly cannot mimic the complex environment found *in vivo*. Complementary data on *ex vivo* or *in vivo* are necessary to assess the net effect of Celastrol within a living environment. This is our next step towards the translational application of the current findings.

Finally, signaling pathways were assessed with our highest dose, Cel 10^{-6} M, which was believed to be able to best highlight the intracellular pathways. The optimal concentration in the viability assessment and the scratch tests differs, however. The viability findings point towards a concentration between Cel 10^{-8} M and 10^{-10}M as the optimal condition for survival in oxidative stress and Cel 10^{-6} M for reendothelialization. In choosing dosing intervals, we decided to test concentrations at 10^{2} increments to cover more testing grounds. Following those results, additional testing for Cel 10^{-7} M should probably be conducted. In choosing between marginal

proliferative effect (for any concentrations lower than Cel 10^{-6} M) versus no significant survival benefits (for Cel 10^{-6} M), the debate is still open.

Chapter 5 – Conclusion

Evidences in the literature show that endothelial integrity is critical for the evolution of vein graft failure. Less damage and loss as well as faster recovery correlate with better outcomes in terms of graft patency and remodeling. Most of the damages occur in the very first few hours to days and the early response to the injury dictates how the graft will evolve. Targeting that key moment to reverse part of the harmful effects can be an efficient strategy to prevent graft failure.

Celastrol is a compound capable of triggering adaptive pathways in endothelial cells. This study shows that Celastrol activates the RISK pathways early. It also shows that, within 1 hour, Celastrol also triggers the HSR as well as an anti-oxidative response through induction of their respective effector proteins (HSP70 and HO-1). This forerunner activation prepares the cells to the following stress event, which reflects an improved survival potential. Celastrol treatment also promotes faster and more complete reendothelialization. Together, these results strongly support Celastrol as a successful pre-conditioning agent for endothelial cells. In the context of vascular graft preservation, where the window of opportunity is narrow, it is recognized as a potential candidate drug for endothelium preservation and protection.

Before clinical application however, one important point to remember is Celastrol's therapeutic window. Although the proposed treatment is beneficial to endothelial cells, the specific dosage and treatment period are critical as higher concentrations (≥ 1uM) and longer periods (≥1 hour) do not guarantee similar responses and may even have opposing effects. Also, this study showed that optimal dosage differs depending on the wanted effect. The specific treatment condition required is still a limitation to the clinical use of Celastrol. Fortunately, a lot of work has been devoted to finding new compounds or creating analogous molecules with the similar or improved therapeutic profile. Although many were proposed as a potential replacement, none has gone through extensive investigations as Celastrol.

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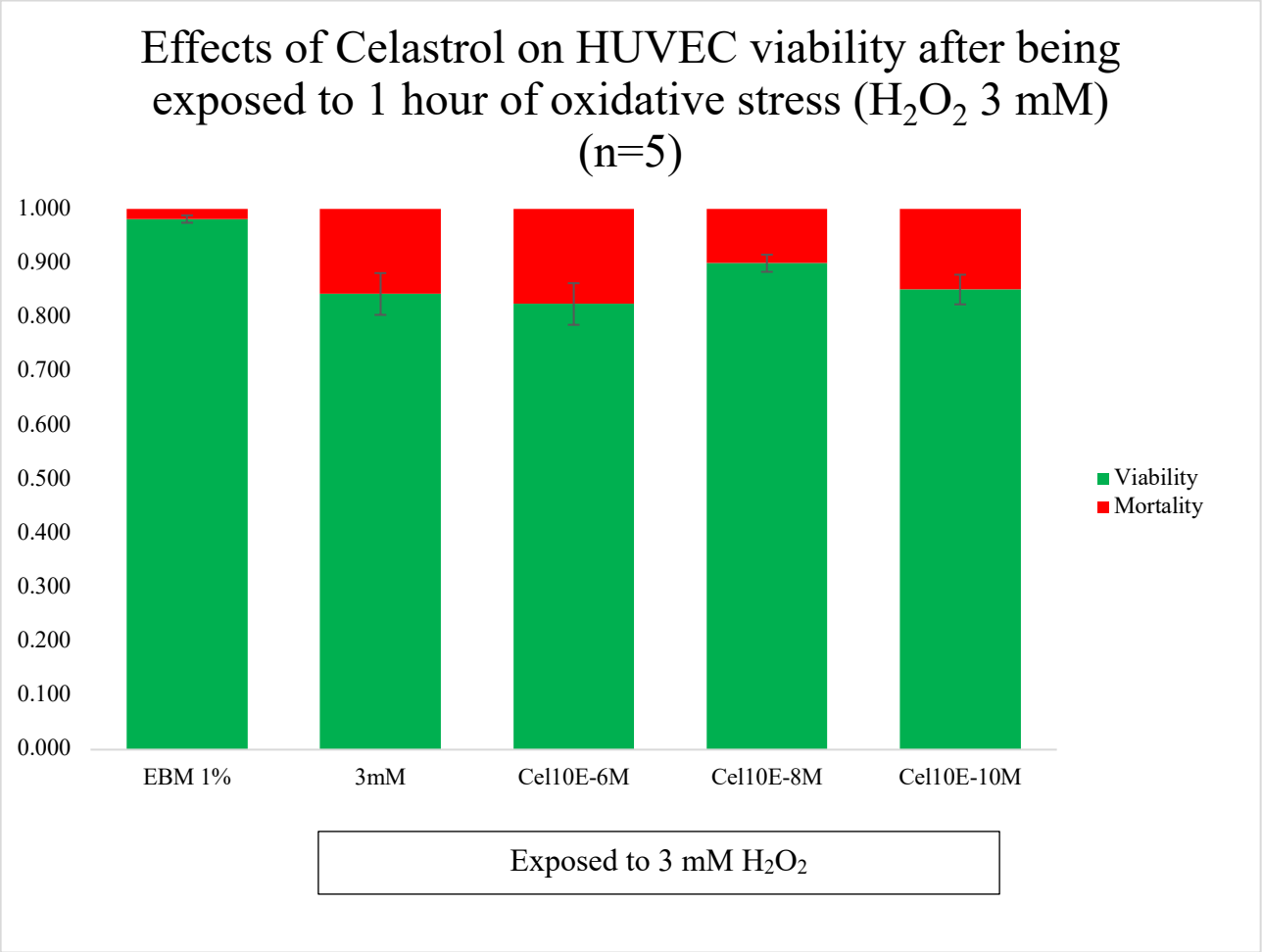
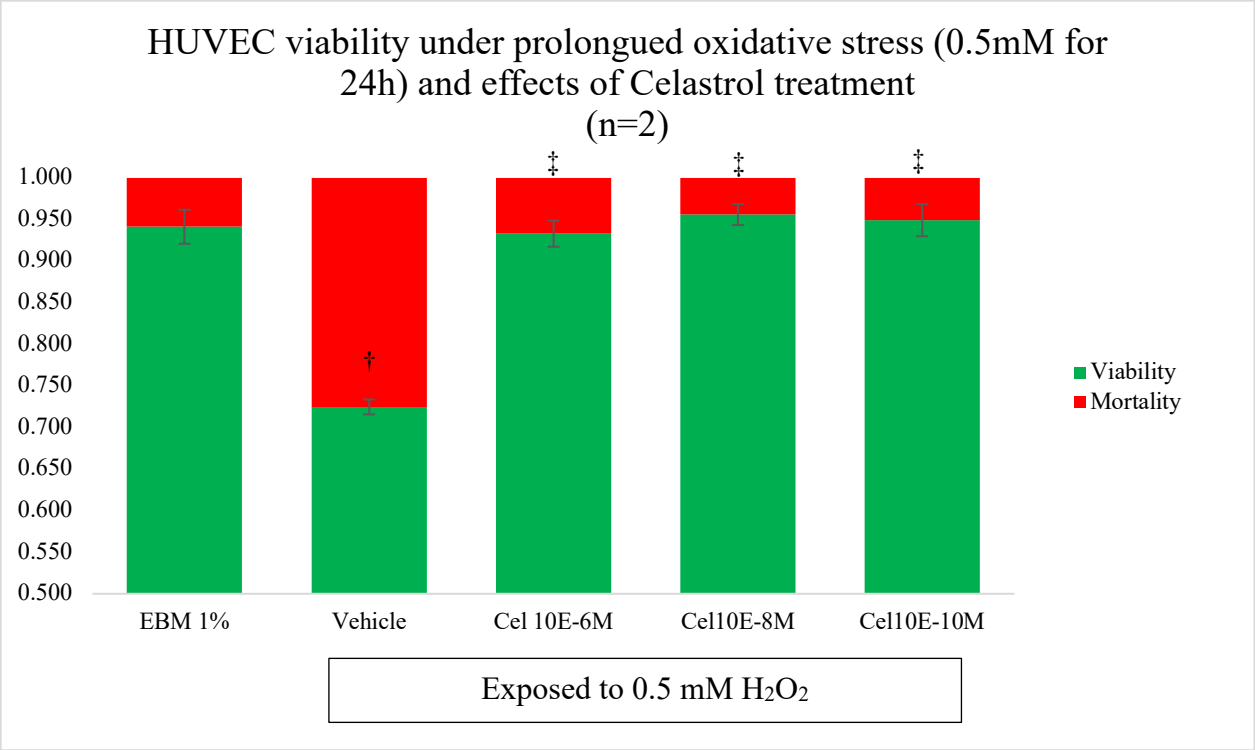
Appendix



This figure shows the compilation of 5 experiments exposing HUVEC to H_2O_2 3 mM. The EBM 1% group was not exposed to Celastrol nor H_2O_2 and served as the control group. The 3 mM group did not receive Celastrol but was exposed to 3 mM H_2O_2 for 1 hour. All groups pre-treated with Celastrol for 1 hour prior to stress.

†Exposure to 1 hour of H_2O_2 3 mM significantly increase mortality compared to EBM 1%; 1.9 vs 15.7% (p=0.0003)

Figure 1 Effects of Celastrol on HUVEC viability after being exposed to 1 hour of oxidative stress (H_2O_2 3mM)

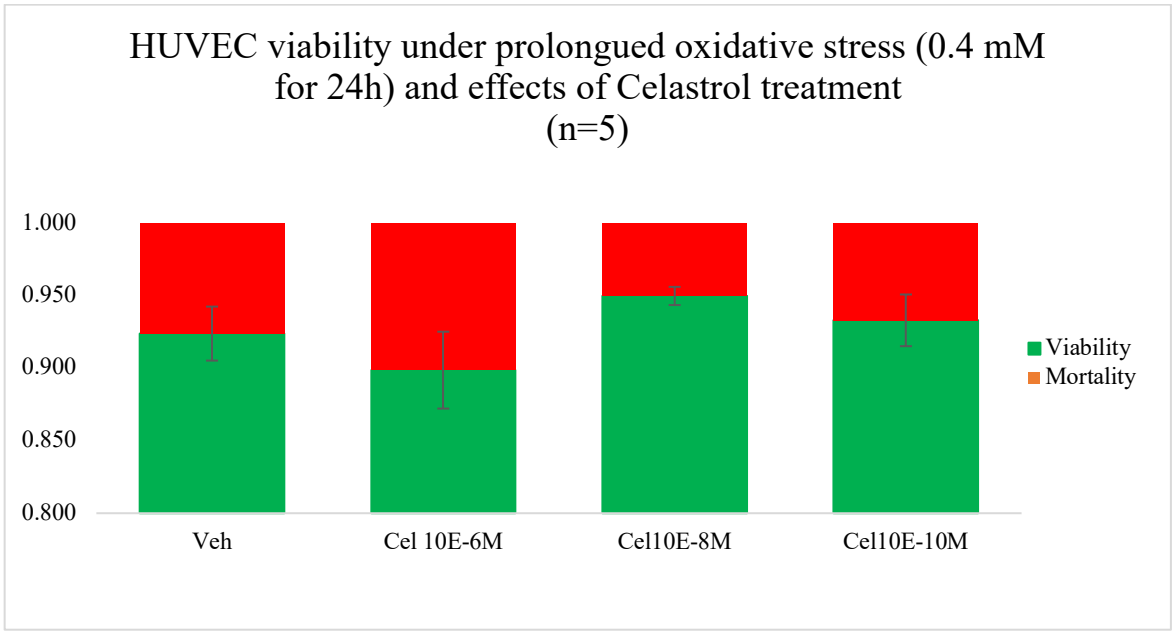


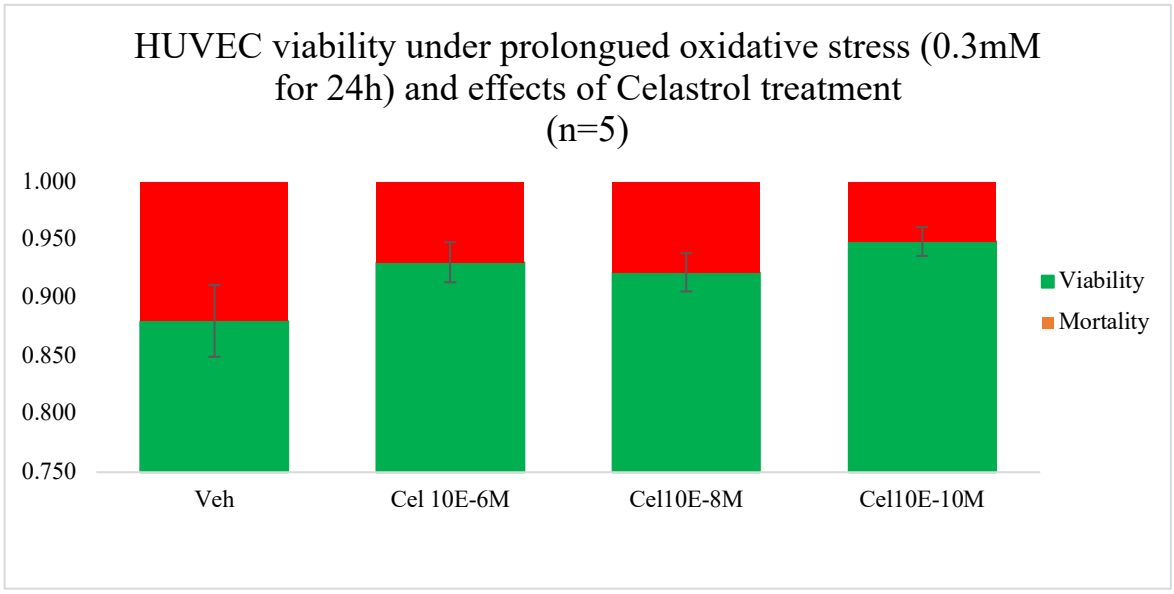
This figure shows the compilation of 2 experiments exposing HUVEC to H_2O_2 0.5 mM for a period of 24 hours. The *Vehicle* group was not treated to Celastrol and serves as the control group. Treatment with Celastrol lasted 1 hour and was done prior to stress. 1 of the initial experiment showed outlier values for the Cel 10^{-6}M which was considered in the main analysis. This graphic shows that without the outlier, benefits were detected in all wells treated with Celastrol.

† Prolonged oxidative stress (H_2O_2 5 mM) significantly reduces viability: 72.5% compared to 94.1% ($p<0.0001$)

‡ HUVEC pre-treated with Cel 10^{-6}M showed a higher viability compared to the stress condition, 93.3% vs 72.5% ($p=0.0007$). HUVEC pre-treated with Cel 10^{-8}M showed a higher viability compared to the stress condition, 95.6% vs 72.5% ($p=0.0004$). HUVEC pre-treated with Cel 10^{-10}M showed a higher viability compared to the stress condition, 94.9% vs 72.5% ($p=0.0004$).

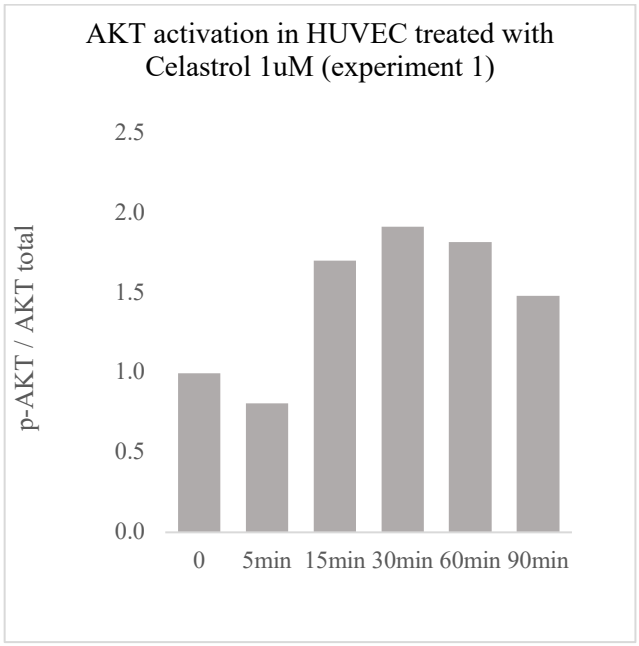
Figure 2

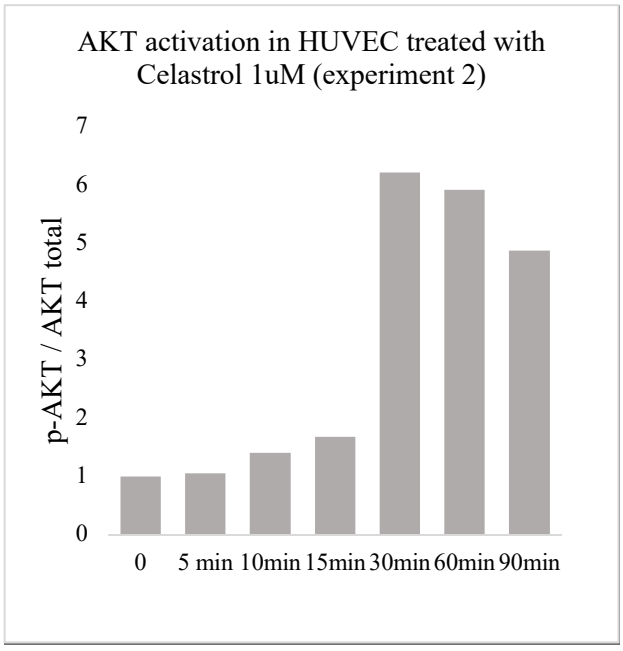




This figure shows the compilation of 5 experiments exposing HUVEC to H_2O_2 0.3 and 0.4 mM for a period of 24 hours. The *Vehicle* group was not treated to Celastrol and serves as the control group. Treatment with Celastrol lasted 1 hour and was done prior to stress. The increased viability in the treated groups did not show significance.

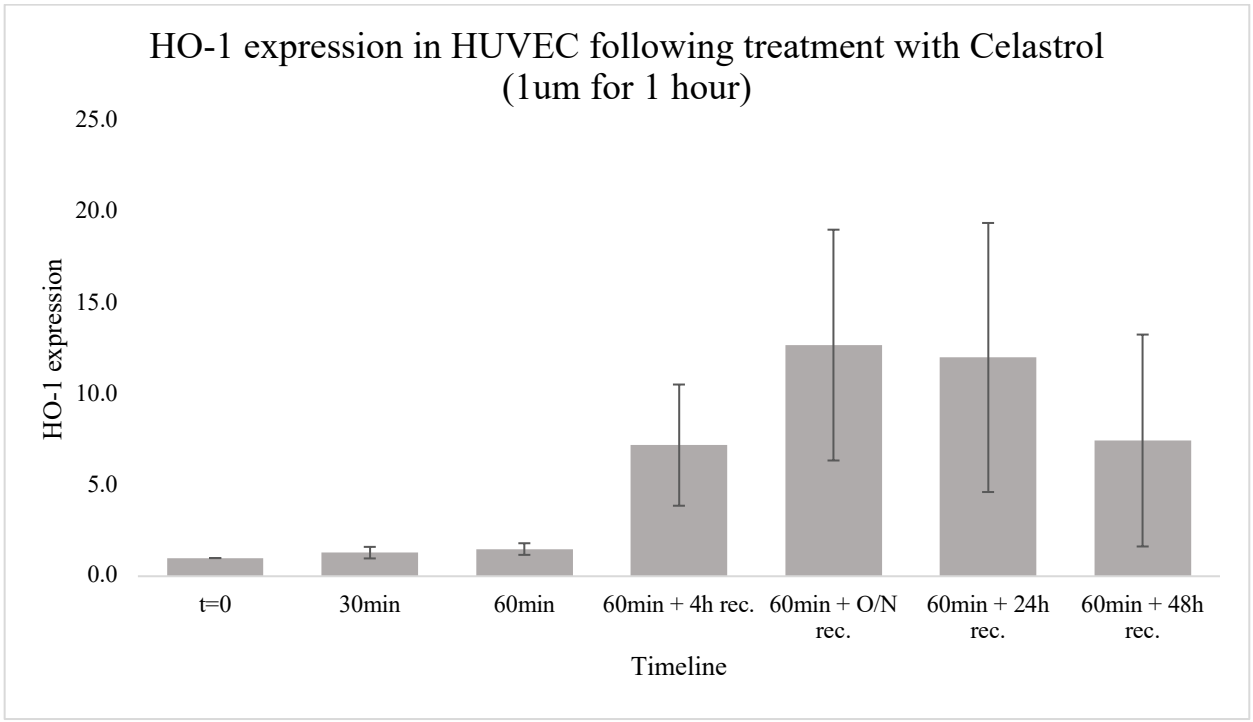
Figure 3





Experiment 2 shows a ratio p-AKT / AKT total 3 times of that of experiment 1 which resulted in high standard deviation and a non-statistical difference ($p>0.05$). Concordant increase in p-Akt is noted starting at 15 minutes and persists up until 90 minutes.

Figure 4



Compilation of 3 experiments. HUVEC were treated with Celastrol 1uM for 1 hour followed by a recuperation period in EGM 5% over a span of 48 hours. Therefore, HUVEC was exposed to Celastrol only in the first 60 minutes. HO-1 expression was assessed at 4 hours, overnight or 16 hours, 24 hours and 48 hours during that recuperation period. (HUVEC, human umbilical vein endothelial cells; rec, recuperation; min, minute; O/N, overnight; HO-1, heme oxygenase 1; GADPH, glyceraldehyde 3-phosphate dehydrogenase; t=0, 0 minute or time prior to treatment)

Figure 5

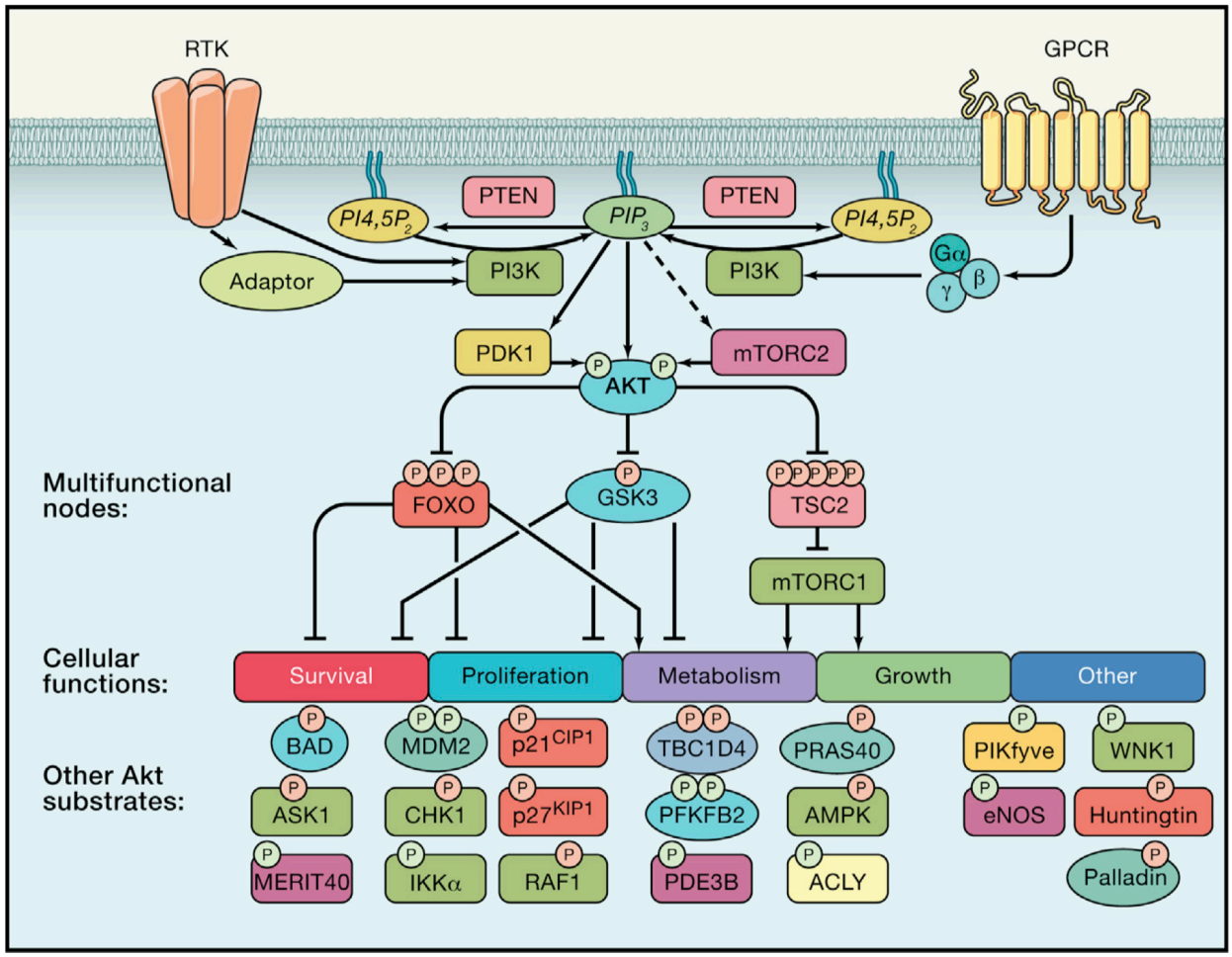


Figure 6 PI3/Akt signalling network [315]